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Archibald J. Motley, Jr. (1891–1981).
Nightlife (1943)
Oil on canvas (91.4 cm × 121.3 cm)
The Art Institute of Chicago.

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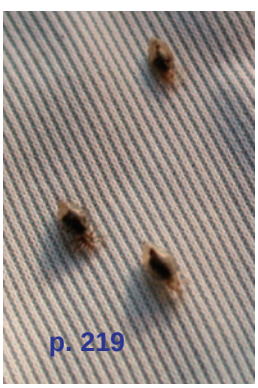

Research

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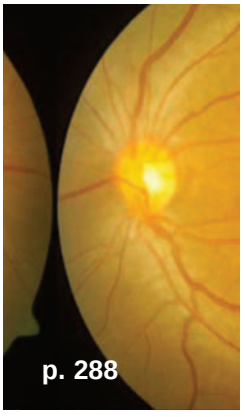

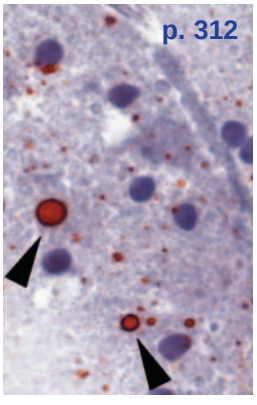

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Antimicrobial Drug Resistance, Regulation, and Research[1]

Joshua P. Metlay,*† John H. Powers,‡ Michael N. Dudley,§ Keryn Christiansen,¶ and Roger G. Finch#**

Innovative regulatory and legislative measures to stimulate and facilitate the development of new antimicrobial drugs are needed. We discuss research approaches that can aid regulatory decision making on the treatment of resistant infections and minimization of resistance selection. We also outline current and future measures that regulatory agencies may employ to help control resistance and promote drug development. Pharmacokinetic/pharmacodynamic research models offer promising approaches to define the determinants of resistance selection and drug doses that optimize efficacy and reduce resistance selection. Internationally, variations exist in how regulators use drug scheduling, subsidy restrictions, central directives, educational guidelines, amendments to prescribing information, and indication review. Recent consultations and collaborations between regulators, academics, and industry are welcome. Efforts to coordinate regulatory measures would benefit from greater levels of international dialogue.

Strategies for addressing antimicrobial drug resistance stress the need for new drugs (*1–3*), and yet the rate of drug development is in decline (Figure 1) (*4*). The Infectious Diseases Society of America (IDSA) (*5*), the World Health Organization (*6*), and other experts (*7*) have drawn attention to this potentially serious threat to public health. Possible reasons include the slow growth in antimicrobial drug sales, caused in part by guidelines for conservative and generic drug prescribing. Resistance limits the market life of antimicrobial drugs, while limited markets exist for agents only active against resistant pathogens. Developers face challenges in demonstrating that new drugs are as safe as established agents. Finally, researchers have found converting pharmacologic targets into commercially viable drugs to be difficult.

Regulatory bodies have roles within collaborative responses to improve the prevention and treatment of infections caused by resistant bacteria. However, in an era of emerging drug resistance, controlled clinical data are often not available to guide regulatory policy. In the first half of this article, we discuss pharmacokinetic/pharmacodynamic (PK/PD) research approaches that can aid regulatory decision making on the treatment of resistant infections and minimization of resistance selection. In the second half, we outline measures that regulatory agencies may use to help control resistance and facilitate drug development.

Scientific Basis for Regulatory Responses to Resistance

Measures to control resistance should be based on scientific evidence concerning its effect on human health and the effectiveness of available interventions. Unfortunately, quantitative data concerning the clinical implications of resistance are lacking for many common infections (*8*). PK/PD models may be used to identify the determinants and implications of resistance, although clinical data on symptom resolution or survival remain the standard (*9*).

PK/PD research aims to identify antimicrobial drug exposures relative to the in vitro MIC that best predicts efficacy and reduced selection of resistance, i.e., the PK/PD index (Figure 2) (*10–13*). The PK/PD index is influenced by bacterial, host, and experimental factors (*12,14*) but tends not to vary among strains of a bacterial species. While absolute doses (in milligrams per kilogram)

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[1]On behalf of the Second Colloquium of the International Forum on Antibiotic Resistance (IFAR), held on September 13, 2003, in Chicago, Illinois, USA. IFAR is a multidisciplinary, international group concerned with evaluating current knowledge regarding antimicrobial drug resistance and the means for its control.

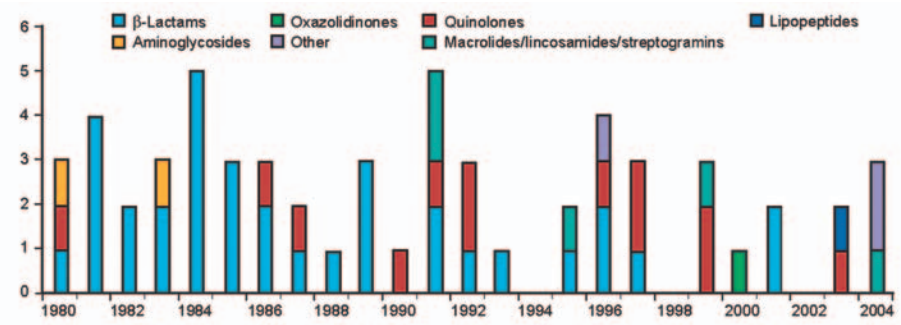

Figure 1. US Food and Drug Administration approvals of systemic antibacterial new molecular entities, 1980–2004. Adapted with permission from Blackwell Scientific (*4*).

associated with efficacy correlate poorly between animal models and humans, parameters of antimicrobial drug exposure relative to MIC can generate clinically relevant PK/PD indices (*11*).

Applying PK/PD Analyses to Doses and Breakpoints

Many existing in vitro MIC susceptibility breakpoints were established both for epidemiologic surveillance and to guide therapy in individual persons. Accumulating evidence supports the use of separate breakpoints for these purposes.

PK/PD data may aid the selection of clinical breakpoints. PK/PD breakpoints represent the highest MIC for which the unbound plasma concentrations of the antimicrobial drug (following standard doses) are sufficient to achieve the PK/PD target against a defined organism and for which adequate clinical data support their use (Figure 3). PK/PD targets are usually derived in vivo by using susceptible strains. The targets for strains with certain resistance mechanisms may differ. However, in several cases, studies have verified that these PK/PD targets apply in less susceptible strains (*15*).

The effects of variability within populations on attaining PK/PD targets can be probed by using Monte Carlo simulation of numerous drug exposures (*10*,*16*,*17*). The fraction of exposures that attain the PK/PD target can be determined across the MIC range of the pathogen and used to help select MIC breakpoints (*17*,*18*). The optimal dose can also be selected by analyzing PK/PD target attainment rates for fixed doses across the MIC range.

Clinical breakpoints may differ substantially from in vitro MIC breakpoints (Figure 3). In 2000, the National Committee for Clinical Laboratory Standards revised the recommended MIC breakpoints for oral β-lactams against *Streptococcus pneumoniae* in light of clinical and PK/PD data (*19*). PK/PD analyses have recently been applied to other breakpoint determinations (*10*,*16*,*19*,*20*). Controlled trials regarding the clinical relevance of discrepancies between current and proposed breakpoints are unavailable. However, case reports indicate a potential increase in treatment failures with some drug classes (and a potential failure to detect these mechanisms with reference microbiologic methods) and suggest the need for better clinical data to reassess susceptibility breakpoints for these agents.

We may also have to challenge the paradigm that interprets breakpoints as dichotomous variables associated with categoric responses such as success and failure. Reductions in susceptibility have graded effects and should instead be interpreted in terms of a reduced relative likelihood of positive outcomes.

PK/PD Targets To Suppress Resistance

Intermediate PK/PD index values may produce antibacterial effects but also select for resistant bacteria (Figure 4). This phenomenon can be conceptually described by considering an infectious bacterial inoculum as a swarm, rather than a clone. A large bacterial load is likely to contain a resistant subpopulation at baseline that is selected during antimicrobial drug therapy. This occurrence can be studied by using a mixed inoculum made up

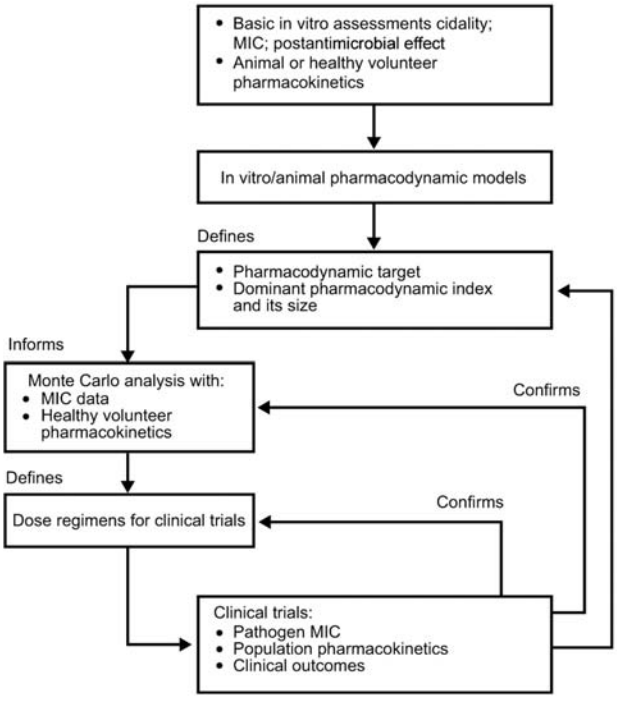

Figure 2. Defining the pharmacodynamic target for therapy. Adapted with permission from Blackwell Scientific (*13*).

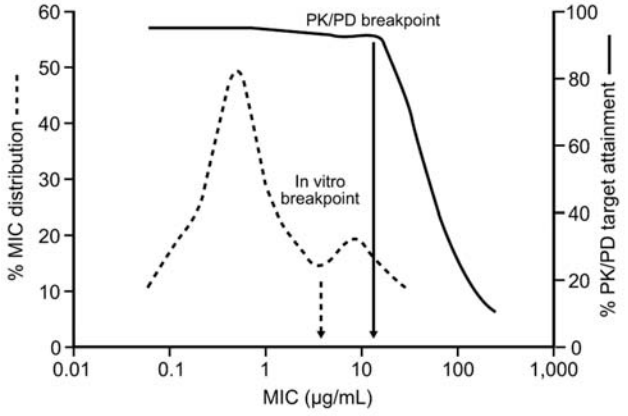

Figure 3. Relationship between MIC and attainment of the pharmacokinetic/pharmacodynamic (PK/PD) target for effect. Accumulating evidence supports the use of separate PK/PD breakpoints for clinical decision making, distinct from in vitro breakpoints used for epidemiologic surveillance. A breakpoint derived from PK/PD data represents the highest MIC for which the unbound plasma concentrations of the drug (after standard doses) are sufficient to achieve the target PK/PD exposure.

of a susceptible population and a small resistant subpopulation (Figure 5) (*16*,*18*).

Mixed-inoculum studies show that the time when the antimicrobial drug concentration exceeds the MIC is the dominant PK/PD index for the selection of penicillin-resistant *S. pneumoniae* (*22*,*23*). The ratio of the area under the concentration-time curve to the MIC (AUC/MIC) predicts fluoroquinolone resistance in this species (*21*,*24*), while the ratio of the maximum concentration and the MIC, and the AUC/MIC, predicts the selection of fluoroquinolone resistance in *Pseudomonas aeruginosa* (*16*,*25*,*26*). In each case, the PK/PD index for resistance selection is the same as that associated with microbiologic activity, although its magnitude may exceed values that can be supplied with usual or safe dosage regimens (*25*,*27*).

Jumbe et al. (*16*) calculated fluoroquinolone PK/PD targets that would amplify or suppress susceptible and resistant populations of *P. aeruginosa* in mice and prospectively validated the resulting dose regimens. These and other data (*23*) underscore the need to determine in clinical studies whether drug regimens should be directed against resistant subpopulations as well as susceptible populations. Such studies would need to correlate bacteriologic treatment failures with initial and posttreatment susceptibility data and antimicrobial drug exposure. Ultimately, they could assess the emergence of resistance among commensal flora.

Future Directions

Although PK/PD data are increasingly valuable, detailed information on the selection and effect of resistance in patients can only be provided by studies designed for this purpose. Such studies should be disease specific and should control for the confounding effect of coexisting conditions (*28*,*29*). Outcomes research would benefit from standardized scoring systems for severity of illness (*30*) and from careful analyses of outcome data in relation to drug exposure. Recent developments in culture sampling, such as nasal catheterization in patients with bacterial sinusitis (*31*), may allow serial observations of antimicrobial drug effects over time and avoid the bias introduced by solely evaluating treatment failures. In principle, continuous sampling of urine in patients with urinary tract infections and the analogous monitoring of drug concentrations and outcomes in middle-ear and lower respiratory infections may also be possible. However, the invasive nature of such studies may preclude a mandatory role in routine antibacterial drug development and licensing.

In April 2004, a workshop cosponsored by the US Food and Drug Administration (FDA) concluded that PK/PD research is useful in dose selection, that modeling and simulation tools may be used to quantitatively predict microbiologic outcomes and account for PK variability, and that PK/PD relationships generated from nonclinical studies should be confirmed in well-designed clinical studies (www.fda.gov/cder/drug/antimicrobial/FDA_IDSA_ISAP_Presentations.htm). As a tool for both regulatory agencies and the pharmaceutical industry, PK/PD studies can provide critical information to help 1) guide the development of optimal dosing schedules for clinical trials and minimize the selection of resistant bacteria during routine clinical use; 2) translate evolving MIC susceptibility data into dosing and treatment recommendations in the absence of data on the clinical effect of resistance; and 3) identify areas where resistance patterns most threaten the efficacy

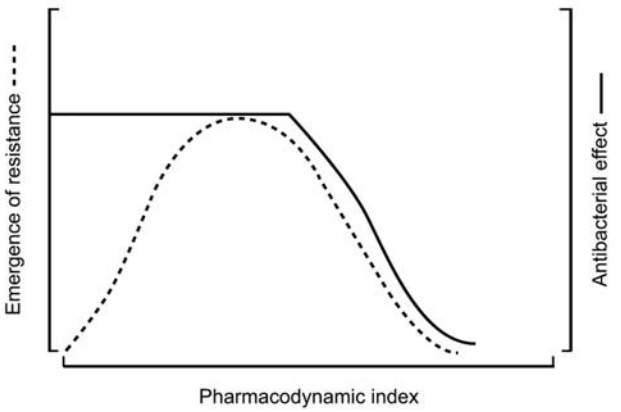

Figure 4. Relationship between the dominant pharmacokinetic/pharmacodynamic (PK/PD) index, efficacy, and resistance emergence in vitro (both quantified by the number of bacterial colony-forming units). The PK/PD index is related to efficacy in a sigmoid curve and the resistance emergence by an inverted U-shaped curve (*21*).

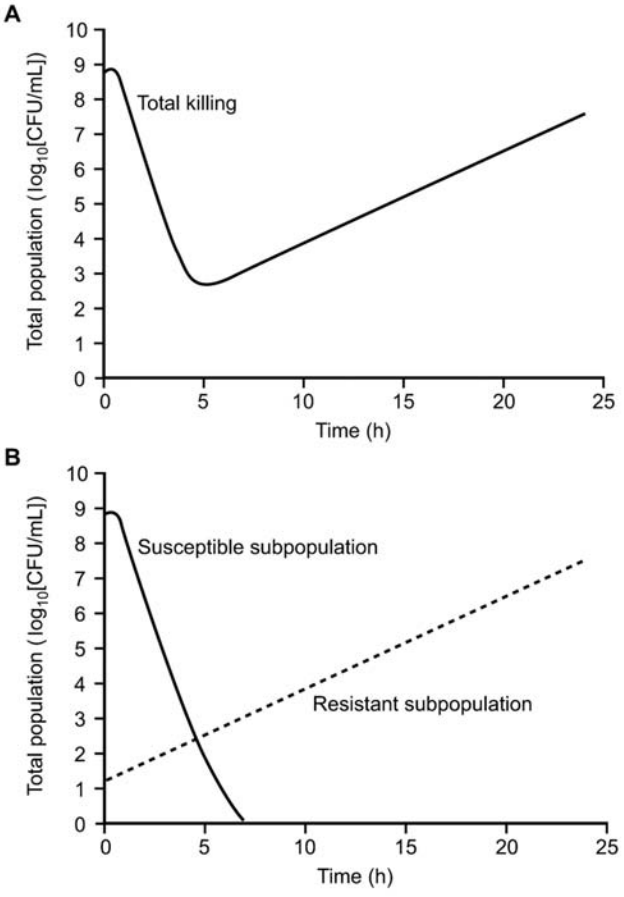

Figure 5. Killing pattern for a fluoroquinolone against *Pseudomonas aeruginosa* that illustrates the initial decline and subsequent regrowth observed in the total number of colony-forming units (A) represent the sum of a decline in the susceptible subpopulation and the uninhibited growth of a resistant subpopulation (B).

of existing therapies and help identify priorities for new drug development.

Regulatory Responses to Resistance

Regulators are primarily concerned with licensing new drugs by verifying their safety, efficacy, and quality. Regulators also have roles that relate to the long-term safety of established agents by responding to postlaunch data. In some countries, licensing authorities regulate the fiscal effect of new therapies, while other countries rely on market forces or employ other agencies to assess cost-effectiveness. In addition, regulators share some responsibility for the sustainability of licensed agents through refinement of indications and recommendations.

The activities of 4 regulatory agencies were discussed during the International Forum on Antibiotic Resistance (IFAR) 2003 colloquium, namely those of Australia, the United States, France, and the European Union (EU) (Table). These activities represent a range of approaches to antimicrobial drug regulation but do not represent a comprehensive sample.

In Australia, registration of drugs for human use is undertaken by the Therapeutic Goods Administration, which is supported by the Australian Drug Evaluation Committee. Both groups are advised by the Expert Advisory Group on Antimicrobial Resistance. In the United States, FDA is responsible for reviewing the safety and efficacy of antimicrobial drugs. When appropriate, FDA solicits input from its Anti-infective Drugs Advisory Committee. The wider issues involving antimicrobial drug resistance, such as surveillance and appropriate use, are the purview of a number of agencies, including FDA, the Centers for Disease Control and Prevention (CDC), and the National Institutes of Health (NIH) (*2*).

Antimicrobial drug licensing at the French Health Products Safety Agency involves an external, multidisciplinary antiinfectives working group, the Groupe de Travail Anti-infectieux. Drug licensing at the EU level is performed either through a centralized procedure mediated by the European Medicines Evaluation Agency (EMEA) or a decentralized procedure based on mutual recognition among member states after the initial step of a national market authorization in a state. Information on drugs registered at the EU level is described in a common European summary of product characteristics document. The EU Committee for Human Medicinal Products guides industry in developing medicines and identifies key information required for licensing (*32*). FDA supplies similar guidance to drug developers (http://www.fda.gov/cder/guidance/index.htm), and guidance on developing agents to treat resistant pathogens is under development.

EMEA (*33*) and FDA encourage drug developers to submit supportive PK/PD data. For example, if in vitro and PK/PD studies show that a drug has similar activity against strains that are susceptible or resistant to existing agents, clinical data against susceptible strains may support efficacy against resistant strains (although clinical data against resistant strains will ultimately be necessary).

Scheduling and Subsidy Restriction

Most developed countries categorize antimicrobial drugs within a "prescription-only" schedule, thereby preventing over-the-counter sales and giving physicians and other healthcare professionals responsibility for their distribution. Restrictions on the subsidization of prescription costs paid by patients in the community may be a means of controlling state-funded drug use. In Australia, prescriptions for certain antimicrobial drugs are not subsidized unless the prescriber gains approval for their use (in specific indications) from the central Pharmaceutical Benefits Scheme. This system has resulted in low levels of fluoro-

Table. Measures taken by selected regulatory agencies before and after licensing to assess and control antimicrobial drug resistance*

Measure	Australia	France	European Commission	United States
Primary drug registration body	Therapeutic Goods Administration, Australian Drug Evaluation Committee	AFSSAPS (French health products safety agency), Commission for Marketing Authorization	European Medicines Evaluation Agency†	Food and Drug Administration‡
Drug resistance advisory resource	EAGAR	GTA, CA-SFM, ONERBA	EARSS	AIDAC
Licensing				
Use of supportive PK/PD data	Yes	Yes	Yes	Yes
Risk assessment	Yes	Yes	Yes	Yes
After licensing				
Prescription-only status	Yes	Yes	NA	Yes
Community drug subsidy restrictions	Yes	No	NA	No
Participation in education (e.g., guidelines)	Yes	Yes	No	Yes
Directives on drug use	Yes	Yes	No	No
Indication review based on resistance	No§	Yes	Yes	Yes
SPC update/ harmonization	Yes¶	Yes	Yes	Yes

*AFSSAPS, Agence Française de Sécurité Sanitaire des Produits de Santé; EAGAR, Expert Advisory Group on Antimicrobial Resistance; GTA, Groupe de Travail Anti-infectieux; CA-SFM, Comité de l'Antibiogramme de la Société Française de Microbiologie; ONERBA, Observatoire National de l'Epidémiologie de la Résistance Bactérienne aux Antibiotiques; EARSS, European Antimicrobial Resistance Surveillance System; AIDAC, Anti-Infective Drugs Advisory Committee; PK/PD, pharmacokinetic/pharmacodynamic; NA, not applicable; SPC, summary of product characteristics.
†Scientific opinions are prepared by committees for human medicinal products, veterinary products, and orphan products.
‡Wider issues involving drug resistance, such as surveillance and appropriate use, are the purview of a number of United States agencies, including the Food and Drug Administration but also the Centers for Disease Control and Prevention, the National Institutes of Health, and other agencies partnering in the United States Public Health Action Plan to Combat Antimicrobial Resistance initiated in 2001 (*2*).
§Only possible for animal antimicrobial drugs.
¶Agreement has been made to update SPCs every 5 years with Australian surveillance data. However, a mechanism for collecting these data has yet to be agreed upon.

quinolone use and resistance (*34*,*35*). However, differential subsidy levels may simply shift drug use toward cheaper agents, and consequently, subsidy restriction may be more useful in controlling the types of drugs prescribed, rather than the gross quantity. In the United States, where cost controls are not used, a decrease in prescribing has been accompanied by an increase in the use of newer, more expensive, and broad-spectrum agents (*36*). However, this increase may be the result of industry marketing forces rather than the lack of subsidy restrictions.

Prescribing Directives and Guidance

Regulators may issue directives to prescribers regarding antimicrobial drug use. However, these must be carefully planned and implemented to avoid disadvantageous effects on prescribing behavior (*37*). FDA issues licensed indications and can create mandatory regulatory policies for certain drugs. It also oversees the content of package inserts and advertisements. However, as in other countries, prescribing practices are at the discretion of the individual clinician.

Regulatory authorities may be involved in educational initiatives to improve antimicrobial drug use. In France, official guidelines on drug use underpin regulation, pharmaceutical promotion, and education. A recent national plan to promote judicious use involved amending antimicrobial drug summaries of product characteristics, as well as amending treatment guidelines and the provision of free streptococcal tests and information for patients and parents (B. Schlemmer, pers. comm.). In the United States, FDA and CDC have partnered on the Get Smart program (www.cdc.gov/getsmart), aimed at fostering appropriate antimicrobial drug use.

Prescribing Information

The usefulness of resistance data within current prescribing information labels may be questioned, given the largely empiric nature of community antimicrobial drug prescribing. FDA has recognized the need to inform clinicians about resistance issues for empirically treated diseases and has designated several drugs, for which adequate clinical data exist, as safe and effective in the treatment of community-acquired pneumonia caused by multidrug-resistant *S. pneumoniae*. Updating labeling is a substantial undertaking. In 2003, labels for 669 drugs had to be changed when FDA amended labeling requirements for antimicrobial drugs (*38*).

In Europe, international disharmony remains in the summaries of product characteristics for older drugs. Efforts to update and harmonize these will require

cooperation between EMEA, national regulatory bodies, and the pharmaceutical industry. Experience from Australia, where the registration system for human antimicrobial drugs has been revised to incorporate resistance risk assessment, suggests that this process will be challenging. As generic manufacturers have no responsibility to provide resistance data for their products, healthcare systems may have to provide resources to collect these data.

Indication Review

Indication review is the process by which regulatory authorities reassess the licensed indications of a drug in light of new data. In some countries (e.g., Australia) indication review may only be performed on the basis of drug safety. In others, it may in principle be performed on resistance grounds. Any decision to change a drug's license should be based on robust clinical evidence of a public hazard. In vitro surveillance data may be insufficient in isolation, as previously discussed. Moreover, uncertainty exists about the threshold resistance prevalence at which indications should be withdrawn.

Incentives to Antimicrobial Drug Development

In principle, the current decline in drug development could be reversed by a number of means. Substantial costs are incurred by the late-stage failure of developmental candidates. Costs may be reduced by efficiently identifying drugs that are more likely to be effective, allowing earlier decisions on development cessation, which is the focus of the FDA Critical Path Initiative (www.fda.gov/oc/initiatives/criticalpath/). Public-industry risk sharing could also be considered for phase III trial funding. Detailed PK/PD investigations could potentially reduce the number of phase I/II studies required (*33*) and facilitate dose selection for phase III trials. Other possible approaches include the use of data in 1 indication to support a license application in another (providing the spectrum of causative pathogens, PK/PD factors, and infection severity is sufficiently similar). Regulatory authorities have offered fast-track designation and priority review for narrow-spectrum antimicrobial drugs and agents active against multidrug-resistant organisms. However, FDA grants priority reviews on the basis of results of clinical trials with a drug, not on in vitro spectrum alone.

Recently, fruitful collaborations have taken place between regulatory agencies, healthcare systems, academia, and industry. FDA has consulted with representatives of the pharmaceutical industry and IDSA and has identified pathogens of primary public health importance (www.fda.gov/ohrms/dockets/ac/03/slides/3931S2_03_Powers_files/frame.htm). IDSA has held preliminary discussions with NIH to explore ways in which trial funding could be shared between public bodies and industry. However, considerable political, logistic, and financial challenges must be overcome if public-private partnership models are to be applied.

Financial incentives could be provided to industry by waiving or reducing the new drug application fee, by extending or renewing patents for antimicrobial drugs of public health priority, or by granting orphan drug status for treatments for serious but rare diseases. "Wild card" measures are an alternative approach, whereby a company can choose which drug in its portfolio is granted exclusivity or patent extension. Considering government contracts with industry for specific agents or guaranteeing markets for niche drugs may have value. More widely, opportunities may exist to reconsider drug pricing structures and tax incentives related to antimicrobial drug revenues. Because regulatory bodies can only act within existing legislation, legislative changes may be required to provide economic incentives to industry.

The provision of such incentives should be dependent on responsible marketing and sales activities by pharmaceutical companies. In the United States, the Department of Health and Human Services Office of the Inspector General has developed guidelines for marketing activities that have been adopted by many companies (http://oig.hhs.gov/authorities/docs/03/050503FRCPGPharmac.pdf).

The development of narrow-spectrum antimicrobial drugs or adjunctive agents that target specific resistance mechanisms will not be viable without effective, low-cost diagnostic methods available at the point of prescribing. Thus, incentives must also be considered for the development and clinical adoption of diagnostic technologies.

Conclusions

Regulatory authorities must balance the requirements for safe and effective medicines, and the need for new antimicrobial drugs effective against resistant pathogens, with the technologic and commercial realities of drug development. We do not know whether the development of new antimicrobial drugs will keep pace with the emergence of resistant pathogens. This uncertainty highlights a need to identify gaps in available drugs and for governments to devise innovative regulatory and legislative measures to stimulate the development of new agents and diagnostic technologies.

PK/PD models that integrate preclinical and clinical data offer a promising approach to defining optimal drug doses for phase III clinical trials. PK/PD data may also help define the determinants of resistance selection, quantify the clinical effect of resistance, and identify where resistance patterns most threaten the efficacy of existing therapies and where priorities for drug development lie.

However, further clinical research is required to correlate microbiologic outcomes based on PK/PD data and clinical outcomes in patients. These trials should exploit recent advances in novel endpoints, sampling techniques, and PK modeling. Potentially, these data may be used in conjunction with outcomes research in determining susceptibility breakpoints for clinical purposes.

Initiatives in Europe and the United States indicate a welcome trend toward greater consultation and collaboration between regulatory authorities, the pharmaceutical industry, and knowledgeable professionals. The role played by regulatory authorities in controlling drug use varies by country. In this context, efforts to improve regulatory measures would benefit from greater international dialogue.

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HIV Drug-resistant Strains as Epidemiologic Sentinels

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Observed declines in drug resistance to nucleoside reverse transcriptase inhibitors among persons recently infected with HIV-1 in monitored subpopulations can be interpreted as a positive sign and lead public health officials to decrease efforts towards HIV prevention. By means of a mathematical model, we identified 3 processes that can account for the observed decline: increase in high-risk behavior, decrease in proportion of acutely infected persons whose conditions are treated, and change in treatment efficacy. These processes, singly or in combination, can lead to increases or decreases in disease and drug-resistance prevalence in the general population. We discuss the most appropriate public health response under each scenario and emphasize how further data collection and analyses are required to more reliably evaluate the observed time trends and the relative importance of forces shaping the epidemic. Our study highlights how drug resistance markers can be used as epidemiologic sentinels to devise public health solutions.

In recent years investigators have begun monitoring the HIV epidemic by reporting changes in the proportion of newly infected persons who are carrying an HIV-1 drug-resistant strain, i.e., the primary or acute resistant fraction. Several studies report decreases in this primary resistant fraction (*1–7*), including transient decreases (*8–14*). Unfortunately, none of these studies included precise longitudinal data on the exact number and type of infected persons or of the fraction of the total population that is screened for acute infection or resistance, and as we show here, making direct interpretations from data collected from a subset of the population can lead to erroneous conclusions. Given the potentially serious clinical implications of drug resistance for HIV-infected persons, public health officials and other authorities need to know whether the decline in drug resistance among acutely infected persons in the monitored subpopulations corresponds to a real decline in drug resistance in the general population and whether this effect is sustainable over time. The decline might be interpreted as a positive sign caused, for example, by less high-risk activity by HIV-positive persons infected with a drug-resistant variant. This explanation could lead public health officials to decrease their support for HIV surveillance and prevention programs targeted at impeding the spread of drug-resistant HIV strains such as drug-resistance testing or adherence counseling. The amount of resources that should be dedicated to drug resistance monitoring and reporting is a controversial issue in light of the recent isolation of a highly virulent multidrug-resistant strain in New York City (*15*). Here we show how drug-resistance data can offer not only clinical information regarding appropriate treatment regimens for individual patients, but also critical insights into an epidemic's course.

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Treatment History of HIV-1 and Its Impact on High-Risk Behavior

Different types of drugs have been developed to fight HIV. Zidovudine (AZT), a nucleoside reverse transcriptase inhibitor (NRTI), was first administered in 1987, and until 1995, monotherapy or dual therapy with NRTIs were the only treatments available. The first protease inhibitor (PI), saquinavir, was approved for treatment in 1995, followed closely in 1996 by a nonnucleoside reverse transcriptase inhibitor (NNRTI), nevirapine. These new drugs generated a major change in the treatment strategy against HIV—highly active antiretroviral therapy (HAART)—that coincided with the start of the monitoring periods in several of the studies mentioned above (1995–1996). With HAART, at least 3 drugs are administered at the same time, which substantially reduces viral load and, compared to results of earlier regimens, increases the life expectancy of patients. These advantages follow because the mutations necessary to confer resistance to HAART are generated at a slower rate and are lost more rapidly than those conferring resist-

ance to monotherapy or dual therapy. Moreover, viral strains resistant to HAART are not as efficient at completing their own life cycle (e.g., their replication rates are lower), they may generate less illness and lower proportion of deaths among infected persons, and the viral strains are less likely to be transmitted to other persons.

The primary resistance time trends observed for NRTIs do not match those observed for the other 2 drug types. For example, in North America some researchers report a decrease in the proportion of persons recently infected with a drug-sensitive HIV strain resistant to NRTIs; this decrease is followed by an increase and subsequent decrease (*11–13*). The pattern is particularly noticeable in the study by Grant et al. (*12*), in which NRTI genotypic resistance decreased from ≈30% in 1997 to 5% in 1999, rose to 20% in 2000, and fell to 15% in 2001. Little et al. also found this trend in primary resistance to NNRTIs (*11*). These 2 studies (*11,12*) also documented a steady increase in the proportion of persons newly infected with a virus strain resistant to PIs. Here we focused on identifying the likely forces responsible for the time trends exhibited by viral strains resistant to NRTIs. We did so because their time trends are expected to provide better insight into the long-term dynamics of the epidemic than strains resistant to PIs or NNRTIs, given that NRTIs have been administered to more HIV-infected persons and for a longer period than the other 2 types of drugs (*16,17*).

Treatment optimism after the initial successes of HAART likely affected the subsequent dynamics of HIV because these favorable treatment outcomes led some persons to increase their high-risk behavior. Later it became apparent that HAART does not completely eliminate HIV from an infected person or impede its transmission. Moreover, when HAART first became implemented, the best strategy was believed to be "hit hard, hit early," because the medical community was trying to limit the expansion of HIV within an infected person's body and ameliorate the gradual deterioration of the patient's immune system. However, HAART can have considerable negative side effects, which affect the functioning of the gastrointestinal system, renal system, pancreas, and liver and produce changes in blood count, allergies, lactic acidosis, and other problems. As a result, treatment began to be delayed to balance the following factors: 1) containing the viral load, 2) minimizing the risk of drug-resistant mutants developing by limiting the amount of treatment time, and 3) reducing negative side effects.

Modeling Drug Resistance in HIV

Empirical studies have shown that antiretroviral treatment (ARV) produces substantial changes in the viral dynamics at the within-host level that translate into substantial changes at the between-host level (*8,18,19*). Mathematical and computational models permit us to create simplified versions of complex realities that we can manipulate to further our understanding of their dynamic behavior. Consequently, numerous theoretical studies have investigated the impact of drug therapy on HIV dynamics at both levels (e.g., *20*). Initial HIV treatment models (e.g., *21–23*) addressed how ARVs might affect the infectiousness of treated persons, and the spread of HIV and its disease-induced deaths. The magnitude of the public health threat created by drug-resistant HIV strains was only recognized later. As a result, Zaric et al. presented a novel model that showed that adhering to treatment regimens would discourage the emergence of multidrug-resistant HIV strains in heterogeneous populations (*24*). Blower et al. developed a relatively simple but revealing deterministic compartmental framework (*25*) that has served as the reference point for most of the modeling studies subsequently done to investigate the effect of ARVs on disease incidence and prevalence, drug-resistance transmission and prevalence, AIDS death rate, and the potential to eradicate the HIV epidemic (*26–31*). Dangerfield et al. built a detailed HAART treatment model that accounts for persons in all 4 HIV stages, and the last is partitioned in early- and late-stage AIDS (*32*). They investigated the effects of HAART on HIV incidence and prevalence, assuming different average efficacious periods and assimilation times for HAART, different infectivity probabilities when receiving HAART, and different increases in the mean number of sexual partners.

Blower and Volberding reviewed mathematical studies used to understand the dynamics of a drug-resistant HIV epidemic, predict the incidence and prevalence of drug-resistant HIV strains, evaluate cost-benefit strategies, and assess the impact of public health policies (*33*). The general approach to these studies had been to construct a descriptive simplification of the epidemic by identifying critical categories and processes and to use this structure to make predictions, given a set of assumptions regarding the parameter values. In this regard, several studies have characterized the epidemic's trends as monotonic, including the fraction of new HIV infections that are drug resistant (e.g., *16,25,28,34*). Goudsmit et al. conducted an analysis in which including changes in treatment rates explained the nonmonotonic trends of zidovudine resistance observed in a cohort of newly infected homosexual men enrolled in the Amsterdam Cohort Study (*1*).

In our previous study (*35*), we extended the basic modeling framework detailed in (*25*) to incorporate additional complexity, including 2–3 separate categories of acutely infected persons, depending on whether a person was infected with a drug-sensitive HIV strain, a strain resistant to monotherapy, or a strain resistant to triple-drug therapy. In doing so, we were able to distinguish among acutely

infected persons, who are clinically and epidemiologically distinct from uninfected and chronically infected persons (e.g., we can consider them to engage more frequently in high-risk behavior (*2*) than chronically infected persons). We also counted these categories separately and tracked their temporal trends and better channeled the different categories through the model, according to the different processes acting on them (such as a decrease in the proportion of persons receiving treatment among those recently infected, an effect that did not occur in persons in the chronic phase). We also created 10 subcategories in the chronically infected stage to more accurately represent the progression of persons from the acute stage of infection to AIDS (*35*). Moreover, because our intent was to explain observed trends rather than to make future predictions, we adopted a specific approach that consisted in altering individually the value of each parameter during a given simulation (as was done in [*1*] with treatment rates of all persons), rather than running simulations with a set of fixed parameters and comparing outcomes across runs.

Primary Resistant Fraction

The most direct explanation for the decrease in the observed proportion of newly infected persons infected with NRTI-resistant HIV-1 strains is that resistance to NRTIs in the recently HIV-infected population is declining. Unfortunately, the data need to be further evaluated because the HIV infection status of every person in the general population has not been monitored. We therefore do not have absolute numbers for these time trends, but only the relative numbers obtained from monitored subpopulations, which consist of consenting persons enrolled in research programs at specific locations. To be eligible to participate in these programs, patients had to display symptoms typical of an acute HIV seroconversion syndrome or have recently engaged in risky activities that could have placed them at risk of contracting HIV. Accordingly, what has decreased is the fraction of drug-resistant carriers among the pool of recently infected HIV patients who are willing to participate in particular research programs and attend clinics involved in these studies. The time trends exhibited by the variable representing the actual counts of all the newly infected persons who are carriers for a resistant strain in the general population may or may not be a direct match to those of the monitored subpopulations.

These points are best illustrated by considering the fraction of recently infected persons who are carriers of a drug-resistant strain (primary resistant fraction, F_R), defined mathematically as

$$F_R = \frac{R}{S+R},$$

where S is the number of persons initially infected with a drug-sensitive strain, and R is the number of persons initially infected with a drug-resistant strain. This fraction may decrease because R decreases (fewer newly infected persons have a strain that is drug resistant), or because S increases (more newly infected persons carry drug-sensitive strains). If both S and R increase or decrease by the same proportion, F_R remains unchanged. As explained below, the benefit of using this variable's time trends to further our understanding of the past, present, and future of the HIV epidemic is that underlying alterations in the relative values of drug-sensitive and drug-resistant strains may arise from a variety of mechanisms with critically different epidemiologic outcomes.

To determine which processes could have caused the observed decrease in F_R, we built a mathematical model of HIV transmission (Figure 1); a more mathematically detailed explanation of our analysis can be found in our previous study (*35*). We then simulated the epidemic using this model and varied each of the parameters shown in Figure 1 (e.g., the average number of high-risk contacts in 1 year, the likelihood of transmitting HIV given a high-risk contact, the fraction of persons with acute or chronic HIV

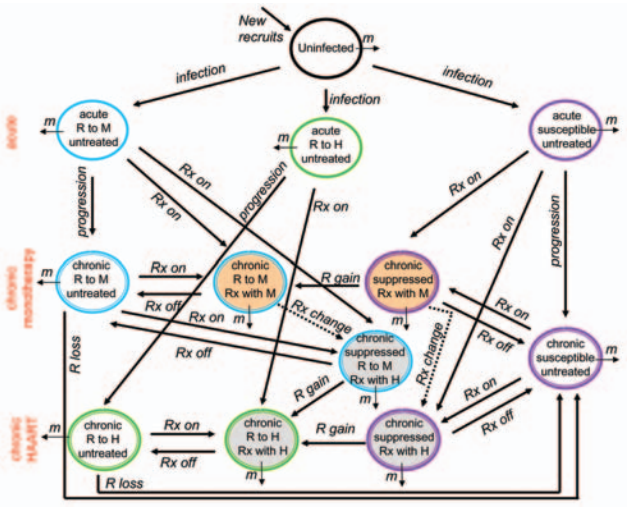

Figure 1. Flow chart of the different categories and flows considered in our model system. For simplicity, we considered 1 type of treatment when analyzing the effects of an increase in high-risk behavior and treatment delay. When considering the effects of overall change in treatment strategy, all categories and flows were included in the analysis. Abbreviations: *m*, mortality (composed of background deaths for all categories, and for persons in the chronic phase of infection, HIV-related deaths are included); Rx, treatment; R, resistance; M, monotherapy; H, highly active antiretroviral therapy (HAART). Color code for the categories' encircling ovals: black (uninfected); purple (wild type); blue (monotherapy resistant strain); green (HAART-resistant strain). Background code of oval categories: no fill (untreated); tan (monotherapy); gray (HAART). Code for the categories' encircling ovals: single, no staging (uninfected and acutely infected); double, staged categories (persons in the chronic phase).

infection that are placed on treatment each year, the likelihood of generating or losing drug resistance in 1 year). Once we determined which processes can cause a decrease in the acute fraction infected with a drug-resistant strain, we evaluated whether the process had occurred in industrialized countries in recent years. If so, we could consider the process as a potential contributor to the observed trends. As a result, we identified 3 independent processes that caused a decrease in F_R and were consistent with the history of the HIV-1 epidemic in industrialized countries from 1995 to 2001: 1) overall increase in risky behavior, 2) decrease in the fraction of individuals in the acute phase who are placed on treatment, and 3) increase in the efficacy of treatment. Goudsmit et al. also found that discontinuation of monotherapy with zidovudine in 1996 explained the observed drop in zidovudine resistance in patients newly infected with HIV in the Amsterdam Cohort Study (*1*).

Figure 2A illustrates the outcomes of running the model given our manipulation of the parameter values characterizing these 3 processes. We obtained the same qualitative patterns across all reasonable combinations of parameter values. When high-risk behavior increases, the drug-sensitive strain has an initial advantage over the drug-resistant strain because of its higher transmission rate, and it increases to its equilibrium prevalence value at a faster pace than the drug-resistant strain. This increase causes a temporary decrease in F_R (solid trajectory, Figure 2A). The decrease is only temporary because the relative equilibrium prevalence value of the strains is independent of the risky behavior rate, and the relative prevalence value among acute F_R returns to its original value before the perturbation (*35*). If fewer persons are treated, fewer patients will be generating and transmitting drug-resistant strains (dashed trajectory, Figure 2A). The change in treatment efficacy also leads to a decrease in F_R because drug-resistant strains are harder to generate and are less likely to be transmitted under treatment with HAART than under monotherapy with AZT (dotted trajectory, Figure 2A). Figure 2A shows that the long-term behavior of the primary resistant fraction is substantially different under the 3 scenarios, even though it initially decreases for all 3.

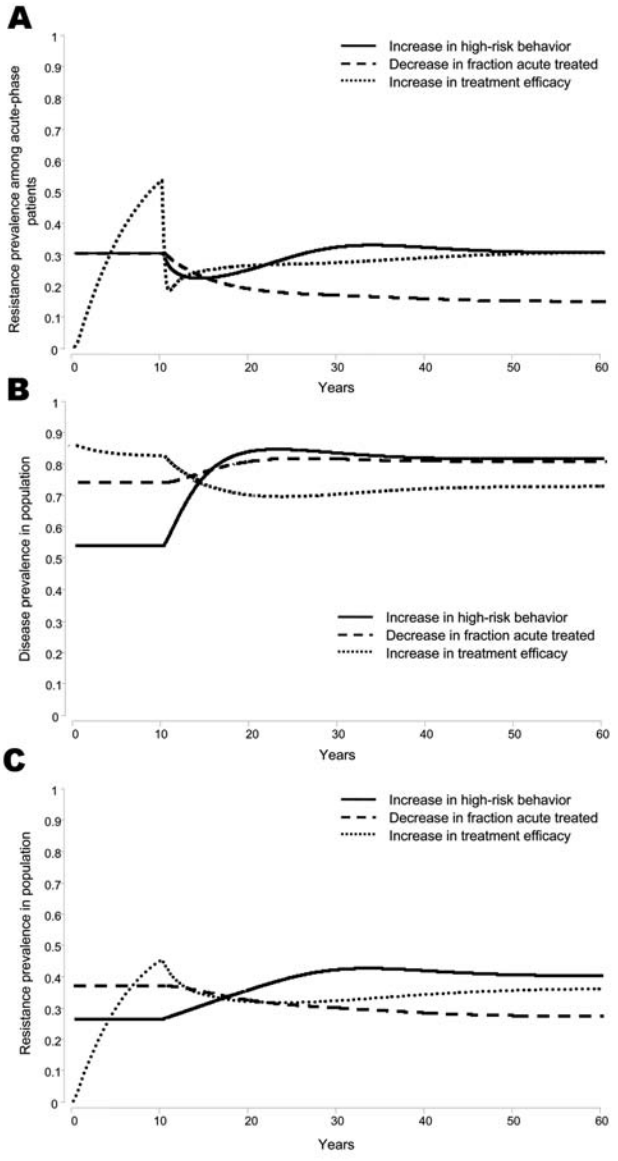

Figure 2. Time trends for A) proportion of persons in the acute phase infected with a resistant viral strain, B) disease prevalence in the population, and C) resistance prevalence in the population. At time t = 10 years, we introduce a 1) increase in high-risk behavior from 2 to 4 contacts/person/year, or 2) decrease in the yearly fraction of acutely infected persons on treatment from 0.4 to 0.1, or 3) increase in treatment efficacy from monotherapy with zidovudine (AZT) to highly active antiretroviral therapy (HAART). All other parameter values and conditions are as reported by Sánchez et al. (*35*). At t = 0, there is 1 infected person in a population of 100,000. For the first 2 processes, we let the simulations reach equilibrium and then introduced the change. The graphs show the trajectories starting at equilibrium and the changes occurring after 10 years. The third process reconstructs San Francisco's historical time frame for the treatment regimen change. Now the epidemic runs without treatment for 30 years, monotherapy with AZT follows for 10 years, and HAART begins at t = 40. To facilitate the comparison with the first 2 processes, we graphed the dynamics of the treatment change from the moment AZT was introduced.

Course of the HIV Epidemic

How do these 3 processes (increase in high-risk behavior, treatment delay, and greater treatment efficacy) impact the course of the HIV epidemic? Are these effects comparable or are they sufficiently different such that the policy implications will vary according to which one we interpret to be the leading cause for the observed decline? To address these questions, we determined the short- and long-term changes induced by these processes on 2 variables of critical public health importance for which we do not have reliable measurements: prevalence of disease

(i.e., overall fraction of persons infected with HIV in the general population) and prevalence of drug resistance (i.e., overall fraction of persons infected with a drug-resistant HIV strain in the general population). By doing so, we use time trend changes in the relative prevalence values of 2 viral strains (i.e., the fraction F_R defined above) to make inferences about changes in the absolute values of disease and resistance prevalence in the population (i.e., actual counts of infected persons and carriers of drug-resistant infections in the general population). Figures 2B and 2C show that the 3 factors we identified as causing a decrease in the primary resistant fraction are predicted to force different and permanent, long-term changes in disease and drug-resistance prevalence. A synopsis of our findings is provided in the Table.

Policy Implications and Comparison with the Data

Our results demonstrate how a decrease in the fraction of persons recently infected with a drug-resistant HIV strain can occur not only when the epidemiologic conditions improve (i.e., disease and drug-resistance prevalence in the population decrease), but also when the epidemic worsens (i.e., disease and drug-resistance prevalence increase). The 3 processes that can generate the decrease in primary resistance are not mutually exclusive, and probably all have contributed to the observed time course of F_R. The challenge now is to identify which one has had the greatest effect on the recent trends of disease and drug-resistance prevalence in the HIV-1 epidemic.

If an increase in high-risk behavior has dominated HIV-1 epidemiology since the onset of HAART, then the decrease in primary resistance, counter to intuition, signals a worsening of the epidemic: a greater number of persons may have become infected, and a greater number of persons may be infected with a viral strain resistant to drug therapy. Other studies have obtained similar conclusions (e.g., *28*,*29*,*31*,*32*). If this is the case, the public health response to the decrease in drug-resistance levels among the acutely infected should be to expand programs aimed at reducing high-risk behavior.

Determining the most appropriate public health response is difficult if a decrease in the fraction of acutely ill persons receiving treatment is the main driving force of the HIV epidemic. Under this scenario, the indications for treating acutely infected persons may need to be modified by taking into account the potential balance between an increased number of infected persons as opposed to a decreased number of carriers of drug-resistant infections. Cost-benefit analyses of this nature are an intrinsic part of public health policy (*33*). In any case, we do not expect this process to be the main driving force responsible for recent trends in disease and drug resistance in the HIV-1 epidemic because the number of persons in the acute phase of HIV infection is much smaller than that in the chronic phase (1), and a large proportion of chronically infected patients received HAART at the beginning of the study period (*36*). Moreover, new treatment regimens, such as structured treatment interruptions and drug holidays, may have affected recent drug-resistance trends (*36*).

The most favorable outcome occurs if the increase in treatment efficacy brought about with HAART is the most important process determining recent HIV-1 trends. Now both the prevalence of the disease and of drug resistance in the population are decreasing, and therefore the decline in drug-resistance prevalence among the acutely infected is a positive sign (*25*,*26*,*32*). Results under this scenario underscore the importance of public health interventions directed toward increasing the number of persons receiving treatment (we must keep in mind that these results assume that the fraction of persons treated remains constant).

However, the uncertainty in the parameters does not allow us to readily distinguish between the 3 likely scenarios. Moreover, we cannot be sure that the trends observed correspond to a real decrease in F_R, or are simply fluctuations due to stochastic or sampling phenomena around a monotonically increasing time trend (*11*,*18*,*25*,*34*,*37–39*). Little et al. and Grant et al. report trends consistent with the first scenario: the decrease in the fraction of persons infected with an NRTI-resistant strain is followed by an increase and subsequent decrease (*11–13*). Several authors report biphasic patterns of alternating trends (*1*,*2*,*5*,*9*,*10*,*14*), which are not necessarily correlated with an increase in non-B subtypes (*8*). Other studies report overall increasing (*38*), stable (*40*), and decreasing (*3*,*4*,*6*,*7*) trends in the proportion of persons recently infected with an NRTI drug-resistant HIV-1 strain. These studies, together with our results, highlight why disease surveillance must be increased, with additional data collection and analyses, to fully understand the present and future course of the HIV epidemic. In this regard, mathematical modeling can provide a crucial tool for the correct

Table. Impact of an increase in high-risk behavior, decrease in the fraction of acute HIV infected persons receiving treatment, and increase in treatment efficacy on the primary resistant fraction, disease, and resistance prevalence in the overall population

	Effect		
Cause	Primary resistant fraction	Disease prevalence	Resistance prevalence
Increase in high-risk behavior	Temporary decrease	Permanent increase	Permanent increase
Decrease in fraction acute treated	Permanent decrease	Permanent increase	Permanent decrease
Increase in treatment efficacy	Permanent decrease	Permanent decrease	Permanent decrease

interpretation of epidemiologic data by identifying the processes responsible for generating observed time trends and characterizing their potential implications for public health programs.

Conclusion

Our mathematical analysis shows that the observed time trends of measurable quantities from particular subgroups of infected persons (such as primary drug resistance in monitored subpopulations) can correspond to different and unexpected time trends of variables of critical public health interest that are not measured directly in the general population. On the other hand, with the appropriate analyses, information on drug resistant strains can be used not only to guide treatment in individual patients, but also as epidemiologic sentinels to help devise public health solutions. Because changes in the relative value of 2 strains that vary in any of their life history traits (such as their ability to be transmitted, to be suppressed when in the presence of drug therapies, or to lose mutations that confer drug resistance) can show information on an epidemic's trends, the reasoning and methods we used in this study can be applied equally well to understand the epidemiology of any genetically variable microbe.

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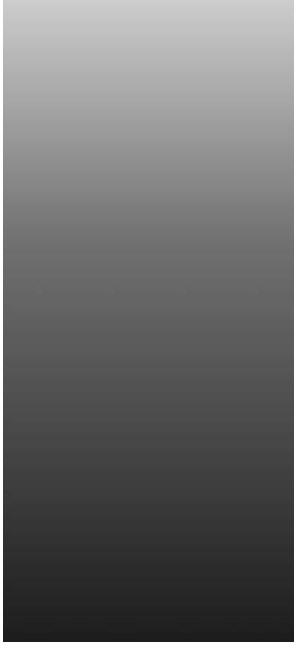

Detecting Hepatitis B Surface Antigen Mutants

Paul F. Coleman*

Hepatitis B viral mutants can emerge in patients as a result of selection pressure from either immune response or treatment options. Mutations that occur within the immunodominant epitopes of hepatitis B surface antigen (HBsAg) allow mutant virus to propagate in the presence of a neutralizing immune response, while wild-type virus is reduced to undetectable levels. HBsAg mutants present as false-negative results in some immunoassays. An understanding of immunoassay reactivity with HBsAg mutants is key to establishing an appropriate testing algorithm for hepatitis B virus detection programs.

Over the past decade, the importance of hepatitis B virus (HBV) mutants has made a transition from an academic phenomenon of unknown prevalence to a factor for consideration during disease diagnosis. HBV infection has a major effect on world health care: more than one third of the world's population has been infected at some point; ≈350 million people are currently infected (*1*). This immense worldwide reservoir of infection serves as the basis for the generation of HBV mutants because of the unique molecular biology of this virus. Since the late l980s, we have seen the emergence of mutants across the entire HBV genome as the virus responds to selective pressures, such as vaccination and antiviral therapy. Viral adaptation through mutation will continue as new treatment options are employed and current treatment options are expanded into areas of endemic infection. HBV mutant surveillance and understanding of HBV mutant impact on disease diagnosis will pose a challenge to global health care for the foreseeable future. Thus, diagnosticians and the healthcare industry need to increase their awareness of HBV mutants and how these mutants may alter current diagnostic and treatment algorithms. This article addresses recent information concerning the emergence of hepatitis B surface antigen (HBsAg) mutants, their impact on viral antigen presentation, latest prevalence data, and discussion of the issues associated with detection of mutants in healthcare settings.

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Mechanism of HBV Mutant Generation

HBV belongs to the genus *Orthohepadnavirus*, family *Hepadnaviridae*. This virus has a small circular DNA genome, ≈3.2 kb in length, that contains 4 genes with partially overlapping open reading frames (ORFs). These ORFs encode the polymerase protein (Pol gene); core antigen and e antigen (C gene); large, medium, and small surface-antigen proteins (S gene); and the X protein (X gene). From a relatively small genome, these overlapping ORFs generate 7 proteins. While this gene overlap may constrain some viral variability, mutant or variant forms have been identified for all 4 genes (*2*). HBV analysis has transitioned from the serologic subtype classification of the early 1970s to the more precise genotype genetic classification. HBV has been classified into 8 genotypes (A–H) on the basis of intergenotypic difference of >8% in the entire nucleotide sequence (*3*). HBV genotypes demonstrate geographic diversity. However, distinct genotypes have evolved in more remote areas, as evidenced by genotype E, localized in Madagascar, and genotype F, localized in South America. This diversity of the HBV genome is generated by the same mechanism that drives the emergence of mutants, replication.

The replication of HBV DNA proceeds through a RNA reverse transcriptase intermediary step. HBV variants are generated during this process. Since the reverse transcriptase activity of the HBV polymerase protein lacks a proofreading function, random mis-incorporation of bases into the replicating DNA strand occurs. This mismatch leads to the generation of multiple variant transcripts from a single template and the formation of a quasispecies pool (*4*). This quasispecies pool provides the source material for the emergence of a mutant when selection pressure is applied (*5*). A mutation selected for in 1 gene can potentially lead to an amino acid change in the overlapping reading frame. Replication of the hepatitis B virion is, therefore, the sole requirement for generating these nucleotide mismatch sequences. The number of viral particles generated in some infected persons can be as high as 10^{11} viral particles per day. Because of the polymerase reverse transcription

error rate (1 error per 10^7 bases), in active infection, 10^7 base-pairing errors can be generated per day over the 3,200-bp genome (*6*). While most of these new sequences are nonviable or fail to effectively compete with wild-type virus, they provide a starting point for the emergence of mutants when selection pressure is applied. HBV mutants can be expected to emerge in any geographic area where populations of infected persons are exposed to a selective pressure.

New treatment regimens developed over the past 2 decades have successfully reduced overall HBV infection rates, but they have also exerted powerful selection pressures for the emergence of HBV mutants. Treatments that have selected for mutants include immunotherapy (vaccination, administration of HBV immune globulin) and nucleoside analogs (e.g., lamivudine, lobucavir, famciclovir, adefovir) to inhibit polymerase activity. These treatment options can suppress wild-type HBV to undetectable levels, allowing a mutant HBV strain to emerge as the predominant form. Emergence of a mutant species can be monitored by using such techniques as real-time polymerase chain reaction (PCR) assays, restriction length polymorphism assays, quantitative fragment analysis, and primer extension assays. These powerful techniques can detect trace mutant sequences in clinical samples with a preponderance of wild-type virus, while conventional DNA sequencing cannot (*7*). Mixed infection samples (i.e., low-level HBV chronic infections) that contain a preponderance of wild-type HBsAg present a challenge to immunoassay sensitivity, not epitope recognition. We only address the detection of HBsAg mutants in clinical samples that appear to be homogenous and therefore specifically challenge immunoassay epitope recognition. Replication-defective mutants, intracellular accumulation of normally secreted antigens, and tissue localization can also affect mutant detection in clinical samples.

Surface Antigen Structure

The translational products of the surface antigen gene consist of 3 proteins that have different initiation sites with the same termination site. The most important of these proteins, from a diagnostic standpoint, is the small HBsAg (sHBsAg) protein, which is composed of 226 amino acids (aa). sHBsAg is the major structural protein of the hepatitis B viral envelope. Most HBsAg in the plasma of HBV-infected persons consists of 22-nm spherical particles composed of ≈100 HBsAg monomers each (*8*). Initial studies noted that HBsAg has a complex structure with discontinuous epitopes. The possibility of multiple antigenic conformations or intermolecular epitopes cannot be ruled out when considering surface antigen structure. This antigenic complexity has impeded elucidation of HBsAg structure.

The HBsAg amino acid sequence contains a highly conformational, hydrophilic domain from positions 100 to 160 referred to as the "a" determinant. The "a" determinant represents the immunodominant region of HBsAg. The reagents used in many HBsAg diagnostic assays are directed against epitopes in the "a" determinant. The "a" determinant conformational epitopes are stabilized by a backbone of conserved disulfide-bonded cysteine residues. Alteration of residues in the "a" determinant can result in reduced antigenicity and reduced levels of protein expression (*9*). Using a combination of conformational peptides (*10*) and phage display experiments (*11*), we constructed a working model of the "a" determinant (Figure). The key features of this model include a large laminar loop stabilized by bonding between cysteine residues 108–138 with a fingerlike projection stabilized by disulfide-bonded 121–124 cysteine residues. While other cysteine residues affect antigenicity when mutated, a double mutation of these 121–124 cysteine residues has physical properties similar to those of wild-type virus (*12*). These data indicate that the fingerlike projection at aa 121–124 forms an epitope that is relatively isolated from other substitutions in the "a" determinant. The model also includes a second loop, which projects from the viral membrane and is stabilized by bonding between cysteine pairs 136–149 and 139–147. The human immune response to HBsAg is primarily directed against disulfide-bonded conformational epitopes of the "a" determinant and can be classified into a limited number of epitopes (*13–15*). Alteration of these conformational epitopes not only can result in failure to neutralize viral infection but also can affect diagnostic assay detection, depending on the epitopes recognized by the assay reagent configuration.

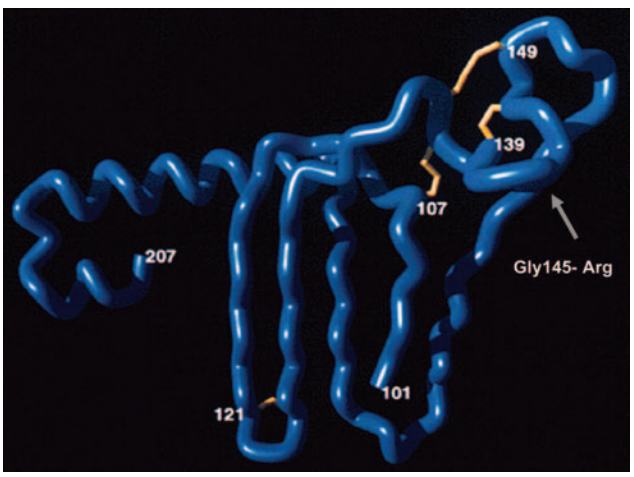

Figure. Gly/Arg 145 mutant in the projecting amino acid 139–147 antigenic loop of the "a" determinant. This mutant produces false-negative results in some commercial assays. Image courtesy of Y. C. Chen et al. (*11*).

Surface Antigen Mutants

The initial description of an HBsAg mutant was made in the breakthrough infection of a child born to a HBV-positive mother (*16*). The virus was vertically transmitted despite the child's being vaccinated and passively immunized against HBV. The breakthrough viral strain was DNA sequenced and shown to contain a substitution mutation of glycine to arginine at HBsAg aa position 145 (Gly/Arg 145) (*17*). The child subsequently remained both DNA- and HBsAg-positive for this Gly/Arg 145 mutant for >12 years, despite having protective antibody to surface antigen (anti-HBs) titer against the wild-type virus. The Gly/Arg 145 substitution alters the projecting loop (aa 139–147) of the "a" determinant such that neutralizing antibody induced by vaccination no longer recognizes the mutated epitope, hence the term vaccine-escape mutant. Wild-type HBsAg is reduced to undetectable levels in these patient samples. For the vaccine-escape mutant to emerge, the patient's anti-HBs response must be localized to the aa 139–147 region; the Gly/Arg 145 substitution thus confers a selective advantage in viral replication, and the mutant becomes the dominant form of the virus (*18*). The replication of Gly/Arg 145 mutants has been investigated with chimpanzee infection models. In the first study, a wild-type HBV infection developed in chimpanzees inoculated with a human sample of Gly/Arg 145 HBV; only samples diluted $\geq 10^{-6}$ established mutant infection (*19*). Since the pol gene ORF partially overlaps the S gene, the Gly/Arg 145 mutation in the S gene sequence corresponds to a Trp/Gln 153 mutation in the pol gene sequence, which results in the expression of an altered polymerase gene product. This altered polymerase is replication competent but has reduced replication efficiency (*6*). When anti-HBs selection pressure is removed, wild-type HBV returns as the predominant infectious form because of the impeded replication of the Gly/Arg 145 mutant. These facts may explain why transmission studies have failed to show mutant transmission to vaccinated animals (*20*). If the recipient animal had an anti-HBs response directed against an epitope outside the aa 139–147 region, the mutant inoculum would be neutralized by anti-HBs binding to epitopes unaffected by the Gly/Arg 145 escape mutation. In this case, no HBV infection would be established. Since the emergence of the Gly/Arg 145 mutant is constrained by requiring the host antibody response to be directed solely against the aa 139–147 region, whether the Gly/Arg 145 mutant will become the predominant infectious form of HBV in the future, as some models have predicted (*21*), is questionable.

The Gly/Arg 145 substitution remains by far the predominant HBsAg mutant described in the literature (*22*). However, a wide range of mutants have been described in the past 10 years, including many amino acid substitution mutants across the "a" determinant (*23*), amino acid insertions into the "a" determinant (*24*,*25*), and deletion mutants (*7*,*26*). Some of these substitution mutants appear to be of academic interest as they occur at very low levels in long-term HBV carriers and have only been identified by highly directed DNA amplification techniques that used primers specific for mutant sequence detection. The conditions for performing highly amplified PCRs must include controls to ensure that any sequence changes found are not an artifact of PCR fidelity itself (*27*). Some HBV isolates found in screening studies may be infrequently occurring natural variants (*28*). Given the diversity of HBV genotypes, the categorization of a novel HBsAg amino acid change as a mutant should hinge on a tangible alteration in viral function, such as antigenicity, infectivity, replication, and morphology, which is attributable to the specific change. One method for establishing a mutant is to introduce the suspected amino acid change into a wild-type backbone sequence and demonstrate altered function.

Important to the healthcare management of HBV infection is detection of HBsAg mutants by diagnostic assays. HBsAg is a sentinel marker in blood bank donor screening to prevent transmission of HBV infection in patients receiving transfusions. A diagnostic assay used for HBV screening may show false-negative results if the assay configuration cannot detect mutants in the "a" determinant. Initial reactivity data on 9 HBsAg assay configurations determined for 28 defined and quantitated HBsAg recombinant mutant antigens (*29*) have been confirmed by several groups. In these studies, recombinant HBsAg antigens containing a single amino acid substitution in an otherwise wild-type sequence were tested for immunoassay reactivity. Since the level of protein expression varies greatly for each recombinant HBsAg mutation, diluting each recombinant mutant protein to a known concentration before immunoassay testing was important. By setting the concentration of each recombinant mutant sample well above the antigen endpoint detection of the assays tested, the possibility of false-negative results caused by assay sensitivity was eliminated. Therefore, false-negative results were due to failure to detect the mutated epitopes of the recombinant antigen. Recombinant HBsAg that represents common mutants found in neonatal breakthrough infections was tested with different immunoassay formats (Table 1). Substitution mutants in the projecting loop of the aa 139–147 region were not detected by some commercial assays. Later generation HBsAg assays have enhanced reagent configurations that allow them to detect not only the common HBsAg mutants but also the rare mutations that occur in the aa 121–124 region such as the Arg + Ala 123 insertion mutant (*29*). This mutant produces a 228-aa surface antigen (instead of the wild-type 226 aa antigen) with gross alteration of "a" determinant epitopes. This

Table 1. Detection of most common hepatitis B surface antigen (HBsAg) mutants by 9 commercial assays (*29*)*†

Assay configuration	Ausria poly/poly	Auszyme mono/mono	IMx HBsAg mono/poly	AxSYM HBsAg mono/poly	PRISM HBsAg mono/poly	ARCHITECT HBsAg mono/poly	Commercial assay A mono/mono	Commercial assay B mono/mono	Commercial assay C poly/mono
HBsAg mutants									
Wildtype	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
Thr126- Ser	+ +	+	+	+ +	+ +	+ +	+	+ +	+ +
Gln129- His	+ +	+	+	+ +	+ +	+ +	+	+ +	+ +
Met133- Leu	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
Asp144- Ala	+ +	+ +	+ +	+ +	+ +	+ +	–	+ +	+ +
Gly145- Arg	+ +	+ +	+ +	+ +	+ +	+ +	–	–	–
Thr126- Ser + Gly145- Arg	+ +	+ +	+	+ +	+ +	+ +	–	–	–
Pro142- Leu + Gly145- Arg	+ +	+ +	+ +	+ +	+ +	+ +	–	–	–
Pro142- Ser + Gly145- Arg	+ +	+ +	+ +	+ +	+ +	+ +	–	–	–
Asp144- Ala + Gly145- Arg	+ +	+ +	+ +	+ +	+ +	+	–	–	–

*All positive samples confirmed in their respective assays.
†+ +, equivalent detection to wild-type antigen; +, detection less than wild-type antigen; –, not detected.

mutant is one of the most challenging to detect by an immunoassay format. In addition to the recombinant antigens, 3 corresponding patient samples containing the native HBsAg mutants were also available for testing. The data indicated that the immunoreactivity of both the recombinant antigen and the original patient sample were the same. No wild-type antigen was detectable in the original patient samples. Not quantitating recombinant HBsAg mutant antigens before immunoassay evaluation can account for some conflicting immunoassay detection results published in subsequent studies (*30*). Moerman et al. (*31*) have recently published an expanded selection of immunoassays and their detection of the more common HBsAg mutants (Table 2). Four commercially available assays were tested with both recombinant antigens containing defined mutations within the "a" determinant (samples 1–10) and with actual serum samples containing HBsAg mutants (samples 11–14). Several assays detected all of the mutant panel members, while others failed to detect ≥1 panel member. The detection of recombinant antigens paralleled the detection of patient serum samples. Furthermore, only mutant HBsAg appears in the false-negative clinical samples, as wild-type antigen would have been detected by the corresponding assays if present at sufficient levels. Other investigators have also confirmed the findings that some immunoassays are susceptible to the common "a" determinant mutants and produce false-negative results (*32*).

Case reports of false-negative diagnostic results due to HBsAg mutants have been described in blood bank (*33*) and hospital settings (*34*). The blood bank sample is of special importance since this patient sample (containing a Thr/Leu 143 mutant) was reported as HBsAg positive by 1 screening immunoassay, while a second screening immunoassay reported the same sample as false-negative. The Thr/Leu 143 mutant may be more prevalent than originally thought, as another occurrence has been recently reported in Europe (*35*). Screening efforts should be undertaken to establish the prevalence of this apparently emerging mutant and to establish its mechanism of selection.

In most cases, investigators reporting false-negative results due to HBsAg mutants recommend that laboratory users of HBsAg assays be aware of a given assay's ability to detect mutants. An expert advisory meeting has recently issued a consensus report on emerging HBsAg mutants (*36*). The meeting participants concluded that the prevalence of HBsAg mutants is probably higher than previously believed. The participants called for enhanced surveillance efforts and data collection for mutants and recommended using assays that detect the most frequently observed mutants at aa positions 139–145. In addition, users should develop an appropriate testing and confirmatory algorithm to ensure mutant detection. The prevalence of HBsAg mutants can be established in laboratories that perform sequential testing of a sample using 2 assays, each with differing susceptibility to mutant false-negative results. Discordant positive samples would be PCR amplified and sequenced to determine if a mutant sequence is present. In a study in Singapore, the Gly/Arg 145 mutation was present alone or in combination with other mutations in 70% of the isolated HBsAg mutants from neonatal breakthrough infections, for an overall mutant prevalence of 4.6% in this population (*37*). A screening program for school-age children in Taiwan found 27/3,849 patient samples with "a" determinant mutants for a prevalence of 0.7% (*38*). In India, testing of an HBV chronic carrier's household contacts found what might be the first documented case of Gly/Arg 145 horizontal transmission (*39*).

Table 2. Detection of hepatitis B surface antigen (HBsAg) mutants by 4 commercial assays (*30*)

Capture/detection	Abbott AxSYM HBsAg mono/poly	Bayer Centaur HBsAg mono/mono	Ortho Vitros ECi HBsAg mono/mono	Roche Elecsys HBsAg mono/mono
Recombinant samples	(s/co)	(s/co)	(s/co)	(s/co)
Wildtype	5.45	15.17	15.95	9.77
Thr126- Ser	4.74	20.17	12.50	8.64
Gln129- His	4.48	20.12	13.85	8.61
Met133- Leu	4.84	12.72	12.15	8.58
Asp144- Ala	3.65	6.47	0.12	6.55
Gly145- Arg	3.85	<0.10	0.06	0.56
Thr126- Ser + Gly145- Arg	3.36	<0.10	0.05	0.51
Pro142- Leu + Gly145- Arg	3.77	<0.10	0.05	0.55
Pro142- Ser + Gly145- Arg	4.08	<0.10	0.06	0.54
Asp144- Ala + Gly145- Arg	3.62	<0.10	0.06	0.52
Clinical samples				
Gly145 - Arg	5.85	<0.10	0.40	0.70
Pro120-Gln/Thr-131Lys/Gly145-Arg	2.48	<0.10	0.14	0.62
Thr118-Val/Met133-Ile/Phe134-Asn/Pro142-Ser/ Thr143-Leu/Gly145-Arg	17.60	<0.10	0.11	0.67
Thr115-Asn/Pro120-Leu/Met133-Ile/Phe134-His/ Asp144-Val/Ser154-Pro	2.73	<0.10	0.10	0.58

Therefore, the Gly/Arg 145 mutant occurs at a significant rate in some populations and appears to be horizontally transmissible, which suggests that HBV surveillance programs should use diagnostic methods capable of detecting this mutant.

In contrast, substitutions at positions outside of the "a" determinant appear to be readily detected by current commercially available HBsAg immunoassays. For example, mutations near the carboxy terminus of the small HBsAg protein occur when polymerase mutations are selected for in the YMDD reverse transcriptase domain (again well outside the "a" determinant). Of greater interest are the secondary compensatory changes emerging in polymerase mutants (*6*). These "polymerase stabilizing" mutations are expressed in HBsAg close to or in the "a" determinant and reduce HBsAg immunoreactivity (*40*). The risk of a "stabilized" polymerase mutant with altered HBsAg epitopes (presumably from a patient on long-term nucleoside analog treatment) being transmitted to a compatible recipient is a key issue for diagnosticians to monitor in the future. These mutants would potentially produce false-negative test results in susceptible HBsAg immunoassays and yet have the capacity to replicate in a manner similar to that of wild-type virus. Reporting mutant occurrence at the national level by using data-tracking to monitor regional exposure would mitigate such a risk.

These studies of recombinant surface antigen mutants underscore the usefulness of mapping the epitope susceptibility of various commercially available HBsAg assays. While testing of mutant panels is voluntary in some countries, certain regulatory agencies are becoming increasingly aware of HBsAg mutants. In the United States, manufacturers of new HBsAg assays must address mutant detection in their package inserts. With a firm understanding of immunoassay mutant detection, the diagnostician can select the appropriate HBsAg screening algorithm to minimize the impact of mutants in sentinel screening programs.

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Detecting Emerging Diseases in Farm Animals through Clinical Observations

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Predicting emerging diseases is among the most difficult challenges facing researchers and health managers. We present available approaches and tools to detect emerging diseases in animals based on clinical observations of farm animals by veterinarians. Three information systems are described and discussed: Veterinary Practitioner Aided Disease Surveillance in New Zealand, the Rapid Syndrome Validation Project—Animal in the United States, and "émergences" in France. These systems are based on syndromic surveillance with the notification of every case or of specific clinical syndromes or on the notification of atypical clinical cases. Data are entered by field veterinarians into forms available through Internet-accessible devices. Beyond challenges of implementing new information systems, minimizing economic and health effects from emerging diseases in animals requires strong synergies across a group of field partners, in research, and in international animal and public health customs and practices.

After the discovery of antimicrobial drugs, the increased knowledge in pathogenesis, and the improvement of health management, infectious diseases were thought to be a concern restricted to the application of known control measures. However, the dramatic spread of highly pathogenic diseases such as AIDS and multidrug-resistant bacterial infections led the scientific community to seriously examine emerging infectious diseases (*1*). Additionally, most of the emerging issues for humans are zoonotic (*2*) (e.g., avian influenza, bovine spongiform encephalopathy [BSE], severe acute respiratory syndrome [SARS], West Nile virus fever). Consequently, emerging diseases are now being addressed in domestic animals and wildlife with greater interest (*3*).

Emerging diseases in animals, especially farm animals, involve economic losses through direct (deaths, culls, movement restriction, laboratory tests) and indirect (decreased consumption of animal products, tourism decline) costs. For example, the cost of the BSE epidemic in the United Kingdom has been high, both for control measures and through lost trade, >£740 million in 1997 alone (http://www.defra.gov.uk/animalh/bse/general/qa/section9.html, accessed 9 May 2005). In addition, BSE has been implicated in the deaths of 150 persons in the United Kingdom to date (http://www.cjd.ed.ac.uk/figures.htm, accessed 9 May 2005). In 1997 and 2004, outbreaks of avian influenza A (H5N1) in Asia, with transmission to humans, led to massive destruction of poultry to avert a pandemic (*4*).

Because diseases will continue to emerge, the potential unexpected or atypical features of future health problems makes surveillance particularly challenging (*5*). No single data source captures all the information required for surveillance. Early clinical detection is one of the cornerstones (*6*) regarding unexpected diseases insofar as the surveillance activities of the veterinarians can be focused and systematized. This article presents approaches and tools focused on detecting potentially emerging diseases in farm animals through 3 information systems being tested in New Zealand, the United States, and France.

Approaches To Detect Clinical Emerging Issues

Most surveillance programs deal with a restricted set of known diseases that fail to address the challenges of looking for the unknown. However, in the United States, many new human infectious diseases have been recognized by examining illnesses without identified cause (*7*).

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Furthermore, in Great Britain, the unusual neurologic clinical signs in cattle forewarned of a new disease, BSE (*8*). Developing the ability to detect atypical syndromes in a timely fashion is critical to reducing the impact of disease emergence.

Programs targeted to detect atypical animal diseases follow 2 approaches. The first approach, syndromic surveillance, monitors disease trends by grouping clinical diseases into syndromes on the basis of clinical features rather than specific diagnoses (*9*). Even though syndromic surveillance systems seek to minimize the amount of data collected from each case, their main drawback is the heavy reporting load and requirement for disciplined reporting of recognized case data.

The second approach focuses on detecting individual atypical cases. Based on how previous emerging diseases have been detected (Table 1), atypical cases can arise from a new disease that shows clinical signs the clinician cannot link to a known disease. Alternatively, they arise from a known disease expressed atypically through unusual clinical signs, atypical region or species, or increased severity. An atypical case can also result from the detection of a rare or inadequately documented sporadic disease. Detection focused on atypical cases requires a lighter reporting load than syndromic surveillance, but the practitioner response is likely to be variable and require regular prompting.

Information Systems To Analyze Clinical Data from Farm Animals

Advances in information technology have allowed novel uses of Web and pocket personal computer applications, which provide speed, efficiency, interactivity, and security. In 1997 in Colorado, veterinarians provided information regarding unusual clinical events through the Internet (*22*); however, the program was discontinued because of poor user response. Subsequent approaches and tools to clinically detect potential emerging diseases in farm animals are presented here through 3 prototype information systems: the Veterinary Practitioner Aided Disease Surveillance System (VetPAD, New-Zealand) (*23*), which is in its third year with 7 pilot veterinarians; the Rapid Syndrome Validation Project—Animal (RSVP-A, USA) (*24*), which has been piloted among 17 veterinarians in Kansas since 2003 and 10 veterinarians in New Mexico since 2005; and the "émergences" system (available from http://www.inra.fr/maladies-emergentes) (*25*), which was

Table 1. Examples of emerging diseases and how they were detected and identified in farm animals in the last 20 years

Emerging disease (etiology)	Species	Location, date	Detection keys at time of emergence	Ref.
Blue tongue (*Reoviridae*)	Sheep	Mediterranean basin, 1998–2001	Disease normally occurring south of the Mediterranean basin	(*10*)
Border (*Flaviviridae*)	Sheep	France, 1994	Unusual death rates and clinical signs for the region: abortion, nervous signs, hydrocephalus	(*11*)
Bovine leukocyte adhesion deficiency (CD 18 gene mutation)	Holstein cattle	Different countries, 1980s	Unusual death rates in calves with recurrent infections	(*12*)
Bovine spongiform encephalopathy (prion)	Cattle	Great Britain, 1980	Unusual clinical and pathologic signs in the species: progressive neurologic disorders, gray matter vacuolation and scrapie associated fibrils	(*8*)
Complex vertebral malformation (SLC35A3 gene mutation)	Dairy cattle	Denmark, 2000	Unknown lethal congenital defect	(*13*)
Epizootic rabbit enteropathy (unidentified virus)	Rabbits	Europe, 1996	Unknown disease: serious enteritis, highly contagious, often fatal	(*14*)
Hendra virus disease (Paramyxovirus)	Horses, humans	Australia, Papua New Guinea, 1994	Sudden outbreak of acute respiratory syndrome in horses	(*15*)
Highly pathogenic avian influenza (H5N1 virus)	Poultry, humans	Southeast Asian countries, 2003–2004	Outbreak of highly pathogenic avian influenza in poultry	(*16*)
Nipah virus disease (Paramyxovirus)	Swine, humans	Malaysia and Singapore, 1998	Outbreak of unknown highly contagious disease in pigs: acute fever, respiratory signs, neurologic signs; encephalitis in humans	(*17*)
Porcine dermatitis and nephropathy syndrome (suspected porcine circovirus 2)	Swine	United Kingdom, 1993	Unusual clinical signs: unusual skin lesions in patches and plaques	(*18*)
Porcine reproductive and respiratory syndrome (*Arteriviridae*)	Swine	North America, 1987	Unusual association of: swine infertility, respiratory problems, abortion, and cyanotic ears	(*19*)
Post-weaning multisystemic wasting syndrome (suspected porcine circovirus 2)	Swine	Canada, 1990	Unusual association of: wasting, dyspnea, enlarged lymph nodes, diarrhea, pallor, and jaundice	(*18*)
Rabbit hemorrhagic disease (*Caliciviridae*)	Rabbits	China, 1984	Unusual high death rate and hemorrhage	(*20*)
West Nile fever (*Flaviviridae*)	Humans, crows	United States, 1999	Unusual cluster of human encephalitis, extensive death rate in crows, deaths of exotic birds in a zoo	(*21*)

pilot tested with 12 veterinarians in 2003 and has been pilot tested with 30 veterinarians since September 2005 (Table 2). All systems are being tested in cattle because veterinary practitioners have high rates of on-farm contact with bovine herds.

Data Capture and Strategies

All 3 systems work from the premise that practicing veterinarians hold key animal health information, which could improve means for early detection of emerging disease if aggregated efficiently through advanced information technology. While all systems capture basic epidemiologic data, they each represent a different approach to emerging disease surveillance.

VetPAD has a syndromic surveillance approach. It can include all farm animals. It collects data describing every case. Cases are categorized by using dropdown lists, check boxes, and a clinical diagnosis. Based on the categorizations, cases can be flexibly aggregated for syndromic surveillance. The strategy to minimize the surveillance reporting impact is to provide a tool capturing the ordinary business data veterinarians must manage anyway (medical records, inventory, and accounts). Surveillance data are a subset of these other data.

The RSVP-A system employs an aggregation-based syndromic surveillance but focuses on a restricted set of syndromes (nonneonatal diarrhea, neurologic dysfunction or recumbency, abortion or birth defect, unexpected death, erosive or ulcerative lesions, and unexplained feed refusal or weight loss). These syndromes are defined to cover clinical signs of emerging disease other than the common production problems on which most livestock enterprises are focused. Practitioners determine the specific syndrome each case best fits and record demographic data about the diseased animals. The RSVP-A system also requests additional clinical observations potentially useful to further characterize incident patterns. The strategy to minimize the reporting impact is to focus on less common clinical syndromes and to make data capture for each case require ≤1 minute.

"émergences" has a different approach as it targets atypical cases and specific diseases, which correspond to

Table 2. Comparison of 3 information systems to analyze animal disease through clinical observations*

	VetPAD	RSVP-A	"émergences"
General information			
Country of origin	New Zealand	United States	France (available in French, Spanish, English)
Species targeted/ where applied	Farm animals/dairy cattle	Cattle/cattle	Domestic animals/cattle
Means of recording	Pocket PC	Palm device, PC with Internet, wireless microbrowser	PC with Internet, cell phone
Pilot tests	7 veterinarians in New Zealand, 2004–2005	1) 17 veterinarians in Kansas, 2003–06; 2) 10 veterinarians in New Mexico, 2005–2006	1) 12 veterinarians in France, 2003; 2) 30 veterinarians in 2 French counties, 2005–2007
Record			
Type of clinical data	Syndromic surveillance: all clinical cases	Syndromic surveillance: 6 syndromes (see text)	Atypical syndromes and customized targeted diseases, record of the absence of cases
Main epidemiologic data	Farm localization and ownership, number affected, dead, and at risk	Type of farm, production stage, localization, number affected, dead, and at risk	Type of farm, production stage, localization, contact with other animals, number affected, dead, and at risk
Main data related to the disease	Clinical syndrome/specific clinical diagnosis	Type of syndrome, some additional clinical observation	Reasons for notification, main clinical characteristics
Type of data field	Pick-up lists, check boxes, free text fields	Pick-up lists, check boxes, free text fields	Pick-up lists, check boxes, free text fields
Other record	Photos		Photos, epidemiologic questionnaires
Output			
Related to epidemiologic surveillance	Analysis and reporting at the practitioner, regional, and national levels	Incident pattern reports from coverage areas defined by practitioners, maps	Practice statistics, statistics with all reported cases, access to all reports
Other outputs	Visit management, list of remedies, printouts for clients (wireless technology)		
Further technical developments			
	GPS capability, linkage of clinical to laboratory diagnosis, barcode scanning	GPS capability	Implementation of anatomo-pathology and laboratory analyses

*VetPAD, Veterinary Practitioner Aided Disease; RSVP-A, Rapid Syndrome Validation Project – Animal; "émergences," information system in France; PC, personal computer; GPS, global positioning system.

known diseases hypothesized to be emerging. Forms are available (see an example of atypical case form, Figure) for reporting epidemiologic and clinical data. The system requests a follow-up description of each case's evolution and monthly confirmations of vigilance from veterinarians reporting no cases. Moreover, atypical cases can be categorized by the system administrator according to clinical description similarities to facilitate exploration of their potential links. The system has generic features, making it available for any country, any disease, and any domestic species. Description of atypical cases for "émergences" is a less frequent and more open process than the syndromic surveillance methods.

In all these systems, routine data recording is simplified by the use of pick-up lists. However, free text fields are also available, as the unexpected often does not fit in predefined fields. VetPAD and RSVP-A use mobile telephones or personal data assistants for data capture. "émergences" primarily uses the Internet.

Output and Statistics

A successful surveillance system must be able to keep veterinarians engaged and continuing to submit data after the novelty of the new system wears off. Systems can provide value to a veterinarian with useful management tools, which are available in VetPAD, and by enhancing their clinical expertise and intellectual curiosity. To trigger interactions and learning from participants' experiences, practitioners participating in "émergences" have access to all case descriptions. In addition, illness and death rates are available in real time either at the clientele level ("émergences") or at a custom-made level ("émergences," RSVP-A). In VetPAD, customized reports are available to involved parties.

One output of these surveillance systems is an indication of unusual events that require additional investigation. This investigation might include communication with other veterinarians to find additional cases, targeted epidemiologic studies, research projects, or control programs.

Other outputs are data upon which analyses can be conducted. A challenge is the categorization of reports to identify possible etiologic links. Procedures based on contextual analysis must be developed to analyze pick-up list data as well as free text (*26*). Each system must also address the challenge of detecting increased incidence of a rare event. Two types of situations can be considered. The first is the emergence from a "zero case" situation (e.g., BSE occurred probably as erratic cases before its amplification [*27*]). Incidence threshold analysis needed for this situation requires methods such as the evaluation of record process (*28*). Moreover, the constructed statistics should be robust with a small number of cases and allow differentiation of sporadic cases from emergence (*29*). The second

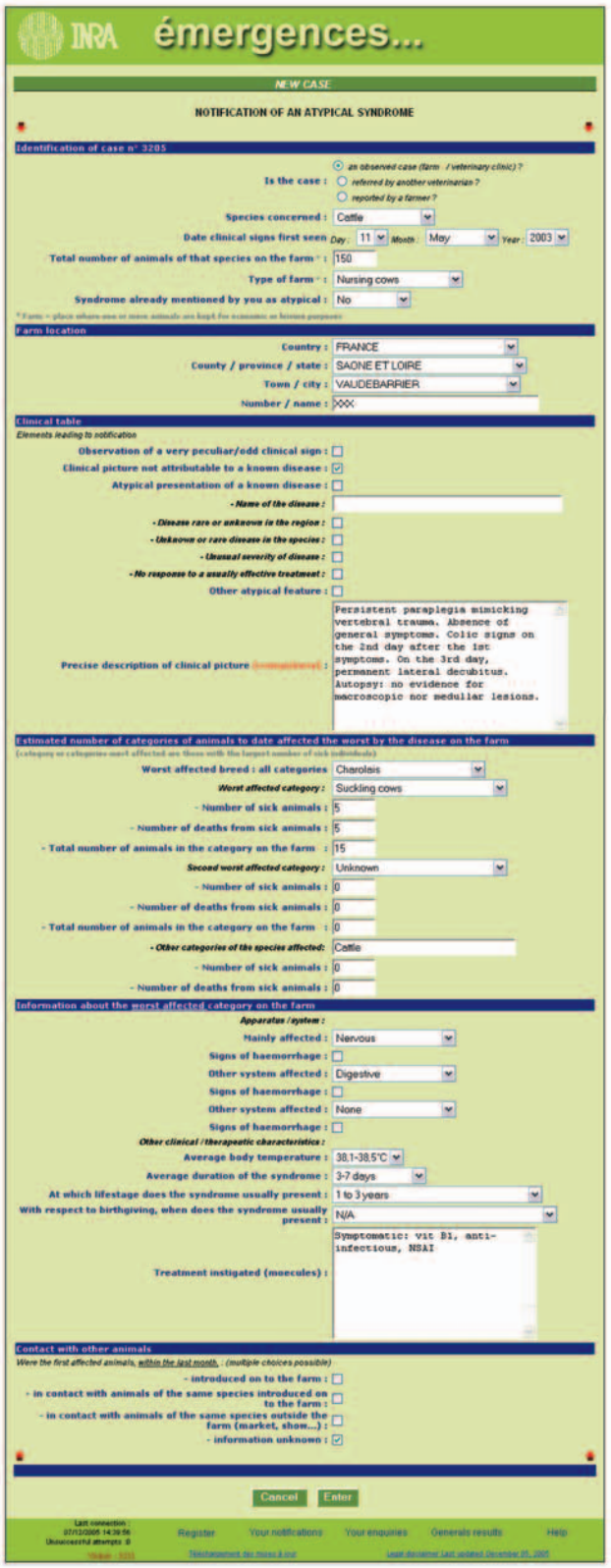

Figure. Sample of online form reporting epidemiologic and clinical data.

situation is the emergence of clusters of highly pathogenic variants of an endemic disease. Spatial-temporal analysis can provide helpful insights concerning baseline patterns of clinical syndromes and aberrations from them, which can trigger further investigation.

Limitations and Evaluation of Systems Based on Clinical Observation

Limitations

Atypical case detection is limited by practitioners' experience, knowledge, vigilance, and willingness to report findings (*30*). Multiple, similar reports of atypical cases improve confidence that a new disease is emerging. Making case data available through surveillance systems, such as the 3 we have indicated, will also foster basic common knowledge and shared practical experience among veterinarians. Because surveillance for the unknown requires a mindset different from surveillance of the known, notification quality and vigilance should be enhanced by specific training courses (*31*).

A substantial limitation of syndromic surveillance is the need to establish baseline levels for defined syndromes. This step requires time and resources; however, without them, we cannot know when the incidence of a syndrome has significantly increased. VetPAD and RSVP-A are developing such baselines.

Economic consideration leaves few alternatives to clinical detection of farm animal diseases. Laboratory analyses are infrequently performed and generally more basic compared to human medicine (*32*). However, slaughterhouses and other assembly points do provide surveillance opportunities.

Finally, a clinical reporting tool alone is only the first step to determine if the cases share an etiologic pathway. Review by expert clinicians, necropsy findings, immunologic screenings, and focused epidemiologic studies play key roles in such determination (*33*). Similarities between distinct submitted atypical cases provide additional evidence. For example, BSE was identified as a novel syndrome through epidemiologic, clinical, and pathologic findings (*8*).

Evaluation

To determine whether to extend an information system, several points must be reviewed. First, the activity and number of participating veterinarians can be evaluated by quantifying indicators such as number of entries submitted, number of atypical cases entered, and participants' levels of accessing posted results. Moreover, all systems include reference diseases or symptoms for which descriptive statistics are available, which can serve to check quality recording (e.g., babesiosis in the "émergences" pilot study). In addition, the likelihood of detecting an emerging event is high. Many rare diseases are not defined in cattle, so a dedicated information system should detect $\geq$1 unexpected event over the test period. For example, the initial "émergences" pilot found 3 sets of clinical signs not linked to a known disease (persistent, ultimately fatal paraplegia, without general clinical signs [Figure]; weight loss, depilation at the extremities leading to death; and congenital cataract neither linked to bovine virus diarrhea nor familial history) and 1 rare known syndrome (facial eczema). Finally, the decision to extend a detection system will depend largely on the interest veterinarians hold and on the inclusion of new diseases as a national surveillance objective (*6*,*34*).

Other Systems To Capture Clinical Data

We have presented examples of clinical data capture from cattle herds at the veterinary level, in which sufficient individual health data are available. For species concerned by herd health approaches (sheep, poultry), initiatives have been taken for information systems through online questionnaires answered by farmers (*35*). In 1 such system, New Zealand producers must complete questionnaires targeted on diseases that occurred in the previous 12 months and have clinical signs similar to exotic diseases. The ultimate research goal is to develop a disease sentinel Web module to integrate with veterinary practice Web sites. The main problem is the disparity in response quality between farmers.

The reality of an emergence can be tested by survey of a set of representative herds. In the United States, the National Animal Health Monitoring System is not designed to collect information regarding emerging diseases per se; however, questions about a previously identified emerging disease have been inserted into surveys. In addition, the National Animal Health Monitoring System has provided baseline data on emerging disease analysis and assessment. In France, the Central Service for Survey and Statistical Studies, which runs economic surveys among a representative national sample of herds, has added specific questions regarding animal health issues (*36*).

In addition to farm animals, pets, zoo animals, and wildlife must be considered as sources of transmission and reservoirs for emerging diseases. For pets and zoo animals, tools similar to the ones proposed can be adapted because these animals are regularly seen by veterinarians. Wildlife can be a source of new farm animal or human diseases and is affected by many farm animal diseases (Table 1). Thus, all observations of health problems in wildlife can potentially contribute relevant information for human or domestic animal populations (*37*). However, the ability to closely monitor clinical signs is lacking. Death rate is the most feasible way to monitor wildlife health and has indeed

been the detection trigger of many emerging diseases (*38*). Testing sampled healthy animals for a set of diseases is another strategy, but few disease surveillance programs not targeted at specific diseases are in place (e.g., "marine mammal strandings" project in United Kingdom [*39*]). One of the key challenges remains to bring professional and amateur outdoorsmen to report wildlife health observations through an information system flexible enough to encompass all species and situations. New forms dedicated to wildlife with appropriate location (instead of client or farm) could be added to the information systems already adapted to several species (VetPAD and "émergences"). Alternatives such as monitoring risk factors for emergence (e.g., encroachment of habitats), as well as minimizing contact between domestic and wild species by good, on-farm biosecurity, could reduce the likelihood of new domestic animal or human diseases emerging from wildlife reservoirs. In all cases, approaches must seek to increase collaboration among wildlife and domestic animals health workers to break down traditional boundaries between fields.

Conclusion and Interest for Human Health

Much effort is being put into developing new tools to detect emerging diseases through veterinary practitioners. If successful, this effort will also define the "normal" clinical baseline for syndromes and rare diseases, allowing statistical confirmation that an atypical syndrome is emerging. In addition to building new information technologies, early disease identification with timely responses requires synergy across a group of partners, including those who traditionally interact in animal health management as well as in public health (*40*) and across geopolitical boundaries. Although human and animal worlds remain fairly separated, initiatives are narrowing this separation. For instance, integration of emerging animal disease surveillance systems with those in the human arena is proposed in the UK's "RADAR" veterinary surveillance information management system (*41*). Furthermore, during the "émergences" test phase, the Health National Institute agreed to cooperate in the event an animal issue with potential public health implications was identified. Finally, the most relevant challenge is to promote joint human-animal projects concerning potentially common emerging diseases, such as the avian-porcine-human influenza complex. Effective combination of such emerging disease surveillance systems would result in earlier identification of potential issues, providing opportunity for quicker response.

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Systematic Review of Antimicrobial Drug Prescribing in Hospitals

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Prudent prescribing of antimicrobial drugs to hospital inpatients may reduce incidences of antimicrobial drug resistance and healthcare-associated infection. We reviewed the literature from January 1980 to November 2003 to identify rigorous evaluations of interventions to improve hospital prescribing of antimicrobial drugs. We identified 66 studies with interpretable data, of which 16 reported 20 microbiologic outcomes: gram-negative resistant bacteria, 10 studies; *Clostridium difficile*–associated diarrhea, 5 studies; vancomycin-resistant enterococci, 3 studies; and methicillin-resistant *Staphylococcus aureus*, 2 studies. Four studies provided strong evidence that the intervention changed microbial outcomes with low risk for alternative explanations, 8 studies provided less convincing evidence, and 4 studies provided no evidence. The strongest and most consistent evidence was for *C. difficile*–associated diarrhea, but we were able to analyze only the immediate impact of interventions because of nonstandardized durations of follow-up. The ability to compare results of studies could be substantially improved by standardizing methods and reporting.

Despite strenuous efforts to control antimicrobial drug use and promote optimal prescribing, practitioners continue to prescribe excessively; it is estimated that up to 50% of antimicrobial drug use in hospitals is inappropriate (*1*–*3*). Antimicrobial drug resistance is largely a consequence of the selective pressures of antimicrobial drug use. Reducing these pressures by the judicious administration of these drugs should facilitate a return of susceptible bacteria or, at least, prevent or slow the pace of the emergence of drug-resistant strains (*4*,*5*). Furthermore, *Clostridium difficile*–associated diarrhea (CDAD) is a hospital-acquired infection associated with use of antimicrobial drugs (*6*–*8*) and reducing the incidences of CDAD is an additional potential benefit of improving hospital antimicrobial drug prescribing.

Implementing and monitoring interventions to optimize prescribing of antimicrobial drugs place a burden on hospital resources and their efficacies need to be confirmed (*9*). We have conducted a systematic review of interventions to improve antimicrobial drug–prescribing practices for hospital inpatients using the methods of the Cochrane Effective Practice and Organization of Care Group to assess validity (*10*). In this study, our primary objective was to evaluate the impact of interventions on reducing the incidence of colonization with or infection caused by antimicrobial drug–resistant pathogens or CDAD. In addition to the usual threats to the validity of interventions to change health care, infection control interventions are particularly prone to regression to the mean (*11*). This refers to the natural tendency of extreme observations to return towards the average (mean) over time. An epidemic or outbreak is a sequence of unusually large number of cases of infection, so that the natural history of an epidemic is to increase, peak, and then decrease. Consequently, regression to the mean is always a threat to the validity of evaluations of unplanned interventions that are initiated in response to an outbreak.

Methods

The full protocol is available in the Cochrane Library (*10*). We searched Medline, EMBASE, the Cochrane database, and the Effective Practice and Organisation of Care specialized register for studies from January 1, 1980, to November 30, 2003, relating to prescribing of antimicrobial

drugs to hospital inpatients. Additional studies were obtained from the bibliographies of retrieved articles, the Scientific Citation Index, and personal files. We requested additional data from the authors when necessary. There were no language limitations for the literature review. We included all randomized and controlled clinical trials (RCT/CCT, designs where allocation to the intervention is determined either by an explicit random process [RCT] or by a nonrandom process such as date of birth or case note number) before and after studies (a design with contemporaneous data collection before and after the intervention and an appropriate control site or activity) and interrupted time series (ITS, a clearly defined point in time when the intervention occurred and at least 3 data points before and 3 after the intervention). Data about microbiologic outcomes were considered reliable if they met the same criteria. For example, if a paper included prescribing data that met the criteria for an ITS but provided only mean data about microbiologic outcomes before and after the intervention, then the microbiologic data were not considered reliable. Two reviewers independently extracted data and assessed the quality of each study with the standardized criteria.

Statistical Considerations

Many statistical methods can be used to analyze ITS designs (e.g., ARIMA modeling or time series regression). However, the design is often analyzed inappropriately, which makes interpretation of individual studies difficult (*12*). Methods of analyzing ITS data were examined critically (*12*). The preferred method for short time series is segmented time series regression analysis, which is a statistical comparison of time trends before and after the intervention to identify either an immediate change in the level of the regression line or a sustained change in the slope of the line (*12*,*13*). In this report, we have distinguished 2 intervention effects: immediate (a sudden change in the level of the regression line at the point of intervention) and sustained (a sustained change in the slope of the regression line from the start of the intervention phase). If the original report did not include an appropriate analysis, data were reanalyzed by using segmented time series regression.

The following model was specified: $Y_t = B_0 + B_1 \times \text{preslope} + B_2 \times \text{postslope} + B_3 \times \text{intervention} + e_t$, where Y_t is the outcome (e.g., CDAD incidence) in month *t*, preslope is a continuous variable indicating time from the start of the study up to the last point in the preintervention phase and coded constant thereafter, postslope is coded 0 to and including the first point postintervention and coded sequentially from 1 thereafter, and intervention is coded 0 for preintervention time points and 1 for postintervention time points. In this model, B_1 estimates the slope of the preintervention data, B_2 estimates the slope of the postintervention data, and B_3 estimates the change in level of outcome as the difference between the estimated first point postintervention and the extrapolated first point postintervention if the preintervention line was continued into the postintervention phase. The difference in slope is calculated by $B_2 - B_1$. The error term e_t was assumed to be first-order autoregressive. Confidence intervals (95%) were calculated for all effect measures.

Formal metaanalysis of results was not attempted given the differences in context, setting, and type of outcomes. However, to gain an overall summary picture of the heterogeneity of effect sizes we standardized all measures so that they were all on the same scale. To do this, we divided the change in level and the change in slope by the preintervention standard deviation (SD) in each study. The resulting metric has no unit, it is known in standard metaanalysis as the standardized mean difference. Standardized effect sizes of 2 to 3 SD were considered large, whereas an effect size <0.5 SD was considered of questionable clinical significance even if statistically significant (*14*). To visually display the heterogeneity of the standardized effect sizes, graphic plots of level effects versus slope effects for each study (with associated 95% confidence intervals) were generated.

Other Criteria for Assessing Evidence

The statistical analysis assessed how likely it was that study results could simply have happened by chance, and the Cochrane quality criteria assessed common threats to the validity of interventions to change practice or organization of care. To assess other threats to the validity of infection control interventions, we used the format for reporting the results of included studies recommended by guidelines derived from a recent systematic review of isolation measures to control methicillin-resistant *Staphylococcus aureus* (MRSA) (*15*). We required studies to provide reliable data about the effect of interventions on both microbial and drug outcomes with clear case definition, description of infection control measures, and other variables such as bed occupancy or staffing levels that could provide plausible alternative explanations for changes in microbial outcomes. We have provided a summary of detailed information from the included studies (online Appendix Table, available from http://www.cdc.gov/ncidod/EID/vol12no02/05-0145.htm#apptable). Additional information is available from the British Society for Antimicrobial Chemotherapy (www.bsac.org.uk). We classified case definitions into colonization, infection or clinical isolates, or a combination of ≥2 with the following definitions.

Colonization was defined as a microorganism, usually detected by screening, at a host site (normally nonsterile, although the urine of a catheterized patient may be an

exception) without causing systemic signs of infection or a specific immune response. Colonization by case note review was established by excluding infection diagnosed according to criteria adopted by the authors or defined by appropriate bodies, e.g., the Centers for Disease Control and Prevention criteria for diagnosing nosocomial infections. Infection was established by case note review according to criteria adopted by the authors or defined by appropriate bodies or by recording specific symptoms and/or signs, such as diarrhea in patients with CDAD. Clinical isolates were defined as the recovery of a microorganism after culture of a clinical (not screening) specimen without specifying whether it represents colonization or infection.

Results

We identified 66 intervention studies to improve prescribing of antimicrobial drugs to hospital inpatients that met our inclusion criteria (*16*) and excluded 243 studies that were uncontrolled before and after studies (n = 164) or inadequate ITS studies (n = 79). Of the 66 studies, 16 reported reliable data about 20 microbiologic outcomes: gram-negative resistant bacteria (GNRB), 10 studies; CDAD, 5 studies; vancomycin-resistant enterococci (VRE), 3 studies; and MRSA, 2 studies (online Appendix Table). The setting for the intervention was the entire hospital in 8 studies (*17–24*), a single service in 2 studies (*25*,*26*), and a unit or ward in 6 studies (*27–32*). One intervention was educational with advice about changes in antimicrobial drugs (*17*); the other 15 interventions were restrictive (online Appendix Table). Two studies were RCTs (*31*,*32*) and 1 study was a CCT (*30*); the remaining 13 studies used an ITS design.

Statistical Validity

All 3 clinical trials reported appropriate statistical analysis (*30–32*), whereas only 2 of the 13 ITS studies reported appropriate statistical analysis (*17*,*27*). Of the remaining 11 ITS studies, 5 did not report statistical analysis; 6 reported inappropriate statistical analysis by using tests such as χ^2 or *t* tests that assume independence between observations and do not account for time trends. Data from these 11 studies were reanalyzed.

Effectiveness of Interventions

Overall, 4 studies provided strong evidence of control of the microbial outcome by change in prescribing (*17*,*27*,*30*,*31*). All of these studies provided reliable data about antimicrobial drug prescribing, with significant changes in both microbial and drug outcomes after planned interventions. In addition, 2 studies provided further protection against regression to the mean by using a crossover design (*27*,*30*). Three of these studies have rigorous case definitions based on prospective screening cultures plus full description of infection control measures.

Eight studies provided less convincing evidence. Two studies showed significant changes in prescribing that were associated with nonsignficant changes in CDAD (*20*,*26*). An additional 6 studies reported statistically significant improvement in microbial outcome but without reliable data about the effect of the intervention on prescribing (*18*,*19*,*23*,*24*,*28*,*29*). The importance of this omission is confirmed by the 6 studies that included reliable data about prescribing because all showed that there was some prescription of restricted drugs during the intervention phase (*17*,*20*,*26*,*27*,*30*,*31*)

Four studies had negative results (*21*,*22*,*25*,*32*). One study provided strong evidence of failure to control microbial outcomes despite a successful change in prescribing (*32*). One study reported an intervention that failed to change use of vancomycin (*22*). The remaining 2 studies showed no change in microbial outcome but did not provide reliable data about the effect of the intervention on prescribing (*21*,*25*).

CDAD

The most consistent evidence was for the 5 interventions designed to reduce the incidence of CDAD. Four were implemented throughout the hospital (*17*,*18*,*20*,*24*) and 1 was implemented in the elderly care service (*26*); all 5 targeted prescribing of cephalosporin or clindamycin. All of the interventions were associated with a change in the expected direction (Figure part A), which was a change in the incidence of CDAD in the same direction to a change in use of cephalosporin or clindamycin. For 1 intervention, the expected direction was an increase in CDAD incidence after the introduction of ceftriaxone (*20*); for all other interventions a decrease in CDAD incidence was expected to accompany a decrease in use of cephalosporin or clindamycin. These 5 studies reported 7 interventions. The immediate effect after 6 of the 7 interventions was at least 0.5 SDs; 5 of these 7 immediate effects were statistically significant (Figure part A). Sustained changes after the intervention were more modest, but all were in the expected directions and 4 of 7 were statistically significant (Figure part A.). The 5 CDAD studies had results expressed in different units: cases per month (*24*,*26*); cases per quarter (*18*,*20*); or cases per 1,000 admissions per year (*17*). Consequently, we were only able to compare effect sizes in numbers of CDAD cases per quarter by recalculating results from 2 studies (*24*,*26*). The antimicrobial drug intervention was associated with a mean immediate reduction of 15.0 CDAD cases per quarter (range 6–26) and a median sustained reduction of 3.2 CDAD cases per quarter (range 1–6).

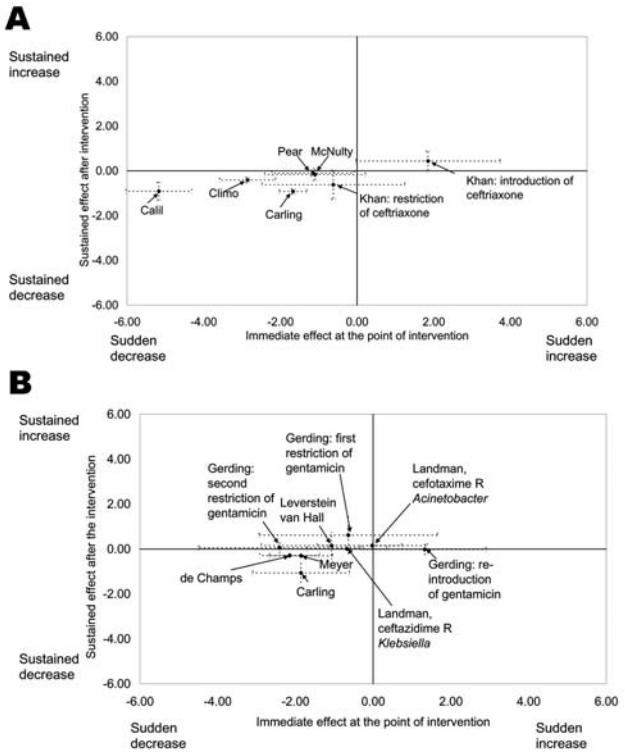

Figure. A) Standardized immediate and sustained effects for *Clostridium difficile*–associated diarrhea. B) Standardized immediate and sustained effects for resistant gram-negative bacteria.

Resistant Gram-negative Bacteria

The results of the 10 interventions designed to reduce the incidences of GNRB were less consistent. Three were implemented throughout the hospital (*19*,*21*,*23*), 1 was implemented in the neurology and neurosurgery service (*25*), and 5 were implemented in a single intensive care unit (ICU), which included 4 with pediatric patients (*28*–*30*,*32*) and 1 with adult patients (*31*). One intervention was designed to reduce the duration of treatment with any antimicrobial drug for ICU patients at low risk for pneumonia; this was associated with a significant reduction in the incidence of colonization by any GNRB and exposure to antimicrobial drugs (*31*). The remaining 9 interventions involved changes in antimicrobial drug treatment, mainly aminoglycosides or cephalosporins. One RCT provided no evidence that antimicrobial drug cycling reduced the incidence of GNRB in a neonatal ICU (*32*). The 8 ITS studies reported 9 outcomes (Figure part B). The expected direction of effect from a change in aminoglycoside or cephalosporin prescribing was usually a reduction in GNRB. For 1 intervention, the expected direction of effect was an increase in the incidence of GNRB after gentamicin was reintroduced (*19*). The expected direction for all 9 outcomes changed, but the effect size was <0.5 SD in 2 studies and not statistically significant in 5 studies (Figure part B). In 3 studies the changes in slope were in the expected direction and in 1 the changes were both statistically significant and >0.5 SD, which is likely clinically important. Unlike with CDAD data, effects cannot be expressed in a common unit. Some studies measured colonization and others examined infection. Units of measurement were also variable (e.g., number of isolates, percentage of isolates, number of cases, and number of cases per time period).

Gram-positive Bacteria

Data for gram-positive bacteria were very limited. One study provided strong evidence that restricting ceftazidime in a hematology unit was associated with significant reduction in risk for colonization by VRE (*27*). However, reduction of cephalosporin use in a hospital was not associated with any change in the prevalence of VRE isolates (*17*). A third study targeted at VRE showed that implementation of a vancomycin order form had no significant impact on vancomycin prescribing, with a trend in the unintended direction (*22*). Two studies report effects on MRSA prevalence (*17*,*21*). Our segmented regression analysis showed no significant change in response to a reduction in use of third-generation cephalosporins (online Appendix Table), although 1 of the reports claimed that a change did occur (*21*).

Discussion

Our primary conclusion is that 4 of the 16 studies provided strong evidence that changes in prescribing antimicrobial drugs to hospital inpatients can improve microbial outcomes (*17*,*27*,*30*,*31*). Eight of the remaining studies provided some evidence that antimicrobial drug–prescribing interventions can improve microbial outcomes, but flaws in their design indicated that there were plausible alternative explanations for the results (*18*–*20*,*23*, *24*,*26*,*28*,*29*). The remaining 4 studies were unequivocally negative (*21*,*22*,*25*,*32*).

Estimation of overall effect size was only possible for reduction in CDAD, where the evidence suggested that restriction of clindamycin or third-generation cephalosporins resulted in an immediate reduction in prevalence by 15 cases per quarter, with an additional sustained reduction by 3 cases per quarter. Prevalence is usually adjusted for clinical activity, e.g., cases per 1,000 admissions per quarter (*7*), but only 1 study provided this information (*17*). Furthermore, potentially important differences in the case definitions of CDAD occurred between the studies in our review.

Finding valid studies required painstaking analysis of a huge volume of literature, most of which is fundamentally flawed (*16*). The included studies could be dramatically improved by following guidelines for standardized reporting (*15*). In particular, the unequal duration of postinterven-

tion phases made it difficult to reliably compare the sustained effects of interventions, these being the most important outcome measures. The short and unequal duration of preintervention phases provides limited information about underlying preintervention trends. To understand how much of a change in prescribing is required to change outcome, the intervention must be independent of other control measures and be accompanied by reliable data about both prescribing and microbial outcomes.

Only 1 of the interventions was designed to reduce overall exposure to antimicrobial drugs (*31*). All of the other studies targeted the choice of antimicrobial drug (e.g., by restricting access to third-generation cephalosporins in favor of drugs recommended by the hospital antimicrobial drug policy) but did not aim to shorten the duration of treatment. This intervention (*31*) shortened the duration of antimicrobial drug treatment for ICU patients at low risk for ventilator-associated pneumonia. This study was conducted in an ICU with adult patients. However, the same principle of using clinical scores to identify low-risk patients, in whom antimicrobial drug therapy could be stopped, has been developed in other clinical settings (*33–35*), and the impact on microbiologic outcomes should be investigated.

None of the studies provided evidence for cost-effectiveness or clinical outcome. The study designs likely did not have sufficient power to measure these outcomes. Few studies provided data about multiple microbiologic species and 1 of these endpoints (incidence of cefotaxime-resistant *Acinetobacter* spp.) was opposite to that which was expected (*21*). Future studies should provide more data about cost and clinical outcomes. Notably, evidence is needed to show that interventions do not have adverse outcomes.

The potential for the success of antimicrobial drug interventions likely varies by organism (*36*,*37*). Antimicrobial drugs are likely to play a large role in the selection of enterobacteria expressing extended-spectrum β-lactamases, a minimal role in the selection and transmission of MRSA, and an intermediate role in VRE. However, the available evidence is not sufficient to investigate these hypotheses.

Implications for Practice

The evidence supports the theory that limiting the use of specific antimicrobial drugs will reduce the prevalences of resistant gram-negative bacteria and CDAD. For gram-positive bacteria, there is a lack of evidence rather than evidence of no effect. Hospitals would like to know how much they should limit their antimicrobial drug prescriptions and what is the minimum that will show a real effect. Unfortunately, the available evidence is too limited to provide definitive answers to these issues. Thus, hospitals must estimate the effect of their own interventions. The good news is that the data required for ITS analysis of the incidences of drug-resistant bacteria or CDAD should be readily available in most hospitals. Healthcare providers need to invest in data analysis so that evaluation of antimicrobial drug control in hospitals becomes a routine measure of the quality of care rather than a research project.

Standardized reporting of outbreaks and interventions to control the incidence of antimicrobial drug resistance or hospital-acquired infection would greatly enhance the ability to combine results from hospitals in metaanalyses. Key issues include full description of other infection control measures, consistent and reproducible case definitions, the length of preintervention and postintervention phases, and the intervals between data points (*15*). Ideally, data should be made available in a way that allows reanalysis and, where appropriate, metaanalysis. Metaanalysis of single hospital studies is no substitute for good multicenter studies, but it could be used to provide some evidence of reproducibility and thus to prioritize targets for definitive trials.

Priorities for Research

The research agenda needs to move to multicenter studies with randomized allocation to interventions. This will provide better evidence of external validity as well as the power to measure cost-effectiveness and exclude important unintended adverse clinical outcomes. Development and pilot testing of the effectiveness of clinical decisions for reducing unnecessary exposure to antimicrobial drugs should be a priority for research in hospitals.

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Bartonella quintana Characteristics and Clinical Management

Cédric Foucault,* Philippe Brouqui,* and Didier Raoult*

Bartonella quintana, a pathogen that is restricted to human hosts and louse vectors, was first characterized as the agent of trench fever. The disease was described in 1915 on the basis of natural and experimental infections in soldiers. It is now recognized as a reemerging pathogen among homeless populations in cities in the United States and Europe and is responsible for a wide spectrum of conditions, including chronic bacteremia, endocarditis, and bacillary angiomatosis. Diagnosis is based on serologic analysis, culture, and molecular biology. Recent characterization of its genome allowed the development of modern diagnosis and typing methods. Guidelines for the treatment of *B. quintana* infections are presented.

Trench fever, the first clinical manifestation attributed to *Bartonella quintana*, affected an estimated >1 million people during World War I (*1*,*2*). The name "trench fever" was mentioned for the first time in 1915 (*3*,*4*). In 1916, McNee et al. described 2 types of the disease (*5*). The first was characterized by a sudden onset of headache, dizziness, pain in the shins, and elevated temperature (39°C–40°C). Between days 3 and 7, temperature would suddenly drop to normal or subnormal. Thereafter, temperature rose sharply before falling again. The second manifestation of the disease was characterized by a shorter initial period and frequent relapses. In 1919, 200 consecutive cases were recorded by Byam et al., and transmission by human body lice was demonstrated, but the nature of the trench fever agent was still unknown (*4*).

Trench fever was precisely described based on experimental infections in volunteer soldiers (*4*). The first experiments consisted of transmitting whole blood from typical cases to volunteers, which reproduced natural infection. Byam confirmed in 1919 the others' work, showing that "rickettsia bodies" were present in lice, their excreta, and their guts when they were collected from trench fever patients. In 1949, Kostrzewski precisely described trench fever after an accidental epidemic spread among louse-feeders in laboratories that produced typhus vaccine (*6*). Of 104 persons who worked with lice, 90 contracted symptomatic trench fever, and 5 were asymptomatic carriers. Three different courses of trench fever were described by Kostrzewski: the classic relapsing form associated with shin pain, headaches, and dizziness; the typhoidal form characterized by a prolonged fever, splenomegaly, and rash; and the abortive form, characterized by a brief, less intense course.

After World War I, the incidence of trench fever decreased dramatically, but during World War II, epidemics were again reported (*6*). More recently, reports have indicated the reemergence of *B. quintana* infections among the homeless population in cities in both Europe and the United States (*7*,*8*). Major predisposing factors for new *B. quintana* infections include poor living conditions and chronic alcoholism (*8*). Epidemics of trench fever were also recently reported in particular conditions, such as in refugee camps in Burundi in 1997, where pediculosis was prevalent (*1*).

The Bacterium

Taxonomy

When trench fever was first described in 1915, its etiologic agent was called *Rickettsia quintana* or *R. volhynica* (*6*). At the same time, other names were also proposed, i.e., *R. pediculi*, *R. weigli*, and *R. rocha-limae* (*1*). The 1984 edition of Bergey's Manual of Systematic Bacteriology combines *Rickettsiaceae*, *Bartonellaceae*, and *Anaplasmataceae* families into the *Rickettsiales* order. *R. quintana* was classified in the genus *Rochalimaea*, tribe *Rickettsiae*, family *Rickettsiaceae*. In 1993, Brenner et al. proposed unifying the genera *Bartonella* and *Rochalimaea* (*9*). They also proposed removing the unified genus *Bartonella* from the *Rickettsiales* order. The new unified

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genus thus contained 5 species: *B. bacilliformis*, *B. quintana*, *B. vinsonii*, *B. henselae*, and *B. elizabethae*. In 1995, Birtles et al. proposed unifying the *Bartonella* and *Grahamella* genera (*10*), and the 2 established *Grahamella* species, *Grahamella talpae* and *G. peromysci*, were renamed as *Bartonella* species. Birtles et al. also described 3 new species within *Bartonella*: *B. grahami*, *B. taylorii*, and *B. doshiae* (*3*,*10*).

Characteristics

B. quintana is a facultative, intracellular, gram-negative rod belonging to the α2 subgroup of proteobacteria (*3*). It is a short rod, 0.3–0.5 μm wide and 1–1.7 μm long. Catalase and oxidase reactions are negative. The bacterium can be grown on axenic media and cocultivated in cell culture (*3*,*11*). When grown on blood agar, rough colonies embedded in the agar are obtained after 12 to 14 days, but prolonged incubation may be necessary, up to 45 days for primary isolation. Subcultures reduce the time to obtain colonies to only 3–5 days (*11*). Humans are the reservoir of the bacterium (*12*), and the human body louse, *Pediculus humanus corporis*, is its usual vector (*1*). *B. quintana* is located in erythrocytes during asymptomatic bacteremia (*13*) and has been observed in erythroblasts in bone marrow in bacteremic patients (*14*). The bacterium has a tropism for endothelial cells, leading to angioproliferative lesions, as observed in bacillary angiomatosis (*15*).

Genome

The 1.6-Mb genome of *B. quintana* has recently been sequenced and was found to be a derivate of the larger 1.9-Mb genome of *B. henselae*; the main difference between the species is the absence of genomic islands in *B. quintana* (*16*). Both *B. quintana* and *B. henselae* genomes are shortened versions of chromosome I from *Brucella melitensis*, a phylogenetically highly related bacterium (*16*). The comparison of *B. henselae* and *B. quintana* genome and the specialization of the latter to its human reservoir and louse vector suggest that use of host-restricted vectors is associated with accelerated rates of genome degradation (*16*).

Epidemiologic Features and Natural History

Transmission

B. quintana is transmitted by the human body louse, *P. humanus corporis* (Figure 1), which lives in clothes (Figure 2) and is associated with poverty, lack of hygiene, and cold weather. In our cohort of 930 homeless persons, lice infestation was present in 22% and was associated with hypereosinophilia (*17*). Pediculosis (lice infestation) is transmitted by contact with clothes or bedding, is prevalent in the homeless population. *B. quintana* multiplies in the louse's intestine and is transmitted to humans by feces through altered skin (*1*). Body lice usually feed 5 times a day and inject their bites with biological proteins, including an anesthetic that provokes an allergic reaction and leads to pruritus and scratching (Figure 3), which facilitates the fecal transmission of *B. quintana*, and persistent *B. quintana* bacteremia facilitates its spread by lice (*12*). Body lice are probably not the only vectors of *B. quintana*. The bacterium was recently detected in cat fleas (*18*) and in cat dental pulp (*19*), which suggests bacteremia in cats, and has been isolated in a patient who owned a cat and sought treatment for chronic adenopathy (*20*). These data suggest a transmission mode similar to that observed for *B. henselae* in cat-scratch disease.

Figure 1. *Pediculus humanus corporis*, the human body louse, viewed with electron microscope at magnification ×120.

Natural History of *B. quintana* Infection

After trench fever (which corresponds to the primary infection) resolves, chronic bacteremia will develop in some patients (*7*). Overproduction of interleukin-10 could be partially responsible for persistence of bacteremia. The link between chronic bacteremia and *B. quintana* endocarditis has not been clearly shown, but it likely exists. Chronic asymptomatic bacteremia in humans indicates that they may be the natural reservoir of *B. quintana* (*12*), as this condition occurs with other *Bartonella* species in their reservoirs, i.e., *B. henselae* in cats, *B. alsatica* in rabbits, and *B. tribocorum* in rats. However, other reservoirs should be investigated for *B. quintana* because the bacterium has been detected in cat fleas (*18*), cat teeth (*19*), and

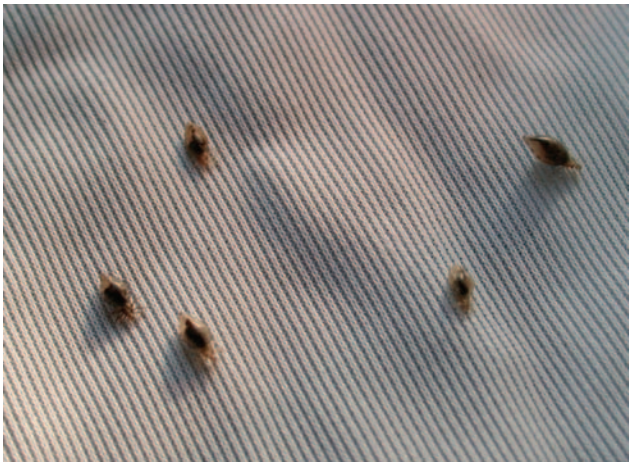

Figure 2. Human body lice in clothes.

monkey fleas (unpub. data). Recently, an animal model of *B. quintana* infection in rhesus macaques has been developed to reproduce the prolonged bacteremia that is observed in humans (*21*).

Surveys in Homeless Shelters

A substantial seroprevalence of *B. quintana* has been reported in France (*7*) and the United States (*22*). An epidemiologic survey conducted in emergency rooms of the University Hospital in Marseilles, France, showed that 30% of 71 tested homeless persons had antibody titers against *B. quintana* and that 14% were bacteremic (*7*). We found that 50 (5.4%) of 930 nonhospitalized, homeless persons tested during 4 years (2000–2003) were bacteremic (*17*).

Clinical Manifestations

Trench Fever

Trench fever is characterized by attacks of fever that last 1–3 days; are associated with headache, shin pain, and dizziness; and recur every 4–6 days (*2*,*23*), although each succeeding attack is usually less severe. The incubation period typically varies from 15 to 25 days but may be reduced to 6 days in experimental infections (*3*,*4*). Although trench fever often results in prolonged disability, no deaths have been reported (*1*,*3*).

Chronic Bacteremia

Persistent bacteremia has long been associated with *B. quintana* infection (*2*,*4*). Kostrzewski showed that *B. quintana* was present in the blood of trench fever patients up to 8 years after initial infection (*6*). More recently, asymptomatic and prolonged bacteremia was confirmed in 16 of 42 patients with positive blood cultures (*12*). Chronic bacteremia persisted for 78 weeks in 1 of those patients, for 53 and 17 weeks in 2 other patients, and for 1 to 8 weeks in the remaining 13. Intermittent bacteremia was also observed over periods of 4 to 58 weeks.

Endocarditis

Cases and series of *Bartonella* endocarditis have been widely reported (*2*,*24*), including a report of 48 cases, 38 of *B. quintana* infection, and 10 of *B. henselae* infection (*24*). Patients appeared to have chronic, blood culture–negative endocarditis; fever was usually present (90%), a vegetation was usually observed on echocardiograph (90%), and >90% of patients required valvular surgery. *B. quintana* endocarditis mostly develops in persons without any previous valvular injuries; known risk factors are alcoholism, homelessness, and body lice infestation. *B. henselae* endocarditis patients frequently have a previous valvulopathy, and disease is associated with cat bites or scratches and cat flea exposure (*24*). Treatment and outcome of *Bartonella* endocarditis were examined on the basis of 101 cases (*25*). Patients who received an aminoglycoside were more likely to fully recover, and those treated with aminoglycosides for $\geq$14 days were more likely to survive than those treated for a shorter duration. Of the 101 patients with *Bartonella* endocarditis, 12 (11.9%) died despite antimicrobial drug therapy and valvular surgery, 10 of acute heart failure and 2 of multiorgan failure (*25*).

Bacillary Angiomatosis

Bacillary angiomatosis was first described early in the HIV epidemic (*2*,*26*). It is a proliferative vascular disease recognized in both immunocompetent and immunodeficient patients (mostly HIV-infected persons) (*3*,*27*). *B. quintana* and *B. henselae* are the 2 etiologic agents (*26*). Various organs may be affected, including the liver, spleen, bone marrow, and lymph nodes, but the skin is most often involved (*3*). Cutaneous lesions may be solitary or multiple and may bleed profusely when punctured. They may be

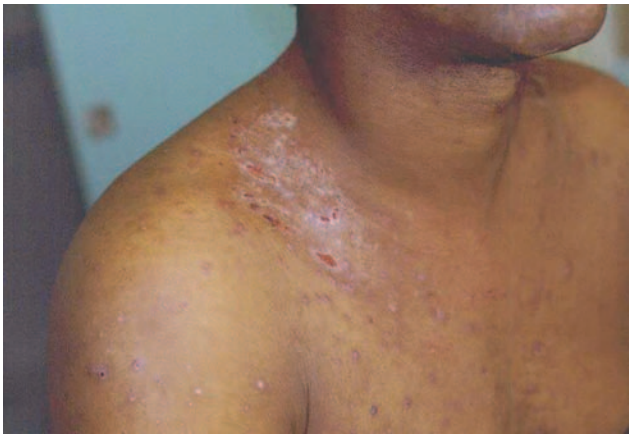

Figure 3. Lesions from scratching induced by body lice infestation.

superficial, dermal, or subcutaneous. Superficial lesions may be red, purple, or colorless. Deep lesions are not usually colored and are either mobile or fixed to underlying structures. The oral, anal, and gastrointestinal mucosa may also be involved (*3*). Molecular epidemiologic features of *Bartonella* infections in HIV-infected patients with bacillary angiomatosis were investigated (*26*); bone lesions and subcutaneous masses were associated with *B. quintana*, whereas hepatic peliosis and lymph node lesions were associated with *B. henselae*. Bacillary angiomatosis may be life-threatening in untreated patients.

Lymphadenopathy

B. quintana has been reported to cause lymphadenopathy. A 30-year-old woman with isolated chronic, afebrile, cervical, and mediastinal adenopathy was the first reported patient (*20*). Histologic examination of the cervical lymph node showed a granulomatous reaction, and *B. quintana* was isolated from blood cultures. A second case was later reported in a hemodialysis patient with Sjögren syndrome who had mediastinal lymphadenopathies and secondary pancytopenia (*28*). *B. quintana* was isolated from a bone marrow biopsy specimen, and the bacterium was identified by using molecular biologic methods. His serum showed an antibody titer of 1:50 against *B. quintana*. In 2003, a coinfection with *B. quintana* and *Mycobacterium tuberculosis* was reported in an HIV-infected patient with supraclavicular inflammatory lymphadenitis. The 2 microorganisms were isolated from lymph nodes (*29*).

Diagnosis

Serologic Tests

Serologic testing is the most widely used method to diagnose *Bartonella* infection. Indirect immunofluorescence is the reference method. Variability in antibody titers may occur when different methods of antigen preparation are used for the assay. Moreover, cross-reactions have been reported with *Coxiella burnetii* and *Chlamydia pneumoniae* (*1*,*3*). Western blot and cross-adsorption resolve this problem and can be used to determine the species involved (Figure 4) (*30*). On indirect immunofluorescence, immunoglobulin G titers ≥1:50 indicate *Bartonella* infection, and titers ≥1:800 predict endocarditis (*31*).

Culture

B. quintana was first cultivated in axenic media by Vinson in the early 1960s (*32*). To date, the most widely used methods for isolation are direct plating onto solid media, blood culture in broth, and cocultivation in cell culture (*11*). From 1993 to 1998, we correlated the results of *Bartonella*-positive cultures with the type of sample, the culture procedure, and polymerase chain reaction (PCR)–based genomic detection (*11*). During this period, we received 2,043 samples of *Bartonella* species for culture and obtained 72 isolates of *B. quintana*. The most efficient culture method in patients with endocarditis was to subculture blood culture broth into shell vials. For samples from homeless patients with *B. quintana* bacteremia, subculturing blood culture broth onto agar was more efficient than direct blood plating (*11*). Lysis centrifugation has been shown to enhance the recovery of *Bartonella* species as well as sample congelation from blood. Primary isolates are typically obtained after 12–14 days, although an incubation period of up to 45 days may be necessary (*11*).

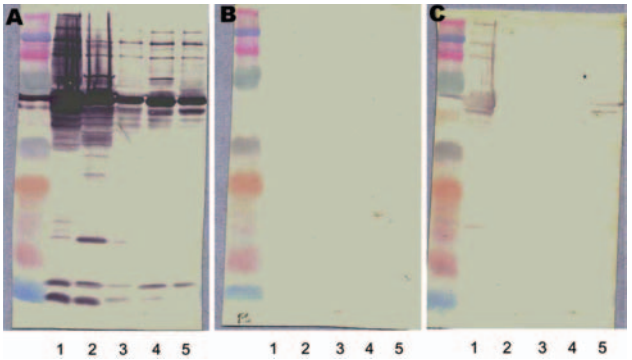

Figure 4. Western blot and cross-adsorption results in a patient with *Bartonella quintana* endocarditis. A) Nonadsorbed. B) Adsorbed with *B. quintana*. C) Adsorbed with *B. henselae*. Lane 1, *B. quintana*; lane 2, *B. henselae*; lane 3, *B. elizabethae*; lane 4, *B. vinsonii* subsp. *Berkhoffi*; lane 5, *B. vinsonii* subsp. *Arupensis*. Before adsorption (A), antibodies are detected against all species (1, 2, 3, 4, and 5). After adsorption with *B. quintana* antigen (B), all antibodies disappear. After adsorption with *B. henselae* antigen (C), antibodies against *B. quintana* (*1*) persist. This reaction shows *B. quintana* infection.

Molecular Biology

Bartonella species can be detected from blood and tissues by using PCR. Various tissues may be used, including lymph node, cardiac valve, skin, and liver. Although fresh tissues are more convenient, formalin-fixed, paraffin-embedded tissues may be used for PCR-based assay as well. Using universal primers to amplify the 16S rRNA gene is not a convenient method for diagnosing *Bartonella* infection to the species level because the 16S rRNA genes of *Bartonella* species are >97.8% similar. New targets have been used to detect *Bartonella* species with a PCR-based assay (see online Appendix Table available from http://www.cdc.gov/ncidod/EID/vol12no02/05-0874_app.htm) (*33*).

Immunohistochemistry and Immunofluorescence

Immunohistochemistry is a convenient tool for detecting *B. quintana* in tissues. Positive detection has been

reported in valvular tissue (Figure 5) (*34*) and in skin biopsies of patients with bacillary angiomatosis (*35*). On immunohistochemical tests, *Bartonella* species are observed in proliferative endothelial cells localized in the upper reticular dermis in patients with bacillary angiomatosis (*35*). In patients with *Bartonella* endocarditis, clusters of bacteria, mainly in the valvular vegetation, occupy an extracellular location in the fibrin deposits (*34*).

B. quintana can be detected in erythrocytes by using immunofluorescence (*13*). The bacterium is observed in thin blood smears from fresh blood fixed with methanol and stained with mouse monoclonal antibody (*13*). The intraerythrocytic location of *B. quintana* can be confirmed by using confocal microscopy (Figure 6) (*13*).

What Diagnostic Modality To Choose

Serologic testing and blood culture should be performed as described above when *B. quintana* infection is suspected, regardless of the clinical signs and symptoms. When endocarditis is suspected, culture, immunohistochemical tests, and PCR should be performed on the cardiac valve when available. When bacillary angiomatosis is suspected, culture, immunohistochemical tests, and PCR may be performed on a skin biopsy specimen.

Typing

Pulsed-field Gel Electrophoresis (PFGE)

Until recently, the only available method for typing *B. quintana* was PFGE, which allowed defining a specific pattern for each of 7 tested isolates (*36*). However, the PFGE profile does not correlate with that of the sequenced-based typing method; variability in PFGE patterns could be explained by frequent genome rearrangements in *B. quintana* (*37*). This finding was supported by the fact that the PFGE profile of 1 strain was modified after 9 subcultures while the profile from sequence-based typing remained identical (*37*), which showed that this method was unreliable in assessing epidemiologic aspects of *B. quintana*.

Multispacer Typing

Recently, we proposed a new typing method for *B. quintana*, multispacer typing (MST) (*37*). This method, first used for *Yersinia pestis* (*38*), is based on comparing spacers, i.e., intergenic zones. Surprisingly, MST allowed us to determine only 5 different sequence types among *B. quintana* isolates. The finding of few sequence polymorphisms in the noncoding DNA of *B. quintana* agrees with findings from a previous study in which 16S–23S spacer sequencing allowed a specific sequence of each of the tested *B. henselae* isolates to be identified, while only 2 different types were identified for *B. quintana* (*36*). As *B. quintana* is a genomic derivate of *B. henselae* (*16*), its oligoclonality could have been caused by a very recent adaptation to its unique human host and louse vector.

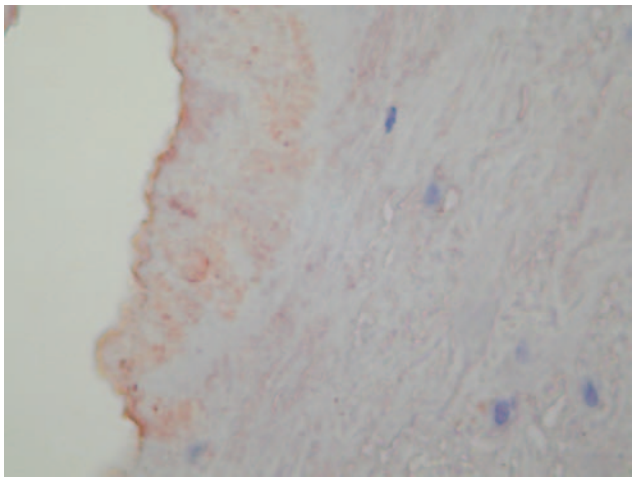

Figure 5. Immunohistochemical demonstration of *Bartonella* sp. in a cardiac valve of a patient with endocarditis. Magnification ×400.

Treatment

Pediculosis

Pediculosis can be treated with insecticides, i.e., treating all clothing with 10% DDT, 1% malathion, or 1% permethrin dust (*1*). Because body lice live in clothing, lay their eggs in clothing, and only visit human skin to feed, the patient's body does not need to be deloused. Boiling infested clothes is also efficient (*1*). Changing a person's clothing, including underwear, is the simplest method for delousing, but it is, however, not always practical (*1*). Recently, oral agents have been evaluated, and ivermectin has been efficient in delousing homeless persons in shelters, without other measures (*39*). Bedding at shelters is a major source of infestation and should be treated with insecticides or by boiling the sheets.

Antimicrobial Drug Susceptibility of *B. quintana*

Evaluation of susceptibilities to antimicrobial drugs has been performed in both axenic media and cell culture. Bacteria of the genus *Bartonella* are susceptible to a wide range of agents, including penicillins, cephalosporins, aminoglycosides, chloramphenicol, tetracyclines, macrolides, rifampin, fluoroquinolones, and cotrimoxazole (*40*). However, only aminoglycosides have a bactericidal effect. MICs correlate poorly with the in vivo efficacies of antimicrobial drugs in patients with *B. quintana* infection, and this discrepancy may be explained by the lack of bactericidal effect of most compounds and by sequestration of the bacterium in erythrocytes (*40*).

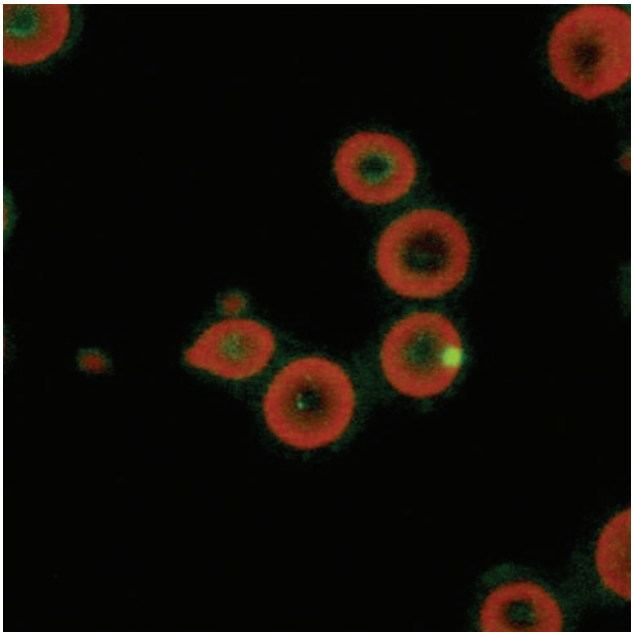

Figure 6. Laser confocal microscopy showing the intraerythrocytic location of *Bartonella quintana*. Magnification x400.

Chronic Bacteremia

In 2003, a randomized trial showed that doxycycline in combination with gentamicin was effective in treating chronic bacteremia. Treatment was given as follows: doxycycline 200 mg/day in 1 oral daily dose for 28 days combined with gentamicin 3 mg/kg/day in 1 intravenous daily dose for 14 days (*40*). This treatment is efficient at the individual level, but *B. quintana* remains endemic in the homeless persons in shelters in Marseilles, France, although bacteremic persons have been treated for 4 years (*17*). Considering the poor adherence of homeless persons to medical care and treatment, a 1-month treatment, including 14 days of intravenous treatment, is difficult to manage in this population. Shorter durations should be evaluated in the future.

Endocarditis

The efficiency of antimicrobial drugs to treat *Bartonella* endocarditis has recently been evaluated (*25*,*40*). Patients receiving an aminoglycoside are more likely to fully recover ($p = 0.02$), and those treated with aminoglycosides for ≥14 days are more likely to survive than those who undergo therapy of shorter duration ($p = 0.02$) (*25*). The recommended treatment for *B. quintana* endocarditis is as follows: doxycycline 100 mg 2×/day orally for 6 weeks in combination with gentamicin 3 mg/kg/day in 1 intravenous daily dose for 14 days (*40*).

Bacillary Angiomatosis

Drug treatment of bacillary angiomatosis has not been studied systematically to date, but erythromycin is reported to be efficient and is currently the firstline agent of choice (*40*). Doxycycline has also been recommended, and successful treatment has been reported with ceftriaxone or fluoroquinolones, but treatment with ciprofloxacin was unsuccessful (*40*). The recommended treatment for bacillary angiomatosis is erythromycin 500 mg 4×/day orally for 3 months. In patients with a contraindication to macrolides, doxycycline 100 mg 2×/day orally for 3 months should be considered (*40*). The dramatic efficiency of erythromycin in bacillary angiomatosis is linked to its antiangiogenic effect, rather than to its antimicrobial effect (*15*).

Conclusion

B. quintana infection has long been disconcerting for physicians and researchers. The uncommon quintan fever has been a subject of medical curiosity since 1915, and its clinical course and pathologic features have been understood only after numerous experimental infections in volunteers. The clinical spectrum of *B. quintana* infection includes various manifestations such as bacillary angiomatosis and endocarditis. Treatment of these infections has also been debated and has recently been codified. To date, some points still remain to be elucidated. *B. quintana* was thought to be strictly a human pathogen but was recently detected in cat fleas and monkey fleas, which reopens the debate on the existence of an animal reservoir. The availability of the complete genome sequence of *B. quintana* allows sequence-based typing, which has created convenient tools for molecular epidemiology that are necessary to determine the natural history of *B. quintana* infection.

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Dr Foucault is a specialist in infectious diseases in Marseilles, France. His research interests are in the management of infectious diseases in the homeless population and vectorborne diseases, particularly *B. quintana* infection.

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Rickettsia africae in the West Indies

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Rickettsia africae is the agent of African tick-bite fever, a mild but common disease of local persons and tourists in Africa. The major vector of this spotted fever group rickettsia is most likely *Amblyomma variegatum*, the tropical bont tick, which has become widely distributed through the West Indies in the last 30 years. This report reviews all available information on *R. africae* in the West Indies.

Rickettsia africae is a recently described spotted fever group (SFG) rickettsia that is the agent of African tick-bite fever (ATBF), a mild but common tickborne disease of local persons and tourists, in particular, in sub-Saharan Africa. The clinical and laboratory features of ATBF have recently been reviewed (*1*), as has the diagnosis of the disease (*2*). In Africa, the tropical bont tick, *Amblyomma variegatum*, is commonly infected with *R. africae* and is likely the major vector of the organism (*3*). This tick was introduced from Africa (Senegal) into the West Indies (Guadeloupe) in the early 1800s but has only spread widely and become endemic on many islands in the last 30 years (*4*). This spread was probably due to an increase in the between-island movement of livestock, major hosts of *A. variegatum* (*5*), and the introduction and spread of the cattle egret (*Bubulcus ibis*), a host of the immature stages of *A. variegatum* (*5*). Recent studies have demonstrated *R. africae* infections in *A. variegatum*, persons, and animals in the West Indies. In this report, the available information on *R. africae* in the region is reviewed.

Epidemiology of *R. africae*

Early studies in southern Africa showed the bont tick, *A. hebraeum*, was commonly infected with *R. africae* (*6*). In feeding experiments, *R. africae* was maintained transtadially and transovarially in *A. hebraeum*, and the tick transmitted the organism at each feeding stage (*7*). Cattle and goats are common hosts of *A. hebraeum* and, when infected with *R. africae*, show no clinical or laboratory signs of disease. They are, however, intermittently rickettsemic and may then be sources of infection for ticks (*8*,*9*). While *A. hebraeum* is the most common vector of *R. africae* in southern Africa, epidemiologic evidence indicates that *A. variegatum* is the predominant vector in the rest of sub-Saharan Africa. This tick readily feeds on people (*10*,*11*) and is commonly infected with *R. africae* (16%–75%) in widely separated areas in Africa (*6*,*12*–*14*).

Although *R. africae* is widely distributed in Africa, and serosurveys have shown infections are extremely common in humans (up to 100%) (*1*), reports of ATBF in indigenous people are unexpectedly rare. This finding could be because they are generally infected at a young age, when the disease might be very mild or subclinical, and medical attention is not sought. Also, inoculation eschars are difficult to see in pigmented skin, and definitive diagnosis of ATBF requires sophisticated diagnostic tests not available in developing countries. The disease, however, is quite common in international travelers; up to 11% of visitors to disease-endemic areas have evidence of infection (*15*,*16*).

R. africae in the West Indies

The first suspected cases of human spotted fever were reported from Guadeloupe in the 1960s (*1*). The patients had a history of tick bites and antibodies against SFG rickettsiae. Although rickettsiae were isolated from *A. variegatum* on the island, they were never definitively identified, and samples have been lost (*17*).

In 1998, Parola et al. (*18*) described a French woman who was bitten on the foot by a tick while visiting Guadeloupe. An erythematous nodule subsequently developed at the site as well as fever, elevated liver enzyme levels, and leukopenia. Serologic and adsorption studies suggested that she had been infected with *R. africae*. She recovered slowly when she was treated with doxycycline for 3 weeks. Subsequently, further human infections were documented on the island (*19*), and *R. africae* was detected in 27% of *A. variegatum* used for isolation experiments

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or polymerase chain reaction (PCR) analysis with *rOmpA* primers (*20*).

In 2002, Robinson et al. (*21*) used PCR with *rOmpA* and *gltA* primers to show that 84% of 75 *A. variegatum* collected from cattle in Antigua contained DNA of *R. africae.* In 2003, Kelly et al. (*22*) found 41% of *A. variegatum* from Saint Kitts and Nevis were positive for DNA of *R. africae* in PCRs in which *rOmpA* primers were used for the SFG rickettsiae. Positive ticks were found at 7 of 8 sites sampled, with prevalences varying from 14% to 71%. In the same year, Parola et al. (*23*) reported finding DNA of the *ompA* gene of *R. africae* in 7 (56%) of 12 *A. variegatum* tested from Martinique.

Although *Rhipicephalus* (*Boophilus*) *microplus*, the tropical/southern cattle tick, and *Rhipicephalus sanguineus*, the brown dog tick, are widespread in the Caribbean (*24*), they have not been implicated as vectors of *R. africae*. PCR with rO*mpA* primers of 52 *R. sanguineus* and 16 *R. microplus* from Saint Kitts and Nevis did not show DNA of SFG rickettsiae (unpub. data). Similarly, SFG rickettsial DNA was not identified in 6 *R. microplus* and 11 *R. sanguineus* from Martinique (*23*) or in 6 *R. microplus* from Antigua (*21*).

The studies described show that *A. variegatum* is commonly infected with *R. africae* in the West Indies. In the only published serosurvey conducted in the region (*20*), high prevalences of antibodies to *R. africae* were found in Guadeloupean cattle (81%) and goats (87%), which are common hosts of *A. variegatum* (*24*). Antibodies to *R. africae* were also highly prevalent (49%) in local people from Guadeloupe. The prevalence in men was significantly higher than in women, possibly because men were more likely to be exposed while working outdoors. The West Indian population, then, appears to be commonly exposed to *A. variegatum* that transmits *R. africae*. As is the case in Africa, however, clinical cases of ATBF in local persons are unexpectedly rare; the only reported cases of ATBF contracted in the region have been in tourists (*18*,*19*).

Recently, programs have been introduced to eradicate *A. variegatum* from the Caribbean (*25*–*27*). The principal justification for the projects has been the economic loses to island economies caused by animal diseases associated with *A. variegatum*, mainly heartwater and dermatophilosis. Also of great concern have been the huge economic loses that would be anticipated if the tick and its animal diseases were introduced into South, Central, and North America (*28*,*29*). The programs have met with mixed success, and although some islands have been certified provisionally free of the tick, others remain infested or have reinfestations or recrudescences of *A. variegatum*. No attempts appear to have been made to control the populations of cattle egrets, which are hosts of *A. variegatum* and can migrate long distances, even as far as the Florida Keys (*30*).

Conclusions

Until *A. variegatum* is eradicated from the West Indies, local health workers and those treating persons who have traveled to the area should suspect ATBF in patients who seek treatment with a history of tick bites and clinical signs of fever, headache, and multiple eschars. Further, vigilance is required to prevent transportation of *A. variegatum* or rickettsemic animals to the mainlands of North, South, and Central America because this importation might enable *R. africae* and ATBF to become established in these areas. The potential impact of *R. africae* on the health of indigenous people and tourists in the West Indies and its potential introduction into the Americas further justifies the eradication of *A. variegatum* from the region.

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Free-grazing Ducks and Highly Pathogenic Avian Influenza, Thailand

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Thailand has recently had 3 epidemic waves of highly pathogenic avian influenza (HPAI); virus was again detected in July 2005. Risk factors need to be identified to better understand disease ecology and assist HPAI surveillance and detection. This study analyzed the spatial distribution of HPAI outbreaks in relation to poultry, land use, and other anthropogenic variables from the start of the second epidemic wave (July 2004–May 2005). Results demonstrate a strong association between H5N1 virus in Thailand and abundance of free-grazing ducks and, to a lesser extent, native chickens, cocks, wetlands, and humans. Wetlands used for double-crop rice production, where free-grazing duck feed year round in rice paddies, appear to be a critical factor in HPAI persistence and spread. This finding could be important for other duck-producing regions in eastern and southeastern Asian countries affected by HPAI.

Despite fears of an emerging influenza pandemic, human cases observed in Vietnam, Thailand, and Cambodia (*1*), and the severe socioeconomic losses in the poultry industry, the principal risk factors associated with the highly pathogenic avian influenza (HPAI) epidemic, which started in 2003 in eastern and southeastern Asia, are still poorly understood. Reports on the start of the epidemic in China indicated that a variety of H5N1 viruses circulated in domestic ducks in the coastal and southern parts of the country until the dominant Z strain emerged and caused a subcontinental-scale epidemic (*2*,*3*). Areas where both extensive and semi-intensive poultry production systems coexist were believed to be particularly at risk, while larger scale commercial and industrial poultry plants remained relatively unexposed (*4*,*5*). Recent studies found that ducks infected with H5N1 showed few clinical signs of disease (*3*,*6*,*7*) but were capable of shedding appreciable amounts of virus and may therefore form a potential reservoir or permanent source of infection. Trade and movements of live birds, including fighting cocks, and live-bird markets have also been identified as potential risk factors in the spread of HPAI caused by H5N1 (*5*).

Between January 2004 and early 2005, Thailand had 2 major HPAI epidemics (*8*). The first peaked at the end of January 2004 and a second, which may have started in July 2004, assumed epidemic proportions only after the end of September 2004 (Figure 1). On September 28, 2004, the Thai Government launched a nationwide survey (the X-ray survey) to produce a composite picture of HPAI situation in Thailand, reduce disease incidence, and when possible, halt virus circulation. This survey involved the participation of hundreds of thousands of inspectors searching door to door for evidence of HPAI. All sick and dead poultry in the villages suspected of HPAI infection were reported to local authorities.

After initial training of inspectors, this operation was fully implemented in the second week of October 2004 until early November. This unprecedented increase in intensity of surveillance complicated the interpretation of records of disease outbreaks. The intensity of the second-wave epidemic was likely modulated by the x-ray survey because the increase in case detection activity contributed to a higher than usual number of reported HPAI outbreaks. Conversely, because of more intensive inspection and culling of infected birds, a more effective disruption of transmission cycles probably occurred, which contributed to a relatively strong decrease in incidence. However, the increase in reported cases just before the onset of the x-ray survey suggests that a serious outbreak was occurring. The

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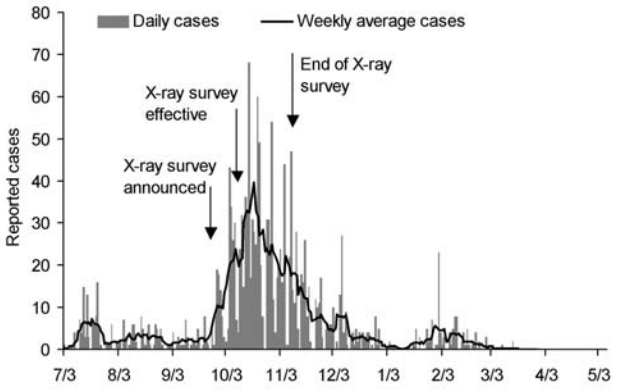

Figure 1. Number of daily highly pathogenic avian influenza outbreaks, Thailand, July 3, 2004–May 5, 2005. Shown are laboratory-confirmed H5N1 cases only, with the dates matching actual detection of clinical disease.

weekly incidence of HPAI started to decrease at the end of October 2004, and the weekly number of disease outbreaks has continued to decrease progressively.

The aim of this study was to analyze the HPAI spatial distribution based on laboratory-confirmed H5N1 outbreaks recorded during the second epidemic. To identify the risk factors associated with HPAI, we applied autologistic multiple regression to relate HPAI to the geographic distribution of the main poultry species, relevant land-use features, and other environmental or anthropogenic variables.

Materials and Methods

Data

Data on HPAI outbreaks caused by H5N1 consisted of 1,716 laboratory-confirmed cases reported from July 3, 2004, to May 3, 2005, by the Department of Livestock Development, Ministry of Agriculture and Cooperatives, Bangkok, Thailand. These data were pooled for the entire time series and converted into presence or absence of HPAI within each of the 8,089 subdistricts of Thailand (online Appendix Figure 1, available from http://www.cdc.gov/ncidod/EID/vol12no02/05-0640_appG1.htm) for analysis at the national level and within each of the 913 villages in Suphanburi Province for analysis at the local level. Poultry census data were collected simultaneously in the x-ray survey from all Thai villages countrywide during October to November 2004. Poultry data comprised bird numbers and categories by subdistrict for analysis at the national level or by village for analysis in Suphanburi Province.

Poultry categories considered in the analysis were farm chickens (including broilers and layer hens), native chickens, farm ducks (including meat and layer ducks), free-grazing ducks (domestic ducks raised in the open in flocks of >1,000 birds for egg production and, to a lesser extent, for meat; see Discussion for a more detailed description of this type of husbandry), cocks, and other poultry. Native chickens and free-grazing ducks form separate categories as because both groups are raised in the open and are more exposed to prevailing pathogens. In contrast, variable levels of biosecurity measures may apply to chickens and ducks that are raised in farms.

In addition to poultry data (Table 1), we obtained relevant variables describing road network, land use, and physical environment (*9*). These variables were used to analyze 1) possible disease introduction and propagation through waterfowl's frequenting rice paddy fields and wetlands (thus the choice of variables relating to the rice fields, wetlands, and topography), and 2) the role of human activities in the spread of disease associated with live-bird trade and traffic (thus the choice of variables on human population and roads).

Statistical Analysis

Preliminary analysis on HPAI distribution in Thailand indicated that Suphanburi Province accounted for nearly 50% of all outbreaks in ducks (outbreaks in ducks refer to outbreaks reported in all type of domestic ducks). This province had the highest cumulative number of outbreaks and a large population of free-grazing ducks. We thus decided to conduct a follow-up analysis of HPAI distribution in Suphanburi Province using village-level data on HPAI presence or absence. Therefore, this 2-scale analysis, in addition to considering an identical analytical approach for 2 different levels of resolution, also compares results obtained at the national level, including areas where HPAI outbreaks were never reported, with those obtained of the epicenter of HPAI in Thailand.

The association between HPAI occurrence, either at the subdistrict or village level, and the poultry and environmental variables was explored by using stepwise multiple logistic regressions. Linear model statistics are affected by spatial autocorrelation in response and predictor variables, i.e., the tendency for the value of neighboring points to be more similar than those from distant points. This tendency, known as spatial autocorrelation, contradicts the assumption of independence among samples replicated through space (*10*). We accounted for spatial autocorrelation in the general model by applying an autologistic approach (*11*,*12*), in which an autologistic term was added as a covariate to the logistic model (the autologistic term averages the probability of HPAI presence among a set of neighbors, defined by the limit of autocorrelation and weighted by the inverse of the Euclidean distance). The extent of the autocorrelation of the response variable was obtained from the spatial correlogram $\rho(h)$ (*13*) of HPAI presence or absence. The inverted correlogram $1 - \rho(h)$

Table 1. Variables used in the analysis of highly pathogenic avian influenza distribution in Thailand (subdistrict level) and Suphanburi Province (village level)

Category	Description	Abbreviation
Poultry*	No. broilers and layer hens	BrLaCh
	No. native chickens	NaCh
	No. meat and layer ducks	MeLaDu
	No. free-grazing ducks	FgDu
	No. cocks	Co
	No. other poultry	Ot
Land use/water†	Proportion of land occupied by wetland in the subdistrict, and in a 1-, 2-, 5-, and 10-km radius neighborhood	Pwet, Pwet1K, Pwet2K, Pwet5K, Pwet10K
	Proportion of land occupied by rice fields in the subdistrict, and in a 1-, 2-, 5-, and 10- km radius neighborhood	Price, Price1K, Price2K, Price5K, Price10K
	River/stream density (per km^2) in the subdistrict and in a 1-, 2-, 5-, and 10-km radius neighborhood	Sd, Sd1K, Sd2K, Sd5K, Sd10K
People/roads‡	No. humans	Hpop
	No. roads	Nroads
Topographic§	Elevation, m	Alt
	Slope, degrees	Slp

*Census in the subdistrict or village.
†Estimate in the subdistrict was not used for the village-level analysis; distance-based variables between the 1- and 10-km neighborhood were estimated around the village point or subdistrict centroid.
‡No. roads in the subdistrict or connected to the village.
§These variables were averaged in the subdistrict polygon for subdistrict level analysis or extracted at the point of the village for village-level analysis.

was modeled by using a spherical model (*14*), and the parameters for the model (termed nugget, scale, and range, respectively) were obtained by using nonlinear regression with bootstrapped estimates of the standard errors (SPSS version 12.0; SPSS Inc., Chicago, IL, USA). The autoregressive term was built by using a neighborhood determined by the range of the spatial correlogram model and was estimated as the average number of HPAI instances in this neighborhood weighted by the inverse distance. The autoregressive term was then added to each tested model.

A first ranking and selection of variables consisted of testing the HPAI status separately against each variable (with the autoregressive term included), and variables yielding nonsignificant changes in log-likelihood were excluded. Next, a stepwise multiple logistic regression with forward entry mode was carried out by using the subset of variables and entering the variable accounting for the highest change in the model log-likelihood. This procedure was repeated until no additional significant variable could be added (likelihood ratio test; decision rule: $p<0.01$ for entry, $p>0.05$ for removal). The regression with the subset of variables was also run in backward mode, and the most parsimonious model included the variables found significant, and with the same sign, using the 2 approaches.

The performance of the models was assessed by determining the area under the curve (AUC) of the receiver operating characteristics plots. AUC is a quantitative measure of the overall fit of the model that varies from 0.5 (chance event) to 1.0 (perfect fit) (*15*). This measure is independent of the threshold value (*16*) and has the advantage of being independent of presence rarity, which is not the case with Cohen's kappa index.

Results

Most H5N1 outbreaks in poultry in Thailand were recorded in chickens (*8*). However, the distribution of these clinical outbreaks in chickens did not match the distribution of native, backyard chickens (Figure 2). Instead, the national distribution of HPAI outbreaks shows the strongest association with the distribution of free-grazing ducks (Figure 2). This result is quantified in Tables 2 and 3, which shows the number of free-grazing ducks as the most important risk factor associated with HPAI presence (as quantified by the Wald statistic). HPAI presence is also, but to a lesser extent, associated with number of native chickens, land elevation, number of cocks, and size of the human population. Elevation is the only variable that shows a negative association with HPAI presence, which shows that most outbreaks occurred in the lower plains.

When results are further analyzed in terms of chicken and duck HPAI outbreaks separately, for outbreaks in ducks, the association with native chickens is no longer present, while a positive association is observed with the proportion of rice paddy fields in the 10-km range neighborhood and the number of farms and free-grazing ducks. When the analysis was carried for Suphanburi Province at the village level, results were consistent with those obtained in the national-level analysis. This analysis included the association with the number of ducks (free-grazing ducks and meat and layer ducks), proportion of rice paddy fields in the 5-km neighborhood, and the number of chicken, both for all outbreaks and for chicken outbreaks only.

These results, particularly the association of HPAI with free-grazing ducks, are maintained when the analysis was

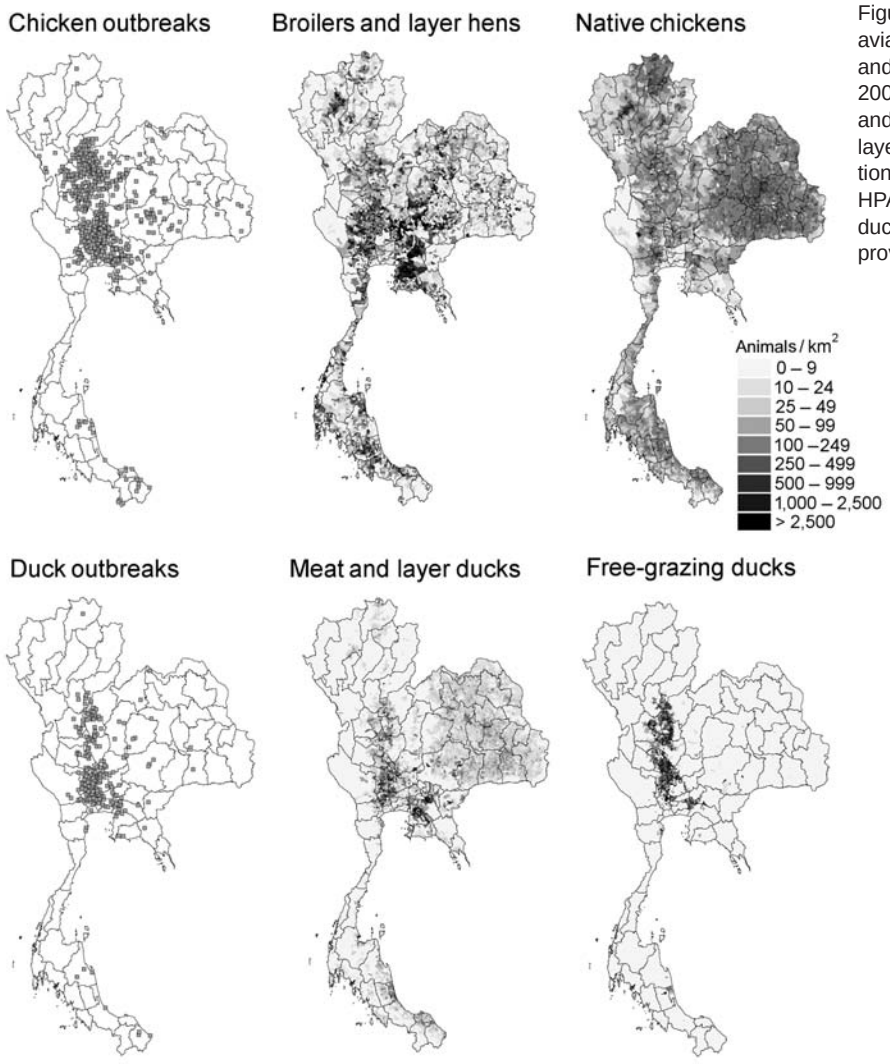

Figure 2. Distribution of highly pathogenic avian influenza (HPAI) outbreaks in chickens and ducks, Thailand, July 3, 2004–May 5, 2005, and respective distribution of broilers and layers hens, native chicken, meat and layer ducks, and free-grazing duck populations, highlighting the correlation between HPAI outbreak distribution and free-grazing duck populations. The divisions are Thailand provinces.

stratified for 3 study periods: the period before the start of the x-ray survey (July 3–September 28, 2004), during the x-ray survey period (September 28–November 10, 2004), and beyond (November 10, 2004–May 5, 2005). The spatial structure of HPAI presence or absence as quantified by their spatial correlograms (online Appendix Figure 2 and Appendix Table, available from http://www.cdc.gov/ncidod/EID/vol12no02/05-0640_appG2.htm and http://www.cdc.gov/ncidod/EID/vol12no02/05-0640.htm#apptable). This was characterized by a relatively weak spatial dependence with all scale parameters estimated as <0.25 (scale parameter measures the intensity of spatial autocorrelation and ranges between 0 and 1) and an estimated range between 20 km and 72 km (range parameter measures the geographic extent of the spatial autocorrelation).

Discussion

Although most HPAI outbreaks during the second epidemic in Thailand occurred in chickens, the spatial distribution of these outbreaks does not correspond to areas with high densities of chickens. For example, northeastern Thailand has many native chickens that are not protected by biosecurity measures. However, apart from incidental HPAI outbreaks, this disease never showed a marked increase in this area (Figure 2). Instead, the distribution pattern suggests an important role of free-grazing ducks in rice paddies as in the central plains of Thailand. The variable genetic susceptibility of different poultry species, breeds, or races to HPAI may have created a bias in the recorded results, given that clinical detection of HPAI was the measure of HPAI presence.

Although subsequent H5N1 verification was carried out for all reported outbreaks, virus circulation in native chickens may have remained unnoticed because disease presence was not prominent. Furthermore, the reduced susceptibility of ducks has likely contributed to underreporting of HPAI virus because ducks may carry virus but have no signs of disease (*3,6,17*). Nevertheless, the results

substantiate the claim that the geographic pattern of HPAI outbreaks in Thailand is not primarily driven by long-distance transmission between chicken productions units or villages, which would have resulted in more outbreaks in areas with high densities of chickens. In the national level analysis, free-grazing ducks constitute the most important poultry-associated variable associated with HPAI in either ducks or chicken (Table 2). The significant but weaker association found for native chickens, both in the analysis of all HPAI reports and of HPAI reports in only chickens, may reflect infections in areas with a higher abundance of the host. This association is confirmed because this variable was replaced by duck numbers in the analysis of HPAI presence in ducks. The pattern that emerges is that free-grazing ducks form a HPAI risk factor both in chicken and ducks, which suggests that they may form a reservoir of

Table 2. Parameters of autologistic regression models of highly pathogenic avian influenza presence or absence as a function of selected poultry and other environmental variables

Model	Variable*	Parameter	SE	Wald statistic	p value
Thailand, all outbreaks					
	Art	37.03	1.69	482.5	<0.001
	FgDu	2.47×10^{-5}	3.67×10^{-6}	45.3	<0.001
	NaCh	1.63×10^{-5}	4.18×10^{-6}	15.1	<0.001
	Alt	-3.60×10^{-3}	1.00×10^{-3}	12.9	<0.001
	Co	1.12×10^{-4}	3.21×10^{-5}	12.1	<0.001
	Hpop	1.03×10^{-5}	3.10×10^{-6}	10.9	<0.001
	Alt2	2.40×10^{-6}	1.01×10^{-6}	5.7	0.017
	Intercept	–2.93	0.16	317.9	<0.001
Thailand, outbreaks in chicken					
	Art	44.49	2.33	363.2	<0.001
	FgDu	1.76×10^{-5}	3.25×10^{-6}	29.5	<0.001
	NaCh	1.62×10^{-5}	4.23×10^{-6}	14.7	<0.001
	Alt	-3.91×10^{-3}	1.03×10^{-3}	14.6	<0.001
	Alt2	2.73×10^{-6}	9.96×10^{-7}	7.5	0.006
	Co	7.55×10^{-5}	2.98×10^{-5}	6.4	0.011
	Hpop	7.85×10^{-6}	3.50×10^{-6}	5.0	0.025
	Intercept	–2.92	0.17	302.9	<0.001
Thailand, outbreaks in ducks					
	Art	41.60	2.87	209.9	<0.001
	FgDu	2.94×10^{-5}	3.65×10^{-6}	64.9	<0.001
	Alt	-1.15×10^{-2}	2.16×10^{-3}	28.4	<0.001
	Alt2	7.63×10^{-6}	1.83×10^{-6}	17.4	<0.001
	Price10K	9.24×10^{-1}	2.39×10^{-1}	14.9	<0.001
	Co	4.78×10^{-5}	1.66×10^{-5}	8.2	0.004
	Hpop	1.22×10^{-5}	4.65×10^{-6}	6.9	0.009
	MeLaDu	5.96×10^{-6}	2.66×10^{-6}	5.0	0.025
	Intercept	–3.33	0.31	112.2	<0.001
Suphanburi Province, all outbreaks					
	Price5K	3.70	0.621	35.6	<0.001
	FgDu	9.46×10^{-5}	2.03×10^{-5}	21.7	<0.001
	MeLaDu	7.47×10^{-5}	2.52×10^{-5}	8.8	0.003
	NaCh	2.09×10^{-4}	8.27×10^{-5}	6.4	0.012
	Intercept	–4.89	0.511	91.6	<0.001
Suphanburi Province, outbreaks in chickens					
	Price5K	4.64	1.135	16.7	<0.001
	NaCh	3.01×10^{-4}	1.00×10^{-4}	9.0	0.003
	MeLaDu	7.00×10^{-5}	2.60×10^{-5}	7.2	0.007
	FgDu	4.54×10^{-5}	1.81×10^{-5}	6.3	0.012
	Intercept	–6.74	0.960	49.3	<0.001
Suphanburi Province, outbreaks in ducks					
	Price5K	3.76	0.742	25.7	<0.001
	FgDu	8.67×10^{-5}	1.87×10^{-5}	21.4	<0.001
	MeLaDu	7.16×10^{-5}	2.39×10^{-5}	9.0	0.003
	Intercept	–5.16	0.604	73.1	<0.001

*SE, standard error; Art, autoregressive term. For descriptions and abbreviations of other variables, see Table 1.

Table 3. Results of autologistic regression models of H5N1 highly pathogenic avian influenza as a function of variables shown in Table 2

Model	–2 log likelihood	χ^2	p value	AUC*
All Thailand, all outbreaks	3,812.8	1,294.3	<0.001	0.854 ± 0.014
All Thailand, outbreaks in chickens	3,455.9	812.9	<0.001	0.828 ± 0.018
All Thailand, outbreaks in ducks	1,634.7	781.7	<0.001	0.894 ± 0.021
Suphanburi Province, all outbreaks	691.4	135.8	<0.001	0.783 ± 0.039
Suphanburi Province, outbreaks in chickens	374	63.05	<0.001	0.794 ± 0.061
Suphanburi Province, outbreaks in ducks	585.8	106.8	<0.001	0.767 ± 0.045

*Area under the curve of the receiver-operating characteristics plot.

HPAI virus. Conversely, chicken and duck numbers are associated with the probability of an outbreak in each respective category, i.e., they are related to the occurrence of infections. The robust association between HPAI and free-grazing ducks at the national level (Figure 2) corroborates the results obtained for Suphanburi Province (Figure 3). Ducks are the type of poultry most strongly associated with HPAI presence in villages.

Traditional free-grazing duck husbandry in Thailand is characterized by the practice of frequent rotation of duck flocks in rice paddy fields after the harvest, in which they are moved from 1 field to another every 2 days to feed on leftover rice grains, insects, and snails. Duck husbandry involves frequent field movements of flocks that are brought together in shelters often located within villages; with marketing of live birds and eggs extending beyond villages, apparently healthy ducks may play an important role in virus transmission, which explains the observed spatial pattern of HPAI. Infectious poultry or livestock diseases can be transmitted either locally through contagion between adjacent production units; by direct contact; by wind, insects, or rodents; or over a long distance by movements of animals, persons, or infected material (*18*). Local spread typically results in a strong spatial clustering of cases, whereas long-distance spread produces a distance-independent distribution of cases. The weak spatial autocorrelation in HPAI presence or absence, in particular in Suphanburi Province, indicates a weak clustering of HPAI. This finding suggests a relatively important contribution of long-distance movements of animals and infected materials.

The duck production cycle is closely connected with rice crops because rice provides duck feed. Figure 4 shows the distribution of duck and rice paddy fields. Most rice fields in eastern Thailand produce 1 crop per year, but areas in the central plains (Figure 4B) produce 2 or even 3 crops per year. Single-crop areas are associated with duck farming, but fewer ducks are present because of the shorter

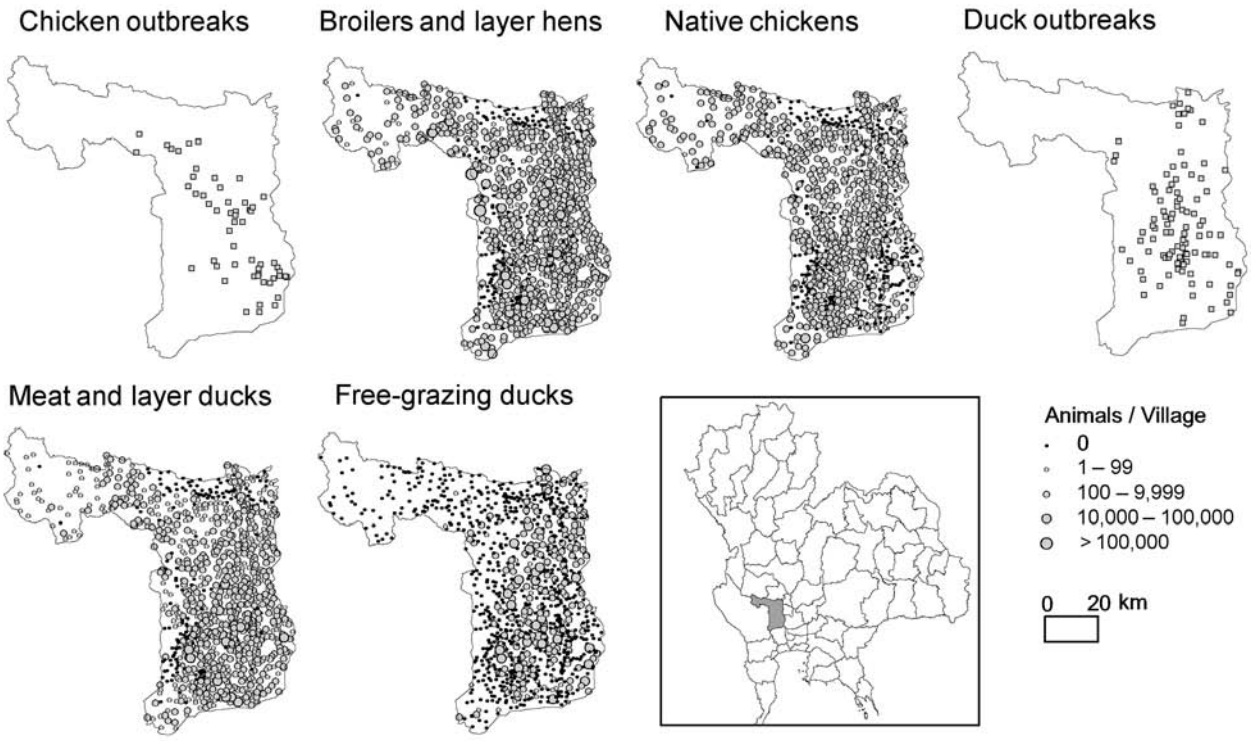

Figure 3. Distribution of highly pathogenic avian influenza (HPAI) outbreaks in chickens and ducks, Thailand, July 3, 2004–May 5, 2005, and respective distribution of broilers and layers hens, native chicken, meat and layer ducks, and free-grazing duck populations.

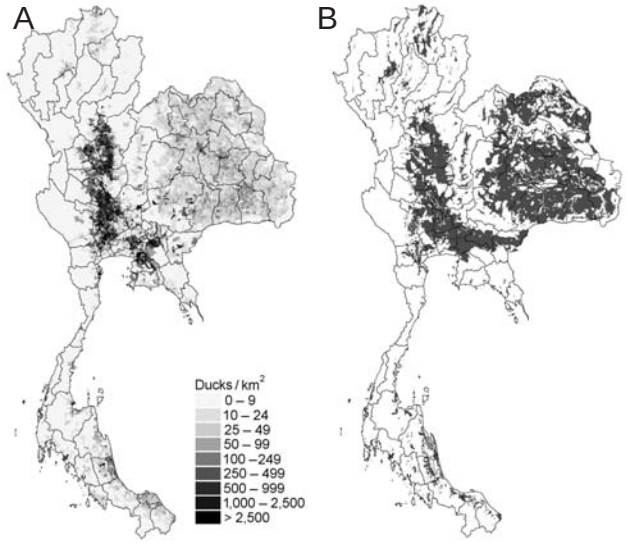

Figure 4. Distribution of A) duck and B) rice production areas in Thailand.

period of rice harvest. In contrast, in double-crop areas, rice paddy fields are available year round after harvest. This availability sustains the low-input, low-output, free-grazing duck farming system and represents a large proportion of total ducks. As shown in Figure 4, there is a good correlation between the distribution of ducks and rice paddy fields and a strong correlation between free-grazing duck areas and double rice–crop areas in the central plains.

The 2-crop rice production system in the central plains is facilitated by local hydrology because irrigation systems provide enough water and wetland to produce a second crop outside the monsoon period. These wetlands and feed in the paddy fields are also attractive to migratory waterfowl and create a meeting point for wild and domestic aquatic bird species. The coexistence of free-grazing ducks and waterfowl during a defined period of the year (mainly November to February) may have provided an entry point or an index case for HPAI in poultry population in Thailand. The positive association between HPAI in villages in Suphanburi Province and the proportion of rice fields around the village, and the negative association with elevation (reflecting that HPAI was more frequently found in lower wetlands) suggest that wetland-rice-duck systems increase the risk for HPAI outbreaks, even after the effect of free-grazing ducks has been considered.

The strong association between ducks and rice crops facilitates application of remote sensing to identify rice-crop areas and patterns that may sustain forms of duck husbandry prone to HPAI outbreaks. All currently affected countries are known for their rice and duck production. For example, similar associations between rice and duck farming occur in Vietnam, where HPAI-affected areas coincide with river delta areas with year-round rice production. With duck populations remaining relatively healthy while excreting sufficient amount of virus to sustain transmission (*6*), wetlands with duck-production areas may act as a reservoir from which the virus can spread to distant aquatic duck farms and terrestrial chicken farms.

Other factors have been proposed as potential pathways for the spread of HPAI. These include migratory birds and introductory spread of virus from disease-endemic sources in China (*19*), trade of live animals and animal products (e.g., restocking, movements to slaughterhouses) within and away from infected areas, and movement of fighting cocks. Insufficient information exists to discern the possible role of migratory birds. However, several hypotheses have been proposed regarding their role and contribution to observed patterns of HPAI outbreaks. For example, a plausible scenario is that migratory birds initially spread H5N1 virus genotype Z virus over wide areas, but HPAI increased only after transmission to free-grazing ducks through water contamination, resulting in local amplification, persistence, and secondary spread to terrestrial poultry.

We found significant associations at the national level between HPAI and the overall number of cocks used in cock fights. The results at the national level also suggest that human activities may have played a role through a higher risk for transmission in more densely populated areas where poultry-related trade and traffic are more intensive. However, since these results were less strongly associated with HPAI and were not important at the village level, follow-up and local analysis of disease hotspots are needed to confirm that these 2 factors substantially contributed to transmission of HPAI, mainly within terrestrial poultry.

Options are available to veterinary authorities to further contain HPAI persistence in the central plains, address frequent movement of duck flocks in rice paddy fields, especially at the time of wild-bird migration, and actively encourage duck production in farms with adequate biosecurity. In 2005, a number of new control measures were introduced to enhance HPAI prevention, persistence, and spread nationwide. Some of these control measures specifically target free-grazing duck husbandry. These measures included registration and surveillance of all flocks (culling infected animals and compensating their owners), premovement testing, and incentives for improving biosecurity and shifting from free-grazing duck husbandry to farm production systems. These measures were effective in reducing the number of HPAI outbreaks in 2005. A total of 1,064 outbreaks were reported from July 3 to October 31, 2004 (second epidemic wave), but only 64 outbreaks were recorded during the same period in 2005. These results show that HPAI was still in Thailand in late 2005. Whether these outbreaks result from year-round persistence of

HPAI within Thailand or from new introductions from external sources remains to be established. The reduced number of outbreaks suggests an overall reduction in circulation of the virus in free-grazing ducks and terrestrial poultry and a reduced risk for spread to birds or mammals.

In conclusion, our results highlight that free-grazing ducks were a critical factor in HPAI persistence and spread in Thailand during the second HPAI epidemic in 2004 at a time when there was little regulation concerning their movements and potential transmission to terrestrial poultry. This finding is of particular importance to duck-producing regions in other countries affected by HPAI.

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Nipah Virus-associated Encephalitis Outbreak, Siliguri, India

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During January and February 2001, an outbreak of febrile illness associated with altered sensorium was observed in Siliguri, West Bengal, India. Laboratory investigations at the time of the outbreak did not identify an infectious agent. Because Siliguri is in close proximity to Bangladesh, where outbreaks of Nipah virus (NiV) infection were recently described, clinical material obtained during the Siliguri outbreak was retrospectively analyzed for evidence of NiV infection. NiV-specific immunoglobulin M (IgM) and IgG antibodies were detected in 9 of 18 patients. Reverse transcription–polymerase chain reaction (RT-PCR) assays detected RNA from NiV in urine samples from 5 patients. Sequence analysis confirmed that the PCR products were derived from NiV RNA and suggested that the NiV from Siliguri was more closely related to NiV isolates from Bangladesh than to NiV isolates from Malaysia. NiV infection has not been previously detected in India.

During January and February of 2001, an outbreak of febrile illness with altered sensorium was observed in Siliguri, West Bengal, India. Siliguri is an important commercial center with a population of ≈500,000. It is near borders with China, Bangladesh, Nepal, and Sikkim. The outbreak occurred among hospitalized patients, family contacts of the patients, and medical staff of 4 hospitals. Japanese encephalitis, which is endemic in this area, was initially suspected, but the age group affected and the epidemiologic features suggested a different disease. Laboratory investigations conducted at the time of the outbreak failed to identify an infectious agent (*1*).

Nipah virus (NiV), a recently emergent, zoonotic paramyxovirus (*2*), was implicated as the cause of a highly fatal (case-fatality ratio 38%–75%), febrile human encephalitis in Malaysia and Singapore in 1999 (*1*) and in Bangladesh during the winters of 2001, 2003, and 2004 (*3–6*). The natural reservoir of NiV is presumed to be fruit bats of the genus *Pteropus*. Evidence of NiV infection was detected in these bats in Malaysia, Bangladesh, and Cambodia (*7–10*). In the Malaysian outbreak, NiV was introduced into the pig population, and most of the human cases resulted from exposure to ill pigs (*2*). However, an intermediate animal host was not identified during the Bangladesh outbreaks, which suggests that the virus was transmitted either directly or indirectly from infected bats to humans. Human-to-human transmission of NiV was also documented during the outbreak in Faridpur, Bangladesh (*4,5*). Because the clinical manifestations of the cases in Siliguri were similar to those of NiV cases in Bangladesh (*3–6*), and because Siliguri is near affected areas in Bangladesh, a retrospective analysis of clinical samples was undertaken to determine if NiV was associated with the Siliguri outbreak.

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Methods

Case Definition and Clinical Sample Collection

A team of physicians and epidemiologists from the National Institute of Virology, Pune, India, along with local public health authorities, visited Siliguri. Investigations were conducted with the assistance of health authorities from West Bengal State and staff from the North Bengal Medical College Hospital. Medical records of patients who were hospitalized during the study period were examined, and their family members or caretakers were interviewed. Areas of the town in which cases occurred and the houses of patients who died were visited. Contacts and family members of patients who died were also interviewed.

A broad working case definition was used for case detection. The case definition evolved over the course of the outbreak on the basis of information from case-patients admitted to different hospitals, review of the line list of patients, and interviews with contacts in the community. A suspected patient was one >15 years of age with acute

onset of high-grade fever and headache. A probable patient was one >15 years of age who had high-grade fever and altered sensorium and encephalitis of unknown origin. Blood samples were available for 18 hospitalized patients and for 13 family contacts of patients who died 2–3 weeks earlier. Six urine samples (5 samples had corresponding serum samples) were also collected.

Serologic Testing

State health authorities conducted laboratory tests to rule out malaria and bacterial infections. Serologic tests to detect infection by Japanese encephalitis virus, West Nile virus, measles virus, dengue virus, *Leptospira* spp., and hantavirus were carried out at the National Institute of Virology.

Serum samples were gamma-irradiated at the Centers for Disease Control and Prevention (CDC) before being tested for immunoglobulin G (IgG) and IgM antibodies to NiV and measles by enzyme-linked immunosorbent assay (ELISA), as previously described (*2,11,12*). Briefly, inactivated antigens for these ELISAs were prepared from gamma (^{60}Co)-irradiated NiV-infected or mock-infected Vero E6 cells. Serum samples were tested in 4-fold dilutions from 1:100 to 1:6,400. Samples were considered positive for the IgM assay if the sum of the adjusted optical densities (OD) from all of the dilutions (OD from infected antigen well minus OD from the mock-infected antigen) was >0.75 through the entire dilution series, and the titer was ≥1:400. Similarly, samples were considered positive for IgG if the sum for the adjusted OD from all the dilutions was >0.90 through the entire dilution series, and the titer was ≥1:400.

Detection of NiV by RT-PCR and Virus Isolation

RNA was extracted from urine samples by using a Qiagen (Valencia, CA, USA) RNA extraction kit. Reverse transcription–polymerase chain reaction (RT-PCR) was performed with the SuperScript One-Step RT-PCR kit with Platinum Taq (Invitrogen, Carlsbad, CA, USA), as previously described (*13,14*). Reaction products were analyzed by agarose gel electrophoresis and ethidium bromide straining. PCR products were sequenced by using a cycle sequencing reaction with fluorescent dye terminators (Perkin-Elmer, Applied Biosystems Division, Foster City, CA, USA), and reaction products were analyzed with an ABI 3100 (Perkin-Elmer) automatic sequencer. Sequence data from multiple reactions were analyzed by using version 10.1 of the Genetics Computer Group Package (Accelrys, San Diego, CA, USA). Phylogenetic analyses were performed with PAUP version 4.01 (Sinauer Associates, Sunderland, MA, USA). Two sets of primers were used for RT-PCR reactions. Primer set NVNBF-4 (5′-GGAGTTATCAATCTAAGTTAG-3′) and NVNBR4 (5′-CATAGAGATGAGTGTAAAAGC-3′) amplified a 159-nucleotide (nt) region of the N gene of NiV. Primer set NVBMFC1 (5′-CAATGGAGCCAGACATCAAGAG-3′) and NVBMFR2 (5′-CGGAGAGTAGGAGTTCTAGAAG-3′) amplified a 320-nt region of the M gene. Virus isolation was attempted from the urine samples on Vero E6 cells as previously described (*2*).

Results

The outbreak of fever with altered sensorium began in late January 2001 and peaked in mid-February. No cases were reported after February 23 (Figure 1). All of the patients were residents of Siliguri, and some clustering of cases was observed around the Medinova Hospital, since the staff of this hospital resided in the area. Based on the case definition, 66 cases of encephalitis were identified, and the case-fatality ratio was ≈74%. The outcome of 61 cases was known; the remaining 5 patients were discharged from the hospital against medical advice. All patients were >15 years of age; the male-to-female ratio was 1.4:1. Forty-five (75%) of the 60 patients had a history of hospital exposure, i.e., they were members of the hospital staff or had attended to or visited patients in the hospital. The outbreak began at a single hospital, and cases were subsequently detected at 3 other hospitals. No definitive information about the possible index case exists. Five families had >1 case, but all of the persons affected had a history of hospital visits and had onset of illness 2 or 3 days apart from each other. The sequence of events is shown in Figure 2.

The patients initially had fever (100%), headache and myalgia (57%), vomiting (19%), altered sensorium (confusion to coma, 97%), respiratory symptoms (tachypnea to acute respiratory distress, 51%), and involuntary movements or convulsions (43%). No neck rigidity or cranial nerve involvement was observed in the 16 patients who were examined. Pupils were bilaterally dilated and reactive to light. Deep tendon reflexes were diminished or absent.

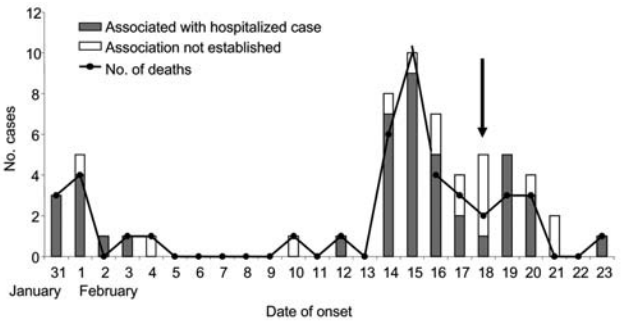

Figure 1. Epidemic curve of outbreak of febrile encephalitis in Siliguri, India, January though February 2001, by number of hospital-associated and nonhospital-associated cases and deaths. The vertical, black arrow indicates when barrier methods were introduced for case management.

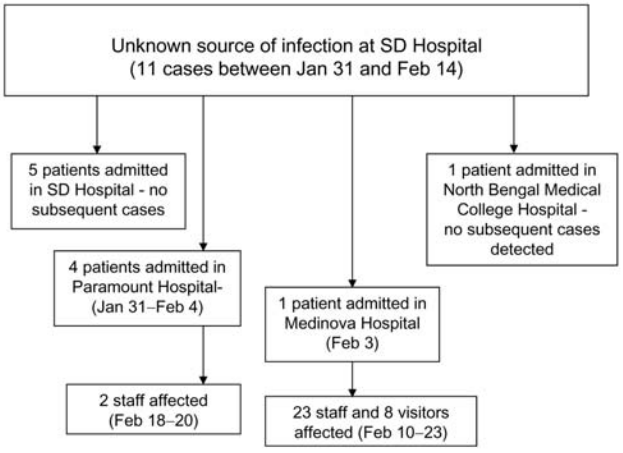

Figure 2. Sequence of events in the Siliguri (SD) outbreak.

Abnormal plantar reflexes (extensor plantar response) were elicited in 11 patients. Patients were normotensive at admission but became hypertensive before death. Death occurred within 1 week of onset of disease in 10 patients (62.5%), within 2 weeks in 5 (32.8%) patients, and on day 30 after onset for 2 patients.

Before the outbreak, the staff did not routinely use personal protective equipment or barrier nursing methods. Use of surgical masks was minimal on wards, except in the intensive-care units. Certain universal precautions, such as hand washing and use of gloves, were partially adhered to when staff were carrying out invasive procedures. Patients were housed on wards with ≥4 patients in a single room and could be visited or be attended to by their family and others. Patients did not wear masks on wards or when being transported for procedures (e.g., x-ray examination). Disposal of waste, collection of soiled linen, laundry, and cleaning of floors and other surfaces in the wards were carried out by personnel who did not follow infection control practices.

Once the outbreak of encephalitis was established, stringent infection control practices were introduced (Figure 1), including isolating patients in the Medical College Hospital, where 2 wards were established, one for suspected and the other for probable cases. Barrier nursing techniques were initiated, and housekeeping procedures and waste management were improved.

Cerebrospinal fluid was obtained from all patients. Analysis showed that the fluid in all cases was under pressure and clear with <5 lymphocytes/mm^3 (normal range 0–5 cells/mm^3). These samples were not available for further analysis.

Laboratory testing during and immediately after the outbreak did not identify a likely etiologic agent. Patient serum samples were tested for IgM antibodies to Japanese encephalitis, West Nile, dengue, and measles viruses as well as for *Leptospira* spp. Serum samples were also tested for IgG antibody to hantavirus. All serologic tests were negative, and no likely viral or bacterial agents were detected. All serum samples tested positive for IgG to measles virus.

Because NiV was identified as the cause of encephalitis outbreaks in Bangladesh, the Siliguri samples were tested for evidence of NiV infection. In all, 17 serum samples were available from 18 patients from Siliguri. All were tested for IgG and IgM antibodies to NiV by ELISA. The 6 urine samples collected from these 18 patients were tested for NiV RNA by RT-PCR, and aliquots were inoculated onto Vero E6 cells in an attempt to isolate NiV.

NiV-specific IgM and IgG were detected in 9 of 17 serum samples; 1 sample was positive for IgG and negative for IgM (Table). RT-PCR assays detected RNA from the N gene of NiV in 4 urine samples from NiV antibody–positive patients and in 1 urine sample from a NiV antibody–negative patient. RNA from the M gene was detected in 3 of these 5 samples (Table). No viral isolates were obtained from the 6 urine samples.

Sequence analysis confirmed that the PCR products were derived from NiV RNA (Figures 3 and 4). Partial N-gene sequences (159 nt) from 2 of 5 Siliguri samples were identical, and the other 3 sequences differed by no more than 1 nt, although unresolved sequence heterogeneity occurred at 2 positions (A or G) in 3 of the Siliguri N-gene sequences (Figure 3). Comparison of the Siliguri N-gene sequences to the N-gene sequences from NiV samples isolated in Bangladesh in 2004 and Malaysia in 1999 showed an overall level of nucleotide identity of 97.5%. Siliguri N-gene sequences were more closely related to the N-gene sequence from the Bangladesh isolate than to the sequences from the Malaysian isolates. Two of the Siliguri N-gene sequences were identical to the Bangladesh N-gene sequence.

Comparison of the partial M gene sequence amplified from the specimens from Siliguri to the M gene sequences from NiV isolated in Malaysia and Bangladesh (Figure 4) showed identity at 302 (94%) of 320 nt positions. Again, the Siliguri M gene sequences were more closely related to the M gene sequences from Bangladesh (99% identity) than to the sequences from Malaysia (94% identity).

Discussion

This retrospective study provides evidence of NiV infection during a 2001 outbreak of febrile encephalitis in Siliguri. Nine of 18 of the patients tested had IgM and IgG antibodies; 1 had IgG antibodies only to NiV. Urine samples from 4 of these patients contained NiV RNA. One other patient had NiV RNA in the urine but lacked a detectable IgM and IgG response. In this case, the serum

Table. Serologic and PCR test results for clinical material from patients with encephalitis, Siliguri, India*

Patient no.	Days after onset of fever	Serology†		PCR (urine)	
		IgM	IgG	N gene	M gene
1	10	+	+	+	NA
2	5	+	+		NA
3	9	+	+	+	NA
4	10	+	+	NA	NA
5	9	–	–	NA	NA
6	10	–	–	+	+
7	3	–	–	NA	NA
8	7	–	–	NA	NA
9	Unknown	–	–	NA	NA
10	1	–	+	NA	NA
11	3	+	+	NA	NA
12	5	+	+	+	+
13	7	–	–	NA	NA
14	6	+	+	NA	NA
15	3	+	+	NA	NA
16	8	–	–	NA	NA
17	8	+	+	+	+
18	2	NA†	NA	–	–

*PCR, polymerase chain reaction; IgM, immunoglobulin M; IgG, immunoglobulin G; NA, no sample available.
†Nipah virus–specific IgM or IgM by enzyme-linked immunosorbent assay.

sample may have been obtained early in infection before antibodies to NiV were present. These laboratory results, along with the observation that the symptoms in the Siliguri patients were consistent with those described for patients during NiV outbreaks in Bangladesh and Malaysia (*3–5*,*15–17*), provide strong evidence that NiV caused the outbreak in Siliguri. Failure to detect evidence of NiV or NiV-specific antibodies in some patients may have been due to early sample collection or to inclusion of encephalitides of other causes because of the broad case definition. One patient was IgG-positive but had no detectable IgM, which suggests past infection by NiV. Unfortunately, because no case control and population-based studies were undertaken during this outbreak, interpreting this result is difficult.

The main reservoir for NiV is believed to be fruit bats of the genus *Pteropus*. NiV was isolated from fruit bats in Malaysia and Cambodia, and seropositive bats have been detected in other parts of Southeast Asia (*7–10*). In the Malaysian outbreak, commercially raised pigs were believed to be intermediate hosts. Presumably, the pigs were infected by virus shed from fruit bats and then transmitted the virus to humans. Although fruit bats with antibodies to NiV were captured in the outbreak areas of Bangladesh, no intermediate animal host was identified. In Bangladesh, NiV might have been transmitted to humans by direct contact with bats or indirectly by contact with material contaminated by bats. Person-to-person spread was also noted during the 2004 NiV outbreak in Faridpur, Bangladesh (*4*,*5*). The range of *Pteropus giganteus*, one of the flying foxes commonly found in south Asia (*18*), includes West Bengal. Therefore, the range of the proposed

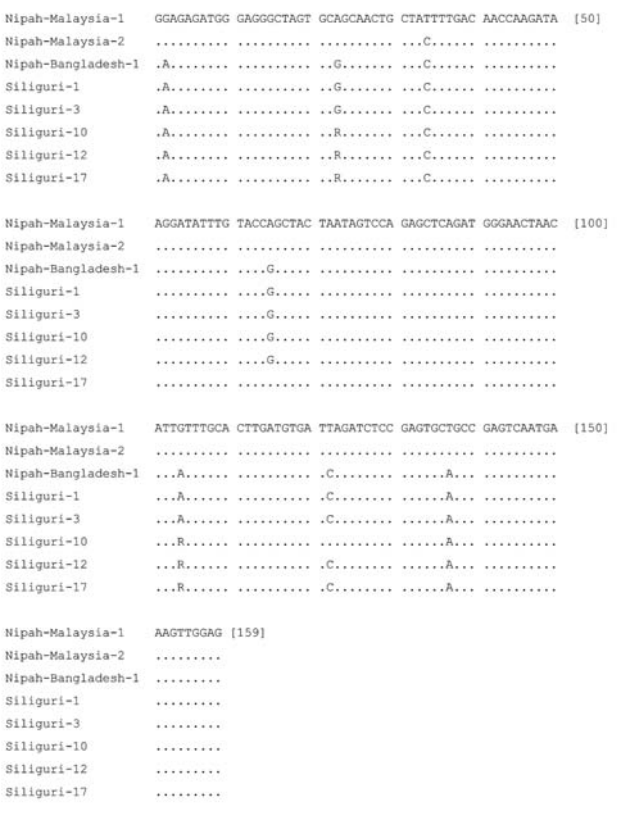

Figure 3. Comparison of partial N-gene nucleotide sequences obtained from the Siliguri specimens (by patient number, see Table) to sequences obtained from Nipah virus isolates from Bangladesh (AY988601) and Malaysia (AF212302, AF376747). Letters indicate positions that differ from the reference sequence on the top line, Nipah-malaysia-1. Dots indicate nucleotide identity. R indicates A or G.

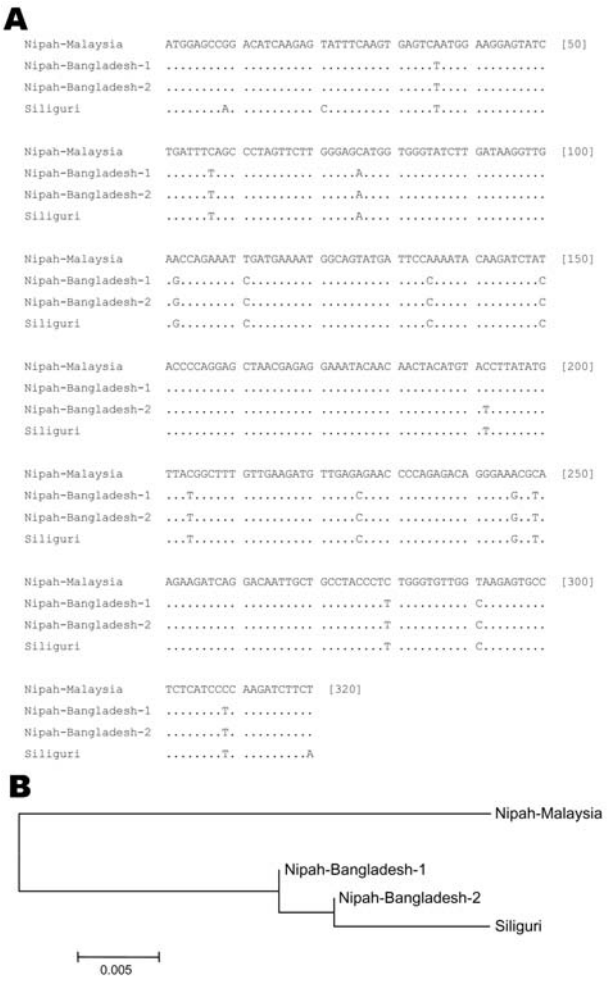

Figure 4. A) Comparison of partial M-gene nucleotide sequences of Siliguri specimens to Nipah virus isolates from Bangladesh (Bangladesh-1:AY988601, Bangladesh-2:unpublished) and Malaysia (AF212302). Letters indicate positions that differ from the reference sequence on the top line, Nipah-Malaysia. Dots indicate nucleotide identity. B) Phylogenetic tree based on the sequence alignment shown in panel A.

natural reservoir for NiV extends into northeastern India, and since the geographic features of West Bengal are similar to those of Bangladesh, environmental circumstances that favor transmission of NiV to humans would likely also be found in West Bengal.

Many of the epidemiologic features of the outbreak in Siliguri were similar to those of the recent NiV outbreaks in Bangladesh. In Bangladesh, no intermediate animal host was identified, whereas in Siliguri studies to detect an intermediate host were not conducted. In Siliguri, no samples were obtained from local wildlife or domestic animals. In both outbreaks, transmission occurred in healthcare settings through contact with infected persons. In Siliguri, the observation that only adults were affected supported the nosocomial transmission theory, as the number of children on the wards of hospitals was minimal. During infection, NiV is present in respiratory secretions and urine (*19*) and in both outbreaks, failure to use personal protective equipment probably contributed to the spread of the virus. Many of the patients had nasogastric tubes inserted or were intubated, procedures which made exposure to respiratory secretions more likely. Initiating adequate barrier nursing techniques helped to curtail further spread of infection.

Sequence analysis of PCR products confirmed NiV RNA. Unfortunately, no virus was isolated, and only limited sequence data could be obtained from the available clinical material. Analysis of the limited sequence data suggested that the NiV strains associated with the outbreak were more closely related to NiV isolated in Bangladesh than to NiV isolated in Malaysia. These data extend the previous observation that viruses circulating in different areas have unique genetic signatures (*10,14*) and suggest that these strains may have co-evolved within local natural reservoirs.

To our knowledge, NiV infection has not occurred previously in India; however, given the proximity of Siliguri to the areas of Bangladesh that experienced NiV outbreaks in 2001, 2002, and 2004, the outbreak is not surprising. Given the distribution of the locally abundant *P. giganteus*, the apparent natural reservoir of NiV in this area, outbreaks of NiV will likely continue to occur in Bangladesh and northern India. Establishing appropriate surveillance systems in these areas will be necessary so that NiV outbreaks can be detected quickly and appropriate control measures initiated. When NiV infections are suspected, infection control practices must be strengthened to avoid outbreaks in hospital settings, as apparently occurred in Siliguri.

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Methicillin-resistant *Staphylococcus aureus* Clones, Western Australia

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Community-associated methicillin-resistant *Staphylococcus aureus* (MRSA) was first reported in Western Australia in the early 1990s from indigenous peoples living in remote areas. Although a statewide policy of screening all hospital patients and staff who have lived outside the state for MRSA has prevented the establishment of multidrug-resistant epidemic MRSA, the policy has not prevented SCC*mec* type IV and type V MRSA clones from becoming established. Of the 4,099 MRSA isolates analyzed (referred to the Gram-positive Bacteria Typing and Research Unit) from July 2003 to December 2004, 77.5% were community-associated MRSA (CA-MRSA). Using multilocus sequence/staphylococcal chromosome cassette *mec* typing, 22 CA-MRSA clones were characterized. Of these isolates, 55.5% were resistant to ≥1 non–β-lactam antimicrobial drug. Five Panton-Valentine leukocidin (PVL)–positive CA-MRSA clones were identified. The emergence of multidrug-resistant CA-MRSA clones and the detection of PVL toxin genes in clones previously reported as PVL negative is a major public health concern.

Staphylococcus aureus successfully colonizes humans, contaminates the hospital environment, and has the genetic versatility to acquire resistance to multiple antimicrobial agents. Methicillin-resistant *S. aureus* (MRSA) was first detected soon after the introduction of methicillin in 1960, and isolation rates increased until the early 1970s (*1*). These earlier "classic" MRSA strains were genetically similar to each other and may have evolved from a single clone (*2*). In 1976, the first outbreak of gentamicin-resistant MRSA in Australia (*3*) was reported, and by 1981 extensive outbreaks occurred in several countries. In 1985, it became evident that these "modern" strains of MRSA carried epidemic potential not possessed by MRSA isolated in the 1960s and early 1970s and that they were genetically different from the earlier classic MRSA (*4*). Since 1990, international and intercontinental spread of MRSA (known as epidemic MRSA or EMRSA) has increased. In 2002, Enright et al., using multilocus sequence typing (MLST) combined with staphylococcal chromosome cassette *mec* (SCC*mec)* typing, established that relatively few major EMRSA clones existed (*5*). These clones emerged either as descendants of preexisting EMRSA clones or by horizontal transfer of the *mec* determinants into methicillin-susceptible *S. aureus*.

EMRSA became endemic in hospitals in eastern Australian states (New South Wales, Victoria, and Queensland) in the late 1980s and 1990s, with some spread to hospitals in South Australia, the Northern Territory, and Tasmania (*6*). However, a statewide MRSA policy, introduced in 1982, prevented these strains from becoming established in Western Australia (WA) hospitals. This policy required MRSA screening of anyone who had been hospitalized or had been a healthcare worker in a hospital outside of WA in the previous 12 months. MRSA-positive patients were isolated in the hospital, and staff with MRSA-positive test results received decolonization treatment. Imported MRSA still occasionally caused single-strain outbreaks in hospitals; however, infection control interventions contained them.

In the early 1990s, nonmultidrug-resistant MRSA (nmMRSA) were observed in WA, initially from indigenous people in remote communities (*7*) but subsequently in Perth, the state capital. These strains became known as "WA-MRSA." Although WA-MRSA did not readily spread in WA hospitals, 1 strain was responsible for an outbreak of hospital-acquired infection (*8*). Strains of nmMRSA have recently been reported in the eastern

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Australian states, and studies in Queensland and New South Wales showed a strong association between community-acquired infection with nmMRSA and Polynesian ethnicity. Isolates causing these infections were indistinguishable by phage typing and pulsed-field gel electrophoresis from those previously reported in New Zealand (*9*,*10*). Subsequently, a second strain (WA-MRSA-7 or Qld MRSA) has been associated with community-acquired infections in Caucasians in Queensland (*11*).

The emergence of nmMRSA has also been reported in other parts of the world, including North America (*12*) and Europe (*13*). Although nmMRSA strains appear to have originated in the community, they may include nmEMRSA strains that have been associated with healthcare facilities (e.g., EMRSA-15, EMRSA-16, and the New York/Japan EMRSA) or nonmultidrug-resistant sporadic hospital MRSA strains that have been taken into the community.

In 1997, the Department of Health WA, in collaboration with the Department of Microbiology and Infectious Diseases at Royal Perth Hospital, PathWest Laboratory Medicine WA, and the School of Biomedical Sciences at Curtin University of Technology established the Gram-positive Bacteria Typing and Research Unit to assist in controlling MRSA in WA. Since then, all MRSA isolated in WA have been referred to the unit for epidemiologic typing. This study describes the different epidemic and CA-MRSA clones isolated in WA and establishes their genetic relatedness.

Materials and Methods

MRSA Isolates

All MRSA isolated in WA between July 1, 2003, and December 31, 2004, were included in this study. Isolates were recovered from clinical and infection control screening specimens. For the purpose of this study, duplicate isolates from the same patient (as determined by their antimicrobial drug susceptibility phenotype) were excluded.

Antimicrobial Susceptibility Testing

A test for oxacillin susceptibility was performed on Mueller-Hinton agar by the disk diffusion method according to Clinical Laboratory Standards Institute (CLSI) recommendations by using a 1-μg oxacillin disk (*14*). Oxacillin susceptibility results discrepant with those of the referring laboratory were confirmed by the detection of the *mecA* gene by polymerase chain reaction (PCR) (*15*).

An antibiogram was performed on Mueller-Hinton agar by the disk diffusion method according to CLSI recommendations, against a panel of 8 antimicrobial drugs (*14*): erythromycin (15 μg), tetracycline (30 μg), trimethoprim (5 μg), ciprofloxacin (5 μg), gentamicin (10 μg), rifampin (5 μg), fusidic acid (10 μg), and mupirocin (5 μg). The French CA-SFM susceptibility testing interpretive criterion was used for fusidic acid (*16*), and the suggested interpretive criterion by Finlay et al. was used for mupirocin (*17*). CLSI interpretive criteria were used for the remaining antimicrobial drugs (*18*). MRSA that were resistant to ≥3 of the 8 antimicrobial drugs listed were defined as mMRSA and those resistant to <3 drugs were defined as nmMRSA (*8*). Urease production was performed by Christensen's urea slopes incubated for 24 h at 37°C.

Typing Methods

Resistogram typing was performed by disk diffusion against a panel of 6 chemicals and dyes: cadmium acetate (10 mmol/L), sodium arsenate (0.2 μmol/L), ethidium bromide (15 mmol/L), propamidine isethionate (2% [wt/vol]), mercuric chloride (0.4 μmol/L), and phenylmercuric acetate (5 mmol/L) as previously described (*19*,*20*).

Coagulase gene restriction fragment length polymorphism (RFLP) typing was performed as previously described (*21*). Contour-clamped homogeneous electric field electrophoresis (CHEF) was performed as previously described with the CHEF DR III System (Bio-Rad Laboratories Pty Ltd, Regents Park, New South Wales) (*8*). Chromosomal patterns were examined visually, scanned with a Fluor-S Multimager (Bio-Rad Laboratories), and digitally analyzed with Multi-Analyst/PC (Bio-Rad Laboratories). CHEF patterns were grouped according to the criteria of Tenover et al. (*22*) and using a dendrogram similarity of ≥80% to assign strain relatedness. *S. aureus* NCTC8325 was used as the size marker.

Multilocus sequence typing (MLST) was performed as specified by Enright et al. (*23*) on randomly selected isolates within each pulsotype. To assign a sequence type (ST) the sequences obtained were compared with the sequences described on the MLST website (http:www.mlst.net/). Using the MLST database, clones were subsequently grouped into clonal complexes (CC).

SCC*mec* typing was performed by PCR by using previously published primers that identified the class of *mec* complex and type of cassette chromosome recombinase (*ccr*) encoded on the element (*24*,*25*). The presence of the Panton-Valentine leukocidin (PVL) determinants were detected by PCR by using previously published primers (*26*) and confirmed by sequencing the products.

Results

A total of 4,099 MRSA isolates were studied. All isolates were initially grouped according to their antibiogram and urease production. Isolates within a group were then further characterized by using coagulase PCR-RFLP and CHEF analysis. MLST/SCC*mec* typing and PVL detection were performed on randomly selected isolates within each

pulsotype. Twenty-nine clones were identified, including 7 (22.5%) EMRSA clones and 22 (77.5%) CA-MRSA clones.

EMRSA Clones

Table 1 shows the 7 EMRSA clones identified: ST22-MRSA-IV (EMRSA-15), ST239-MRSA-III (Aus-2 and Aus-3 EMRSA), ST8-MRSA-$IV_{pediatric}$ (Irish-2 EMRSA), ST36-MRSA-II (EMRSA-16), ST5-MRSA-II (New York/Japan EMRSA), ST8-MRSA-$II_{variant}$ (Irish-1 EMRSA), and the classic MRSA clone ST250-MRSA-I. Only 3 EMRSA clones were typically multidrug-resistant: ST239-MRSA-III (resistant to tetracycline, erythromycin, trimethoprim, ciprofloxacin, and gentamicin), ST8-MRSA-$IV_{pediatric}$ (resistant to erythromycin, trimethoprim, and ciprofloxacin), and ST8-MRSA-$II_{variant}$ (resistant to tetracycline, erythromycin, trimethoprim, ciprofloxacin, gentamicin and mupirocin).

Overall, 94.6% of EMRSA were identified either as ST22-MRSA-IV (78.1%), a urease-negative nmEMRSA clone (resistant to erythromycin and ciprofloxacin) or ST239-MRSA-III (16.5%). By using CHEF electrophoresis and resistogram typing, ST239-MRSA-III could be further classified into 2 subclones: Aus-2 EMRSA (susceptible to mercuric chloride and phenylmercuric acetate) and Aus-3 EMRSA (resistant to mercuric chloride and phenylmercuric acetate).

CA-MRSA

Of the 22 identified clones of CA-MRSA, 21 were WA-MRSA and 1 was Western Samoan phage pattern (WSPP) MRSA (Table 2). By using CHEF electrophoresis, ST8-MRSA-IV and ST5-MRSA-V could be further classified into WA-MRSA-5 and WA-MRSA-12, and WA-MRSA-11 and WA-MRSA-14 pulsotypes, respectively.

Overall, 93.7% of CA-MRSA were classified into 3 clones: ST1-MRSA-IV (55.3%), ST129-MRSA-IV (29.8%), and ST5-MRSA-IV (8.6%). Of the CA-MRSA, 97.3% were SCC*mec* type IV and 2.6% SCC*mec* type V. Four isolates carrying novel SCC*mec* type(s) were found in 2 STs (ST5 and ST8). Using the MLST database, the 22 clones were grouped into 10 CCs and 2 singletons.

Five clones (2.3% of CA-MRSA) were PVL positive including ST30-MRSA-IV and ST93-MRSA-IV. These 2 clones were originally reported outside WA, ST30-MRSA-IV in New Zealand and ST93-MRSA-IV in Queensland. The remaining 3 clones, ST8-MRSA-IV (MRSA-12 pulsotype), ST59-MRSA-IV, and ST583-MRSA-IV accounted for only 0.3% of CA-MRSA isolated in WA.

CA-MRSA Antibiograms

The online Appendix Table (available from http://www.cdc.gov/ncidod/eid/vol12no02/05-0454_app.htm) shows 6 of the 22 CA-MRSA clones (17 isolates) were predictably resistant to β-lactam antimicrobial drugs only. In the remaining 16 clones, 55.9% of isolates were resistant to at least 1 non–β-lactam antimicrobial drug, including 47.7% to erythromycin, 10.8% to fusidic acid, 2.9% to ciprofloxacin, 2.5% to trimethoprim, 1.8% to tetracycline, 1.6% to gentamicin, 1.3% to mupirocin, and 0.2% to rifampin. In 6 of these clones, 1.5% of isolates were classified as multidrug-resistant. None of the 73 isolates found in PVL-positive clones were multidrug-resistant: 7% of ST30-MRSA-IV were resistant to rifampin, 15% of ST93-MRSA-IV were resistant to erythromycin, 50% of ST583-MRSA-IV were resistant to fusidic acid and tetracycline, and 50% were resistant to fusidic acid and erythromycin; all the ST59-MRSA-V were resistant to tetracycline and erythromycin, and 75% and 25% of ST8-MRSA-IV (MRSA-12 pulsotype) were resistant to erythromycin and tetracycline, respectively. The 11 strains classified as ST5-MRSA-V (WA-MRSA-11 pulsotype) were all resistant to gentamicin. This strain was involved in a single strain outbreak in a burn unit.

Discussion

In WA, colonization or infection with MRSA has been a notifiable condition since 1982, which has enabled the rapid and widespread emergence of CA-MRSA to be monitored. In rural areas, the overall MRSA notification rate

Table 1. Characteristics of EMRSA clones* (of 4,099 total MRSA isolates), Western Australia, July 1, 2003–December 31, 2004

Clone	CHEF pattern (pulsotypes)	n (% of total MRSA)	CC	Urease	Coagulase PCR RFLP pattern	PVL toxin
ST22-MRSA-IV	EMRSA-15	719 (17.54)	22	Neg	22	Neg
ST239-MRSA-III	Aus-2 EMRSA	95 (2.32)	8	Pos	24	Neg
	Aus-3 EMRSA	57 (1.39)	8	Pos	24	Neg
ST8-MRSA-IVp	Irish-2 EMRSA	20 (0.49)	8	Neg	18	Neg
ST36-MRSA-II	EMRSA-16	16 (0.39)	30	Pos	18	Neg
ST5-MRSA-II	New York/Japan EMRSA	11 (0.27)	5	Pos	36	Neg
ST8-MRSA-IIv	Irish-1 EMRSA	2 (0.05)	8	Neg	18	Neg
ST250-MRSA-I	Classic MRSA	1 (0.02)	8	Pos	18	Neg
Total		921 (22.47)				

*EMRSA, epidemic methicillin-resistant *Staphylococcus aureus*; CHEF, contour-clamped homogeneous electric field; CC, clonal complex; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphisms; PVL, Panton-Valentine leukocidin; MRSA, methicillin-resistant *S. aureus;* p, pediatric; v, variant.

Table 2. Characteristics of community MRSA clones* (of 4,099 total MRSA isolates), Western Australia, July 1, 2003–December 31, 2004

Clone	CHEF pattern (pulsotypes)	n (% of total MRSA)	CC	Urease	Coagulase PCR RFLP pattern	PVL toxin
ST1-MRSA-IV	WA MRSA-1	1,757 (42.86)	1	Pos	20	Neg
ST129-MRSA-IV	WA MRSA-2	947 (23.10)	78	Pos	258	Neg
ST5-MRSA-IV	WA MRSA-3	273 (6.66)	5	Pos	36	Neg
ST45-MRSA-V	WA MRSA-4	60 (1.46)	45	Pos	DNC	Neg
ST8-MRSA-IV	WA MRSA-5	27 (0.66)	8	Pos	18	Neg
	WA MRSA-12	4 (0.1)	8	Pos	18	Pos
ST93-MRSA-IV	WA MRSA-7	34 (0.83)	S	Pos	32	Pos
ST75-MRSA-IV	WA MRSA-8	9 (0.22)	S	Pos	DNA	Neg
ST59-MRSA-V	WA MRSA-9	3 (0.07)	59	Pos	40	Pos
ST573-MRSA-V	WA MRSA-10	2 (0.05)	1	Pos	34	Neg
ST5-MRSA-V	WA MRSA-11	11 (0.27)	5	Pos	34	Neg
	WA MRSA-14	4 (0.09)	5	Pos	40	Neg
ST584-MRSA-IV	WA MRSA-13	1 (0.2)	9	Pos	32	Neg
ST59-MRSA-IV	WA MRSA-15	3 (0.07)	59	Pos	40	Neg
ST8-MRSA-Novel	WA MRSA-16	1 (0.2)	8	Neg	18	Neg
ST583-MRSA-IV	WA MRSA-17	2 (0.5)	80	Pos	DNC	Pos
ST5-MRSA-Novel	WA MRSA-18	1 (0.02)	5	Pos	36	Neg
ST609-MRSA-IV	WA MRSA-19	1 (0.02)	8	Neg	18	Neg
ST5-MRSA-Novel	WA MRSA-21	2 (0.5)	5	Pos	34	Neg
ST577-MRSA-V	WA MRSA-22	3 (0.07)	121	Pos	42	Neg
ST45-MRSA-IV	WA MRSA-23	1 (0.02)	45	Neg	22	Neg
ST87-MRSA-IV	WA MRSA-24	1 (0.02)	59	Pos	40	Neg
ST575-MRSA-IV	WA MRSA-25	1 (0.02)	5	Pos	256	Neg
ST30-MRSA-IV	WSPP MRSA	30 (0.73)	30	Pos	24	Pos
Total		3,178 (77.53)				

*MRSA, methicillin-resistant *Staphylococcus aureus*; CHEF, contour-clamped homogeneous electric field; CC, clonal complex; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphisms; PVL, Panton-Valentine leukocidin; WA MRSA, Western Australia MRSA; DNC, did not cut; S, singleton; DNA, did not amplify; WSPP MRSA, Western Samoan phage pattern MRSA.

has increased from 10/100,000 persons in 1983 to 542/100,000 persons in 2002. Similarly, in the same period rates increased in the Perth metropolitan area from 7/100,000 to 520/100,000. Although part of this increase in the metropolitan area from 1998 was due to an increase in EMRSA notifications, most can be attributed to CA-MRSA (unpub. data).

In this study, 22.5% (n = 921) of MRSA isolates were classified as EMRSA. Six international epidemic clones and the classic MRSA clone, ST250-MRSA-I, were identified (Table 1). More than 78% of EMRSA isolates were identified as ST22-MRSA-IV (EMRSA-15), an nmEMRSA with the community type IV SCC*mec*. Originally described in the United Kingdom in the early 1990s and now the predominant epidemic strain in that country (*27*), ST22-MRSA-IV was first isolated in WA in 1997 in preemployment screening of healthcare workers coming from the United Kingdom and Ireland (*28*). Notifications have increased from 55 in 1998 to 383 in 2002 (unpub. data). Although recent national surveillance studies have also reported the emergence of ST22-MRSA-IV in other Australian states (*29*), the predominant EMRSA in most Australian capital cities is ST239-MRSA-III (*30*). The 6 epidemic clones reported in this study have 6 STs which can be grouped into 4 of the 5 major epidemic CCs described by Enright et al. (*5*), CC5, CC8, CC22, and CC30. CC8 also includes the ancestral MRSA genotype ST250-MRSA-I, which evolved from the methicillin-susceptible strain ST250-MSSA, which is thought to have arisen from ST8-MSSA by a chromosomal mutation (*5*). Other STs isolated in this study, which form part of CC8, were ST8-MRSA-IV$_{pediatric}$, ST8-MRSA-II$_{variant}$, and ST239-MRSA-III. ST5-MRSA-II, ST22-MRSA-IV, and ST36-MRSA-II belong to CC5, CC22, and CC30, respectively.

CA-MRSA made up 77.5% of the isolates. These MRSA have several characteristics that differentiate them from most nosocomial MRSA. They harbor a smaller, SCC*mec* (SCC*mec* IV and V), are susceptible to most antimicrobial drugs other than the β-lactam agents, and are more likely to encode the virulence factor PVL (*31*).

In this study, 21 clones of WA-MRSA were identified by MLST/SCC*mec* typing with further delineation into 23 chromosomal DNA pulsotypes and numerous pulsosubtypes by CHEF. Also identified was the Western Pacific CA-MRSA (ST30-MRSA-IV) first isolated in Auckland, New Zealand (*32*). CA-MRSA from different parts of the world has been reported with varied genetic backgrounds (*24*). The results presented here demonstrate that this is also the case for CA-MRSA isolated within a single

state of Australia (WA). The 22 STs were grouped into 10 CCs (CC1, CC5, CC8, CC9, CC30, CC45, CC59, CC78, CC80, and CC121) and 2 singletons (ST75-MRSA-IV and ST93-MRSA-IV). The 10 CCs identified include 4 of the 5 major epidemic CCs (CC5, CC8, CC30, and CC45). Five CCs had >1 clone (2 clones in CC1 and CC45, 3 clones in CC8 and CC59, and 5 clones in CC5).

The 2 predominant CA-MRSA clones isolated were ST1-MRSA-IV and ST129-MRSA-IV (55.3% and 29.8% of CA-MRSA, respectively). ST1-MRSA-IV belongs to CC1, which has the same allelic profile as the *S. aureus* that is the proposed ancestor of MW2 CA-MRSA that was responsible for the deaths of 4 children in the United States (*33*). CC1 CA-MRSA has also been reported in France (*13*) and other areas of Australia (*24*), which indicates that this clone is particularly successful. ST129-MRSA-IV belongs to CC78, a smaller CC that includes strains isolated elsewhere in Australia, Portugal, and Japan (http://www.mlst.net).

Despite the diversity of CCs, the CA-MRSA strains were remarkably uniform in their SCC*mec* allotypes. SCC*mec* IV was identified in 14 STs and SCC*mec* V was identified in 5. This suggests that in WA these 2 allotypes are well adapted to the community environment. Two STs were found to have novel SCC*mec* types.

Unlike SCC*mec* types II and III, which carry a number of inserted plasmids and transposons downstream of the *mecA* complex, community-associated SCC*mec* types IV and V are smaller and lack other resistance genes. However, resistance may be encoded elsewhere on the chromosome or the isolate may carry resistance plasmids. Although CA-MRSA isolated in WA is typically nonmultidrug-resistant, all strains harbor a large plasmid that varies in size (*34*). This plasmid encodes determinants for β-lactamase production and cadmium resistance. In addition, some isolates have been reported to carry a 41.4-kb plasmid that also encodes β-lactamase and resistance to mupirocin, tetracycline, trimethoprim, and cadmium and a smaller plasmid (2 kb) that encodes inducible erythromycin resistance (*34*). Chromosomal fusidic acid and tetracycline resistance determinants have also been reported (*34*); however, the location of these determinants on the chromosome is unknown. In this study, 44% of CA-MRSA were resistant to β-lactam antimicrobial drugs only. Of the remaining 56%, 54.5% were also resistant to 1–2 non–β-lactam agents, and 1.5% to ≥3 non–β-lactam agents, including 3 isolates resistant to 5 additional antimicrobial drugs (online Appendix Table).

CA-MRSA have been shown to express several virulence genes, including the determinants for PVL (*35*). PVL is a necrotizing toxin that causes leukocyte destruction and tissue necrosis and is associated with abscesses and severe pneumonia. PVL is present in most of the CA-MRSA studied in Europe and the United States (*13*). In WA, CA-MRSA infrequently carries the genes encoding PVL (*34*); however, 2 CA-MRSA clones, ST30-MRSA-IV, and ST93-MRSA-IV, more commonly isolated in eastern Australia are PVL positive. ST30-MRSA-IV was first noted in Australia in 1997 in the Polynesian population living in the eastern Australian states and the Australian Capital Territory (*9*). ST93-MRSA-IV was first identified as a cause of community-acquired infection in the Caucasian population in Ipswich, Queensland, in 2000 (*11*). Both clones are now frequently isolated in several areas of Australia (*29*). In WA, ST30-MRSA-IV and ST93-MRSA-IV were first isolated in 2001. In this study, PVL was detected in 5 MRSA clones, including ST30-MRSA-IV, ST93-MRSA-IV, ST8-MRSA-IV (pulsotype WA-MRSA-12), ST59-MRSA-V, and ST583-MRSA-IV. However, these 5 clones were infrequently isolated and accounted for only 2.3% of all CA-MRSA. PVL genes have been transmitted by a temperate phage designated ϕPVL (*36*), which indicates that the PVL determinants are transferable. Recently, a PVL-positive ST1-MRSA-IV strain was isolated in Queensland (*37*) and New South Wales (*38*), Australian states that have reported an increasing incidence of ST30-MRSA-IV and ST93-MRSA-IV (*9–11*). This finding suggests that the PVL determinants are being transferred and raises the prospect that more CA-MRSA in WA may acquire PVL determinants in the future.

Some researchers have proposed that CA-MRSA may arise either by hospital strains escaping into the community, where they spread person to person, or de novo when the SCC*mec* complex is acquired by a methicillin-susceptible *S. aureus* isolate (*24*,*39*). In WA, the genetic background of nosocomial MRSA is different from that of CA-MRSA, and therefore, community strains have likely evolved independently of hospital strains. In addition, in WA hospitals, apart from 2 single-strain outbreaks in a large metropolitan hospital (ST1-MRSA-IV [*13*] and ST5-MRSA-V (WA-MRSA-11 pulsotype), little evidence has been found of CA-MRSA spreading within healthcare facilities. Although person-to-person spread most likely occurs in the community, the increasing number of MRSA in the WA community may also be due to mobility of the community SCC*mec* types. The genetic diversity of CA-MRSA isolated in WA and the presence of at least 3 SCC*mec* types also support this possibility.

Conclusions

Although a comprehensive MRSA screening and control program has prevented the mEMRSA from emerging, it has not prevented SCC*mec* type IV and type V MRSA clones, including nmEMRSA (ST22-MRSA-IV) and CA-MRSA, from becoming established in WA. SCC*mec* types IV and V are now found in MRSA with distantly related

genetic backgrounds. In addition, at least 1 novel SCC*mec* type has been detected. Initially nonmultidrug-resistant, many of these CA-MRSA clones have acquired plasmids and chromosomal resistance determinants allowing some strains to become resistant to up to 5 non–β-lactam antimicrobial agents, including erythromycin, tetracycline, trimethoprim, ciprofloxacin, gentamicin, rifampin, fusidic acid, and mupirocin. With the detection of 5 PVL-positive clones and the recent emergence of PVL in a previously reported PVL-negative CA-MRSA clone, more severe staphylococcal disease caused by CA-MRSA can be expected in the future. SCC*mec* types that can be acquired by multiple genotypes of *S. aureus* over a short period and the isolation of multidrug-resistant or PVL-positive CA-MRSA are major public health concerns and emphasize the importance of typing in tracing the origin of isolates and in designing antimicrobial drug prescribing policies for their control, if possible, in the community.

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Ameba-associated Microorganisms and Diagnosis of Nosocomial Pneumonia

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To elucidate the role of ameba-associated microorganisms (AAMs) as etiologic agents of pneumonia, we screened for *Legionella* spp., *Parachlamydia acanthamoeba*, *Afipia* sp., *Bosea* spp., *Bradyrhizobium* spp., *Mesorhizobium amorphae*, *Rasbo bacterium*, *Azorhizobium caulinodans*, *Acanthamoeba polyphaga mimivirus*, and conventional microorganisms in 210 pneumonia patients in intensive-care units by using culture, polymerase chain reaction, and serologic testing. These resulted in 59 diagnoses in 40 patients. AAMs and non-AAMs were implicated in 10.5% of the patients. The infectious agents were identified in 15 patients: *Acanthamoeba polyphaga mimivirus*, 8; *Legionella pneumophila*, 3; *L. anisa*, 1; *Parachlamydia* sp., 1; *Bosea massiliensis*, *L. worsleiensis*, *L. quinlivanii*, and *L. rubrilucens*, 1; and *M. amorphae* and *R. bacterium*, 1. *A. polyphaga mimivirus* was the fourth most common etiologic agent, with a higher seroprevalence than noted in healthy controls. This finding suggested its clinical relevance. Therefore, AAM might cause nosocomial pneumonia and should be suspected when conventional microbiologic results are negative.

Pneumonia is a major cause of illness and death throughout the world (*1*). Approximately 600,000 persons with pneumonia are hospitalized each year, and 64 million days of restricted activity occur because of this disease (*2*). Pneumonia is associated with high death rates, in particular, 30% for community-acquired pneumonia (*3*). Hospital-acquired pneumonia occurs in 0.5% to 1% of hospitalized patients, which represents 10%–15% of all nosocomial infections; pneumonia is the most common cause of nosocomial infection in intensive-care units (ICUs) (*4*). The etiologic agent of community-acquired pneumonia remains unknown in 20% to 50% of cases (*5*), and several pathogens that may cause pneumonia seem to be underestimated (*6–8*).

Microbiologically contaminated water distribution systems have been linked to outbreaks of hospital- and community-acquired pneumonia (*9,10*). Water-associated microorganisms, such as *Legionella* spp., *Pseudomonas* spp., *Stenotrophomonas* spp., *Burkholderia* spp., and *Acinetobacter* spp., colonize hospital water supplies and have been causally associated with cases of hospital-acquired pneumonia (*10*). There is also a growing concern that water–associated microorganisms, for example, *Legionella* spp., *Afipia* spp., *Bosea* spp., *Bradyrhizobium* spp., *Mesorhizobium* spp., *Rasbo bacterium, Parachlamydia* spp., and *Acanthamoeba polyphaga mimivirus*, may be associated with amebas (*11–13*). We previously demonstrated that patients with nosocomial pneumonia who received care in a hospital near a contaminated water distribution system showed strong serologic evidence of exposure to these microorganisms (*14*). Specimens from 12 (40%) of 30 patients in an ICU seroconverted to microorganisms known to survive in an aquatic environment in the intracellular niche provided by free-living *Acanthamoebae* (*15*). These seroconversions were associated with ventilator-associated pneumonia, especially in patients for whom no etiologic agent was found by usual microbiologic investigations. We have also reported serologic evidence of exposure to an emerging giant virus that is resistant to phagocytic destruction by ameba, which we named *A. polyphaga mimivirus* (www-micro.msb.le.ac.uk/3035/VirusGroups.html), in 26 patients with ventilator-associated pneumonia at another ICU (*12,13,16*). Using this rationale, we evaluated infections with ameba-associated microorganisms (AAM) in a larger series of patients with pneumonia hospitalized in Marseille, France. Our goal was to identify typical pathogens as well as emerging AAMs (*12–15,17–20*).

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Materials and Methods

Study Population

All patients admitted to the ICU of Sainte-Marguerite Hospital in Marseille, France, with clinically suspected pneumonia over an 18-month period ending in June 2003 were enrolled in a prospective study. For all patients, the clinical suspicion of pneumonia was based on the presence of new or progressive pulmonary infiltrates on chest radiograph along with 2 of the following features: pyrexia with a temperature ≥38.5°C, purulent tracheobronchial secretions, and leukocytosis with a total peripheral leukocyte count ≥12, 000/mm^3. All episodes of suspected ventilator-associated pneumonia with fever and pulmonary density were retrospectively reevaluated and all differential diagnoses were excluded (*21*). Since patients might have been treated for pneumonia more than once during the 18-month study period, episodes rather than individual patients were the unit of analysis. Beginning from the time of admission, all occasions on which pneumonia had been diagnosed in individual patients were considered a single episode of pneumonia unless the interval between 2 such occasions exceeded 30 days. Excluded from the final analyses were patients who did not recover between 2 episodes of pneumonia.

Data Collection

Samples used for this study resulted from the current residual sampling strategy of the ICU; no supplement sampling was performed for the study. The identity of patients who provided samples and questionnaire information before the study remained confidential according to French law. We collected clinical data by using a standardized questionnaire that included sociodemographic data (age, sex), medical history (chronic obstructive bronchopneumonia, asthma, cystic fibrosis, smoking and alcohol habits, immunosuppression, cancer, HIV infection, malnutrition, tuberculosis), hospitalization data (surgery, inhalation therapy, duration of ventilation, and antimicrobial drug use), and type of pneumonia (nosocomial or community acquired). Acute respiratory distress syndrome (ARDS) was defined according to the American-European consensus (*22*). Information on immunosuppression was obtained for patients with a history of cancer, organ transplants, splenectomy, HIV infection (when the CD4+ T-cell count was <200 cells/μL), and immunosuppressor or steroid treatment (≥0.5 mg/kg prednisone for ≥30 days or ≥5 mg/kg prednisone for ≥5 days).

Study Design

The diagnostic strategy included bronchoalveolar lavage (BAL) fluid, blood cultures, and serologic and urine samples. BAL was performed by wedging the bronchoscope into a subsegment of the area of the lung when greatest abnormality was seen on a radiograph, or when the disease was diffuse, into the lingual or right middle lobe. Normal saline was sequentially instilled in 20-mL aliquots and sectioned into sterile traps for microbiologic testing for AAMs. This testing included culturing onto an agar base containing buffered charcoal yeast extract and enriched with α-ketoglutarate and L-cysteine (*23*) (Oxoid, Dardilly, France) with cefamandole, polymyxin B, and anisomycin for *Legionella* spp. cultivation; coculture with amebas as previously reported (*24*,*25*) for AAM; and TaqMan real-time polymerase chain reaction assay for enhanced detection of AAMs (*Legionella pneumophila*, *L. anisa*, *Parachlamydia* spp., *Bosea* spp., and *A. polyphaga mimivirus*). DNA was extracted from BAL samples by using the QIAMP tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Acute- and convalescent-phase serum samples were drawn into vacutainer tubes (Becton Dickinson, Rutherford, NJ, USA) and tested by immunofluorescence assay for antibodies to *L. pneumophila*, *L. anisa*, *L. bozemanii*, *L. longbeachae*, *L. micdadei*, and other AAMs (*15*,*18*), including *Parachlamydia acanthamoeba* (strain BN 9 and "Hall's coccus"), *Afipia birgiae*, *A. broomeae*, *A. clevelandensis*, *A. felis*, *A. felis* genospecies A, *Afipia* genospecies 1–3, *A. massilliae*, *Azorhizobium caulinodans*, *Bosea eneae*, *B. massiliensis*, *B. thiooxydans*, *B. vestrisii*, *Bradyrhizobium japonicum*, *B. liaoningense*, *L. quinlivanii*, *L. rubrilucens*, *L. worsleiensis*, *Mesorhizobium amorphae*, *Rasbo bacterium*, and *Acanthamoeba polyphaga mimivirus* (*13*,*15*). A urine sample was tested for *L. pneumophila* serogroup 1 antigen by enzyme-linked immunosorbent assay (*26*) (Binax, Inc., Portland, ME, USA). Data on non-AAMs isolated from BAL or blood cultures were obtained by conventional or specific procedures (culture performed on Löwenstein-Jensen medium, shell-vial culture for cytomegalovirus, and inoculation onto continuous cell lines for indirect immunofluorescence assay for herpes simplex virus). *Mycoplasma pneumonia*, *Chlamydia pneumoniae*, *C. psittacci*, *Coxiella burnetii*, influenza viruses A and B, and adenovirus were also identified.

Main Outcome Measures

Two groups of microorganisms were defined. The first was AAM (*Legionella* spp., *Afipia* spp., *Bosea* spp., *Bradyrhizobium* spp., *Mesorhizobium* spp., *Rasbo bacterium*, *Parachlamydia* spp., and *A. polyphaga mimivirus*). The second group was other water-associated microorganisms (*Pseudomonas aeruginosa* and AAM). Diagnosis of AAM infection was classified as having a strong or low level of evidence. The role of an infectious agent in the diagnosis reflected several factors, which included the relationship of the anatomic site of detection to the lung,

reliability of the method, and whether the putative agent was a known cause of pneumonia.

Strong evidence for AAM included 1) positive BAL culture, 2) 4-fold increase in antibody titer between acute- and convalescent-phase serum samples or seroconversion from 0 to 1:128 for *L. pneumophila*, *L. anisa*, *L. bozemanii*, *L. micdadei*, and *L. longbeachae*; and from 0 to 1:100 for *L. quinlivanii*, *L. rubrilucens*, *L. worsleiensis*, *Afipia* spp., *Bosea* spp., *Bradyrhizobium* spp., *Mesorhizobium* spp., *Parachlamydia* spp., *R. bacterium*, and *A. polyphaga mimivirus*; and 3) positive results for *L. pneumophila* antigen.

A low level of evidence for AAM included a stable antibody titer of ≥1:256 for *L. pneumophila*, *L. anisa*, *L. bozemanii*, *L. micdadei*, and *L. longbeachae*; ≥1:400 for *L. quinlivanii*, *L. rubrilucens*, *L. worsleiensis*, *Afipia* spp., *Bosea* spp., *Bradyrhizobium* spp., *Mesorhizobium* spp., *R. bacterium*, and *A. polyphaga mimivirus*; and ≥1:200 for *P. acanthamoeba*.

Statistical Analysis

Results are expressed as mean ± standard deviation (SD). Continuous variables were compared by using the Student t-test or the nonparametric Mann Whitney U test when they could not be judged normal. Categoric variables were compared by using the χ^2 test or Fisher exact test when appropriate. Statistical significance was established at $p<0.05$. All analyses were performed with SPSS version 10 software (SPSS Inc., Chicago, IL, USA).

Results

A total of 157 patients with 210 episodes of pneumonia were included in the study. The frequency of pneumonia episodes per patient was 1 in 123 patients, 2 in 22 patients, 3 in 7 patients, 4 in 3 patients, and 5 in 2 patients. The mean ± SD age was 61.6 ± 16.1 years (range 19–99) and 73.8% of the patients were male. Samples were collected in 62 episodes of community-acquired pneumonia, 120 episodes of nosocomial pneumonia, and 28 episodes of mixed pneumonia (community-acquired, complicated with a nosocomial infection). Data collected for 201 episodes of pneumonia indicated a prevalence of 18.4% with chronic obstructive bronchopneumonia, 6.5% with asthma, 0.5% with cystic fibrosis, 41.8% with smoking habits (19% in males vs. 26% in females; $p = 0.004$), 17.4% with alcohol consumption, 37.3% with immunosuppression (3 cases of prolonged steroid treatment for inflammatory disease [1.5%], 3 HIV infections [1.5%], 2 splenectomies [1.0%], 3 lung transplants [1.5%], 8 kidney transplants [4.0%] and 57 cancers [28.4%]), 6.0% with malnutrition, 5.5% with a history of tuberculosis, 29.8% with previous surgery (62/208), 38.4% with probable or certain inhalation therapy (78/203), and 38.9% with antimicrobial drug therapy for >1 week (70/180). The mean ± SD duration of hospitalization and ventilation was 22.9 ± 32.6 days (range 0–371) and 16.3 ± 19.9 days (range 0–101 days), respectively. Data on antimicrobial treatment before BAL was available for 208 patients. Of these, 116 (55.8%) received an antimicrobial drug, 16 (7.7%) an antiviral drug, and 18 (8.7%) an antimycotic drug.

Some patients had several definite or possible pathogens. A total of 230 documentations corresponding to 40 etiologic agents were identified in 152 (72.4%) of 210 episodes of pneumonia. Eighty-six (41.0%) BAL specimens were contaminated with fungi. Table 1 summarizes the non-AAMs identified as definite (28 in 27 [12.9%] of 210 episodes) or possible (143 in 115 [54.8%] of 210 episodes).

Laboratory investigations for AAMs detected 59 diagnoses in 40 (19.0%) patients. More than 1 AAM was observed in 56 episodes of pneumonia (26.7%); 39 (18.6%) had 2 AAMs, 11 (5.2%) had 3 AAMs, 3 (1.4%) had 4 AAMs, 2 (1.0%) had 5 AAMs, and 1 (0.5%) had 6 AAMs. Ten patients had serologic evidence of mixed infections with AAMs. Of the 40 patients with documented AAM infections, 18 (8.6% of our series) had evidence of AAMs (Table 2); 9 of these patients showed a high level of evidence. Evidence of pneumonia caused by unique AAMs was obtained in 13 patients. Of these, 5 had pneumonia caused by *A. polyphaga mimivirus*, 5 caused by *L. pneumophila*, 2 caused by *L. bozemanii*, and 1 caused by *Parachlamydia* sp. Mixed infections in these patients with 2, 3, and 5 AAMs were observed in 1, 2, and 2 patients, respectively. A unique AAM was observed in 13 patients (5 with *A. polyphaga mimivirus*, 5 with *L. pneumophila*, 2 with *L. bozemanii*, and 1 with *Parachlamydia* sp.).

A total of 22 (10.5%) of 210 episodes of pneumonia were observed in which both AAMs and conventional microorganisms were detected (Table 3). Six patients had diagnoses of AAM infection with a high level of evidence. Three of these 6 patients also had definite diagnoses of pneumonia caused by AAMs, and 3 others had a possible diagnosis of pneumonia caused by non-AAMs. Sixteen patients had diagnoses of pneumonia caused by AAMs with a low level of evidence. Three of these patients also had definite diagnoses of pneumonia caused by non-AAMs, and 13 had possible diagnoses of pneumonia caused by non-AAMs.

Fifteen patients were identified as having definite cases of pneumonia caused by AAMs. This subgroup (of whom 1 had a definite diagnosis of *S. aureus* infection and 1 of *C. pneumoniae* infection), included 8 patients with pneumonia caused by *A. polyphaga mimivirus*, 3 with pneumonia caused by *L. pneumophila*, and 5 who seroconverted. Those who seroconverted included any patient with seroconversion for *L. anisa*, *Parachlamydia* sp., *B. massiliensis*,

L. worsleiensis, *L. quinlivanii*, *L. rubrilucens*, *M. amorphae*, and *R. bacterium*. In addition, 1 who seroconverted also had a diagnosis of infection with *P. aeruginosa* and *B. alpica*. Eleven patients had possible infections with *Legionella* sp. (*L. pneumophila* in 7, *L. bozemanii* in 3, and *L. anisa* in 1), and 19 patients had possible infections with atypical organisms (*A. polyphaga mimivirus* in 7, *B. japonicum* in 6, *B. massiliensis* in 4, *B. liaoningense* in 3.

Table 1. Identification of 26 nonameba-associated microorganisms in 210 episodes of pneumonia

Microorganism	Definite,* no. (%)	Possible,† no. (%)	Total, no. (%)
Community-acquired pneumonia			
Bacteria			
Acinetobacter baumanii		2 (1.4)	2 (1.2)
Chlamydia pneumoniae	1 (3.6)	2 (1.4)	3 (1.8)
C. psittacci	1 (3.6)	2 (1.4)	3 (1.8)
Enterobacter cloacae		1 (0.7)	1 (0.6)
E. faecalis		1 (0.7)	1 (0.6)
Escherichia coli	2 (7.1)	2 (1.4)	4 (2.3)
Haemophilus influenzae	1 (3.6)	3 (2.1)	4 (2.3)
Mycobacterium tuberculosis	1 (3.6)		1 (0.6)
Pseudomonas aeruginosa	1 (3.6)	2 (1.4)	3 (1.8)
Serratia marcescens		1 (0.7)	1 (0.6)
Staphylococcus aureus	1 (3.6)	5 (3.5)	6 (3.6)
Streptococcus agalactiae		1 (0.7)	1 (0.6)
S. pneumoniae		3 (2.1)	3 (1.8)
Fungi			
Pneumocystis carinii	3 (10.7)		3 (1.8)
Viruses‡			
Cytomegalovirus		2 (1.4)	2 (1.2)
Herpes simplex virus 1		4 (2.8)	4 (2.3)
Ventilator-associated pneumonia			
Bacteria			
A. baumanii		1 (0.7)	1 (0.6)
Balneatrix alpica	1 (3.6)		1 (0.6)
C. pneumoniae		1(0.7)	1 (0.6)
Citrobacter koseri		1 (0.7)	1 (0.6)
Clostridium freundii		1 (0.7)	1 (0.6)
Coxiella burnetii		1 (0.7)	1 (0.6)
Enterobacter aerogenes		4 (2.8)	4 (2.3)
E. cloacae		5 (3.5)	5 (2.9)
E. coli	2 (7.1)	6 (4.2)	8 (4.7)
H. influenzae		1 (0.7)	1 (0.6)
Proteus mirabilis		3 (2.1)	3 (1.8)
Pseudomonas aeruginosa	9 (32.1)	31 (21.7)	40 (23.4)
Raoultella ornithinolytica		1 (0.7)	1 (0.6)
S. marcescens		2 (1.4)	2 (1.2)
S. aureus	2 (7.1)	21 (14.7)	23 (13.6)
S. epidermidis	1 (3.6)	1 (0.7)	2 (1.2)
Stenotrophomonas maltophilia		5 (3.5)	5 (2.9)
S. agalactiae		1 (0.7)	1 (0.6)
S. pneumoniae	1 (3.6)	2 (1.4)	3 (1.8)
Fungi			
Candida albicans	1 (3.6)		1 (0.6)
Viruses‡			
Cytomegalovirus		11 (7.7)	11 (6.4)
Herpes simplex virus 1		13 (9.1)	13 (7.6)
Total	28 (100.0)	143 (100.0)	171 (100.0)

*Detection of *M. tuberculosis* or *P. carinii* by bronchioalveolar lavage (BAL); simultaneous positive culture with BAL and blood culture; positive for influenza viruses A and B, adenovirus, or *C. burnetii* (immunoglobulin G2 [IgG2] titer ≥1:200, IgM2 ≥1:50); 4-fold increase in antibody titer between acute- and convalescent-phase serum; or seroconversion from 0 to 1:128 for *C. psittacci*, from 0 to 1:256 for *C. pneumoniae*, from 0 to 1 for *M. pneumoniae*, and from 0 to 1:100 for *B. alpica*.

†Detection of a potentially pathogenic microorganism (*M. tuberculosis* and *P. carinii*) by BAL and single or stable antibody titer ≥1:512 for *Chlamydia* spp., ≥1:2 for *M. pneumoniae*, and ≥1:400 for *B. alpica*.

‡No epidemic of influenza virus A/B or adenovirus was observed during the 18-month study period.

Table 2. Description of 18 cases of pneumonia with only identification of ameba-associated microorganisms

High level of evidence	Low level of evidence	No.
Acanthamoeba polyphaga mimivirus		5*
Legionella pneumophila		1†
L. pneumophila	*L. anisa*	1
Parachlamydia sp.	*Bosea thiooxydans*, *L. boozemanii*	1
Bosea massiliensis, *L. quinlivanii*, *L. rubrilucens*, *L. worsleiensis*	*Bradyrhizobium japonicum*	1
	L. pneumophila	4*
	L. bozemanii	2
	Parachlamydia sp.	1
	B. thiooxydans, *B. japonicum*, *Rasbo bacterium*	1
	L. pneumophila, *L. rubrilucens*, *B. massiliensis*, *B. japonicum*, *R. bacterium*	1

*One case of community-acquired pneumonia.
†Community-acquired pneumonia.

B. thiooxydans in 3, *R. bacterium* in 3, *Parachlamydiae* sp. in 2, and *L. rubrilucens* in 1).

The frequency of infections with AAMs is summarized in Table 4. *A. polyphaga mimivirus*, which was identified in 15 (7.1%) of 210 episodes of pneumonia, was the most common AAM. *Legionella* sp. were identified in 14 episodes. Three of these patients had mixed infections (*L. pneumophila* and *L. anisa* in 1, *L. pneumophila* and *L. rubrilucens* in 1, and *L. quilivanii*, *L. rubrilucens*, and *L. worsleiensis* in 1). *L. pneumophila*, which was identified in 10 (4.8%) of 210 episodes, was the second most frequently documented AAM. *Bradyrhizobium* sp. was identified in 9 patients; 6 of them were also infected with *B. japonicum*. Five of 8 patients infected with *Bosea* sp. were also infected with *B. massiliensis*. Four patients had serologic evidence of mixed infection with *B. japonicum* and *B. massiliensis*. The 7 most common etiologic agents were *P. aeruginosa* (20.5%), *S. aureus* (13.8%), herpes simplex virus (8.1%), *A. polyphaga mimivirus* (7.1%), cytomegalovirus (6.2%), *Escherichia coli* (5.7%), and *L. pneumophila* (4.8%). If one considers only diagnoses with a high level of evidence, the 4 most common etiologic

Table 3. Description of 22 cases of pneumonia with identification of ameba-associated and nonameba-associated microorganisms*

Ameba-associated level of evidence		Nonameba-associated identification	
High	Low	Definite	Possible
Acanthamoeba polyphaga mimivirus†		*Staphylococcus aureus*†	
A. polyphaga mimivirus		*Chlamydia pneumoniae*	
Mesorhizobium amorphae, *Rasbo bacterium*		*Pseudomonas aeruginosa*, *Balneatrix alpica*	
Legionella pneumophila			HSV1
L. anisa†			*Serratia marcescens*†
A. polyphaga mimivirus			*P. aeruginosa*
	L. pneumophila†	*P. aeruginosa*†	
	L. pneumophila	*Candida albicans*	
	Bosea massiliensis, *Bradyrhizobium japonicum*†	*Escherichia coli*†	
	Parachlamydia sp.		*Streptococcus pneumoniae*
	A. polyphaga mimivirus		*Enterobacter cloacae*, *C. pneumoniae*
	A. polyphaga mimivirus		CMV
	A. polyphaga mimivirus		*E. cloacae*
	A. polyphaga mimivirus		*E. aerogenes*, *P. aeruginosa*
	A. polyphaga mimivirus		*P. aeruginosa*
	A. polyphaga mimivirus		*S. aureus*
	Bosea thiooxydans, *A. polyphaga mimivirus*		*S. aureus*
	B. massiliensis, *B. japonicum*, *R. bacterium*		*Proteus mirabilis*, *S. aureus*
	B. massilensis, *B. japonicum*		HSV1
	Bradyrhizobium liaoningense		*S. aureus*
	B. liaoningense		*P. aeruginosa*
	B. liaoningense		*Stenotrophomonas maltophilia*

*HSV1, herpes simplex virus 1; CMV, cytomegalovirus.
†Community-acquired pneumonia.

agents were *P. aeruginosa* (4.8%), *A. polyphaga mimivirus* (3.8%), *E. coli* (1.9%), and *L. pneumophila* (1.4%).

A diagnosis was more frequent in a nosocomial context than outside a hospital (79.1% vs. 54.8%, $p<10^{-3}$), especially for *P. aeruginosa* ($p<10^{-6}$). Water-associated microorganisms were less likely to be identified in a community-acquired context than in a nosocomial context (30% vs. 50%, p = 0.005). Duration of hospitalization and ventilation were longer for patients infected with the water-associated microorganisms than for patients not infected (29 days vs. 19 days p = 0.015 and 21 days vs. 13 days, p = 0.008, respectively). Therapy with antimicrobial agents and a history of cancer were also more frequent in patients infected with water-associated microorganisms (54% vs. 30%, p = 0.001 and 39% vs. 22%, p = 0.014, respectively). Patients who seroconverted for *A. polyphaga mimivirus* used alcohol more frequently than others in the study (44% vs. 18%, p = 0.05).

Discussion

We conducted this study to determine the role of AAMs as causative agents of pneumonia in patients in an ICU. Concerns have been reported about the role of inline medication nebulizers contaminated with water-associated microorganisms, AAMs, or both (*11*,*14*,*15*). Other microorganisms, including *Legionella*-like amebal pathogens, *P. acanthamoeba*, *Afipia* sp., *Bosea* sp., *Bradyrhizobium* sp., *Mesorhizobium* sp., and *A. polyphaga mimivirus*, have also been reported (*14*,*19*,*20*,*27*). Our results indicate that AAMs represented 25.3% (59/233) of all documented causes of pneumonia and that 19.0% (40/210) of all episodes of pneumonia were associated with AAMs.

Marrie et al. reported that *Legionella*-like amebal pathogens might play a role in pneumonia, usually as coinfecting organisms (*18*). In 18 patients (8.6%), the role of AAMs were well documented. Nine of these patients had a high level of evidence for AAMs. Both conventional microorganisms and AAMs were implicated in 22 (10.5%) cases. However, 6 of them had high levels of evidence for AAM infections. Three of these 6 patients had documented infections with *L. pneumophila*, *L. anisa*, and *A. polyphaga mimivirus* and low levels of infection with herpes simplex virus, *S. marcescens*, and *P. aeruginosa.* The serologic evidence obtained from these patients demonstrates only that they were infected by these bacteria or a cross-reactive microorganism, not that these bacteria caused their pneumonia. However, the fact that only 8.6% had only indirect evidence of AAM infection raises questions about the potential pathogenic role of AAMs in pneumonia.

A. polyphaga mimivirus was the fourth most common cause of pneumonia in our study. This finding suggests that this organism may be clinically relevant. However, several lines of evidence now indicate that ameba-resisting microorganisms other than *Legionella* sp. are associated with both community- and hospital-acquired pneumonia (*19*,*28*). La Scola et al. (*13*) and Marrie et al. (*18*) have reported that the seroprevalence of *Legionella* was higher than that of other AAMs. Except for *L. pneumophila* findings, our results agree. The seroprevalence of *Legionella* (7.1%) in our series was lower than that reported by others in community-acquired (9.7%) and hospital-acquired (19.2%) pneumonia (*13*). However, this prevalence was significantly higher ($p<0.002$) than that observed (2.3%) in a healthy control population (*13*). These data also suggest that some patients with ventilator-associated pneumonia might have been in contact with *A. polyphaga mimivirus* or other cross-reactive antigens. These results raise questions about the pathogenic potential of the largest virus known or cross-reactive antibodies to an unknown organism (*13*).

We observed a significantly lower prevalence of seroconversion ($p<10^{-2}$) for other AAMs than was found in a previous series: 32 (15.2%) of 210 serologically diagnosed cases of AAM pneumonia compared with 12 (40.0%)

Table 4. Identification of ameba-associated microorganisms in pneumonia and level of evidence

Microorganism	High, no. (%)	Low, no. (%)	Total, no. (%)
Community-acquired pneumonia			
Bacteria			
Bosea massiliensis		1 (2.5)	1 (1.7)
Bradyrhizobium japonicum		1 (2.5)	1 (1.7)
Legionella anisa	1 (5.3)		1 (1.7)
L. pneumophila	1 (5.3)	2 (5.0)	3 (5.1)
Virus			
Acanthamoeba polyphaga mimivirus	2 (10.5)		2 (3.4)
Ventilator-associated pneumonia			
Bacteria			
B. massiliensis	1 (5.3)	3 (7.5)	4 (6.8)
B. thiooxydans		3 (7.5)	3 (5.1)
B. japonicum		5 (12.5)	5 (8.5)
B. liaoningense		3 (7.5)	3 (5.1)
L. anisa		1 (2.5)	1 (1.7)
L. bozemanii		3 (7.5)	3 (5.1)
L. pneumophila	2 (10.5)	5 (12.5)	7 (11.9)
L. quinlivanii	1 (5.3)		1 (1.7)
L. rubrilucens	1 (5.3)	1 (2.5)	2 (3.4)
L. worsleiensis	1 (5.3)		1 (1.7)
Mesorhizobium amorphae	1 (5.3)		1 (1.7)
Parachlamydiae acanthamoebae	1 (5.3)	2 (5.0)	3 (5.1)
Rasbo bacterium	1 (5.3)	3 (7.5)	4 (6.8)
Virus			
A. polyphaga mimivirus	6 (31.6)	7 (17.5)	13 (22.0)
Total	19 (100)	40 (100)	59 (100)

patients hospitalized in another ICU (*15*). The serologic evidence (e.g., seroconversion) obtained in this study strongly suggests that this patient population may have been exposed to the most common water ameba-associated bacteria in their environment (*15*). No environmental investigations were performed in our epidemiologic survey. The lower seroprevalence of AAMs in our patients suggest that they may have had less exposure in our hospital ICU compared with that observed in previous studies.

Interest in free-living amebas has grown over the last decade with reports of their pathogenic potential (*11*,*29*) and the role of amebas as reservoirs for *L. pneumophila* and other AAMs (*12*–*15*,*17*,*27*,*30*,*31*). Since respiratory care protocols use only sterile water, 2 possible routes of infection with AAMs include a breach in protocol enforcement and handborne AAMs. Adherence to these protocols and use of water filters ensures better protection of water supplies, as is the case in our ICU.

An interesting finding was that ≈44.8% of the patients with severe pneumonia had mixed causes. AAM was implicated in 12.9% of these patients. Fagon et al. reported that only one third of the therapeutic regimens proposed for pneumonia patients needing ventilators were effective (*32*). Because the recommended empiric approaches in guidelines are based on microbial patterns derived from several epidemiologic surveys (*33*), clinicians need to know the local, regional, and global patterns of microbial populations and the possibility of emerging pathogens such as AAMs. If these microorganisms are human pathogens, they will influence the choice of antimicrobial drugs for empiric treatment because most are resistant to carboxypenicillins, ureidopenicillins, third-generation cephalosporins, and fluoroquinolones, which are commonly used in the ICU.

AAMs may cause ventilator-associated pneumonia and should be suspected when results of conventional microbiologic investigations are negative (*11*,*15*,*19*,*28*). A diagnosis is rarely available at the time treatment with antimicrobial agents is begun. Thus, the prevailing situation warrants better diagnosis of pneumonia and identification of new lung pathogens such as AAMs. Recognizing the emerging pathogens responsible for pneumonia should be a major public health concern because the knowledge of predominant microbial patterns will help provide the basis for rational empiric antimicrobial treatment.

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Rickettsial Infections and Fever, Vientiane, Laos

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Rickettsial diseases have not been described previously from Laos, but in a prospective study, acute rickettsial infection was identified as the cause of fever in 115 (27%) of 427 adults with negative blood cultures admitted to Mahosot Hospital in Vientiane, Laos. The organisms identified by serologic analysis were *Orientia tsutsugamushi* (14.8%), *Rickettsia typhi* (9.6%), and spotted fever group rickettsia (2.6% [8 *R. helvetica*, 1 *R. felis*, 1 *R. conorii* subsp. *indica*, and 1 *Rickettsia* "AT1"]). Patients with murine typhus had a lower frequency of peripheral lymphadenopathy than those with scrub typhus (3% vs. 46%, p<0.001). Rickettsioses are an underrecognized cause of undifferentiated febrile illnesses among adults in Laos. This finding has implications for the local empiric treatment of fever.

The Lao People's Democratic Republic (Laos) is situated mostly east of the Mekong River and borders Thailand, Cambodia, Burma (Myanmar), China, and Vietnam. Most (83%) of the population of 5.2 million are rural rice farmers, the per capita income is US $326/year, and life expectancy is 54 years (*1*). Although more data have been obtained in wealthier countries in Asia, minimal information exists on the clinical epidemiology of infectious disease in Laos.

The etiology of fever in Laos usually remains obscure because of limited laboratory diagnostic facilities. In 2000, the main differential diagnoses for adults admitted with fever to the hospital in Vientiane, the capital, were slide-positive malaria or slide-negative *syndrôme paludéen*, or malaria syndrome: both were treated with antimalarial drugs and the latter with additional antimicrobial drugs (unpub. data). Rickettsial diseases, caused by *Orientia tsutsugamushi* (scrub typhus), *Rickettsia typhi* (murine typhus), and members of the spotted fever group (SFG), cause fever in Thailand, Malaysia, China, and Vietnam (*2–5*), and their public health consequences have recently been emphasized in Sri Lanka (*6*) and Nepal (*7*). Indonesian peacekeeping troops seroconverted to *O. tsutsugamushi* and *R. typhi* during their residence in Cambodia (*8*), but acute, symptomatic infections with rickettsia have not been described there since the 1930s (*9*).

No studies that examined the causes of fever in Laos, which has economic, cultural, and geographic differences from adjoining countries, have been published. Such information is crucial in developing appropriate diagnostic tests and guidelines, determining empiric treatment for nonmalarious fever, and planning public health interventions. The mite vectors of scrub typhus have been described from Laos (*10*), but no rickettsial disease has been described from the country, apart from the seroconversion of US troops to *O. tsutsugamushi* (*11*). Therefore, we conducted a 2-year prospective study of the causes of fever among adults admitted to Mahosot Hospital, who were both blood-culture and malaria-smear negative, to determine the causes of *syndrôme paludéen*. We describe the serologic test results for rickettsiae.

Methods

Study Site and Patients

The study was conducted at Mahosot Hospital, Vientiane, a 365-bed primary- to tertiary-care hospital that

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specializes in internal medicine, which has ≈1,200 admissions per month. This hospital, along with 4 other major hospitals (1,210 beds total) and local provincial and district hospitals, serves a population of ≈900,000 people, including the urban population of Vientiane City and surrounding farming communities of Vientiane Province, and less frequently, outlying provinces. We recruited patients admitted from November 2001 to October 2003 on all 4 adult medical wards (including an adult intensive care unit), making up 91 beds. Ethical clearance was granted by the Faculty of Medical Sciences Ethical Review Committee, National University of Laos.

Clinical Procedures

All adults (>15 years of age) admitted with fever had blood cultures taken if community-acquired septicemia was suspected and they gave verbal informed consent. If the patient came from an area of Laos with endemic malaria, Giemsa-stained malaria thick and thin films were examined. If the blood culture showed no clinically meaningful growth after 3 days of incubation, the malaria film was negative, and the patient gave verbal informed consent, a 5-mL whole blood sample was taken for serum analysis. An additional 5-mL convalescent-phase venous blood sample was collected ≈1 week later. The presence of eschars was not recorded systematically, since without evidence of rickettsial infection they were not routinely looked for. Patients' conditions were further investigated and treated according to local hospital practice.

Laboratory Procedures

Serum samples were stored at –80°C until analysis. Specific microimunofluorescence (IFA) assays were performed in Marseille, France, by using whole-cell antigens of *O. tsutsugamushi* serotypes Karp, Kato, Gilliam, and Kawasaki (*12*) and with *Bartonella henselae*, *Coxiella burnetii*, *R. conorii* subsp. *indica*, *R. felis*, *R. heilongjiangensis*, *R. helvetica*, *R. honei*, *R. japonica*, *Rickettsia* "ATI," *R. slovaca*, and *R. typhi* (*13–15*). An IFA result was considered positive if any of the following were detected: 1) positive antibody titers >1:128 for immunoglobulin G (IgG) and >1:64 for IgM, 2) seroconversion, or 3) ≥4-fold increase in titers between acute- and the convalescent-phase serum (*5,13*). Western immunoblotting was performed on samples positive for *Rickettsia* spp. both before and after cross-absorption with relevant antigens (*12,13*). Full blood counts (n = 364) and serum biochemical test results (n = 352) were analyzed on Abx MICROSOT (Abx Hematologie, Montpellier, France) and Cobas Integra (Roche Co. & Tegimenta Ltd, Rotkreuz, Switzerland) analyzers, respectively.

Statistical Analysis

Analysis was performed by using Stata v. 8 (StataCorp LP, College Station, TX, USA). Categoric variables were compared with Fisher exact test and continuous variables by Student *t* test and Mann-Whitney U test as appropriate. Multivariate logistic regression (backwards) was performed to evaluate variables associated with serologic diagnoses.

Results

Serology

During the 2 years of the study, 466 adults were recruited; clinical and laboratory data, including rickettsial serology, were available for 427. Forty-five patients (12.6%) did not have a prior blood culture, and 218 (51%) had a convalescent-phase serum sample taken (median 5 [range 1–50] days after the admission sample).

Of 427 patients, serologic evidence for acute rickettsial infections were found in 115 (26.9%): *O. tsutsugamushi* in 63 (14.8%), *R. typhi* in 41 (9.6%), and SFG rickettsiae in 11 (2.6% [8 *R. helvetica*, 1 *Rickettsia* "AT1," 1 *R. felis*, and 1 *R. conorii* subsp. *indica*]). No serologic evidence was found for acute *B. henselae*, *C. burnetii*, *R. heilongjiangensis*, *R. honei*, *R. japonica*, or *R. slovaca* infection. Of the 63 patients with serologic evidence of infection with *O. tsutsugamushi*, the highest titers were with the Gilliam serotype for 9 patients, the Gilliam or Kawasaki serotype in 9, the Gilliam or Kato serotype in 6, and all 3 serotypes in 39.

Clinical Features

Patients with scrub typhus could not be distinguished reliably from those with murine typhus at the bedside or in retrospective review of all clinical and laboratory details (Table 1). Patients with scrub typhus had a higher frequency of lymphadenopathy and abnormal chest examination than patients with murine typhus ($p<0.001$ and $p = 0.002$, respectively). The respiratory rate was faster for those with scrub typhus than those with murine typhus ($p = 0.0012$). Multiple logistic regression suggested that, in comparison to patients with murine typhus, the presence of lymphadenopathy and a faster respiratory rate were independently associated with scrub typhus (lymphadenopathy, abnormal chest examination, and respiratory rate were entered). Raised (>90 IU/L) serum creatinine kinase concentrations were found in 273 (63.9%) of patients in the serologic study: 57% with scrub typhus, 63% with murine typhus, and 50% with positive *R. helvetica* serologic test results. Patients with rickettsioses who had myalgia on admission had significantly higher serum creatinine kinase (geometric mean 119 IU/L, 95% confidence interval [CI]

Table 1. Admission clinical features of 104 Lao adults with serologic evidence of acute murine and scrub typhus*

Variable	Murine typhus (n = 41)†	Scrub typhus (n = 63)†	p value	Reference range
Age, (y)‡	40 (17–70)	31 (16–73)	0.5	
No. (%) male	26 (63)	40 (63)	0.6	
No. days ill‡	11 (3–30)	10 (2–42)	0.2	
Headache (%)	38 (95) (n = 40)	60 (95)	1.0	
Abdominal pain (%)	17 (43) (n = 40)	22 (35)	0.5	
Nausea (%)	18 (45) (n = 40)	39 (62)	0.09	
Vomiting (%)	11 (28) (n = 40)	25 (40)	0.3	
Diarrhea (%)	7 (18) (n = 40)	22 (35)	0.07	
Cough (%)	14 (35) (n = 40)	24 (38)	0.8	
Sputum (%)	8 (20) (n = 40)	13 (21)	1.0	
Dyspnea (%)	5 (13) (n = 40)	7 (11)	1.0	
Chest pain (%)	3 (8) (n = 40)	13 (21)	0.1	
Back pain (%)	15 (38) (n = 40)	19 (30)	0.5	
Dysuria (%)	3 (8) (n = 40)	2 (3)	0.4	
Arthralgia (%)	10 (25) (n = 40)	13 (21) (n = 62)	0.6	
Myalgia (%)	34 (85) (n = 40)	59 (95) (n = 62)	0.1	
Sore throat (%)	3 (8) (n = 40)	12 (19)	0.2	
Lymphadenopathy (%)	1 (3) (n = 38)	27 (46) (n = 59)	<0.001	
Bleeding (%)	2 (5) (n = 39)	4 (6) (n = 58)	1.0	
Convulsions (%)	0 (n = 40)	1 (2) (n = 62)	1.0	
Rash (%)	5 (13) (n = 38)	16 (27) (n = 59)	0.1	
Abnormal chest exam (%)	1 (3) (n = 38)	16 (27) (n = 59)	0.002	
Abdominal tenderness (%)	1 (3) (n = 39)	6 (10) (n = 59)	0.2	
Liver palpable (%)	27 (73) (n = 37)	30 (52) (n = 58)	0.05	
Spleen palpable (%)	6 (15) (n = 39)	9 (15) (n = 59)	1.0	
Temperature (°C)‡	38.5 (38.2–38.8) (n = 36)	38.6 (38.4–38.9) (n = 56)	0.3	
Pulse/min§	91.1 (87.1–95.1) (n = 36)	95.7 (91.8–99.6) (n = 54)	0.09	
Systolic blood pressure (mm Hg)‡	100 (80–130) (n = 36)	100 (90–150) (n = 54)	0.6	
Diastolic blood pressure (mm Hg)‡	70 (50–80) (n = 36)	65 (50–110) (n = 54)	0.8	
Respiratory rate/min§	20.7 (19.9–21.6) (n = 36)	22.9 (22.0–23.8) (n = 58)	0.0012	
Glasgow Coma Score‡	15 (15) (n = 38)	15 (7–15) (n = 59)	0.3	
Meningism (%)	2 (5) (n = 39)	7 (12) (n = 59)	0.3	
Hematocrit (%)‡	40 (13–48) (n = 35)	40 (23–50) (n = 53)	0.7	
Leukocyte count ($\times 10^9$/L)‡	10.4 (3.1–38) (n = 37)	11.8 (0.7–26.3) (n = 54)	0.1	4.0–11.0
Neutrophils (%)‡	68 (26-86) (n = 37)	70 (0–93) (n = 54)	0.2	
Platelets ($\times 10^9$/L)§	190 (23–350) (n = 37)	200 (192–208) (n = 49)	0.4	150–400
Serum creatinine (μmol/L)‡	106 (70–466) (n = 32)	106 (70–783) (n = 53)	0.7	53–123
Serum AST (IU/L)‡	87 (32–789) (n = 31)	86 (16–437) (n = 52)	0.6	7–35
Serum ALT (IU/L)‡	39 (20–234) (n = 31)	48 (12–180) (n = 52)	0.7	7–35
Serum albumin (g/L)‡	38 (26–50) (n = 32)	35 (22–49) (n = 53)	0.2	35–50
Serum creatinine kinase (IU/L)¶	113 (80–159) (n = 32)	121 (70–210) (n = 53)	0.8	24–190
Serum alkaline phosphatase (IU/L)‡	156 (47–532) (n = 32)	175 (55–745) (n = 53)	0.5	120–290
Serum direct bilirubin (μmol/L)‡	8.3 (1.7–60.4) (n = 32)	8.3 (2.6–83.0) (n = 52)	0.9	0.5–8.8
Serum total bilirubin (μmol/L)‡	17.9 (7.7–109) (n = 32)	18.4 (7.7–131) (n = 52)	0.4	1.7–20
No. patients serum total bilirubin >50 μmol/L (%)	2 (6) (n = 32)	4 (8) (n = 52)	1.0	
No. patients serum AST >105 IU/L (%)	11 (36) (n = 31)	18 (35) (n = 52)	1.0	
No. patients serum ALT >105 IU/L (%)	6 (19) (n = 31)	5 (10) (n = 52)	0.3	
Deaths (%)	0	1 (1.5)		

*AST, aspartate aminotransferase; ALT, alanine aminotransferase; CI, confidence interval.
†The available sample size is given in parentheses where the entire sample was not available for a given variable.
‡Median (range).
§Mean (95% CI).
¶Geometric mean (95% CI).

90–158) concentrations than those who did not (geometric mean 47 IU/L, 95% CI 26–87) (p = 0.02).

Seventeen patients with scrub typhus (27.0%) had evidence for severe organ dysfunction; 7 (11.9%) of 59 had meningismus, 7 (11.1%) of 63 had dyspnea, and 7 (13.2%) of 53 had a serum creatinine level >133 μmol/L. While 4 (7.7%) of 52 patients had a total serum bilirubin level >50 μmol/L, 18 (34.6%) of 52 had a serum aspartate aminotransferase (AST) level >3 times the upper limit of the reference range. Nine patients with murine typhus and severe organ dysfunction (22.5% of 40 patients with data) were also encountered; 2 (5.1%) of 39 had meningism, 5 (12.5%) of 40 had dyspnea, and 2 (6.3%) of 32 had a serum creatinine level >133 μmol/L.

Of the 8 patients with serologic evidence of acute *R. helvetica* infection, 6 had headache, 4 had vomiting, 1 had diarrhea, 2 had cough, 2 had dyspnea, 7 had myalgia, 4 had a palpable liver, and none had palpable lymphadenopathy or splenomegaly (Tables 2 and 3). One had a petechial rash at admission, and rash developed in 1 patient 2 days after

Table 2. Clinical features of patients with serologic evidence for acute spotted fever rickettsioses admitted to Mahosot Hospital*

Patient no.	Age (y), sex	Occupation	Month of onset of illness	Clinical features	Home
45	30, male	Construction worker	March	18-day fever, myalgia, nausea, epistaxis, vomiting, abdominal pain, petechial rash on trunk and legs; liver and spleen not palpable; treated with ampicillin and gentamicin	Vientiane City
72	35, female	Teacher	April	13-day fever, chills, headache, nausea, myalgia, vomiting, conjunctival suffusion, dyspnea, 12-cm liver; treated with ofloxacin	Vientiane City
86	25, male	Health worker	May	11-day fever, headache, nausea, vomiting, abdominal pain, 10-cm liver	Vientiane City
114	18, male	Student	June	14-day fever, chill, headache, arthralgia, myalgia, rash developed 2 days after admission, 12-cm liver; treated with ofloxacin	Vientiane Province
198	50, male	Government official	September	24-day fever, headache, arthralgia, myalgia, vertigo, epistaxis, diarrhea; abdominal CT suggested hepatic carcinoma; no antimicrobial drug	Xieng Khuang Province
237	64, male	Government official	September	21-day fever, myalgia, arthralgia, abdominal pain, sore throat, cough, dyspnea; chest exam abnormal	Vientiane City
290	24, female	Construction worker	March	7-day fever, headache, vomiting, myalgia, unproductive cough, diarrhea; treated with doxycycline	Vientiane City
362	23, female	Student	June	10-day fever, myalgia, headache, conjunctival suffusion, 8-cm liver; treated with doxycycline	Vientiane City
297	43, female	Housewife	March	14-day fever, headache, jaundice, RUQ pain, myalgia, 8-cm hepatomegaly; abdominal CT suggested tumor of intrahepatic bile ducts (cholangiocarcinoma?); treated with ampicillin and gentamicin	Xieng Khuang Province
55	34, female	Housewife	April	7-day fever, chills, headache, myalgia, diarrhea, abdominal pain, nausea, vomiting, rash on arms and abdomen; treated with oral chloramphenicol	Vientiane Province
239	46, male	Merchant	November	6-day fever, headache, myalgia, arthralgia, nausea, abdominal pain, diarrhea, dyspnea, dry cough, and sore throat; treated with doxycycline	Vientiane City

*CT, computed tomographic scan; RUQ, right upper quadrant.

admission. The median (range) serum biochemistry results for patients with *R. helvetica* infection were creatinine 85 (67–142) μmol/L, AST 84 (35–118) IU/L, alanine aminotransferase (ALT) 50 (14–87) IU/L, albumin 39 (23–45) g/L, creatinine kinase 49 (16–125) IU/L, alkaline phosphatase 115 (96–217) IU/L, direct bilirubin 4.8 (3.7–7.3) μmol/L, and total bilirubin 9.5 (8.8–16.8) μmol/L. None of the 8 patients had a bilirubin level >50 μmol/L or an ALT level >3 times the upper limit of the reference range, but 2 patients had an AST level >3 times the upper limit of the reference range.

Geographic Distribution

Districts in which patients lived were recorded for 417 (98%) patients in the serologic study; 73% lived in Vientiane City, and 22% Vientiane Province. The proportion of patients with a home address in Vientiane City was 71% for scrub typhus and 55% for murine typhus patients. Outside Vientiane City and Province, patients with scrub typhus came from Houaphanh and Borikhamxay Provinces, and patients with murine typhus came from Borikhamxay and Luang Prabang Provinces. Of the 11 patients with serologic evidence of spotted fever rickettsiosis, 7 were from Vientiane City, 2 from Vientiane Province, and 2 from Xieng Khuang Province.

Outcome

Of 63 patients with scrub typhus for whom outcome is known, 1 (1.6%) died in the hospital. This 23-year-old housewife died 14 days after delivering a healthy girl at home; she had gone to the hospital with a 1-week history of fever before parturition. Pneumonia, vaginal bleeding from retained placenta, and hypotension developed; her Glasgow Coma Score was 7 of 15. In the hospital, she underwent uterine curettage and received ampicillin, gentamicin, azithromycin, ceftriaxone, and metronidazole. Fever developed in the daughter, and she died 4 days after her mother. The death rate among adults with serologic evidence of an acute rickettsiosis was therefore 1 in 115 (0.9%).

Discussion

These serologic data suggest that scrub typhus and murine typhus are underrecognized causes of fever among adults in Vientiane. A wide diversity of rickettsiae were identified for the first time in Laos. Scrub typhus was the most common rickettsiosis identified. The patients tended to be young adult males presenting with fever, headache, nausea, myalgia, lymphadenopathy, and a palpable liver. Seventeen (27%) patients with scrub typhus had severe disease, and 18 (34.6%) had a liver biochemistry profile

Table 3. Serologic results of patients with serologic evidence for acute spotted fever rickettsioses admitted to Mahosot Hospital

Patient no.	Immunofluorescence results (IgG/IgM admission sample, IgG/IgM convalescent-phase sample)*							
	Rickettsia japonica	*R. helvetica*	*R. heilongjiangensis*	*R. slovaca*	*R. felis*	*R. honei*	*R. conorii*†	"AT1"‡
45	0/0, 1:256/1:128	**0/0, 1:1,024/1:256**	0/0, 1:1,024/1:128	0/0, 1:1,024/1:128	0/0, 1:256/1:256	0/0, 1:256/1:256	0/0, 0/1:256	0/0, 1:256/1:256
72	1:64/1:32	**1:128/1:32**	1:64/1:32	1:128/1:32	0/0	0/0	0/0	0/0
86	0/0, 1:64/0	**0/0, 1:64/1:128**	0/0, 1:64/0	0/0, 1:64/1:128	0/1:128, 0/1:128	0/0, 0/1:32	0/0, 0/1:32	1:128/0, 1:128/1:32
114	1:128/1:64	**1:256/1:512**	1:128/1:64	1:256/1:512	0/1:32	0/0	0/0	0/0
198	0/0	**1:128/1:64**	0/0	0/1:32	0/0	1:128/0	1:64/0	1:256/0
237	1:128/0	**1:256/1:32**	1:128/0	1:256/1:32	0/0	0/1:32	0/1:32	0/1:64
290	0/1:32, 0/1:32	**1:64/1:32, 1:64/1:32**	0/0, 0/0	0/0, 0/0	0/0, 0/0	0/0, 0/0	0/0, 0/0	0/1:32, 0/1:32
362	0/0, 0/0	**1:16/1:16, 1:32/1:32**	0/0, 0/0	0/0, 0/0	1:16/1:16, 1:16/1:32	0/0, 0/0	0/0, 0/0	0/0, 1:32/1:32
297	0/0, 0/1:64	0/0, 0/1:64	0/0, 0/1:64	0/0, 0/1:64	0/0, 0/0	0/0, 0/1:64	0/0, 0/0	**0/0, 0/1:64**
55	0/0, 1:64/1:32	0/1:64, 1:64/1:128	0/0, 1:64/1:32	0/1:64, 1:64/1:128	**0/0, 1:256/1:128**	0/0, 1:256/0	0/0, 1:64/0	0/0, 1:256/0
239	0/0	0/0	0/0	0/0	1:64/0	1:64/1:32	**1:64/1:32**	1:64/1:32

*Titers in **boldface** indicate the pathogen considered to be responsible for the serologic response.
†*R. conorii* subsp. *indica*.
‡*Rickettsia* "AT1" from Japan.

consistent with that of hepatitis. In a recent series of 462 patients with scrub typhus from Japan, lymphadenopathy, headache, myalgia, hepatomegaly, and eschar were recorded in 52%, 46%, 16%, 3%, and 87% of patients, respectively. Elevated serum AST and ALT levels were also common (87% and 77%, respectively) among these Japanese patients (*16*). In comparison to Lao patients, Japanese patients had a substantially lower prevalence of myalgia and hepatomegaly. The clinical importance of acute scrub typhus in the death of the Lao patient who also had retained placenta and probable intrauterine infection remains uncertain. Her infant may have died of neonatal scrub typhus (*17*). Of 12 case reports of scrub typhus in pregnancy (*17–19*), 8 recorded stillbirth, miscarriage, neonatal scrub typhus, or neonatal death, but all the mothers survived. During the 2 years of this study, patients with scrub typhus became ill in the late hot weather and monsoon, similar to observations made 60 years ago in Burma (*20*), but different from the geographically variable epidemiologic features noted in Japan (*16*). Recent clinical observations suggest that the prevalence of eschars in Lao patients with serologically confirmed scrub typhus when the entire skin surface is examined is ≈52% (unpub. data) and 0% in patients with confirmed murine typhus. Therefore, a thorough search for eschars will help with the diagnosis of scrub typhus.

Patients with murine typhus also tended to be young adult males with clinical profiles similar to those with scrub typhus but with a strikingly lower frequency of lymphadenopathy (3% vs. 46%). Similar proportions of patients with murine typhus and scrub typhus had raised serum bilirubin and AST levels. In a series of 137 patients with murine typhus in southern Thailand (*21*), 20% had skin rash, 24% had hepatomegaly, and 5% had splenomegaly. In contrast, among 83 Cretans, 80% had a rash, perhaps because it was easier to detect on fairer skin (*22*). A relatively low frequency of lymphadenopathy in patients with murine typhus has been described from Crete (4% [*22*]), Texas (16% of children [*23*]), and Spain (2% [*24*]). In the Lao series, cough was present in 35% of patients with murine typhus. Respiratory symptoms and signs have been reported among murine typhus patients with cough present in 59% (*25*), 15% (children [*23*]), 28% (*21*), and 25% (*24*) of patients. No concurrent comparisons have been made of clinical features of scrub and murine typhus at 1 site, but the Lao data suggest that the presence of peripheral lymphadenopathy, chest signs, and eschars are clinically useful signs that suggest scrub, rather than murine, typhus.

We also found serologic evidence for 4 SFG species. Although Western blotting and cross-absorbance studies were performed, evidence for rickettsiae in Laos is based on serologic methods and therefore, especially for SFG, needs to be confirmed by genetic analysis (*4*). Human SFG *Rickettsia* infections have been described in Thailand, China, Korea, Malaysia, and Japan (*3*,*4*,*26*,*27*) but not in Laos, Vietnam, Burma or Cambodia. Evidence for human *R. helvetica* infections has been found in Europe (*14*,*28*,*29*), Thailand (*5*), and possibly Australia or Japan (*30*). One of the Lao patients with apparent *R. helvetica* infection had a rash, unlike the 8 patients described previously with *R. helvetica* infection (*5*,*28*). Evidence for acute human infection with *R. felis* has been found in North and South America, Europe, and the Thailand/Burma border

(*5*,*31*). The clinical symptoms of the patient described from the Thailand/Burma border were similar to those of our Lao patient, and neither had a rash. Evidence for *R. conorii* has been found in India (*4*,*32*) and on the Thailand/Burma border (*5*). *Rickettsia* "AT1" was originally isolated from Japanese *Amblyomma* ticks, and its genotype is most closely related to rickettsiae from Slovakian *Ixodes* ticks (*33*). The relevance of *Rickettsia* "AT1" to human disease remains uncertain. Although no acute *C. burnetii* infections were found in this series, Q fever has recently been described from northeast Thailand (*34*).

Raised serum creatinine kinase levels have been described in patients with scrub typhus (*35*) and as an apparently nonspecific result of febrile illness (*36*). In a series of patients with fever in Israel, an elevated creatinine kinase level was associated with increased blood urea, low serum phosphate, reduced consciousness, tremor, and muscle tenderness. Alcoholism and high body temperature may also be associated factors (*36*). In Laos, a rise in creatinine kinase level may also have been a consequence of the common practice of administering intramuscular injections before hospital (unpub. data). Because serum creatinine kinase concentrations are higher in patients with rickettsioses who have myalgia than in those without, muscle pain is likely to be associated with mild muscle damage.

This study is of similar design to a recent investigation of the causes of fever in adults living in and around another tropical capital city, Kathmandu, although the Nepalese study included outpatients and sampled 4 months of 1 year (*7*). The frequency of rickettsioses was lower in patients in Kathmandu, with serologic evidence of acute infection with murine typhus in 11% and scrub typhus in 3%. The high incidence of patients in Vientiane who have diseases for which the vectors, such as chiggers and ticks, are likely to be predominantly rural is not surprising. Many inhabitants of the city visit farms in rural areas, and persons with occupations that would not conventionally be regarded as of high risk for rickettsioses may be exposed. In addition, suburban scrub typhus has been described (*37*).

The decision to enter a particular patient into the study was the responsibility of many doctors, and some infected patients may not have been recruited. Only 11% of the Lao population live in the relatively urbanized areas of Vientiane City, and the results of this study are unlikely to be applicable to the rest of the country, which is diverse in geography and ethnicity. A hospital-based study such as this will tend to underestimate the incidence of disease, and infections, such as scrub typhus, which tend to affect farmers, will be more common in rural Laos. Additional limitations of the study are that we did not perform serologic analysis on all patients who did not have a clinically meaningful blood culture during the study period, that the median interval between acute- and convalescent-phase serum samples was relatively short (5 days), and that 49% of patients did not have a convalescent-phase sample.

These data have affected local clinical practice. With the realization that scrub typhus is an important disease, patients' skin surfaces are now routinely completely examined for eschars, and doxycycline therapy is added at an earlier stage for patients with headache, fever, and myalgia. The drugs usually administered for *syndrôme paludéen* were ampicillin or cotrimoxazole, both of which are ineffective against rickettsiae. These results suggest that an antirickettsial agent, such as doxycycline, should be included in the empiric treatment of Lao adults with fevers whose clinical features are consistent with a rickettsiosis. However, analysis of the clinical features of patients in this study with rickettsiosis, leptospirosis, dengue, and typhoid (unpub. data) suggest that these diseases are difficult to distinguish reliably on clinical examination and that rapid, inexpensive diagnostic tests will help guide therapy. An oral drug with high efficacy against uncomplicated rickettsiosis, leptospirosis, and typhoid could be of considerable use. Azithromycin is effective in treating uncomplicated typhoid fever in Vietnam (*38*) and scrub typhus in Korea (*39*), and it may be effective against leptospires in vitro (*40*). In parallel with the adoption of effective artemisinin-based combination therapy for malaria in rural Laos, the need is urgent to develop rapid and inexpensive tests to diagnose alternative causes of fever and to improve the treatment of common nonmalarious fevers.

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Helicobacter pullorum in Chickens, Belgium

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A total of 110 broilers from 11 flocks were tested for *Helicobacter pullorum* by polymerase chain reaction; positive samples were reexamined with a conventional isolation method. *H. pullorum* isolates were examined by amplified fragment length polymorphism (AFLP) fingerprinting for interstrain genetic diversity and relatedness. Sixteen isolates from cecal samples from 2 different flocks were obtained. AFLP analysis showed that these isolates and 4 additional isolates from a different flock clustered according to their origin, which indicates that *H. pullorum* colonization may occur with a single strain that disseminates throughout the flock. Strains isolated from different hosts or geographic sources displayed a distinctive pattern. *H. pullorum* is present in approximately one third of live chickens in Belgium and may represent a risk to human health.

Helicobacter pullorum was originally isolated from the feces and damaged livers of broilers and laying hens (*1*,*2*). It was defined as a new species in 1994 by Stanley et al. (*1*). *H. pullorum* is a gram-negative, slightly curved rod with monopolar, nonsheathed flagella. It is bile resistant and requires a microaerobic environment supplemented with H_2 in which growth occurs at 37°C and 42°C (*1*,*3*–*6*). Enterohepatic *Helicobacter* species, including *H. pullorum*, are increasingly recognized as microbial pathogens in humans and animals (*3*,*5*,*7*–*9*). *H. pullorum* has been linked with enteritis and hepatitis in broiler chickens and laying hens and diarrhea, gastroenteritis, and liver disease in humans (*1*,*2*,*5*–*8*,*10*,*11*). *H. pullorum* can contaminate poultry carcasses at the abattoir and can be considered a foodborne human pathogen (*4*,*8*,*12*).

Almost no data are available on the prevalence of this species in poultry. Research that could generate these data is hampered by the fastidious growth requirements of *H. pullorum* and the phenotypic similarity between member species of the genera *Helicobacter* and *Campylobacter* (*3*,*4*,*12*). *H. pullorum* in chickens has been studied on only 2 occasions when the organism was detected by using isolation (*4*,*7*). Furthermore, no valid epidemiologic research methods have been recommended.

This study's objective was to determine the occurrence of *H. pullorum* in broilers by using both polymerase chain reaction (PCR) and isolation. In addition, amplified fragment length polymorphism profiling (AFLP) was conducted to investigate the genetic relatedness between *H. pullorum* isolates.

Methods

Sample Origin

Samples from the gastrointestinal tracts and livers of 110 broiler chickens, 10 per flock (flock number 1–11), collected at a poultry abattoir, were studied. Each gastrointestinal tract and liver sample was deposited in a separate waterproof plastic bag. Samples were taken from the liver, cecum, jejunum, and colon for PCR and isolation within 3 hours after collection. All samples were stored at –20°C and –70°C for PCR and isolation, respectively, until further analysis, as described below.

Sample Processing

PCR and Gel Electrophoresis

DNA was extracted from ≈25 mg cecum, colon, jejunum, and liver tissue with a commercial tissue kit (DNeasy Tissue Kit, Qiagen, Venlo, the Netherlands). A PCR assay amplifying a 447-bp fragment of the 16S rRNA gene of *H. pullorum* was then used for detection purposes (*1*). From each sample, 2 μL template was added to 8 μL PCR mixture containing 0.03 U/μL Taq polymerase Platinum (Invitrogen Life Technologies, Merelbeke, Belgium), 10× PCR Buffer (Invitrogen Life Technologies), 3 mmol $MgCl_2$ (Invitrogen, Life Technologies), 40 μmol/L each of deoxynucleoside triphosphate (Invitrogen Life

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Technologies), a final primer concentration of 0.5 μmol/L, and sterile distilled water. The conditions used for the amplifications were the following: an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 90 s, elongation at 72°C for 90 s, and a final elongation at 72°C for 5 min.

Five microliters of the PCR products of each sample were mixed with 3 μL of sample buffer 5× (50% glycerol, 1 mmol cresol red) and were subjected to electrophoresis through an agarose gel containing 1.5% Multi Purpose agarose (Boehringer, Mannheim, Germany) and 50 ng ethidium bromide in per milliliter 1× Tris-acetate ethylenediaminetetraacetic acid buffer (Amresco, Solon, OH, USA), pH 8. As molecular size marker, the Gene Ruler 100-bp DNA ladder plus (MBI Fermentas, St. Leon-Rot, Germany) was used. Electrophoresis was implemented at a constant voltage of 170 V in 0.5× Tris-acetate ethylenediaminetetraacetic buffer for 75 min. The gels were visualized by using the Image Master VDS (Pharmacia Biotech, Puurs, Belgium).

Isolation of *H. pullorum*

Recovery of *H. pullorum* isolates was attempted on all positive samples in the PCR analysis described above. The samples (200 mg) for isolation of *H. pullorum* were placed in a 1.5-mL tube with 400 μL of a mixture of 7.5 g glucose, 25 mL brain heart infusion broth (Oxoid, Basingstoke, England), and 75 mL sterile inactivated horse serum, and then homogenized. The various isolates were inoculated on brain heart infusion agar that was supplemented with 10% horse blood, amphotericin B 20 μg/mL (Fungizone, Bristol-Myers Squibb, Epernon, France), and Vitox (Oxoid) (blood agar). A modified filter technique of Steele and McDermott (*13*) was then used. Briefly, a sterile cellulose acetate membrane filter (0.45 μm) was applied with a sterile pair of tweezers directly onto the surface of the agar. When the filter was totally absorbed on the agar, ≈300 μL of the mixture was placed in the middle of the filter. After at least 1 hour of incubation at 37°C and 5% CO_2, the filter was removed with a sterile pair of tweezers and the filtrate was streaked on the agar with a loop. Incubation was conducted in microaerobic conditions (5% H_2, 5% CO_2, 5% O_2, and 85% N_2) at 37°C for a minimum of 3 days. Very small, gray-white, hemolytic colonies were selected and purified on a blood agar plate. The colonial form and phenotypic characteristics (gram-negative, slightly curved rod, catalase and oxidase positive, and indoxyl acetate negative) of the isolates were used for presumptive identification. Confirmation was based on PCR and sequencing of a 447-bp fragment of the 16S ribosomal RNA gene, as described below.

Analysis of Nucleotide Sequences

The PCR product of the retrieved *H. pullorum* isolates was purified with the Qiaquick purification kit (Qiagen) and sequenced by using the same primers applied in the assay with the BigDye Terminator cycle sequencing kit (Applied Biosystems, Lennik, Belgium). Sequencing products were run on the ABI prism 3100 Genetic Analyzer (Applied Biosystems) by using 50-cm capillaries filled with Performance-Optimized-Polymer 6. The electrophoregrams were exported and converted to the Kodon software package (Applied Maths, Sint-Martems-Latem, Belgium). Sequences were compared to published *H. pullorum* 16S rRNA sequences obtained from GenBank (accession nos. AY631956, L36143, and L36144) by using BLAST software (available from http://www.ncbi.nlm.nih.gov/blast/).

AFLP

Twenty-two poultry and 3 human isolates were fingerprinted by using AFLP (Table 1). These included 16 isolates from flock numbers 5 and 9 screened in this study. In addition, 4 samples previously isolated from broilers' cecal droppings and the boots from another flock's farmer, 4 reference strains (2 of chicken and 2 of human), and 1 human strain isolated from diarrheic stool in our laboratory were included for comparison.

Restriction Endonuclease Digestion and Ligation of Adaptors for AFLP

DNA of *H. pullorum* isolates was extracted by using a commercial tissue kit (DNeasy Tissue Kit, Qiagen). An aliquot containing 200 ng DNA, determined by optic density (260/280 nm) measurement by using the Spectra Fluor (TECAN, Grödig, Salzburg, Austria), was digested for 2 h at 37°C with *Bgl*II (10U/μL) and *Csp*6I (10U/μL) (MBI Fermentas) in TAC-buffer as described by Vos et al. (*14*). Five microliters of DNA digest was used in a ligation reaction containing 130 μg/mL *Bgl*II adaptor-oligonucleotide and 13 μg/mL *Csp*6I adaptor-oligonucleotide (Invitrogen) (*14*), 10× T4 DNA ligase buffer, T4 DNA ligase (1 U/μL) (Amersham Pharmacia), and TAC-buffer in a final volume of 20 μL. After incubation for 2 h at 25°C, the 20 μL ligation reaction was diluted 25 times.

Direct Selective PCR Amplification of Diluted Ligation

Five microliters of the diluted ligation reaction were applied in the PCR assay. The primers used in this assay were BGL2F-0, 5′-GAG TAC ACT GTC GAT CT-3′ (FAM labeled, 5′-end) and CSP6I-A, 5′-GAG CTC TCC AGT ACT ACA-3′ (*15*). The PCR conditions were as follows: an initial denaturation at 94°C for 3 min; 35 cycles

Table 1. *Helicobacter pullorum* isolates studied by using AFLP*

Strain	Flock	Source	Geographic origin
CE III 2	Flock CLO	Cecal droppings, broiler chicken	Belgium
CE III 3		Cecal droppings, broiler chicken	
CE III 4		Cecal droppings, broiler chicken	
CE III 5		Worker's boot	
CE II 1	Flock no. 5	Cecal tissue, broiler chicken	Belgium
CE II 2		Cecal tissue, broiler chicken	
CE II 3		Cecal tissue, broiler chicken	
CE II 4		Cecal tissue, broiler chicken	
CE II 5		Cecal tissue, broiler chicken	
CE II 6		Cecal tissue, broiler chicken	
CE II 7		Cecal tissue, broiler chicken	
CE II 8		Cecal tissue, broiler chicken	
CE I 1	Flock no. 9	Cecal tissue, broiler chicken	Belgium
CE I 2		Cecal tissue, broiler chicken	
CE I 3		Cecal tissue, broiler chicken	
CE I 4		Cecal tissue, broiler chicken	
CE I 5		Cecal tissue, broiler chicken	
CE I 6		Cecal tissue, broiler chicken	
CE I 7		Cecal tissue, broiler chicken	
CE I 8		Cecal tissue, broiler chicken	
CCUG 33837	NA	Healthy broiler chicken	Switzerland
CCUG 33840	NA	Laying hen, hepatitis	Switzerland
CCUG 33838	NA	Stool, gastroenteritis and hepatitis, human	Switzerland
CCUG 33839	NA	Stool, gastroenteritis, human	Switzerland
G 214	NA	Stool, gastroenteritis, human	Belgium (*11*)

*AFLP, amplified fragment length polymorphism; CLO, Centrum voor Landbouwkundig Onderzoek; CCUG, Culture Collection of the University of Göteborg, NA, not applicable or available.

of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, and elongation at 72°C for 90 s; and a final elongation at 72°C for 10 min.

Capillary Electrophoresis

PCR products were run on the ABI prism 3100 Genetic Analyzer (Applied Biosystems) by using the Fragile X Rox-1000 size standard and 50-cm capillaries filled with Performance-Optimized-Polymer 6. Electropherograms were analyzed with Genemapper U 3.5 Software (Applied Biosystems).

Numerical Analyses of AFLP Profiles

The program BioNumerics version 2.5 (Applied Maths) was used to perform numerical analyses of AFLP profiles. Strain relationships were inferred by use of the Pearson product-moment correlation coefficient and unweighted pair-group with mathematical average (UPGMA) clustering and depicted in a dendrogram (*16*).

Results

PCR

In Table 2, the number of *H. pullorum* DNA–positive samples originating from the intestinal tract and liver is shown. In 4 flocks, all samples were negative for *H. pullorum*. In the other 7 flocks, positive samples were found. In the cecum and colon, a PCR reaction for *H. pullorum* was positive in 33.6% and 31.8% of the samples, respectively. In total, 10.9% of jejunum and 4.6% of liver samples were positive for *H. pullorum*.

Isolation of *H. pullorum*

Eight *H. pullorum* cecum isolates from flock number 5 and 8 *H. pullorum* cecum isolates from flock number 9 were obtained. The sequences of the amplified 447-bp fragment of the *H. pullorum* 16S ribosomal RNA gene isolates showed a similarity of 98%–100% to those from GenBank (accession nos. AY631956, L36142, and L36143).

AFLP

AFLP analysis showed that isolates from each of the individual flocks examined clustered according to their flock of origin. The remaining chicken isolates and human strains each displayed a unique profile (Figure).

Conclusion

This study shows that *H. pullorum* is present in 33.6% of the cecal samples of broiler chickens collected at a poultry slaughterhouse during evisceration by using PCR. This microorganism was found in 7 of 11 flocks; 4 flocks were negative. Burnens et al. found a prevalence rate of 4% upon sampling cecal contents of broilers (*7*). The organism

Table 2. No. *Helicobacter pullorum*–positive poultry tissue samples by polymerase chain reaction

Flock no.	No. positive samples*			
	Cecum	Colon	Jejunum	Liver
1	2	2	1	0
2	3	8	4	0
3	4	1	1	0
4	7	4	1	0
5	8	8	0	0
6	0	0	0	0
7	4	4	0	1
8	0	0	0	0
9	9	8	5	4
10	0	0	0	0
11	0	0	0	0
Total	37	35	12	5

*No. positive animals of 10 screened per flock.

was detected by isolation. Considering the fastidious nature of this organism, this finding could explain this markedly lower percentage of positive birds. Additionally, in our study, cecal tissue, rather than cecal contents, was examined for the organism. Microorganisms related to *H. pullorum* adhere closely to the mucosa of the gastrointestinal tract. The phylogenetically related microorganism, *C. jejuni*, may tightly adhere to the brush borders of the intestine in chickens (*17*,*18*). The same phenomenon has also been documented for *H. pylori* in the stomach (*19*).

Comparing our study results to those obtained by Atabay et al. (*4*), the latter group found a higher occurrence of *H. pullorum* (60%) on poultry carcasses. This apparent discrepancy could be due to cross-contamination with cecal contents on the surface of broiler carcasses during poultry processing (*4*,*8*). Furthermore, contamination of the chicken body surface may occur during transportation to the abattoir. Fecal excretion of *Campylobacter* spp. may be increased because of stress during transportation and consequently may contaminate carcasses (*20*).

H. pullorum DNA was detected in only 5 (4.6%) liver and 11 (10.9%) jejunal samples, as opposed to 35 (31.8%) colonic and 37 (33.6%) cecal samples. Hence, one may assume that the lower segments of the intestinal tract are the predominant colonization sites for *H. pullorum* in broiler chickens. *H. pullorum* may gain access to the liver by retrograde transfer from the duodenum. Alternatively, it may translocate from the gut lumen to the portal circulation.

H. pullorum has been associated with vibrionic hepatitis in laying hens, both macroscopically and microscopically (*7*). In our study, no gross pathologic lesions were seen in the livers during sampling (data not shown).

Our modest isolation rate of *H. pullorum* from cecal samples may have been the result of examining frozen, as opposed to fresh, samples. However, we successfully recovered 16 isolates from 2 flocks, allowing (for the first time, to our knowledge) some analysis of the etiology of *H. pullorum* in broiler flocks to be undertaken. We used AFLP profiling for this purpose, a highly discriminatory method that has been successfully applied to molecular epidemiologic studies of several related species, including *H. pylori* (*21*,*22*), *Arcobacter* spp. (*15*), and *Campylobacter* spp. (*23*,*24*). Isolates from each of the individual flocks clustered according to their flock of origin, indicating a clonal relationship. In contrast, field and reference strains isolated from different hosts or geographic sources displayed a distinctive pattern. These data suggest that AFLP profiling has considerable potential for molecular epidemiologic studies of *H. pullorum* for the noted related species.

Several authors have suggested that *H. pullorum* has zoonotic potential and is involved in the pathogenesis of diarrhea and chronic liver diseases in humans (*2*,*8*,*10*,*11*). Retail raw poultry meats and other poultry products may constitute vehicles for human *H. pullorum* infections through carcass contamination, as previously reported for *Arcobacter* and *Campylobacter* species (*8*,*25*–*27*). Concerning health monitoring, PCR may be helpful in detecting this pathogen not only in intestinal tissue but also in broiler chicken cecal droppings.

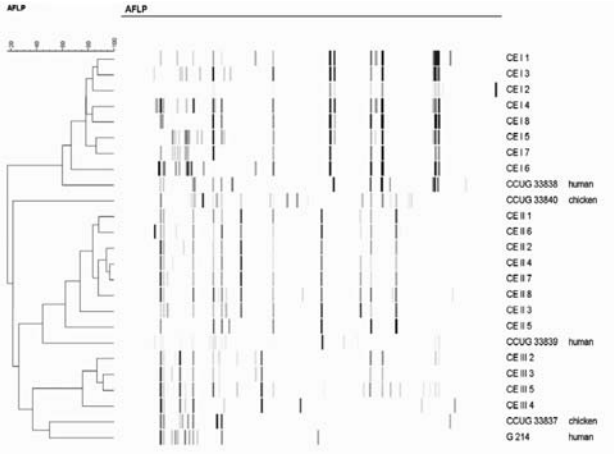

Figure. Chicken isolates and human strains of *Helicobacter pullorum* by amplified fragment length polymorphism.

In conclusion, this study shows that *H. pullorum* is a frequent intestinal colonizer of broiler chickens. PCR and isolation are useful tools to detect the species in intestinal tissue and in cecal droppings. AFLP profiling appears to be useful for molecular epidemiologic studies of this species.

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Ms Ceelen is a veterinary PhD student at Ghent University in Belgium where the work described in this study was performed. Her research interests include bacterial pathogenesis and host-pathogen interactions, with a focus on *Helicobacter* spp.

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Epizootiologic Parameters for Plague in Kazakhstan

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Reliable estimates are lacking of key epizootiologic parameters for plague caused by *Yersinia pestis* infection in its natural reservoirs. We report results of a 3-year longitudinal study of plague dynamics in populations of a maintenance host, the great gerbil (*Rhombomys opimus*), in 2 populations in Kazakhstan. Serologic results suggest a mid-summer peak in the abundance of infectious hosts and possible transmission from the reservoir to humans. Decrease in antibody titer to an undetectable level showed no seasonal pattern. Our findings did not support the use of the nitroblue-tetrazolium test characterization of plague-infected hosts. *Y. pestis* infection reduced survival of otherwise asymptomatic hosts.

Plague, which is caused by infection with *Yersinia pestis* and usually transmitted by fleas, is enzootic in many parts of Asia, Africa, and North and South America. Its natural reservoirs are usually rodent species outside the peridomestic environment, but peridomestic rodents may act as important liaison hosts between the sylvatic reservoir and humans (*1*). In addition to its widely acknowledged historic importance, plague can now be classified as an emerging disease. Land-use changes in many parts of the world are increasing the probability of interaction between sylvatic rodents and humans, and between sylvatic and peridomestic rodents (*1*). Also, throughout much of central Asia, support for surveillance programs that previously screened for and treated plague outbreaks in the rodent reservoirs is now being withdrawn. Furthermore, in the United States in particular, acknowledgement of the risk of plague in humans is increasing, in part because of human cases beyond normal foci (*2*) and in part because of the threat of bioterrorism (*3*).

However, many gaps remain in understanding the dynamics and natural history of plague in its natural reservoirs (*4*,*5*). Thus, problems arise when attempts are made to model plague-flea-rodent systems, which require parameter estimates for important processes (*5*,*7*, S. Park et al., unpub. data), or when these models generate parameter estimates that cannot be checked against independent values more directly derived from field data (S. Park et al., unpub. data). Moreover, work on the epizootiology of plague in different continents has been conducted largely independently, with researchers using different methods (*4*). Under these circumstances, contrasting paradigms accompanied by mutual skepticism may be produced. As Gage and Kosoy (*5*) have commented, "The importance of research on the natural history of plague can hardly be questioned, as it has provided critical information for the development of effective plague prevention and control techniques, but often contradictory results point out the need for studies designed to test specific hypotheses."

Our study is a first attempt to link these independent traditions. We report the results of a study carried out in the plague focus in eastern Kazakhstan, where other data have also been gathered as part of the surveillance system of the former Soviet Union, a function now carried out by the national government. The main reservoir host is the great gerbil, *Rhombomys opimus*, as it is throughout large tracts of central Asia (*8–10*). This animal is an enzootic or maintenance host (*5*) because, although plague is frequently reported in this species, often with a high prevalence (especially serologically), mass deaths of the host are not observed.

Great gerbils live in family groups, typically a single male, 1 or a few females, and their immature offspring, that inhabit and defend discrete, permanent burrow systems (*11*). The number, position, and size of the burrow systems generally do not change over time, but the proportion occupied by family groups (occupancy) may fluctuate dramatically (*12*). The vectors of plague are primarily fleas in the genus *Xenopsylla* that inhabit the burrow systems of the great gerbil (*13*).

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The data were obtained from 2 nearby but independent sites monitored in parallel. Samples were taken repeatedly from the same sites and, when possible, from the same (marked) individual gerbils. Evidence of infection in individual animals was sought both directly, by isolation of *Y. pestis* (though isolates were rare), and by serologic analysis.

The following questions were addressed. First, what accounts for variation in the probability of recapture, and thus of survival, in great gerbils? We attempted to determine whether plague has a detectable effect on survival of asymptomatic gerbils and whether seasonal variations in survival exist to make accurate assumptions in analyses of long-term (e.g., biannual) datasets on infection (S. Park et al., unpub. data).

Second, what accounts for variations in seroprevalence (the proportion of the population seropositive for antibody to plague), variations in the antibody titer of seropositive animals, and variations in the loss of detectable levels of antibody by these animals? These questions address patterns of infection: e.g., is infection acquired during particular seasons and by particular age classes of host? Understanding patterns in the acquisition and loss of antibody may be essential in interpreting long-term data, which are often based on serologic results (S. Park et al., unpub. data).

Third, what accounts for variations in nitroblue-tetrazolium (NBT) test values? The NBT test (*14*) measures the percentage of actively phagocytosing (positively stained) neutrophils in blood smears. Some Russian plague literature relies on it to provide supplementary (indeed crucial) information on infection status in wild rodents, e.g., whether infection is at an early or late stage (*15*). However, the test has not been adopted by plague workers elsewhere, and its value is open to question. We attempted to resolve the issue of its usefulness.

Methods

The 2 study sites were in Kizil-Dzar (site 1; 500 m × 600 m) and Shagildi (site 2; 500 m × 500 m), located ≈40 km apart in an area southeast of Lake Balkhash in eastern Kazakhstan. This area is a desert with sandy soil and sparse vegetation, primarily black saxaul (*Haloxylon aphyllum*), white saxaul (*H. persicum*), a number of grasses (especially *Anabasis ramosissima* and *Ceratocarpus turkestanicus*), and sandy sedge (*Carex physodes)*.

All gerbil colonies at the sites were mapped, although not all were occupied at any time. Site 1 had 76 colonies and site 2 had 87 colonies. The populations at the 2 sites were sampled every month from January 2002 to July 2004, except when problems of access occurred, mostly due to inclement weather, especially during the winter of 2003–2004. Samples were obtained 25 times from each population at the 2 sites. Unbaited wooden traps were placed at entrances to occupied burrows (which showed signs of recently disturbed sand) and checked twice a day over a 3- to 4-day period. Traps were not left in position overnight. Gerbil abundance, as estimated by the proportion of burrows occupied (*12*), increased to a peak each mid-summer. It was somewhat higher and more constant from year to year at site 1 than at site 2, and was lowest at site 2 in 2003. Captured animals were identified by sex, weighed, and classified as juveniles, subadults, or adults on the basis of size, weight, and coat color.

Fleas on the captured gerbils were collected with fine forceps. A blood sample was obtained from the tip of the gerbil's tail, and, if captured for the first time, it was injected subcutaneously with a microchip transponder so that its unique identity (9-number code) could be determined on recapture. Blood samples were analyzed for infection with *Y. pestis* by culturing on Hottinger's agar containing 1% hemolyzed sheep erythrocytes. Colonies obtained were identified as *Y. pestis* by colony morphologic features, sensitivity to diagnostic plague bacteriophage, and the presence of F1 capsular antigen. Blood samples were also tested serologically for F1 antigen by undirected hemagglutination and confirmed by hemagglutination inhibition with F1 antigen (*16*). A blood sample was used for the NBT test (*14*) in which 400 neutrophils from a blood smear were observed under 400× magnification to determine the proportion that were positively stained and showed evidence of active involvement in phagocytosis. Fleas were also monitored for active bacteria; these results will be reported in a subsequent publication.

A field-scale experiment was conducted in which half of the burrows at site 2 were treated with insecticide between November 2003 and the end of the observation period to rid them of fleas. (Detailed results will be described in a subsequent report.) Such treatment was not included as an explanatory variable, but if it affected any of the response variables, this should emerge as a significant site × time interaction.

Five response variables were analyzed by using generalized linear modeling. Recapture, defined as whether an animal was captured subsequently after release, was analyzed with a logit link and binomial errors. This is discussed as a proxy for survival, which clearly codetermines the probability of recapture along with emigration and ease of being caught. Seropositivity, defined as whether an animal exhibited demonstrable levels of antibody, was also analyzed with a logit link and binomial errors. Antibody titer was analyzed with an identity link and Gaussian errors. Loss of seropositivity, defined as whether an animal that was seropositive on release was still seropositive on subsequent recapture, was analyzed by using a logit link and binomial errors. NBT value was analyzed with logit link and binomial errors. For antibody titers, results of 2

laboratory tests showed a strong correlation; thus, results of statistical analyses were always effectively identical. For simplicity, antibody refers to the results of a passive hemagglutination test.

The following explanatory variables were examined initially (when they were not response variables): sex, site, maturity (adult, subadult, or juvenile), recapture, seropositivity, antibody titer, NBT value, year of capture, and month of capture. If a clear pattern was apparent in the full model in the signs of coefficients for different months, then to simplify and detect seasonal patterns, we tested a model with season based on the coefficients against the model that included all months.

Model selection was based on Akaike information criterion (AIC), which attempts to find the simplest model that adequately explains data by trading-off reductions in residual deviance against the number of parameters used in a model (see [*17*] for a review of model selection approaches). Models with a difference in AIC (ΔAIC) <2 can be considered indistinguishable in their explanatory power (*18*). For reasons of practicality in both analysis and interpretation, only 2-way interactions between explanatory variables were examined.

Results

For recapture (as a proxy for survival), initial exploration of competing models of the data (n = 1,360) showed a clear pattern in the sign of the coefficients of different months, which were positive from July through October but negative or zero from November through June, with the exception of April (coefficient = 0.068, standard error [SE] = 0.56). Therefore, a model with July–October classified as summer and November–June as winter (season) subsequently replaced month. The optimal model (AIC = 1,458.5) included season, maturity, year, and seropositivity, along with interactions between season and year and between age and year. Coefficients and their significances are shown in Table 1. The closest model (AIC = 1,460.1, ΔAIC ≈2) excluded seropositivity. Thus, inconclusive evidence supported the conclusion that seropositive animals are less likely to survive than seronegative animals. The probability of recapture was also much lower in the third year of the study and significantly lower in the second year than in the first year. This finding was at least in part an inevitable consequence of many animals released in the third year and even the second year still being alive (but not yet recaptured) when the study terminated. This effect was also responsible for the important interactions in the model. Thus, overall, recapture rates were lower for winter releases but were also low for the summer of the third year, at the end of the study. Similarly, the overall trend was for recapture rates to be lower for animals released as juveniles, but this was marked only in the first year, whereas rates were lowest for adults released in the third year.

For seropositivity (whether animals had detectable levels of plague antibody; n = 1,287), the optimal model (AIC = 1,287.6; Table 2) included maturity, year, site, NBT value, and whether an animal was subsequently recaptured, as well as a large number of interactions. The closest model (AIC = 1,287.7), and the only model to come near the optimal model, also included an interaction between site and whether an animal was recaptured. Subadults were significantly less likely to be seropositive than adults, and juveniles were even less likely, especially in the later years of the study. Seropositivity was significantly associated with high NBT values, especially in subadults and juveniles, and particularly at site 2. A low likelihood of seropositivity was found at site 2 in the third year of the study. Finally, animals that were subsequently recaptured were less likely to be seropositive.

Among seropositive animals, initial exploration of competing models for antibody titer (n = 342) again showed a significant effect of month and a clear pattern in the sign of the coefficients, but in this case September and

Table 1. Estimates of coefficients, standard errors, and significance based on z tests for optimal generalized linear model for whether animals were recaptured after release

Comparator	Effect	Estimate	Standard error	z value	p(>|z|) value*
	Intercept	–0.098	0.17	–0.59	0.56
Summer	Winter	–1.12	0.20	–5.48	4.3×10^{-8}
Adult	Subadult	0.09	0.23	0.41	0.69
Adult	Juvenile	–0.50	0.32	–1.57	0.12
Year 1	Year 2	–1.23	0.25	–5.00	5.5×10^{-7}
Year 1	Year 3	–4.85	1.49	–3.25	0.0011
Antibody negative	Positive	–0.30	0.16	–1.90	0.058
Summer × year 1	Winter × year 2	0.85	0.34	–2.47	0.014
Summer × year 1	Winter × year 3	4.12	1.48	2.80	0.0051
Adult × year 1	Subadult × year 2	0.99	0.35	2.85	0.0044
Adult × year 1	Subadult × year 3	1.32	0.48	2.77	0.0056
Adult × year 1	Juvenile × year 2	2.43	1.55	1.57	0.12
Adult × year 1	Juvenile × year 3	3.70	1.21	3.05	0.0023

*Probability of exceeding the z value by chance alone.

Table 2. Estimates of coefficients, standard errors, and significance based on z tests for optimal generalized linear model for whether animals were seropositive for antibody to *Yersinia pestis**

Comparator	Effect	Estimate	Standard error	z value	p(>|z|) value†
	Intercept	–1.00	0.19	–5.19	2.07×10^{-7}
Adult	Subadult	–0.73	0.31	–2.37	0.018
Adult	Juvenile	–1.94	0.61	–3.19	0.0015
Year 1	Year 2	0.15	0.27	0.55	0.58
Year 1	Year 3	0.534	0.53	1.00	0.32
	NBT	2.54	0.88	2.88	0.0040
Site 1	Site 2	–0.36	0.26	–1.39	0.16
Not recaptured?	Recaptured	–0.35	0.17	–2.08	0.037
Adult × year 1	Subadult × year 2	–1.76	0.50	–3.49	0.00049
Adult × year 1	Juvenile × year 2	–0.11	0.66	–0.16	0.87
Adult × year 1	Subadult × year 3	–2.91	1.09	–2.68	0.0073
Adult × year 1	Juvenile × year 3	–11.90	401.1	–0.03	0.98
Adult × NBT	Subadult × NBT	7.79	2.25	3.47	0.00053
Adult × NBT	Juvenile × NBT	9.47	3.89	2.43	0.015
Year1 × NBT	Year 2 × NBT	–3.60	1.49	–2.41	0.016
Year1 × NBT	Year 3 × NBT	–7.59	3.07	–2.47	0.013
Site1 × year1	Site 2 × year 2	0.26	0.33	0.80	0.42
Site1 × year1	Site 2 × year 3	–2.97	1.14	–2.60	0.0094
Site1 × NBT	Site 2 × NBT	6.42	1.68	3.81	0.00014

*NBT, nitroblue-tetrazolium.
†Probability of exceeding the z value by chance alone.

October had positive coefficients, whereas all others were negative or zero. A model with September and October classified as "autumn" and other months as "other" was far superior (ΔAIC = 14.4), and autumn therefore replaced month subsequently. The model with the lowest AIC (AIC = 1,524.2; Table 3) included autumn, maturity, and whether animals were recaptured subsequently, with no interactions. However, the closest model (AIC = 1,525.0, ΔAIC = 0.8) excluded maturity, which indicated only weak support for a maturity effect. However, antibody titers were significantly more likely to be higher in September and October. They also tended to be higher in animals that were subsequently recaptured.

Among seropositive animals, models that explore factors affecting loss of seropositivity must be interpreted with particular caution because the requirement that animals be recaptured after a seropositive result led to the lowest sample size (n = 81). The optimal model (AIC = 56.3; Table 4) included only antibody titer on release, lower values of which made loss of seropositivity more likely. The only model to come close to this (AIC = 56.9, ΔAIC = 0.6) also included a weak suggestion that a longer gap between release and recapture made loss more likely. Attempts to account for variations in NBT values (n = 1,335) failed to uncover any factors that improved on an intercept-only model; the minimum ΔAIC for a model that also included seropositivity was 2.76.

Discussion

The control of plague, in particular the control of the risk of it spreading from its wildlife hosts into peridomestic animals and humans, depends on understanding the dynamics and natural history of plague in those wildlife hosts. This study investigated 3 aspects of plague in its natural hosts in one of the world's major plague foci: the effect of infection on host survival, the dynamics of the antibody response to infection, and the specificity of the NBT test, which has been used as a measure of rodent infection status in previous studies.

From the point of view of host dynamics, an important finding from these analyses was that seropositive animals were less likely than seronegative animals to be recaptured. (This was mirrored in the analysis of seropositivity: animals subsequently recaptured were less likely to be

Table 3. Estimates of coefficients, standard errors, and significance based on t tests for optimal generalized linear model for variations in antibody titer among seropositive animals

Comparator	Effect	Estimate	Standard error	t value	p(>|t|) value*
	Intercept	8.13	0.38	21.66	$<2 \times 10^{-16}$
Autumn	Other	–1.81	0.36	–5.07	6.74×10^{-7}
Adult	Subadult	0.47	0.34	1.37	0.17
Adult	Juvenile	–0.76	0.52	–1.46	0.15
Not recaptured	Recaptured	0.47	0.26	1.78	0.077

*Probability of exceeding the t value by chance alone.

Table 4. Estimates of coefficients, standard errors, and significance based on z tests for optimal generalized linear model for whether seropositive animals that were released were seronegative on subsequent recapture

Effect	Estimate	Standard error	z value	pr(>|z|) value*
Intercept	1.01	0.70	1.44	0.15
Antibody titer	–0.64	0.18	–3.48	0.00051

*Probability of exceeding the z value by chance alone.

seropositive.) This situation could arise through any combination of the following features: increased death, increased emigration, and greater difficulty in being caught among seropositive animals. However, since greater difficulty in being caught implies abnormal behavior, and rodents leaving their natal territory often have lower survival rates (*19*), these results suggest that death is increased among seropositive animals. Furthermore, the presence of antibody may indicate present or recent infection, or an infection that had been cleared many months previously. Thus, any effect of plague infection on host survival can only be conservatively estimated by an effect of seropositivity. These results further suggest that the effect of plague infection on host survival may be substantial. Plague-induced death in susceptible hosts, such as prairie dogs (*Cynomys* spp.) in the United States, which have extensive "die-backs," is well established (*20*). More subtle effects on asymptomatic animals of resistant species have not been reported for plague (*4*), but they have the potential to affect the dynamics of host populations (*21*). Variations in great gerbil abundance are, in turn, critical in determining the public health risk of plague (*12*), and future development of models designed to predict outbreaks and help manage public health risk (*8,12*) should take such effects into account.

In spite of the influence of inevitably lower recapture rates at the end of the study period (not associated with any decrease in abundance), recapture rates tended to be lower in juveniles, a common finding in rodent populations (*21*), and likely reflected both higher emigration rates and lower survival among young animals yet to establish themselves in the population. The recapture rate also showed seasonal variation; it was higher for animals released from July through October, a period of peak abundance (burrow occupancy) when the population is dominated numerically by adults that will delay breeding until spring of the following year.

Since many factors interacted to determine recapture rates, determining typical recapture rates is not possible. However, the probability of being recaptured for a seropositive animal was ≈84% of that for an equivalent seronegative animal (e.g., for an adult in summer in year 1, ≈40% compared with 48%); the probability of being recaptured if released in winter was ≈50% that if released in summer (for a seronegative adult in year 1, 23% compared with 48%). The probability for juveniles was ≈70% of that for adults and subadults (for seronegative animals in summer in year 1, 35% compared with 50%).

The analysis of seropositivity indicated that adults (who had a longer time to acquire infection) were most likely to be seropositive and that juveniles (who had the least time) were least likely. For animals at site 1 in year 1 who were not subsequently recaptured, the probabilities of being seropositive were 27%, for adults, 15% for subadults, and 5% for juveniles. Furthermore, antibody titers were highest in September and October, soon after the peak period for the acquisition of new infections, after an influx of newborn susceptible animals into the population in mid-summer. For adults who were not subsequently recaptured, average titers were ≈1:2,560 (denoted as 8, the intercept value, in Table 3) in September and October, compared with titers ≈1:640 during the rest of the year. High titers in subadults (Table 3) are likely to reflect the high proportion of recent infections in this age group, whereas low titers in juveniles may reflect samples taken in the very earliest stages of infection, when titers are still increasing. Higher titers in animals recaptured may also reflect a pattern in which the oldest animals were both less likely to survive and more likely to have antibody titers that had decreased to low levels. The lower seroprevalence at site 2 in the third year of the study suggests that treatment of half of this site for fleas in autumn of the second year was successful in reducing plague transmission. Although no seasonal pattern in seropositivity was found, the serologic results suggest a clear mid-summer peak in the abundance of infectious hosts and a possible public health risk because of the sylvatic reservoir. Such risk will also depend on variations in reservoir-human contact rates associated with changes in host and human behavior, which would ideally be included in any risk-management model.

In spite of the inevitably low sample size resulting from stringent criteria for inclusion, the level of antibody was likely to decrease to undetectable levels in animals released with a low titer, particularly if the period of antibody decrease from release to recapture was long. However, no detectable seasonal or age-related effects were detected. Such negative results are important because all existing time series on the dynamics of plague, great gerbils, and fleas in central Asia comprise data collected biannually. Analyses of these data (*7*, S. Park et al., unpub. data) assume a seasonal structure in values of key parameters (loss of antibody, survival), and suggest such a structure as part of a statistical model deemed most likely to account for the biannual time series. Thus, results such as

those reported here, which reflect direct observations at intervals appropriate to the processes being observed, can test the validity of assumptions and seek to confirm (or contradict) statistical models. The observation that survival is lower in winter does not confirm the assumption (S. Park et al., unpub. data) that winter and summer rates are the same, while the absence of a seasonal pattern of antibody loss does not confirm the conclusion from modeling that loss appears to be greater over winter.

Values in the NBT test, which may be an indication of the proportion of neutrophils actively involved in phagocytosis, especially during acute bacterial infections (*15*), have been used in some studies to classify animals positive for antibody to *Y. pestis* into different subgroups. The profile of values within a population would be indicative of different phases of the progression of a plague epizootic and of the consequent risk to humans (*16*). In the present analyses, NBT values were higher in seropositive animals, but none of the factors measured, including seropositivity, accounted for a significant amount of the variation in NBT values. This finding suggests that although activity of neutrophils may respond to *Y. pestis* infection, this activity is also equally or more responsive to other factors not measured here (almost certainly including other infections). Thus, the usefulness of NBT values in determining public health risk must be questioned.

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Blastomycosis in Ontario, 1994–2003

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We describe a case of blastomycosis in an 8-year-old boy with *Blastomyces*-associated osteomyelitis and possible pulmonary involvement. We also identify 309 cases of blastomycosis in Ontario that were seen during a 10-year period, 57% of which occurred from 2001 to 2003. The overall incidence during the study period was 0.30 cases per 100,000 population. Most patients were from north Ontario (n = 188), where the incidence was 2.44 cases per 100,000. The incidence in the Toronto region was 0.29 per 100,000. Thirteen percent of cases occurred in children <19 years of age. These findings substantially increase the number of known cases in Ontario and Canada. Clinicians may encounter persons infected with *Blastomyces dermatitidis* and must be familiar with its signs and symptoms and be aware of locations, such as northwestern Ontario, where disease is endemic or hyperendemic. We advocate resuming blastomycosis as a reportable disease in Ontario to facilitate tracking cases.

First described by Gilchrist in 1894 (*1*), blastomycosis has been documented in Canada since at least 1910 (*2*). The incidence and epidemiologic features of the disease are poorly understood because of underrecognition, difficulty in isolating *Blastomyces dermatitidis* from natural sites, lack of an effective skin test, and because blastomycosis is not nationally reportable in either Canada or the United States (*3*). Blastomycotic infections in Canada have recently been reported in international (*4*) and Canadian (*5*–*7*) literature. We report a case of pediatric blastomycotic osteomyelitis and the results of an almost 10-year review of *Blastomyces* infection in Ontario through reports of laboratory isolates submitted to the Central Public Health Laboratory (CPHL), Ontario Ministry of Health and Long-term Care, Toronto. The objectives of the review were to define in the province of Ontario 1) the geographic epidemiologic features of laboratory-confirmed blastomycosis, 2) changes in the number of cases over time, and 3) demographic characteristics of infected persons. The case report and study were approved by the research ethics board of the Hospital for Sick Children, Toronto, Ontario.

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Case Report

An 8-year-old, previously healthy Caucasian boy, was brought to his family physician with a 2-month history of neck pain and stiffness. The pain was not sufficient to wake the child at night, but it prevented participation in athletics. No history of trauma, fever, weakness, paresthesias, weight loss, or change in bowel or bladder function was noted. The initial diagnosis was muscular strain, and rest and antiinflammatory medication was recommended. When his symptoms did not improve, a cervical spine radiograph showed a lytic lesion of the fifth cervical vertebra.

On hospital admission, physical examination was unremarkable, with the exception of pain on palpation over the posterior cervical spine. Laboratory results at admission showed normal leukocyte count (8.0×10^9/L), differential (polymorphs 4.48×10^9/L, eosinophils 0.08×10^9/L, lymphocytes 2.80×10^9/L, monocytes 0.56×10^9/L), electrolytes, and renal function. Erythrocyte sedimentation rate was mildly elevated at 38 mm/h. Computed tomographic scan of the region showed a well-defined lytic lesion with a "bubbly" appearance involving the posterior elements of the C5 vertebral body. An incidental note was made of a small, nonspecific lesion within the posterior upper lobe of the right lung. Bone scintillography showed positive uptake at C5. Magnetic resonance imaging (MRI) of the cervical spine demonstrated an enhancing mass that involved the posterior aspects of C5 plus an abnormal signal within the adjacent spinous processes.

The patient underwent a C5 laminectomy and a C4 partial hemilaminectomy. During dissection, a small amount

of purulent liquid was extruded from the tissue above C5. Gram stain of the pus aspirate showed no organisms. A calcofluor stain was positive for large, broad-based budding yeast forms. Pathologic examination showed a destructive lesion involving bone and soft tissues with both granulomatous and necrotizing suppurative components. The numerous epithelioid granulomas contained Langhans cells and foreign body giant cells, while the necrotizing lesions contained neutrophilic and eosinophilic infiltrates. Fungal elements highlighted with periodic acid–Schiff and Gomori methenamine silver stains showed double-contoured cell walls surrounding a cytoplasmic mass and proliferation in the form of broad-based buds. On the basis of these features, a presumptive diagnosis of blastomycosis was made. Subsequent fungal cultures grew a filamentous dimorphic fungus identified as *B. dermatitidis* and confirmed by DNA probe (Accuprobe, Gen-Probe Inc., San Diego, CA, USA).

The lung lesion in the right upper lobe could have also been a focus of blastomycotic infection; however, bronchoalveolar lavage or biopsies were not performed. Intravenous amphotericin B, 30 mg every 24 h (1.0 mg/kg/d) was initiated. After 10 days, the dose was reduced by 50% because of renal toxicity. On day 17 of treatment, itraconazole (200 mg orally once per day) was initiated and given concurrently with amphotericin B for 5 days, at which point amphotericin B was discontinued because of laboratory evidence of renal failure. At the time of discontinuation, 480 mg (16 mg/kg) had been given. The patient's clinical status improved, with resolution of pain and a return of full cervical range of motion by time of discharge.

Three weeks after completing a 6-month course of itraconazole, fever associated with vomiting and a sore throat developed in the patient. Chest radiograph showed a consolidation in the upper lobe of the right lung. MRI of the lung fields and cervical spine did not provide evidence of recurrence of blastomycosis. Clarithromycin was initiated and continued for 3 days with no improvement, at which time itraconazole (200 mg orally once per day) was resumed because blastomycosis could not be conclusively ruled out. Antifungal therapy was continued for 6 months, after which the patient was clinically healthy, and radiographs of the cervical spine and chest were normal.

Field Epidemiologic Investigation of Case

The patient had visited a cottage on the lakeshore of an island in the north health region of Ontario 5 months before his hospitalization. Shortly after the owner purchased the property, his dog and an adult male companion both developed laboratory-confirmed, nonfatal blastomycosis. Six months later, another adult male visitor developed a pneumonialike condition and was diagnosed with laboratory-confirmed blastomycosis. A provincial epidemiologic team also determined that a dog belonging to a previous owner of the property died of blastomycosis ≈2 years earlier.

A year and a half after the initial human infection, the cottage owner contacted the mycology laboratory at the Ontario Ministry of Health and requested an environmental investigation. A total of 50 environmental samples were taken from the property, including from a beaver lodge (similar to those previously associated with blastomycosis [*8*]) located underneath a boathouse. The samples were taken ≈3 months after our patient's presumed exposure. Material from the samples was prepared in sterile physiologic saline and injected into 4 mice per sample, as outlined by Ajello and Weeks (*9*) for environmental isolation of *Histoplasma capsulatum*. This technique had previously been used successfully to isolate *B. dermatitidis* (*10*). No mice died of blastomycosis within 6 weeks (autopsy of 2 mice that died showed bacterial infection), and examination of the remaining 198 mice, euthanized after 6 weeks, showed that livers, spleens, and lungs were clear of *B. dermatitidis* by histopathology and by culture on Sabouraud glucose agar with cycloheximide, chloramphenicol, and gentamicin (CCG) and blood agar with CCG and 2.3% egg albumin (*11*,*12*).

Laboratory-based Review of Blastomycosis in Ontario, 1994–2003

Methods

Cases were defined as all positive cultures of *B. dermatitidis* isolated between November 1, 1994, and December 31, 2003. CPHL processes primary cultures cultivated from patient samples sent from referring facilities. It also confirms the identity of cultures sent from referring laboratories. Eleven public health laboratories are in Ontario, and all of these laboratories, except the Thunder Bay laboratory, refer all isolates of *B. dermatitidis* to the CPHL for confirmation. Contact was made with the Northwestern Ontario Health Unit in Kenora, Ontario, to obtain records of confirmed cases of blastomycosis that may not have been sent to CPHL. Some of these cases (and their isolates) are referred to Winnipeg rather than southern Ontario, and some isolates are identified only at the Thunder Bay laboratory. By contacting the specific region that does not refer all cases to the CPHL, a high level of case capturing is ensured. Information obtained regarding each infected person included date of birth, sex, and location of diagnosis. Ontario is divided into 7 health regions (Toronto, southwest, central-south, central-west, central-east, east, and north). Incidence per 100,000 population per year was derived by using population data from Statistics Canada (B. Ball, pers. comm.).

Results

A total of 309 culture-positive cases of blastomycosis were identified in Ontario during the study period. Each of Ontario's 7 health regions had cases identified (Figure 1); 61% (n = 188) of the cases were diagnosed in the north region, and 21% (n = 66) were diagnosed in the Toronto region. Of the north region cases, 89% (n = 167) were from the Northwestern Health Unit's district, which includes Kenora.

The mean number of cases diagnosed per year was 33.7; the fewest number of cases was in 1995 (n = 10), and the greatest number was in 2002 (n = 71) (Figure 2). The number of cases has increased in recent years; 57% (n = 175) were reported in the 3-year period from 2001 to 2003. While cases were diagnosed year-round, 59% (n = 181) occurred in the fall and winter months, from October to March (Figure 3). The age of patients with positive isolates was recorded for 92% (n = 283) of cases; ≈60% were 30–59 years of age (range 6 months to 83 years) (Figure 4). The sex of the affected person was available in 97% (n = 301); 65% (n = 196) were male, and 35% (n = 105) were female. The incidence ranged from a low in the south-central Ontario region of 0.02 cases per year per 100,000 population to a high in the north Ontario region of 2.44 cases per year per 100,000 population (Figure 1). Age-specific incidence rates are shown in Figure 4.

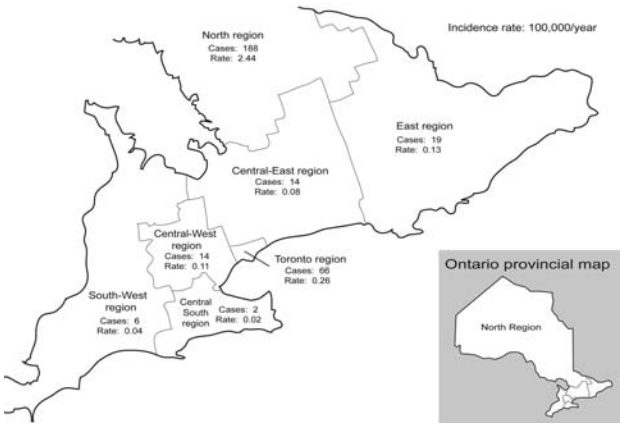

Figure 1. Incidence of blastomycosis by Ontario health region.

Discussion

Case Report

Blastomycosis is a rare but potentially fatal infection caused by the thermally dimorphic fungus *B. dermatitidis*. It is presumed to be a soil organism, and factors that favor it include moisture, high content of organic material, acidic pH, and exposure to animal excreta (*8*,*13*). Primary infection generally follows inhalation of conidia, asexual fungal spores that are shed at maturity. Blastomycosis often has nonspecific initial symptoms: fever, malaise, myalgias, weight loss, cough, and pleuritic chest pain (*14*). The course of illness may be either acute or chronic. While any organ may be involved, lung involvement is most common, manifesting either as a lobar infiltrate resembling a bacterial pneumonia, a miliary infiltration similar to tuberculosis, or as a large mass that is initially suspected of being a bronchogenic carcinoma. The second most common type of disease is cutaneous. Less common clinical syndromes involve bone, the genitourinary system, or the central nervous system (CNS). In contrast to most other invasive fungal infections, blastomycosis most commonly affects immunocompetent persons.

Children make up 2%–11% of cases in previous studies (*15*,*16*). Published case reports and case series of pediatric blastomycosis highlight the potential for pulmonary, bone, and CNS disease, as well as neonatal disease and intrauterine transmission (*6*,*15*–*20*). In our review, 13% of patients were <19 years of age.

Recent guidelines from the National Institute of Allergy and Infectious Diseases Mycoses Study Group and the Infectious Diseases Society of America suggest that life-threatening pulmonary or disseminated non-CNS disease, any CNS disease, and disease in the immunocompromised host be treated with amphotericin B to complete a total dose of 1.5–2.5 g (21–36 mg/kg for a 70-kg person). For patients who do not tolerate amphotericin, it can be used initially until the condition has stabilized at which point itraconazole (for non-CNS disease) or high-dose fluconazole (for CNS disease) can be used as stepdown therapy (*21*). Mild-to-moderate pulmonary and disseminated non-CNS disease can be treated by itraconazole from onset. Duration of therapy with itraconazole should be ≥6 months, except in the case of osteomyelitis, which has a higher rate of relapse (*22*). Bone disease should be treated with ≥1 year of antifungal medications. Relapse is a recognized outcome in blastomycosis and may occur after any treatment regimen (*22*–*24*). Therefore, in our case, given the vertebral involvement, 1 year of itraconazole therapy was warranted.

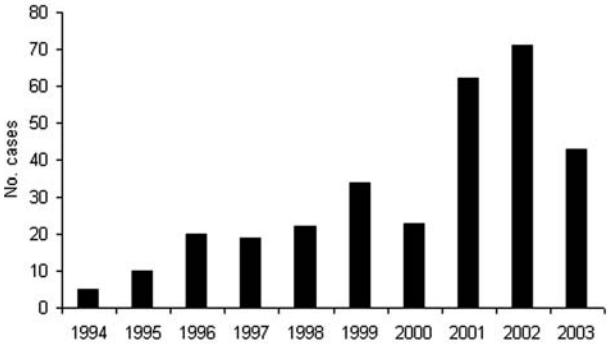

Figure 2. Blastomycosis diagnosed by year, Ontario, 1994–2003.

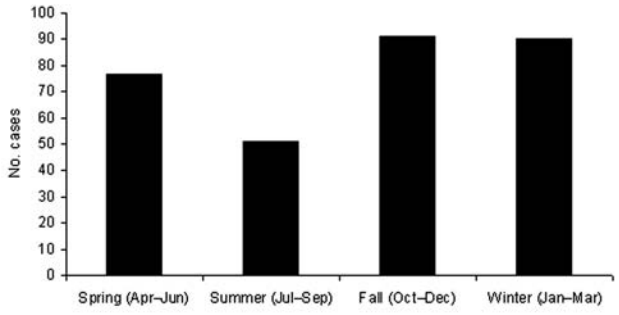

Figure 3. Blastomycosis diagnosed by season, Ontario, 1994–2003.

Epidemiologic Report

B. dermatitidis is endemic in the United States around the Mississippi and Ohio River basins, Midwestern states, Canadian provinces bordering the Great Lakes, and small areas bordering the St. Lawrence River (*25*). Ontario has an area >1 million km^2 and can be divided into 3 natural regions: the rolling uplands of the Canadian shield across the center of the province, the Hudson Bay lowlands to the north, and the Great Lakes and St. Lawrence lowlands to the south. The northwestern portion of the province is largely forested and dotted with lakes and rivers.

While the epidemiologic investigation described in our case failed to yield positive environmental cultures, the link between beaver dwellings and blastomycosis has previously been suggested (*8*). Other outbreaks have been reported in association with construction of a log cabin (*26*), an urban construction site (*27*), outdoor riverside camping (*28*), raccoon hunting (*29*), and travel to a specific small island (*30*).

Blastomycosis was a reportable disease in Ontario until 1989 (*31*). Most Canadian cases have been reported from Ontario and Manitoba (*3,11,31–35*). From 1981 to 1989, before the removal of blastomycosis from the list of reportable diseases, 16 cases were recorded in Ontario, a mean of 1.8 cases per year (*31*). The current review suggests that blastomycosis is more common than previously thought, with a mean of 33.7 cases diagnosed per year in the 10-year study period. Hyperendemicity in the region surrounding Kenora, Ontario, has recently been reported (*35*), with an estimated annual incidence rate of 117.2 cases per 100,000 population (*35*). This amount exceeds the next highest rate reported in North America of 100 cases per 100,000 population in the Eagle River area in Vilas County, Wisconsin (*36*). An increase was noted in the number of cases of blastomycosis identified per year since the late 1990s (Figure 2). Increased awareness in Kenora because of an education campaign aimed at physicians after a fatal case of blastomycosis in 1998 may account in part for the increased number of recognized cases. Our identification of 309 laboratory-confirmed cases of blastomycosis represents the largest group of confirmed cases and substantially increases the total number of known cases in both Ontario and Canada.

During the study period, 66 cases were diagnosed in Toronto. While blastomycosis has previously been reported in persons who have not traveled outside of the greater Toronto area (*5,6*), we could not confirm where infection was acquired in the 66 Toronto patients. The major limitation of this study is that the geographic data are based on location of diagnosis, which may not be reflective of location of infection. Infection may occur in rural regions that are frequent destinations of travel for many Ontario residents.

The preponderance (58%, p = 0.0005) of diagnoses in fall and winter noted in our study has previously been reported (*35*). The incubation period for symptomatic blastomycosis is 1–3 months (*13,37*). Therefore, infection is thought to mainly occur in summer and fall, when persons spend time outdoors, snow cover is minimal, and rainfall is high, thereby increasing exposure to this soil organism (*8,13*).

Early Canadian studies (*2,33*) found most (85%–88%) cases to be in males, and early studies from the United States suggested a male-to-female ratio of 4:1–15:1 (*38*). More recent studies in Canada (*31,35*) and the United States (*39*) suggest that the male sex predominance is not as large. In our study, 65.1% of patients were male, similar to the proportion noted by Crampton et al. (*32*).

Previous studies have suggested increased rates of infection for both African Americans (*40*) and Aboriginals (*35*). Whether this finding is related to genetic or exposure factors is unclear. Given the retrospective nature of this laboratory review, we could not determine the ethnicity of the cases. Future work must identify high-risk groups within the population so that targeted prevention efforts may be put in place.

Despite being the largest series of cases of blastomycosis reported in Ontario, our data may underestimate the

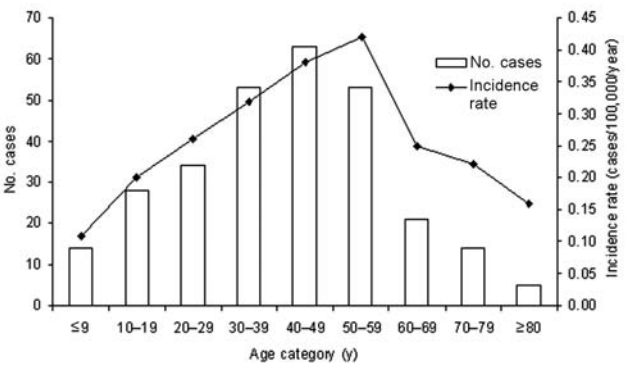

Figure 4. Number of blastomycosis cases and incidence rates by age, Ontario, 1994–2003.

true incidence of disease in the province. The number of cases diagnosed in northwestern Ontario increased after an education campaign. To our knowledge, no similar campaign has been carried out elsewhere in the province, and therefore the diagnosis is likely often missed. Some samples may have been only identified at a regional laboratory and not counted among our data. Because of the geographic proximity, patients in northwestern Ontario are often transferred to Winnipeg, Manitoba, rather than to an Ontario tertiary centre for investigation and treatment. Additionally, persons from outside the province who are infected may be diagnosed in their home provinces. As a result, some cases of blastomycosis acquired in Ontario may be diagnosed in Manitoba or elsewhere and therefore are not included in our data.

Conclusion

The understanding of the natural distribution of blastomycosis and other mycoses endemic in Ontario (such as histoplasmosis) is minimal. This study is the first to describe the Ontario-wide incidence of blastomycosis and to provide incidence rates in each of the 7 provincial health regions. Clinicians practicing throughout the province and country may encounter persons infected with this organism and need to be familiar with its varied clinical signs and symptoms and be aware of regions where disease is endemic or hyperendemic. Our data suggest that the number of diagnoses of blastomycosis has increased over several years. However, the disease likely remains underrecognized. As delay to diagnosis can contribute to illness and death, clinicians should consider blastomycosis in their differential diagnoses of lung, skin, and bone diseases, particularly if the patient does not respond to conventional antimicrobial drug therapy. The lack of rapid and effective diagnostic tools contributes to the underrecognition of blastomycosis. Advances in molecular diagnosis of *B. dermatitidis* (*39*), particularly in regions identified as higher risk, hold the potential for improving case detection and decreasing delay to diagnosis.

Infection by *B. dermatitidis* is more common than was thought before its removal from the list of reportable diseases in Ontario in 1989. Our group advocates strongly for returning blastomycosis to the reportable diseases list in this province. Travel history must be included in the reporting of blastomycosis. While identifying the point of infection in well-traveled individuals may be impossible, a specific or negative travel history would make a valuable contribution to understanding where blastomycosis is contracted in Ontario. Such reporting would facilitate tracking cases and clinical education regarding this potentially fatal invasive fungal infection.

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Fresh Chicken as Main Risk Factor for Campylobacteriosis, Denmark

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We report the findings of a case-control study of risk factors for sporadic cases of human campylobacteriosis in Denmark. In 3 different analytical models, the main domestic risk factor identified was eating fresh, unfrozen chicken. Specifically, 28 of 74 domestically acquired case-patients were exposed to fresh chicken compared with 21 of 114 controls (multivariate matched odds ratio 5.8; 95% confidence interval 2.1–15.9). In contrast, a risk from eating other poultry, including previously frozen chicken, was only indicated from borderline significant 2-factor interactions. The marked increase in consumption of fresh, unfrozen poultry in Denmark during the 1990s likely contributed substantially to the increasing incidence of human campylobacteriosis in this period.

Campylobacter spp. are the most common cause of acute bacterial gastroenteritis in industrialized countries. Although rarely fatal, *Campylobacter* infections cause considerable illness and loss of productivity and may be associated with severe disabling consequences, including arthritis and demyelinating disease (Guillain-Barré syndrome) (*1*).

Denmark is among a limited number of countries worldwide with comprehensive national laboratory-based surveillance of human campylobacteriosis. Denmark, like several other industrialized countries, has recorded a marked increase in the incidence of human campylobacteriosis. From 1980 to 2001 the incidence quadrupled, reaching 86 cases per 100,000 inhabitants in 2001 (Figure).

Most persons who contract *Campylobacter* infections are not part of recognized outbreaks. Risk factors for sporadic *Campylobacter* infections have been investigated in United States, Canada, Australia, New Zealand, and Europe (including Denmark) within the last 20 years (*2*–*7*). Most studies have identified consumption of poultry and poultry products as risk factors. Other domestic risk factors include drinking untreated water; consuming raw or unpasteurized milk; handling and cooking food, particularly raw meat, in relation to barbecuing; and having contact with food-producing animals and pets.

Although the findings from these studies have provided insight to the epidemiology of *Campylobacter* infections, our understanding is still incomplete. The relative importance of the different sources is not well known, and in many countries, no clear explanation for the increasing incidence of *Campylobacter* infections has been determined. Recent experience from Iceland has pointed to an increased consumption of fresh versus frozen poultry as a potential explanation for the increasing disease incidence (*8*).

The first case-control study of risk factors for human campylobacteriosis in Denmark (*6*) did not distinguish clearly between fresh and previously frozen poultry meat. Several factors, for example, consuming undercooked poultry, but not handling and consuming poultry in general, were risks for human campylobacteriosis. Thawing poultry was found to be protective in this study, which might indirectly indicate that fresh, not frozen, poultry was the main poultry-associated risk factor (*6*).

We report the findings of a second case-control study of risk factors for sporadic human campylobacteriosis in Denmark. In this study, unlike the earlier study, we made a clear distinction in the questionnaire between exposure to fresh, unfrozen meat and exposure to previously frozen meat so we could independently assess the risk of the 2 different categories.

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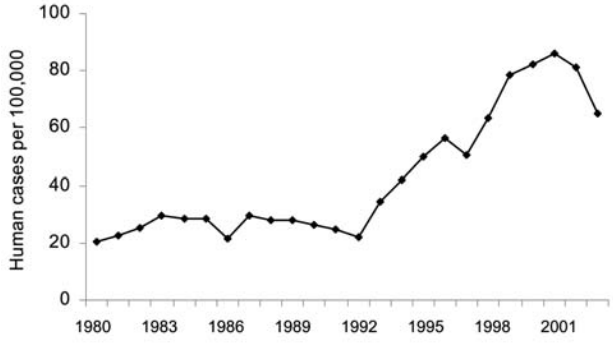

Figure. Laboratory-confirmed human campylobacteriosis in Denmark, 1980–2003 (*13*).

Materials and Methods

From October 2000 to September 2001, the second case-control study on acute sporadic human campylobacteriosis was conducted in Denmark. Participation in the study was voluntary and required written consent. Three groups were interviewed (computer-aided telephone interviews). The first included campylobacteriosis (CB) patients: persons with laboratory-confirmed campylobacteriosis. The second included healthy controls matched to CB patients by age, sex, and geography (6 controls per CB patient were randomly selected through the Danish Civil Registry system after receipt of a signed consent form from a CB patient). Eligible controls received a questionnaire and a consent form by mail. The time between disease onset for the CB patient and the time of interview of controls was sought diminished (mean 27 days). The third group included non-CB bacterial gastroenteritis patients (non-CB controls): patients whose specimens were culture-positive for other zoonotic bacterial infections (mainly *Salmonella* spp.).

Twice a week, 2–4 CB patients and 3 non-CB patients per CB patient were selected in 6 of 16 Danish counties among patients identified the previous week. An approximate match of non-CB controls to CB patients by onset date was obtained (mean 5 days apart). Children <1 year of age were omitted. Recruitment of patients increased during summer when the incidence was higher. A total of 272 persons with *Campylobacter* infection, 786 non-CB controls, and 2,403 healthy controls were invited for the study. The response rates were 50% for CB cases and non-CB controls and 22% for the healthy controls, respectively.

The questionnaire sought data on the following subjects: symptoms; other diseases; use of medications; use of vitamins; consumption of meat (including type, storing [frozen vs. not frozen], and handling); use of barbecue grill; consumption of rice and pasta, fruit, vegetables, cereals, bread, milk, milk products, spices and herbs, and organic products; cooking; kitchen hygiene; vacation or travel experiences; contact with ill persons; information on drinking water, swimming, household, place of residence, and whether a summer house was used; and respondent's occupation, hobbies, and socioeconomic data. More than 350 original or recoded variables were tested in the analysis.

Three analyses were conducted: A) 107 CB patients versus 178 matched healthy controls (including travel-related cases); B) 74 CB patients (domestically acquired only) versus 114 matched healthy controls; and C) 141 CB patients versus 386 non-CB controls matched to patients only on time of disease onset. Initially, variables in analyses A and B were tested by univariate conditional logistic regression (PROC PHREG, SAS Institute, Cary, NC, USA [*9*]). Variables with $p_{(univariate)} \leq 0.30$ and other relevant variables were selected for multivariate conditional logistic regression analysis. Variables with $p<0.01$ were kept in the final models. In analysis B, 2-factor interactions between variables in final model, match variables, and other interactions relevant to the hypotheses were tested; the population attributable risk (PAR) was then estimated (*10*). In analysis C, univariate logistic regression (PROC GENMOD, SAS Institute [*9*]) was used for screening of effect of variables. Effect modification of covariates (age, sex, geography, and season) was tested in analysis C. As the modifying effect was negligible, the results from analyses without covariates is presented (Table 1).

Results

Analysis A (full dataset) identified that travel to southern Europe (odds ratio [OR] 15.81) and outside Europe (16/107 patients exposed vs. 1/178 controls) was associated with *Campylobacter* infection, whereas travel to other parts of Europe was more common in controls than in patients (OR 0.068). Other risk factors identified in analysis A are listed in Tables 1 and 2. Analysis B (domestic cases only) identified eating chicken, bought fresh and not frozen in the home, to be the only significant risk factor for campylobacteriosis (OR 5.80). Some exposures, including pork prepared in large pieces (OR 0.15), eating apples or pears (OR 0.21), eating raw vegetables daily (OR 0.24), and days off (besides weekends) in the week before onset (OR 0.23) were more common in controls than patients ($p<0.01$). Analysis C (CB vs. non-CB patients) found that, among the variables with $p<0.05$ in analysis A or B, only eating chicken, bought fresh and not frozen in the home, was significantly more associated with *Campylobacter* infections than with other bacterial gastrointestinal infections. Contrary to this finding, travel to central and northern Europe and eating pork prepared in large pieces were less associated with *Campylobacter* infection than with other infections. The domestic PAR from chicken bought unfrozen was 23.8% (95% confidence interval 7.98–52.9).

Table 1. Risk factors obtained from 3 analytic approaches for sporadic campylobacteriosis (CB) in Denmark, 2000–2001*

Variables†	Analysis A‡§: 107 CB patients vs. 178 matched controls (multivariate model), OR (95% CI), p	Analysis B§¶: 74 Domestic CB patients vs. 114 matched controls (multivariate model), OR (95% CI), p	Analysis C#: 141 CB patients vs. 386 non-CB controls (univariate analysis), OR (95% CI), p
Travel (14 d)			
Southern Europe	15.81** (2.63–94.9), 0.003	–	0.57 (0.29–1.11), 0.097
Europe, other	0.068** (0.007–0.64), 0.019	–	0.33 (0.11–0.97), 0.044
Outside Europe	16/107 CB, 1/178 controls,** infinite	–	0.93 (0.55–1.55), 0.77
Holiday other than weekend	0.37 (0.13–1.09), 0.072	0.23** (0.083–0.64), 0.005	0.92 (0.62–1.35), 0.67
Chicken bought unfrozen	6.03** (2.17–16.80), 0.0006	5.80** (2.11–15.93), 0.0006	2.91 (1.85–4.59), p<0.0001
Turkey††	0.047** (0.004–0.51), 0.012	1.40 (0.59–3.34), 0.45	0.85 (0.54–1.34), 0.47
Turkey bought fresh	61.1** (4.35–857.6), 0.002	1.75 (0.62–4.93), 0.29	1.43 (0.79–2.52), 0.21
Minced beef/veal at barbecue	0.30 (0.065–1.36), 0.12	0.12 (0.018–0.74), 0.022	0.70 (0.28–1.76), 0.45
Pork prepared in large pieces	0.10** (0.028–0.37), 0.0005	0.15** (0.046–0.49), 0.002	0.53 (0.29–0.96), 0.035
Any white bread without bran or grains last month	2.55 (0.92–7.08), 0.072	3.21 (1.15–8.94), 0.026	1.28 (0.73–2.22), 0.39
White bread with bran or grains >5 times/mo	4.54** (1.83–11.23), 0.001	3.10 (1.30–7.35), 0.0105	1.45 (0.97–2.17), 0.074
Apples and pears	0.12** (0.034–0.40), 0.0007	0.21** (0.07–0.61), 0.004	0.83 (0.54–1.27), 0.39
Strawberries‡‡	0.22**(0.069–0.72), 0.012	1.69 (0.60–4.80), 0.32	1.28 (0.75–2.19), 0.37
Raw vegetables daily	0.16**(0.051–0.51), 0.002	0.24**(0.082–0.71), 0.0099	0.93 (0.55–1.56), 0.78
Chives (fresh)	1.94 (0.76–4.95), 0.17	2.78 (1.10–6.99), 0.030	1.19 (0.79–1.77), 0.40
Urban residency	0.17** (0.050–0.58), 0.004	0.25 (0.074–0.85), 0.027	0.76 (0.45–1.31), 0.32
Tap water from summerhouse	4.68 (1.11–19.8), 0.036	3.00 (0.80–11.3), 0.10	0.80 (0.40–1.61), 0.53

*Variables with p<0.05 in the final models from analyses A, B, or both, are shown.
†Focus period is the week before onset or filling in questionnaire if nothing else is mentioned.
‡All CB patients (including travelers vs. healthy controls).
§Match of patients and controls: age, sex, geography, and as far as possible, to time of disease onset for patient. The risk from factors not included in the final model was achieved by forcing them into the model 1 by 1.
¶Only domestic CB patients vs. healthy controls.
#CB patients vs. non-CB bacterial gastroenteritis patients, match to patients only on time of disease onset.
**Variables in final models A and B.
††Modifies the risk from turkey bought fresh in model A.
‡‡Modifies the risk from travel to southern Europe in model A.

Only in model B (domestic patients) were 2-factor interactions examined. The risk from fresh chicken was significantly increased ($p<0.05$) in summer (vs. winter) and when preparing whole chicken (vs. cuts). The risk was reduced ($p<0.05$) by frequently eating fruits, raw vegetables, high-fiber cereals, vitamins ($p = 0.050$), and acidified milk products ($p = 0.070$). Eating turkey bought fresh and chicken in general interacted borderline significantly with season (chicken: higher risk in summer [$p = 0.078$], turkey: higher risk in winter [$p = 0.056$]). A borderline significant interaction between risk from chicken cuts and barbecuing was found ($p = 0.0502$). Finally, the apparent protection from eating apples or pears was stronger in the cold season ($p = 0.043$).

Discussion and Conclusion

We found that the main domestic risk factor for campylobacteriosis is eating chicken meat that is bought fresh and subsequently not frozen in the home. Eating other poultry meat products and eating previously frozen chicken meat were borderline significant risk factors.

Adding the case-case approach to the risk factor study (CB patients vs. non-CB patients) was expected to highlight risk factors or potentially protective factors, which are specific for campylobacteriosis. Only exposure to unfrozen chicken remained a significant risk factor for campylobacteriosis in the case-case study. The study findings strongly support the contribution of fresh poultry specifically as a source of human campylobacteriosis. In contrast, true common factors for both case groups were expected to be reduced or disappear. Also the apparent effect of factors associated with willingness to participate as a control in the case-control studies was expected to be eliminated. Several significant risk factors from the case-control studies were insignificant or markedly reduced in the case-case study (e.g., apparently protective factors [certain fruits and vegetables] and risk factors [travel, certain types of bread, and fresh turkey]).

The results of the present study are consistent with the hypothesis that a marked increase in the consumption of fresh chicken has been a major driving force behind the increasing incidence of human campylobacteriosis in Denmark during the 1990s. Bacteriologic investigation of fresh and frozen chicken collected at retail outlets in

Table 2. Case-control study on sporadic campylobacteriosis (CB) in Denmark, 2000–2001*

Variables	Exposures					
	Analysis A† (including travelers)		Analysis B‡ (only domestic cases)		Analysis C§ (case-case study)	
	107 CB patients, no. (%)	178 matched controls, no. (%)	74 CB patients, no. (%)	114 matched controls, no. (%)	141 CB patients, no. (%)	386 non-CB controls, no. (%)
Travel (14 d)						
No travel	78 (72.8)	156 (87.6)	74 (100)	114 (100)	100 (70.9)	241 (62.4)
Southern Europe	11 (10.2)	6 (3.3)	–	–	4 (2.8)	29 (7.5)
Europe, other	2 (1.8)	15 (8.4)	–	–	12 (8.5)	51 (13.2)
Outside Europe	16 (14.9)	1 (0.5)	–	–	25 (17.7)	65 (16.8)
Holiday other than weekend	49 (45.7)	91 (51.1)	22 (29.7)	52 (45.6)	65 (46.0)	186 (48.2)
Chicken bought unfrozen	36 (33.6)	40 (22.4)	28 (37.8)	21 (18.4)	46 (32.6)	55 (14.2)
Turkey	29 (27.1)	57 (32.0)	23 (31.0)	33 (28.9)	34 (24.1)	105 (27.2)
Turkey bought fresh	28 (26.1)	35 (19.6)	15 (20.2)	15 (13.1)	21 (14.9)	42 (10.9)
Minced beef/veal at barbecue	4 (3.7)	21 (11.7)	3 (4.0)	9 (7.8)	6 (4.3)	23 (6.0)
Pork prepared in large pieces	11 (10.3)	46 (25.8)	8 (10.8)	28 (24.5)	15 (10.6)	71 (18.4)
Any white bread without bran or grains last month	92 (86.0)	142 (79.7)	65 (87.8)	86 (75.4)	122 (86.5)	322 (83.4)
White bread with bran or grains >5 times/mo	75 (70.0)	100 (56.1)	52 (70.2)	62 (54.3)	94 (66.6)	224 (58.0)
Apples and pears	76 (71.0)	148 (83.1)	55 (74.3)	98 (85.9)	98 (69.5)	283 (73.3)
Strawberries	16 (14.9)	43 (24.1)	18 (24.3)	22 (19.2)	23 (16.3)	51 (13.2)
Raw vegetables daily	16 (14.9)	46 (25.8)	10 (13.5)	29 (25.4)	23 (16.3)	67 (17.4)
Chives (fresh)	38 (35.5)	58 (32.5)	30 (40.5)	34 (29.8)	53 (37.6)	130 (33.7)
Urban residence	88 (82.2)	158 (88.7)	61 (82.4)	99 (86.8)	118 (83.7)	336 (87.0)
Tap water from summerhouse	9 (8.4)	12 (6.7)	8 (10.8)	7 (6.1)	11 (7.8)	37 (9.6)

*Exposures for variables with $p<0.05$ in the final models from analysis A, B, or both.
†Analysis A: all CB patients (including travelers) vs. healthy controls matched to patients by age, sex, geography, and as far as possible, to time of disease onset for the patient.
‡Analysis B: domestic CB patients vs. healthy controls matched to patients by age, sex, geography, and as far as possible, to time of disease onset for the patient.
§Analysis C: CB patients vs. non-CB bacterial gastroenteritis patients, match to patients only on time of disease onset.

Denmark has shown that the number of viable *Campylobacter* bacteria in fresh samples exceeds that of previously frozen chicken. In a survey of chicken meat in retail stores, 194 (79.8%) of 243 samples of frozen chicken harbored <0.4 thermophilic *Campylobacter* bacteria per gram, whereas 134 (46.4%) of 289 samples of fresh chicken were below this level (*11*). This result is because the freezing process reduces the number of viable *Campylobacter* organisms. In the 1990s, the national consumption of poultry meat increased by ≈40% (1991: 63,900 tons, 1998: 93,200 tons) (*12*). The increase was observed for almost all types of chicken and turkey products but most markedly in fresh cuts. In the same period, the incidence of campylobacteriosis increased by >400%, from 20 to 86 cases per 100,000 inhabitants. The bacteriologic data, which show higher loads of *Campylobacter* in fresh poultry, suggest that the exposures to *Campylobacter* spp. have increased much more than the general increase in poultry consumption and thus explains why the increase in human disease incidence has exceeded the increase in poultry consumption.

The Danish broiler industry, in collaboration with governmental institutions, introduced a voluntary control program in 2002–2003, whereby among other initiatives, flocks of chicken are tested for *Campylobacter* spp. immediately before slaughter (*13*). Positive flocks are, to the extent that doing so is logistically feasible, used to produce frozen products, whereas *Campylobacter*-free flocks are primarily used to produce fresh chicken. In the winter, the prevalence of *Campylobacter*-free flocks is sufficiently elevated to enable a near complete separation, but in the summer, when the flock prevalence is high, *Campylobacter*-positive flocks are also included in the fresh product line to some extent. In 2002, the incidence of human campylobacteriosis dropped 5% from the year before and in 2003 another 19%, possibly as a result of the control program (Figure). Thus, the program appears to have a positive effect, which lends further support to the hypothesis.

In conclusion, the results of this study support the hypothesis that fresh chicken is the main risk factor for domestically acquired campylobacteriosis in Denmark. This risk is significantly increased in the summer, when the incidence of infected broiler flocks peak, and when whole chickens are prepared. Travel to southern Europe and travel outside Europe, respectively, were also significant risk factors. The marked increase in consumption of fresh poultry during the 1990s may explain, at least in part,

the increased incidence of human campylobacteriosis in Denmark in this period.

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Ophthalmic Complications of Dengue

The Eye Institute Dengue-Related Ophthalmic Complications Workgroup[1]

We report 13 cases of ophthalmic complications resulting from dengue infection in Singapore. We performed a retrospective analysis of a series of 13 patients with dengue fever who had visual impairment. Investigations included Humphrey automated visual field analyzer, Amsler charting, fundus fluorescein angiography, and optical coherence tomography. Twenty-two eyes of 13 patients were affected. The mean age of patients was 31.7 years. Visual acuity varied from 20/25 to counting fingers only. Twelve patients (92.3%) noted central vision impairment. Onset of visual impairment coincided with the nadir of serum thrombocytopenia. Ophthalmologic findings include macular edema and blot hemorrhages (10), cotton wool spots (1), retinal vasculitis (4), exudative retinal detachment (2), and anterior uveitis (1). All patients recovered visual acuity to 20/30 or better with residual central scotoma by 12 weeks. These new complications suggest a widening spectrum of ophthalmic complications in dengue infection.

Dengue fever (DF) is the most prevalent form of flavivirus infection in humans. Borne by the *Aedes* mosquito, the infection is endemic in the tropics and warm temperate regions of the world. The highest incidence occurs in Southeast Asia, India, and the American tropics. Worldwide cases of illness exceed100 million per year (*1*,*2*).

Dengue hemorrhagic fever (DHF) is a severe and potentially fatal form of the disease. Twenty-five thousand deaths are reported annually to the World Health Organization (WHO). The annual incidence now exceeds 500,000 cases annually and is still rising, despite environmental controls (*3*). DHF is strongly related to previous sensitization of heterologous dengue infection. Increasing endemicity and co-circulation of different serotypes is therefore necessary for the increase in incidence of DHF.

DF is characterized by an abrupt onset of fever after a 2- to 7-day incubation period, with temperatures reaching 41°C. Other symptoms include severe malaise, headaches, and retroorbital and lumbrosacral pain. Patients also experience respiratory symptoms (sore throat, rhinitis, and cough), nausea, anorexia, and altered taste sensation. A transient macular rash is often seen on day 1 to day 2 of illness. This rash disappears, but a second, maculopapular rash appears on days 3–6 of illness. The secondary rash coincides with defervescence and typically involves the trunks, limbs, and face; palms and soles are spared. Blood dyscrasias include thrombocytopenia and neutropenia (leukopenia). The illness is usually self-limiting with minimal systemic sequelae, but it may require prolonged convalescence lasting several weeks.

DHF is defined by WHO as DF associated with thrombocytopenia ($<100 \times 10^9$ cells/L) and hemoconcentration (hematocrit >20% above baseline). Its most severe form, dengue shock syndrome (DSS), is associated with hypotension, narrowing of pulse pressure (<20 mm Hg), and circulatory failure in 30% of cases. The early phase of DHF is indistinguishable from DF. The death rate for untreated DHF/DSS can be as high as 10%–15% in places where emergency supportive treatment with intravenous fluids and platelet replacement is not readily accessible (*4*).

Ophthalmic complications associated with DF and DHF have not been classically described. Within the ophthalmic community, this complication is being observed more frequently in recent times. However, only a few isolated case reports have been published (*5*–*13*). These reports attribute ocular complications to the transient thrombocytopenia and resulting bleeding diathesis. The course of the eye manifestations has also not been well-described. We report a series of 13 patients who had ophthalmic symptoms after DF or DHF, and describe the course, spectrum of manifestations, and prognosis and treatment of these new and emergent complications.

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Methods

We describe a retrospective observational case series of 13 patients who were seen at The Eye Institute (Singapore) over 6 months from September 2004 to February 2005. Patient follow-up varied from 2 weeks to 5 months. Diagnosis was made by a referring infectious disease physician on the basis of characteristic clinical signs and symptoms and confirmed on dengue polymerase chain reaction (PCR), dengue serology (immunoglobulin M [IgM] and IgG seroconversion), or both.

Real-time automated reverse transcriptase (RT)-PCR assay was conducted with the Dengue LC RealArt RT-PCR Kit on the Light Cycler (Roche Diagnostics, Mannheim, Germany) in patients with <5 days of fever. In patients with pyrexia in excess of 5 days, serologic studies were conducted with the PanBio (Sinnamon Park, Queensland, Australia) Dengue Duo IgM and IgG Rapid Strip Test. Classification of DF and DHF was made on the basis of WHO guidelines.

Patients were referred to The Eye Institute following complaints of visual symptoms. All patients had visual acuity measured with a Snellen acuity chart. All underwent a full slit-lamp anterior segment examination as well as dilated fundi examination with slit-lamp biomicroscopy. Upon clinical diagnosis, patients underwent further testing of visual fields (Humphrey automated visual field analyzer [HVF], Amsler charting, and fundal fluorescein angiography [FA]), and measurement of central macular thickness with optical coherence tomography (OCT3, Zeiss, Göttingen, Germany).

Patients were followed up by examination of serial platelet counts until at least 2 consecutive counts showed an upward trend. Retinal findings were documented with serial color fundal photography. Tests (HVF, FA, and OCT) were repeated based on clinical assessment of the patient's response and clinical signs of resolution.

Results

Demographics

Thirteen patients (6 male, 7 female) with ophthalmic symptoms following DF were reviewed. Eleven patients were Singaporeans; the other 2 were Chinese nationals. All cases were contracted in Singapore, based on the absence of travel history 1 month before the illness. All but 1 patient (Malay) was of Chinese race. The ages ranged from 20 to 49 years (mean 31.7 ± 7.9 years, median 31 years) with no age differences between male and female patients. All but 1 patient were classified as having DF (online Appendix Figure, from http://www.cdc.gov/ncidod/EID/vol12no02/05-0274-G_app.htm).

Symptoms

All patients complained of blurring of vision. Nine patients described bilateral visual symptoms in both eyes; 4 (30.7%) noted unilateral visual impairment. Twenty-two eyes from 13 patients were affected. Snellen visual acuity varied from 20/25 to counting fingers only (median 20/40). Seven eyes (31.8%) had vision of 20/100 or worse. Twelve (92.3%) patients described blurring associated with a loss of central vision (relative central scotoma) (Figure 1). This symptom was demonstrated on Amsler charting and automated HVF testing.

Onset of Visual Symptoms

The onset of visual symptoms closely correlated with the nadir of thrombocytopenia associated with DF. Of the 9 patients with available daily serial serum platelet measurements, all had visual symptoms within 1 day of their lowest platelet counts. Five (55.6%) patients complained of visual symptoms on the day of their nadir; 2 patients exhibited this symptom 1 day after their lowest count, and 2 patients had this symptom 1 day before their lowest count (mean 6.8 ± 0.8 days, median 7 days) (Figure 2).

Signs

The most common ophthalmic signs were found on the macular region of the retina (Figure 3). Macular edema was the most common pathology; it occurred in 10 (76.9%) patients. The second most common finding on ophthalmoscopy was macular hemorrhage (9 [69.2%] patients). Characteristically, these took the form of blot hemorrhages. These areas corresponded to the areas of visual scotoma experienced by the patients. Four cases of vasculitis occurred. One involved the macular vasculature, and 3 patients had panretinal vasculitis. Two cases with severe panretinal vasculitis were associated with exudative retinal detachment. Other less common fundus findings include perifoveal telangectasia and cotton wool spots, both at the macula and peripheral retina. Anterior segment findings were relatively uncommon in our series. Only 1 patient had associated anterior uveitis.

Investigations

Diagnosis of dengue was based on a combination of clinical findings correlated to positive results from dengue serology, PCR, or both. Serial serum platelet measurements tracked the thrombocytopenic pattern. All but 4 patients had available serial serum platelet measurements. Three patients had their first platelet measurements only after 1 week of visual symptoms. One patient had been transfused before onset of visual symptoms, but even then, when examined, her platelet count was only 5.0×10^9 cells/L. All but 1 patient was classified as having primary

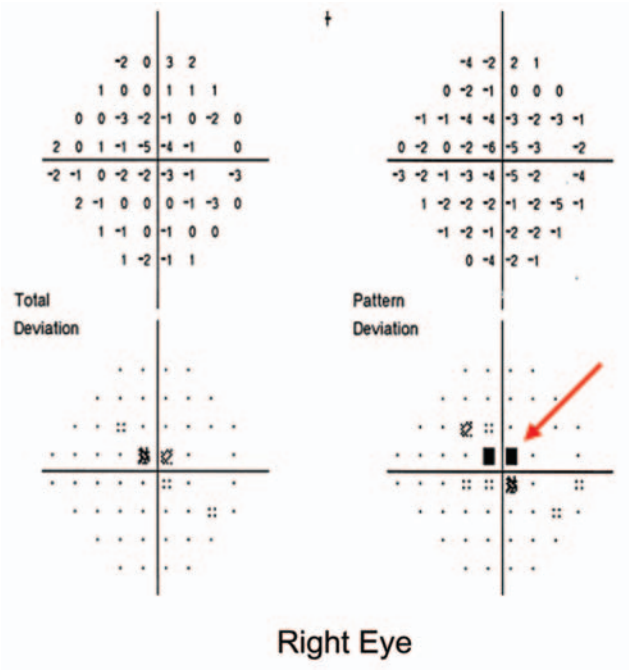

Figure 1. Humphrey visual fields of patient 9 at 1 week after onset of visual symptoms. Central scotoma of the right visual field is denoted as black squares (red arrow).

dengue. Patients with <5 days of dengue symptoms with a positive PCR but negative IgG serologic findings were deemed to have primary dengue. Alternatively, patients with dengue symptoms of >5 days' duration who had a positive IgG serologic finding were classified as having secondary dengue infection.

The mean platelet nadir at the time of onset of visual complaints was $42.8 \pm 20.1 \times 10^9$ cells/L (range $5–77 \times 10^9$ cells/L, normal $160–390 \times 10^9$ cells/L). Complete blood count showed that these findings corresponded to their peak hematocrit of $43.0 \pm 4.3\%$ and a leukopenia nadir of $2.4 \pm 1.0 \times 10^9$ cells/L (range $1.3–3.9 \times 10^9$ cells/L, normal $4–10 \times 10^9$ cells/L). All patients demonstrated central scotoma due to macular pathology by means of Amsler chart reading and automated visual field (HVF) testing. Fundal fluorescein angiography (FA) performed in 4 severe cases demonstrated extensive fluid leakage from retinal vessels corresponding to clinical observation of macular edema (Figure 3B) and peripheral vasculitis. This finding was corroborated on OCT (Figure 3C), which showed thickening of the macula.

Management and Progress of Retinopathy

All but 2 patients were treated conservatively. For these 11 patients, clinical signs resolved spontaneously and rapidly after they recovered from thrombocytopenia (median 3 days). Two patients with extensive panretinal vasculitis and exudative detachment were treated with systemic steroids. One patient was given oral prednisolone at a dose of 1 mg/kg/day for 1 week; this dosage was tailed off slowly over 2 months. The other patient received 6 hourly doses of intravenous methylprednisolone 250 mg for 3 days, followed by oral prednisolone at 1 mg/kg/day for 1 week, tailed off over the next 2 months in a similar manner. None of the patients who had steroid treatment reported adverse effects after steroid treatment. Both patients demonstrated visual recovery with resolution of clinical signs after 1 month. One patient with bilateral anterior uveitis was treated with topical prednisolone 1%. The anterior uveitis resolved by day 7 with no subsequent relapse, and the medication was tapered off (online Appendix Figure).

Outcomes and Prognosis

One patient defaulted follow-up after 2 weeks because vision had returned to normal. The remaining 12 patients had a recovery period between 6 days to 3 months. Resolution of clinical signs was closely followed by improvement of their Snellen acuity back to pre-retinopathy levels. Nine patients (75%) achieved a best corrected visual acuity of 20/25 or better (mean 4.0 weeks). However, despite resolution of ocular signs, all reported residual mild central scotoma that was reflected on HVF as an area of subtle decrease in sensitivity in the central vision (Figure 1). This persisted even up to 3 months after complete systemic recovery.

Discussion

Dengue is the most common mosquitoborne viral disease in humans. In recent years, it has become a major international public health concern. Globally, 2.5 billion people live in areas where dengue viruses can be transmitted (*4,14–16*). Over the past 25 years, the geographic spread of both the mosquito vectors and the viruses has led to the global resurgence of epidemic DF and emergence of DHF; with the development of hyperendemicity in many urban centers of the tropics. Though Southeast Asian in origin, this study would be relevant to clinicians across continents where dengue has taken a foothold.

The spectrum of ophthalmologic manifestations would lead one to conclude that several pathophysiologic processes are involved. The first and most obvious pathogenesis would be the thrombocytopenic state, with its resultant bleeding tendency, which gives rise to increased incidence of hemorrhage. These hemorrhages manifest as retinal blot hemorrhages in the macula and retinal periphery. We believe that the preponderance of cases found with complications located at the macula in our series may be due to the higher likelihood of awareness by the patient of visual impairment resulting from poor central vision. The incidence of dengue-related complications may be higher,

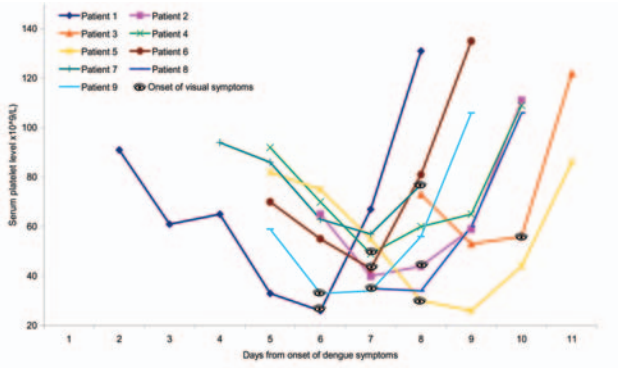

Figure 2. Trend of serial serum platelets after the onset of dengue virus infection.

given that some patients with changes occurring exclusively in the retinal periphery may not have any perceptible visual impairment. Macular edema and occult vascular changes with minimal functional disturbance may also be unreported by the patient. Clinically, these cases may even be missed on examination alone. Investigations such as fundus FA and OCT can help to detect these occult cases. These signs could also lend insight to the microvascular changes that may be occurring in the rest of the body. However, a hypocoagulable state alone would not account for the entire range of complications seen. The presence of periphlebitis, anterior uveitis, and macular edema indicate a hyperpermeable and inflammatory process. Parallels can also be drawn from the observation that the visual symptoms tend to occur and manifest at or close to the moment when the serum platelets and leukocytes levels reach their trough, while the disease is at its peak.

An hypothesis about the pathogenesis of DHF, though proven true in vivo, involves immune clearance by way of induction of cross-reactive T-cell memory, T-cell proliferation, and recognition of dengue viral antigens on infected monocytes by sensitized CD4+CD8– and CD4–CD8+ cytotoxic T cells. This results in the release of cytokines with vasoactive and procoagulant properties (interleukins, tumor necrosis factor, platelet-activating factor, and urokinase) (*17*,*18*). Vasoactive and inflammatory mediators cause capillary leakage, which may form the basis for macular edema and breakdown of the aqueous blood barrier, resulting in anterior uveitis and periphlebitis. In the series reported by Lim et al., ocular complications were mainly confined to the maculae (*5*). However, in our series the extent of involvement includes both the peripheral retina in the posterior segment and the anterior segment (anterior uveitis), which suggests a more widespread inflammatory process in the eye.

The onset of visual symptoms occurs on or close to the day of the lowest serum platelet level. Visual recovery, in the form of improvement of signs and symptoms, usually corresponds to improving platelet levels but may take several weeks to reach a steady state. Most patients report residual visual impairment in the form of central or paracentral scotoma.

The use of systemic steroids in 2 patients did not appear to aggravate the visual complications or the systemic dengue infection. This finding is supportive of an inflammatory or immune-mediated pathophysiology after acute dengue infections. Visual symptoms and visual acuity recovered in the same manner and speed as in patients with milder, untreated cases. However, like the other patients, both also described a persistent central scotoma despite normal functional Snellen visual acuities. However, we were not able to draw any statistical conclusions on the efficacy of treatment outcomes.

Our findings may have arisen as a result of an increase in incidence and awareness of DF in Singapore (*19*). However, we believe that these complications may constitute a change in the pathoimmunology of the disease. The increase in inflammatory response seen in recent DF patients may be due to a change in pathogenicity of the virus, although any viral mutation would be speculative at

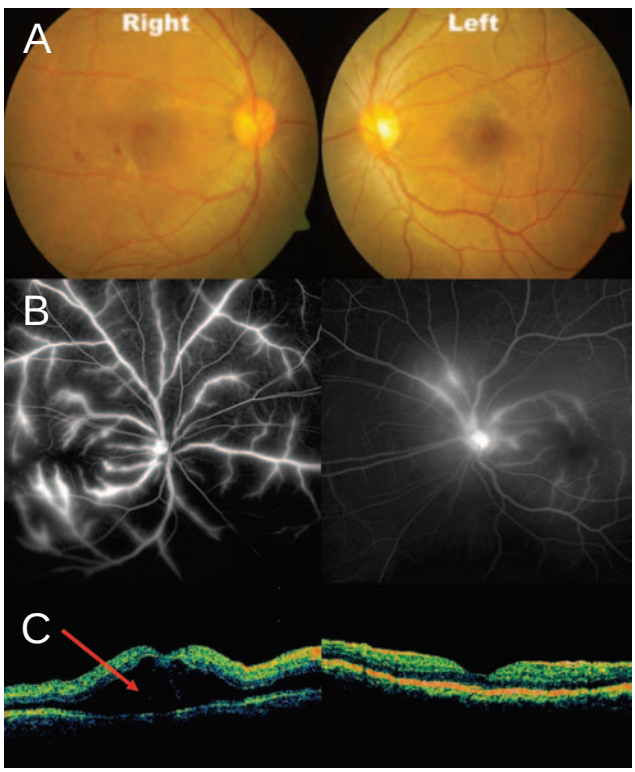

Figure 3. Fundal photos, fundal fluorescein angiography (FA) and optical coherence tomography (OCT) of patient 9. A) areas of blot hemorrhages temporal to the right fovea. B) bilateral dye leakage from the retinal veins, more severe on the right than left. C) OCT gives a 2-dimensional graphic representation of a cross-section of the macular region. The area marked with the red arrow marks the site of exudative retinal detachment. Both sides have marked retinal thickening (edema). Photo: Ken Thian.

best with our current understanding of the disease. Hence, the identification of serotypes or viral RNA epitopes in future studies might identify particular serotype or combinations of serotypes, as in the case of secondary infections, of heterologous dengue serotypes that might be found to confer a higher risk of ocular and possibly systemic complications.

This case series describes the widest variety of ocular complications of dengue infection to date. Although the ophthalmic community has been reporting more of such cases in recent times, the number of cases in this series is still relatively small and represents a limitation to the results of this report. No attempt at randomization had been made with regards to treatment. Management was based on clinical judgment on the progress of pathologic features. However, we feel that the consistency of visual outcomes in these patients still reflects the course of dengue-related ophthalmic complications.

In conclusion, DF and DHF can cause ophthalmic symptoms that were not previously well-described in the medical literature. Blurring of vision typically coincides with the nadir of thrombocytopenia and occurs ≈1 week after onset of fever. Clinical features include retinal edema, blot hemorrhages, and vasculitis. Less common features include exudative retinal detachment, cotton wool spots, and anterior uveitis.

Prognosis is generally good as the disease is often self-limiting, resolving spontaneously even without treatment. However, patients may experience mild relative central scotoma that may persist for months. The use of steroids in treating this inflammatory eye condition is controversial. A randomized controlled trial is under way to evaluate the effect of systemic steroids on dengue retinopathy; results will be reported in due course.

With increasing epidemicity and co-circulation of multiple dengue serotypes, the occurrence of DF and DHF is set to rise. Similarly we expect to see an increase in this newly emergent facet of dengue ophthalmic morbidity. A heightened awareness of dengue-related ophthalmic complications among clinicians involved in the care of patients with dengue would facilitate prompt referral for ophthalmologic assessment and management.

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Scrub Typhus, Republic of Palau

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Scrub typhus, caused by *Orientia tsutsugamushi*, is a severe febrile illness transmitted to humans by trombiculid mites, which normally feed on rodents. The first known outbreak of scrub typhus in Palau occurred in 2001 to 2003 among residents of the remote southwest islands. To determine the extent of scrub typhus distribution in Palau, we tested serum samples from humans and rodents for antibodies to *O. tsutsugamushi*. Of 212 Palau residents surveyed in 2003, 101 (47.6%) had immunoglobulin G (IgG) antibody titers ≥1:64, and 56 (26.4%) had concurrent IgG and IgM antibody titers ≥1:512 and 1:64, respectively. Of 635 banked serum samples collected from Palau residents in 1995, 34 (5.4%) had IgG antibody titers ≥1:64. Sera collected from rodents (*Rattus norvegicus* and *R. rattus*) in 2003 and 2005 were tested, and 18 (28.6%) of 63 had IgG antibody titers ≥1:64. These findings suggest that scrub typhus is endemic in Palau.

Scrub typhus is a zoonotic illness caused by *Orientia tsutsugamushi*. The pathogen is transmitted through the bite of larval mites (chiggers) of the *Trombiculidae* family, which serve as both the vector and the reservoir (*1*,*2*). Rodents of the family *Muridae* (rats and mice) are common hosts for trombiculid mites and may support *O. tsutsugamushi*. Geographically specific foci of scrub typhus are thus determined by the distribution of vector mites and their rodent hosts and by interactions of mites and rodents with humans (*3*). Scrub typhus has been reported from many regions of Asia and the Pacific islands, and known disease-endemic regions extend from Japan and eastern Russia southward to Australia and westward to Pakistan and Afghanistan (*4*,*5*).

Scrub typhus is typically a nonspecific febrile illness; its severity may be influenced by the strain of *O. tsutsugamushi*, a person's immune status, and other factors. Diagnosis may be complicated in areas where the disease has not been documented recently or in regions lacking the capacity for laboratory confirmation. Illness develops after an incubation period of 6 to 21 days and usually begins with an eschar at the site of a chigger bite. Fever, headache, and myalgias are common, and a maculopapular rash may also be present. Nausea, vomiting, diarrhea, or lower respiratory symptoms can also occur. Manifestations such as pneumonitis, meningoencephalitis, jaundice, renal failure, and myocarditis can develop during the prolonged clinical course of untreated illness (*6*). Establishing the diagnosis and initiating prompt antimicrobial drug therapy are important because death rates for untreated scrub typhus patients are 1%–30% (*5*). Scrub typhus is effectively treated with doxycycline, and treatment should begin immediately upon suspicion of illness without awaiting laboratory confirmation.

From October 2001 to October 2003, an outbreak of scrub typhus was confirmed among residents of the Republic of Palau, a Pacific island nation 900 km east of the Philippines (Figure 1). The outbreak occurred among residents of several remote southwest islands (*7*). These islands, ≈300 km from the capital of Koror, are difficult to reach, and affected persons required emergency evacuation by boat to Koror for treatment. This outbreak affected primarily children, and illness was characterized by fever and severe abdominal distress (*7*). Infection with *O. tsutsugamushi* was confirmed by serologic testing at the Centers for Disease Control and Prevention (CDC), where extremely high titers of antibodies to *O. tsutsugamushi* were demonstrated in patient serum specimens (IgG range 1:2,048–1:262,144, IgM range 1:1,024–1:16,384) (*7*). Before this outbreak was confirmed, scrub typhus had not been recognized in Palau. To better direct efforts to control the disease, Palauan public health officials needed to determine whether *O. tsutsugamushi* was restricted to these remote southwest islands or whether the pathogen was present in other parts of Palau. In addition, public health

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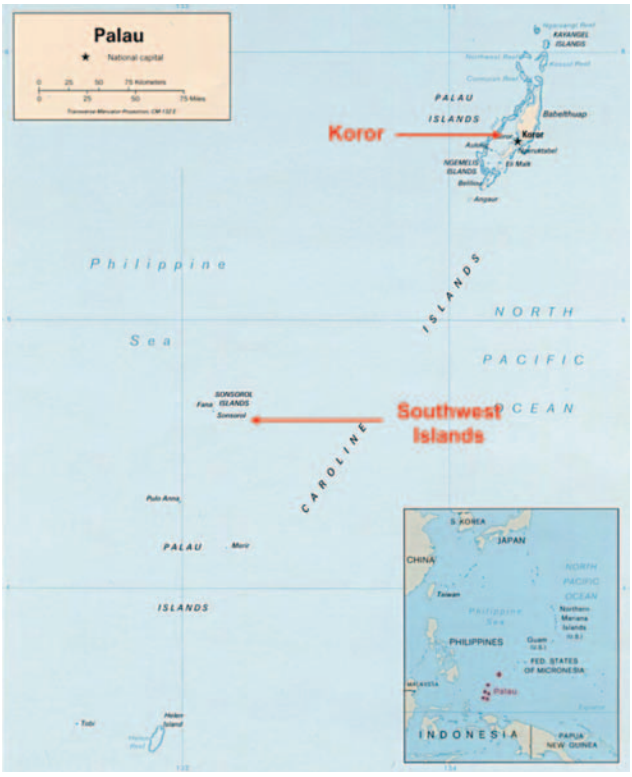

Figure 1. The Palau Islands. Map courtesy of the Central Intelligence Agency, 2004 (available from http://www.cia.gov/cia/publications/factbook/geos/ps.html).

officials wanted to ascertain whether *O. tsutsugamushi* had been recently introduced to Palau or whether it is endemic but poorly recognized. We conducted an investigation in 2003 and 2005 to assess antibodies to *O. tsutsugamushi* among humans and rodents from various regions of Palau. In addition, we assessed the historical presence of scrub typhus by examining banked serum collected from residents of Palau in 1995.

Methods

Human Serosurvey, 2003

A prospective serologic survey was conducted among residents of Palau in December 2003. Three distinct groups were assessed: 1) residents of the southwest islands, 2) residents of Echang hamlet (a community within Koror inhabited by migratory southwest island residents and their families), and 3) residents of other Koror hamlets. Although residents frequently move between the southwest islands and Echang, they seldom migrate from these areas to other hamlets in Koror.

Serum samples from consenting residents were tested for antibodies to *O. tsutsugamushi* (Karp strain) by indirect immunofluorescence assay (IFA) and described previously (*7*,*8*). Antigen suspensions from the Karp strain of *O. tsutsugamushi* were prepared in chicken yolk sac and pipetted onto slides coated with bovine serum albumin (BSA, 1% in sterile water), air dried, fixed with acetone, and stored at –75°C until use. Slides were warmed to room temperature in desiccated conditions. Serial 2-fold dilutions, beginning at 1:16, were made in sample diluent (phosphate-buffered saline [PBS], pH 7.38, with 1% BSA and 1% normal goat serum) and added to slides for 30-min incubation at 37°C, followed by washing in PBS, pH 7.38, for 15 min (3 washes × 5 min). An optimized dilution (1:150) of fluorescein isothiocyanate (FITC)–labeled goat antihuman conjugate IgG (γ-chain-specific) (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA) was then applied to the slides, which were incubated and washed as before; Eriochrome Black T counterstain was added to the middle wash. After glycerol-PBS mounting medium and coverslip were applied, the slides were read at a magnification of 400× with an epifluorescence UV microscope. Any reactive samples were then titrated to endpoint by using IgG-specific (γ) conjugate. Titers were recorded as the reciprocal of the highest dilution displaying specific fluorescence. For IgM testing, the samples were first depleted of IgG by using a recombinant protein G device (Rapi-Sep-M kit, Pan Bio, Columbia, MD, USA). This procedure resulted in a final 1:8 dilution of the serum sample, which was then diluted further in sample diluent and placed onto slides. The protocol is similar to that detailed above for IgG, but it used FITC-labeled, goat antihuman IgM (μ-chain specific) conjugate at a working dilution of 1:100.

For specimens with an anti–*O. tsutsugamushi* IgG antibody titer ≥1:16, endpoint titers were determined for IgG and IgM by serial dilution of samples. An IgG antibody titer ≥1:64 was considered seropositive and indicated past exposure to *O. tsutsugamushi*. Concurrent IgG and IgM antibody titers ≥1:512 and ≥1:64, respectively, were con-

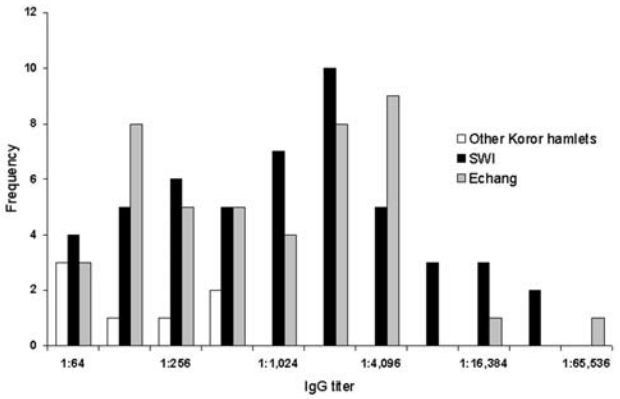

Figure 2. Anti–*Orientia tsutsugamushi* immunoglobulin G antibody titers by indirect immunofluorescent antibody assay for Palau residents, 2003. SWI, southwest islands.

Table 1. Results of *Orientia tsutsugamushi* IFA serologic testing of scrub typhus patients from the southwest islands of Palau, 5 months to 2 years after illness onset*

Patient	Days since illness onset	IgG antibody titer	IgM antibody titer
1	160	1:8,192	1:64
2	245	1:2,048	1:256
3	335	1:512	1:128
4	375	1:2,048	1:128
5	505	1:1,024	1:64
6	785	1:4,096	1:256

*IFA, indirect immunofluorescence assay; Ig, immunoglobulin.

sidered evidence of possible recent exposure to *O. tsutsugamushi*, based on assessment of serum samples collected from southwest islands scrub typhus patients 5 months to 2 years after infection (Table 1).

Questionnaires were administered to residents who provided blood specimens for the serosurvey. We collected information on history of febrile illness and residence or travel history within the past 2 years and on recreational and occupational activities. Epidemiologic and serologic data were analyzed by using EpiInfo 2002 (*9*) and the statistical package SPSS for Windows 12.0 (standard version, SPSS Inc., Chicago, IL, USA). Geometric mean titers (GMTs) were compared between locations by the nonparametric Kruskal-Wallis and Mann-Whitney tests, accounting for multiple comparison groups. All univariate analyses were conducted to account for the cluster design of the survey, with household as the primary sample unit (*10*).

Human Serosurvey, 1995

Serum specimens collected from residents of Palau during a 1995 dengue outbreak investigation were examined retrospectively for antibodies to *O. tsutsugamushi* (*11*). Samples had been stored frozen at –70°C since 1995. Samples were considered exempt from human subjects review after the removal of all identifying information so we could not obtain patient information or epidemiologic data. IFA was performed; IgG antibodies reactive with *O. tsutsugamushi* at a titer ≥1:64 indicated exposure to scrub typhus (*8*).

Rodent Surveys

Rodent trapping and sample collection were conducted in December 2003 and April 2005. Endpoint IgG antibody titers reactive to *O. tsutsugamushi* were determined by serial dilution of samples and IFA similar to that as described above for human serum samples (*8*), using a goat anti-rat IgG (γ) conjugate and positive and negative rat serum as controls. Serum specimens with an IgG antibody titer ≥1:64 were considered seropositive.

In addition, a survey of rodent activity was conducted for households visited during the prospective human serosurvey. Households were scored according to the following 3 observational categories: 1) presence of actual rodent sites, including visible evidence such as footprints, holes, and droppings; 2) appearance of potential rodent sites, including visible evidence of environmental situations that might support rodents, such as piles of debris or trash, unsealed sewers, and refuse pits, and 3) reported rodent activity by household members (reports of sightings, noises, odor, or debris, such as discarded food).

Results

Human Serosurvey, 2003

During the investigation, 212 blood samples were collected from consenting residents of 88 households, including 22 households from the southwest islands, 29 households from Echang, and 37 households from other Koror hamlets. The median age of persons from whom blood was collected was 28 years for the southwest islands, 36 years for Echang, and 36 years for other Koror hamlets; 37 (62.7%), 23 (42.6%), and 53 (53.5%) of persons were male for the southwest island, Echang, and other Koror hamlets, respectively. The average number of persons per household was 3.2, 6.8, and 5.6 for the southwest islands, Echang, and other Koror hamlets, respectively. The proportion of the overall population sampled was ≈80% for the southwest islands, 18% for Echang, and 0.78% for other Koror hamlets.

To demonstrate the range of titers observed and the differences between locations, the frequency of IgG titers in each location is shown in Figure 2. A summary of serologic results is presented in Table 2.

GMTs differed significantly among residents from different locations. Specifically, GMTs for southwest island and Echang residents were significantly higher than those for residents from other Koror hamlets ($p = 0.004$ and $p = 0.002$, respectively). Southwest island residents were significantly more likely than residents of other Koror hamlets to be seropositive (risk ratio [RR] 6.09, 95% confidence interval [CI] 3.33–11.14, $p<0.001$). Echang residents were also significantly more likely to be seropositive than were residents of other Koror hamlets (RR 5.02, 95% CI 2.86–8.80, $p<0.001$). Residents of the southwest islands and Echang did not differ significantly in seropositive status.

Table 2. Results of *Orientia tsutsugamushi* (IFA) serologic testing of Palau residents, 2003*

	SWI, n = 59 (%)	Echang, n = 54 (%)	Other Koror hamlets, n = 99 (%)
No. IgG ≥1:64	50 (84.7)	44 (82)	7 (7.1)
No. IgG ≥1:512 and IgM ≥1:64	32 (54)	22 (41)	2 (2)
Geometric mean IgG titer	996	740	102
Median IgG titer (range)	1:1,024 (1:32–1:32,768)	1:1,024 (1:16–1:65,536)	1:64 (1:16–1:512)

*IFA, indirect immunofluorescence assay; SWI, southwest islands; Ig, immunoglobulin.

The median age of seropositive persons was 30 years for southwest island residents, 35 years for Echang residents, and 30 years for residents of other Koror hamlets. In the southwest islands, residents >18 years of age were significantly more likely to be seropositive than were children (RR 1.35, 95% CI 1.00–1.82). No children were seropositive in Echang, and no significant difference in past exposure between age groups in residents of other Koror hamlets was evident. Among persons with evidence of possible recent exposure (concurrent IgG ≥1:512 and IgM ≥1:64), 25 (78.1%) of 32 southwest island residents, all (100%) Echang residents, and both (100%) residents of other Koror hamlets were adults >18 years old.

Of the 56 Palau residents with evidence of possible recent exposure to scrub typhus (concurrent IgG ≥1:512 and IgM ≥1:64), 15 (26.8%) reported that they had not traveled to the southwest islands or other islands during the past 2 years. In addition, neither of the 2 residents residing within other Koror hamlets with evidence of possible recent exposure to *O. tsutsugamushi* reported visiting Echang hamlet in the past 2 years, which suggests that their exposures occurred elsewhere in Palau.

Human Serosurvey, 1995

Serum samples collected from Palau residents during a 1995 dengue outbreak investigation were also tested for evidence of IgG antibodies to *O. tsutsugamushi*. Of 635 specimens tested, 34 (5.4%) were positive at a titer ≥1:64.

Rodent Survey

A total of 63 rodents were trapped on Palau in 2003 and 2005, including 5 from the southwest islands, 23 from Echang, and 35 from other Koror hamlets. Rodents were identified primarily as *Rattus norvegicus* (Norway or brown rat), although 6 from Echang were identified as *R. rattus* (black or roof rat). All 5 rats (100%) collected in the southwest islands had IgG antibody reactive to *O. tsutsugamushi* at titers ≥1:64 (GMT 1:112, range 1:64–1:8,192). In addition, IgG antibodies to *O. tsutsugamushi* were detected in 4 (17.4%) of 23 rats from Echang (GMT 1:24, range 1:16–1:128) and 9 (25.7%) of 35 rats from other Koror hamlets (GMT 1:32, range 1:16–1:2,048).

A survey to assess rodent activity was conducted at households in the southwest islands, Echang, and other Koror hamlets that were visited as part of the human serosurvey. Significantly more actual and potential rodent sites were observed in the southwest islands and Echang than in other Koror hamlets (Table 3, $p<0.001$).

Discussion

After *O. tsutsugamushi* was identified as the cause of an outbreak of severe illness among residents of Palau from 2001 to 2003 (*7*), officials were concerned about what was perceived to be a newly emergent disease in the remote southwest islands. This investigation was conducted to determine the historical presence and current distribution of scrub typhus among rodent reservoirs and human hosts in Palau to better direct efforts to control disease. We found widespread seroprevalence of antibodies to *O. tsutsugamushi* among both humans and rodents from several areas of Palau, including the southwest islands, Echang, and other Koror hamlets. Although the 2001–2003 outbreak involved only patients from the southwest islands, and to date no patients have been identified from Koror, our data show that scrub typhus is likely endemic in many areas of Palau. We also identified antibodies to *O. tsutsugamushi* among banked serum samples collected from residents of Palau in 1995, which suggests that the disease has been present in the region for at least a decade. Thus, the 2001–2003 outbreak of scrub typhus in the southwest islands is unlikely to be a result of a recent introduction of the pathogen and is probably related to unique host and environmental factors that increased occurrence or recognition of an established disease.

Although the 3 areas had significant differences in seroprevalence, we did not observe any significant differences in individual or household risk factors for seropositive status between these geographic areas (data not shown). We did observe differences in general household environments and individual activities between the 3 geographic areas. Specifically, residents of Echang and the southwest islands appeared to be more frequently exposed to rodents and outdoor environments where mite exposure might be expected to be increased. Because southwest island residents were younger, they may be more likely to engage in recreational activities that place them at increased risk for mite exposures. In addition, residents of Echang and the southwest islands were often fishermen or construction workers and thus more likely to engage in outdoor occupational activities.

These data are subject to several limitations. We did not evaluate the possible influence of immunologic cross-

Table 3. Evidence of rodents around households in Palau, 2003*

Evidence	SWI	Echang	Other Koror hamlets
Average no actual rodent sites per household	1.55†	2.34†	1.17
Average no. potential rodent sites per household	3.41†	2.55†	1.61

*SWI, southwest islands.
†Significantly greater than Koror, p<0.001.

reactivity between *O. tsutsugamushi* and other disease agents; however, *O. tsutsugamushi* is antigenically distinct from other rickettsiae, and cross-reactivity is thought to be minimal. The criteria used to define a possible recent exposure to *O. tsutsugamushi* were determined through assessment of scrub typhus patients from the southwest islands who were tested 6 months to 2 years after infection; however, because the sample size used for this determination was small, we cannot predict the sensitivity of this designation. Furthermore, we cannot rule out the possibility of reexposure as a possible explanation for elevated titers in persons assessed in the serosurvey nor quantify how reexposure may influence our estimation of recent versus past exposure. Finally, the retrospective human serosurvey used specimens collected in 1995 from clinically ill patients as part of a dengue fever outbreak, and long-term storage of these specimens may have influenced detectable antibody titers. In contrast, the 2003 human serosurvey included only healthy residents, and serum samples were tested within 1 year of collection.

Although no human cases of scrub typhus have been recognized to date among residents of the main island of Koror, this investigation indicates that 41% of residents of Echang and 2% of residents of other Koror hamlets had serologic evidence that suggested a possible recent exposure to scrub typhus. The clinical manifestations of scrub typhus are often nonspecific and are similar to those of other endemic zoonotic and vectorborne diseases in Palau, such as leptospirosis and dengue fever. In addition, the severity of disease associated with scrub typhus can be highly variable; the disease may be milder among persons with partial prior immunity. No laboratory testing for scrub typhus was conducted before the 2001–2003 outbreak. Thus, cases of scrub typhus were likely occurring on the main island of Koror but were unrecognized or masked because of the presence of other, clinically similar, endemic diseases.

Eschars or rashes, which are characteristic of scrub typhus infection, may arouse clinical suspicion, but they may be difficult to observe in darker skinned persons, including Pacific islanders. In addition, eschars are less frequently reported in regions where the disease is hyperendemic because of partial immunity from prior exposures (*5*). None of the patients identified during the 2001–2003 outbreak on the southwest islands had an eschar recorded. The absence of severe disease among Palau residents with serologic evidence of recent exposure, as well as the absence of reported eschars among scrub typhus patients from the 2001–2003 outbreak, lends further support to the endemicity of scrub typhus in the region.

The location of Palau and its similarity in terrain and climate to other known disease-endemic regions suggest that this environment might readily support an endemic focus of scrub typhus. The exact role of rodents in distribution and transmission of *O. tsutsugamushi* is not well elucidated, but the detection of rats with antibodies in Palau suggests infected mites and thus indicates a risk for humans to acquire infection (*1–3,12*). Because rats are the common host for the mite that transmits *O. tsutsugamushi*, rodent burrows in close proximity to humans are a substantial and controllable risk factor. This investigation showed that households in the southwest islands and Echang were significantly more likely to have evidence of rodents than were other hamlets in Koror and might benefit from targeted rodent control programs.

The results of our investigation demonstrate the presence of *O. tsutsugamushi* throughout Palau, and historical assessments provide evidence that the disease has been present in the region as early as 1995. Although human cases of scrub typhus appear to be currently limited to the remote southwest islands of Palau, the serologic evidence of exposure to *O. tsutsugamushi* in Echang and other hamlets of Koror indicates that outbreaks could emerge in these locations. Active surveillance for human cases, coupled with appropriate laboratory diagnostics, has been implemented in Palau to detect cases. In addition to aiding physicians in diagnosing and treating scrub typhus patients more effectively, such surveillance ensures that future outbreaks are detected quickly. Continued surveillance for antibodies to *O. tsutsugamushi* among humans and rodents in various locations throughout Palau will help identify foci of infections and direct aggressive rodent and mite control activities.

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etymologia

Orientia tsutsugamushi

[or″e-en′she-ə (t)süt″sə-gə-mü′she]

Etiologic agent of scrub typhus, transmitted by the bite of thrombiculid mite larvae. From the Latin *oriens*, "east" and the Japanese *tsutsuga*, "sickness" plus *mushi*, "insect." The disease was first documented in China in 313 AD and has been a frequent cause of illness in soldiers stationed in the western Pacific. In Vietnam, *O. tsutsugamushi* was among the most common causes of fever in soldiers.

Sources: Dorland's illustrated medical dictionary. 30th ed. Philadelphia: Saunders; 2003; Merriam-Webster's collegiate dictionary. 11th ed. Springfield (MA): Merriam-Webster Incorporated; 2003; and Raoult D. Scrub typhus. In: Mandel GL, Bennett JE, Dolin R, editors. Principles & Practice of Infectious Diseases. 6th ed. Churchill Livingstone; 2004. p. 2309-10.

Global Socioeconomic Impact of Cystic Echinococcosis

Christine M. Budke,* Peter Deplazes,* and Paul R. Torgerson*

Cystic echinococcosis (CE) is an emerging zoonotic parasitic disease throughout the world. Human incidence and livestock prevalence data of CE were gathered from published literature and the Office International des Epizooties databases. Disability-adjusted life years (DALYs) and monetary losses, resulting from human and livestock CE, were calculated from recorded human and livestock cases. Alternative values, assuming substantial underreporting, are also reported. When no underreporting is assumed, the estimated human burden of disease is 285,407 (95% confidence interval [CI] 218,515–366,133) DALYs or an annual loss of US $193,529,740 (95% CI $171,567,331–$217,773,513). When underreporting is accounted for, this amount rises to 1,009,662 (95% CI 862,119–1,175,654) DALYs or US $763,980,979 (95% CI $676,048,731–$857,982,275). An annual livestock production loss of at least US $141,605,195 (95% CI $101,011,553–$183,422,465) and possibly up to US $2,190,132,464 (95% CI $1,572,373,055–$2,951,409,989) is also estimated. This initial valuation demonstrates the necessity for increased monitoring and global control of CE.

Cystic echinococcosis (CE) is a condition of livestock and humans that arises from eating infective eggs of the cestode *Echinococcus granulosus*. Dogs are the primary definitive hosts for this parasite, with livestock acting as intermediate hosts and humans as aberrant intermediate hosts. The outcome of infection in livestock and humans is cyst development in the liver, lungs, or other organ system. The distribution of *E. granulosus* is considered worldwide, with only a few areas such as Iceland, Ireland, and Greenland believed to be free of autochthonous human CE. However, CE is not evenly distributed geographically (Figure 1) (*1*). For example, the United States has few cases in livestock and most human cases are imported. The same is true for regions of Western and Central Europe. In many parts of the world, however, CE is considered an emerging disease. For example, in the former Soviet Union and Eastern Europe, the number of observed cases has dramatically increased in recent years (*2–4*). Additionally, in other regions of the world, such as parts of China, the geographic distribution and extent of CE are greater than previously believed (*5*). CE not only causes severe disease and possible death in humans, but also results in economic losses from treatment costs, lost wages, and livestock-associated production losses. To date, no global estimates exist of CE burden (total health, socioeconomic, and financial cost of a given disease to society) in humans or livestock. Such an estimate is imperative since it can be used as a tool to prioritize control measures for CE, which is essentially a preventable disease.

Two methods previously used to assess disease burden are disability adjusted life years (DALYs) and the calculation of monetary losses (*6*). DALYs were first developed in the 1990s and were used in the Global Burden of Disease (GBD) Study to determine the worldwide burden of disease due to both communicable and noncommunicable causes (*7*). Although the application of DALYs is becoming more

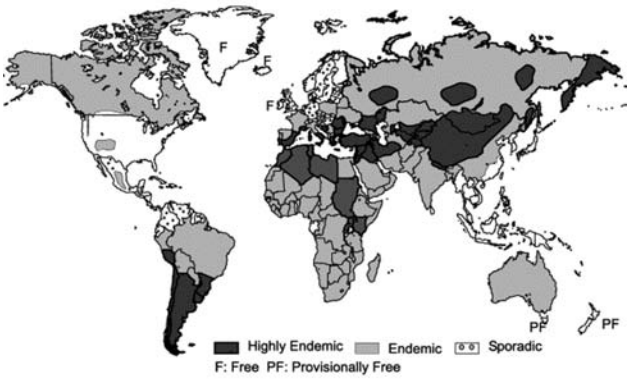

Figure 1. Global distribution of zoonotic strains of *Echinococcus granulosus*. (Adapted from Eckert and Deplazes, 2004 [*1*]. Copyright Institute für Parasitologie, Universität Zürich); used with permission.

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commonplace, the use of DALYs and the methods behind the creation of this measure remain debatable (*8*). The GBD Study was an extensive undertaking; however, echinococcosis was not among the conditions studied. Nevertheless, DALYs have been applied to cystic echinococcosis and alveolar echinococcosis, caused by *E. multilocularis*, on a small scale in western China (*9*). Likewise, monetary evaluations have been applied to CE infections in humans and livestock only at a local level (*10–14*). Global burden indicators not only give an idea of the scope of the disease under study, but can also be used to direct limited financial resources to sites where they can be most effective. Because of the magnitude of applying burden of disease measurements on a global scale, this study must be considered a preliminary estimate. Nevertheless, this report should increase awareness of the global impact of CE by both the public health and livestock sectors.

Materials and Methods

CE Incidence in Humans

Data on country-specific annual reported human CE cases were obtained from the Office International des Epizooties (OIE), World Health Organization Handistatus II database for the years 1996–2003 (*15*). This information was then merged with published case reports from numerous countries and logged into an Excel spreadsheet (Microsoft Corp., Redmond, WA, USA). Type and quality of incidence data varied by country or region; however, most data consisted of annual numbers of detected cases per susceptible population or was converted into this form for analysis purposes. If both an OIE-reported and a literature-based value were available, the larger of the 2 was used. However, if the higher value appeared to be from a survey that evaluated a highly disease-endemic region and was, therefore, not applicable to the entire country, a corresponding adjustment was made. In addition, we assumed that ≈10% of annual cases are not officially diagnosed, and those patients do not receive medical attention because of their socioeconomic status or the subclinical nature of the illness. Based on past studies, this estimate is most likely conservative (*12,14*). For example, in China, mass ultrasound screening in remote areas has shown high prevalence rates of CE (*9*). A number of these patients have advanced clinical disease but would not normally have access to treatment because of poverty and distance from medical facilities. Human cases of CE are also systematically underreported by the healthcare establishment, with up to 75% of clinic or hospital-diagnosed cases never recorded in local or national databases or published reports (*16,17*). Therefore, adjustments were made to account for the substantial underreporting of known treated cases.

CE Prevalence in Livestock

Numbers of annual reported CE cases in slaughtered livestock (sheep, goats, cattle, camels, and swine) for the years 1996–2003 were obtained from the OIE-Handistatus II database (*15*). This information was merged with abattoir studies performed in numerous countries. If data from both sources were available, the larger of the 2 estimates was used. However, if the higher value appeared to be from a region that was highly disease-endemic and was not appropriate for a countrywide estimate, an adjustment was made. Prevalence per species, for each country, was applied to the estimated number of slaughtered animals per year, with 2004 livestock numbers obtained from the FAOSTAT database (*18*). The assumption was made that approximately one fourth of sheep and goat populations, one sixth of cattle and camel populations, and the entire swine population would be slaughtered annually, based on estimated average species' lifespan (e.g., approximately one fourth of a country's sheep population would be slaughtered annually, with a typical animal life expectancy of 4 years). Such a general estimate was used because of the large amount of variation in animal production practices between and within countries. As with the human incidence data, the true number that were positive for *E. granulosus* at slaughter is substantially higher than reported. Therefore, a correction factor was used to estimate true prevalence.

Application of DALYs to Human Incidence Data

The DALY formula (shown below) was applied to global human incidence data.

$$-\left[\frac{DCe^{-\beta a}}{(\beta+r)^2}\left[e^{-(\beta+r)(L)}(1+(\beta+r)(L+a))-(1+(\beta+r)a)\right]\right]$$

In this equation, D is a disability weight, β is an age-weighting function parameter, C is an age-weighting correction constant, r is a discount rate, a is age at clinical onset, and L is the duration of disability or time lost because of death (*7*). Disability weight for CE was assigned a multinomial distribution based on numerous retrospective studies evaluating postoperative outcome (Table 1) (*19–24*). The percentage of patients projected to improve after surgery was assigned a disability weight of 0.200 (Dutch weight for clinically disease-free cancer) for 1 year, the percentage of patients projected to have substantial postsurgical conditions was assigned a disability of 0.239 (GBD weight for preterminal liver cancer) for 5 years, the percentage of patients projected to have recurrent disease was assigned a disability of 0.809 (GBD weight for terminal liver cancer) for 5 years, and the percentage of patients projected to die postoperatively were assigned a disability of 1 (indicating death) for the remainder of their predicted lifespan (*7,25*). An assumption was

Table 1. Outcome of surgery for cystic echinococcosis in humans

Country (y)	No. patients	Cure (%)	Morbidity (%)	Relapse (%)	Death (%)	Reference
Greece (1984–1990)	56	40 (72)	13 (23)	3 (5)	0	(*9*)
Italy (1950–1987)	298	244 (82)	27 (9)	15 (5)	12 (4)	(*20*)
Turkey (1992–1999)	95	32 (34)	38 (40)	24 (25)	1 (1)	(*21*)
Turkey (1990–1995)	108	88 (81)	19 (18)	0	1 (1)	(*22*)
Greece (1985–1990)	67	59 (86)	4 (6)	3 (6)	1 (2)	(*23*)
Italy (1982–1994)	89	70 (79)	17 (19)	1 (1)	1 (1)	(*24*)
Total	713	533 (75)	118 (17)	46 (6)	16 (2)	

also made that ≈10% of cases are not reported and do not receive medical treatment. These cases were assigned a disability weight of 0.200 (Dutch weight for clinically disease-free cancer) for 10 years (*25*). For the GBD Study, a standardized life table was used for *L* (*7*).

Economic Evaluation of Human-associated Losses

Overall cost per human surgical case was based on findings from previous international studies (Table 2) (*11*,*13*,*14*,*26*,*27*). Expenses taken into consideration included diagnostic costs, surgical cost, hospitalization, and postoperative costs. The average cost per surgical patient was shown to be significantly correlated (R^2 = 0.898, $p < 0.05$), with the country-specific per capita gross national income (per capita GNI) (Atlas Method) (Table 2). Therefore, the linear regression coefficient was used as a predictor of treatment costs for each disease-endemic country. In addition to medical costs and single-year wage losses, past studies have indicated an average 2.2% postoperative death rate for surgical patients (Table 1). Approximately 6.5% of cases also are assumed to relapse and require a prolonged recovery time (Table 1) (*11*). Therefore, these outcomes were also taken into account. We assumed that, in addition to surgical cases, ≈10% of cases are not officially diagnosed each year, and those patients never receive treatment. Wage losses for this group were thus taken into consideration. Economic losses in humans were also evaluated, taking into account the nearly 4-fold degree of underreporting of patients who received treatment.

Economic Evaluation of Livestock-associated Losses

Production-based losses attributable to infected sheep, goats, cattle, camels, and pigs were estimated. Losses from liver condemnation, defined as the action of preventing the sale of livers deemed unfit for human consumption (sheep, goats, cattle, pigs, camels), reduction in carcass weight (sheep, goats, cattle), decrease in hide value (sheep, cattle), decrease in milk production (sheep, goats, cattle), and decreased fecundity (sheep, goats, cattle) were taken into account. Only liver-associated losses in camels and pigs are presented since few studies have evaluated production losses from echinococcosis in these species (*28*). Losses from liver condemnation are assumed to occur since hepatic pathology is associated with infection in swine and camels (*29*). Losses from liver condemnation were presumed proportional to those used for the analysis of the economic impact of CE in Jordan (*12*). Decrease in hide value (20%) and decrease in fecundity (11%) were presumed proportional to values suggested by numerous Soviet studies conducted from the 1950s through the 1980s (*28*). Reductions in carcass weight (2.5%) and milk production (2.5%) were also based on previous reports (*30*).

Analysis

Spreadsheet models were constructed in Excel to estimate global impact of CE in terms of DALYs and monetary losses. Total disease effects, in DALYs lost or monetary costs, was calculated by summing all of the constituent components. Uncertainty in parameter estimates was modeled by using Monte Carlo techniques (*6*). Briefly, all parameters were assigned a probability distribution based on the quantity and quality of reported data. Macros were written in Excel to sample across these distributions, with 10,000 iterations of each model calculated. Mean and 95% confidence intervals (CIs) for losses were then determined from these iterations.

Reported global human incidence was assigned a normal distribution, with a standard deviation of 5%. Adjustments were then made to account for the nearly 4-fold degree of underreporting of treated cases believed to occur (*16*,*17*). In addition, cases that would not be official-

Table 2. Average cost per surgical case of cystic echinococcosis

Country	Years	Average cost per case (US $)	% of real per capita GNI* per patient	Reference
Jordan	2002	701.50	40	(*26*)
Spain	1987–2001	10,915.00	76	(*27*)
Tunisia	2000	1,481.00	71	(*11*)
Uruguay	2000	6,721.00	110	(*14*)
Wales, UK	2000	13,600.30	54	(*13*)

*World Bank Atlas method for converting data in national currency to US dollars; GNI, gross national income.

ly acknowledged had to be accounted for, i.e., cases in persons who never receive treatment in a hospital. We therefore assumed that ≈10% (uniform distribution of 8% to 12%) of cases would not be detected. This estimate is conservative compared to other country-specific estimates (*12*,*14*).

The DALY formula was applied to worldwide CE cases in a stochastic manner similar to that used to apply DALYs to echinococcosis cases in a region of western China (*10*). Mean age of clinical onset (*a*) was allocated a uniform distribution of 30 to 40 years, established on the basis of various studies (Table 3) (*4*,*9*,*21*,*31*–*34*). Numerous and varying reports have indicated the sex of CE-positive persons with women tending to be infected at a higher rate than men. Based on these reports, we assigned a uniform distribution of 50% to 60% of infected persons as female (*4*,*35*). Number of DALYs lost, using incidence values corrected and uncorrected for underreporting of surgical incidence, was determined.

Human-associated economic losses were applied in a stochastic manner similar to that used for a region of western China (*10*). Variability in surgical treatment costs, due to CE, was modeled by using a uniform distribution of 50% to 90% of per capita GNI per country and was weighted by each country's contribution to global human CE incidence (*36*). Lower income, higher unemployment, or both has been associated with a diagnosis of CE (*4*,*10*). Consequently, a decrease in wages earned was assumed, at least for the year of initial diagnosis and treatment. Therefore, all patients were assigned a uniform loss of 50% to 90% of country-specific per capita GNI for 1 year (*36*). Approximately 6.5% of patients were also assigned a 50%–90% wage loss for 4 additional years because of relapse and prolonged recovery time. In addition, 2.2% of patients were assigned a 100% wage loss until the expected retirement age of 65 due to postsurgical death. A standard 3% discounting rate was applied to all income losses (*7*). In addition to surgical cases, ≈10% of cases (uniform distribution of 8% to 12%) annually were assumed to not be officially diagnosed. A 25% wage loss for 5 years was consequently assigned to this population. This estimate is conservative and does not take into account income losses attributable to undiagnosed cases with fatal outcomes. Projections were made that assumed the absence and presence of underreporting of surgical incidence (*16*,*17*). In addition to using real per capita GNI (Atlas Method), calculations were also performed by using purchasing power parity (ppp) adjusted per capita GNI.

As with human-associated economic losses, livestock-associated losses were applied in a stochastic manner (*10*). Livestock prices were given uniform distributions of US $30–$60 for sheep, US $15–$30 for goats, US $150–$350 for cattle, US $300–$600 for camels, and US $55–$75 for pigs. Uniform distributions were used because of the large regional variations in prices and assigned in accordance with baseline prices for most affected countries. Production losses were assumed to follow a log-normal distribution; most affected animals were lightly infected, and only a small proportion of animals had severe losses. As with human cases, substantial underreporting of livestock infection was recognized, since official reporting is not mandatory in most countries. Therefore, a uniform correction factor of 1.5 to 2 was used to approximate true economic losses. A large uniform distribution was used because of the lack of information concerning true global prevalence of CE in livestock. This lack will, therefore, be represented in the wide confidence limits obtained.

Results

DALYs

Regional findings for predicted global burden of CE in terms of DALYs lost, with 95% CIs, can be found in Table 4. The most conservative estimate of number of global DALYs lost is 285,407 (95% CI 218,515–366,133), with no consideration for disease underreporting. Estimated number of global DALYs lost, taking into consideration nonreported surgical cases, is 1,009,662 (95% CI 862,119–1,175,654).

Human-associated Economic Losses

Findings for predicted regional burden of human CE in economic terms, with 95% CI, can be found in Table 5. Global losses, assuming no underreporting, are estimated at US $193,529,740 (95% CI $171,567,331–$217,773,513).

Table 3. Average age at ultrasound detection or surgery

Country	Years	Average age at onset/detection (y)	Reference
China	2001–2003	35*	(*9*)
Jordan	1994–2000	31–45†	(*31*)
Kenya (Turkana)	1979–1982	21–30*	(*32*)
Kyrgyzstan	1991–2000	22†	(*4*)
Morocco	2000–2001	32*	(*33*)
Turkey	1992–1999	44†	(*21*)
Uruguay	1991–1992	45*	(*34*)

*Age at time of ultrasound detection.
†Age at surgery.

Table 4. Estimated global impact of cystic echinococcosis in terms of DALYs lost

Region*	Total unadjusted DALYs lost (95% CI)†	Total adjusted DALYs lost (95% CI)
Western Europe, USA, Canada, Australia, New Zealand	11,842 (8,977–15,722)	41,891 (30,949–55,014)
Middle Eastern Crescent	104,503 (79,291–135,722)	370,056 (275,228–486,353)
Formerly socialist economies of Europe and Russia	17,317 (13,129–22,371)	61,369 (45,800–80,077)
China	112,451 (85,001–145,898)	398,015 (295,922–521,879)
Other Asia and Islands	1,130 (851–1,462)	4,003 (2,971–5,256)
Sub-Saharan Africa	2,639 (1,926–3,518)	9,314 (6,664–12,623)
Latin America and the Caribbean	14,834 (11,252–19,241)	52,693 (38,787–69,380)
India	20,691 (15,666–26,822)	73,364 (54,518–96,263)
World	285,407 (218,515–366, 133)	1,009,662 (862,119–1,175,654)

*Regional breakdown of disability-associated life years (DALYs) lost is based on that used in the Global Burden of Disease study (*7*).
†CI, confidence interval.

Losses, adjusted for underreporting, are estimated at US $763,980,979 (95% CI $676,048,731–$857,982,275). When ppp adjusted per capita GNI is used instead of real per capita GNI, estimated annual overall losses, without correction for underreporting, are US $484,878,359 (95% CI $432,898,134–US $542,048,125). When corrected for underreporting, annual losses are estimated at US $1,918,318,955 (95% CI $1,700,574,632–$2,142,268,992) (Table 5).

Livestock-associated Economic Losses

Estimated livestock-associated losses, with 95% CI, can be found in Table 6. Minimal annual losses, assuming liver condemnation alone with no correction for underreporting, is estimated at US $141,605,195 (95% CI $101,011,553–$183,422,465). However, when losses from additional production factors (decreased carcass weight, decreased milk production, decreased hide value, decreased fecundity) are taken into account, losses range from US $1,249,866,660 (95% CI $942,356,157–$1,622,045,957), not taking into account underreporting, up to US $2,190,132,464 (95% CI $1,572,373,055–$2,951,409,989), when underreporting is considered.

Discussion

Even without correcting for the underreporting of human and livestock cases, CE has a substantial global disease impact in terms of DALYs and monetary losses. The importance of using both indicators is illustrated by the proportional difference in DALYS lost versus economic losses per region (Tables 4 and 5). If only monetary losses were evaluated, the severity of the situation in poorer regions would be underestimated because of the decreased income and economic value of livestock products relative to more economically prosperous regions. For example, China is responsible for 40% of the world's CE DALYs but only 19% of human-associated economic losses. However, losses based on ppp-adjusted per capita GNI give a better picture of the relative distribution of disease impact (Table 5). When the number of DALYs lost, taking into account the recognized underreporting of human cases, is compared with those of other parasitic conditions evaluated by the World Health Organization (WHO), worldwide losses due to CE are slightly less than those caused by African trypanosomiasis (1,525,000) and more than those caused by onchocerciasis (484,000) or Chagas disease (667,000) (*37*). Even though estimated number of DALYs lost from

Table 5. Global annual cystic echinococcosis–associated economic losses to humans

Region	Total adjusted economic losses (95% CI)*(US $)	Total adjusted economic losses (95% CI)† (US $)
Western Europe, USA, Canada, Australia, New Zealand	$309,983,585 ($244,256,327–$383,371,741)	$354,460,281 ($277,178,852–$440,438,597)
Middle Eastern Crescent	$197,276,106 ($158,870,204–$240,282,181)	$564,496,304 ($454,402,304–$690,682,060)
Formerly socialist economies of Europe and Russia	$46,896,902 ($37,750,210–$57,355,873)	$143,921,865 ($114,323,294–$176,555,114)
China	$146,129,578 ($114,279,187–$181,937,463)	$663,712,150 ($516,048,103–$826,353,341)
Other Asia and Islands	$1,535,990 ($1,159,946–$1,946,632)	$2,412,386 ($1,826,342–$3,074,240)
Sub-Saharan Africa	$832,295 ($649,915–$1,035,681)	$5,176,229 ($3,710,869–$6,969,680)
Latin America and the Caribbean	$48,396,449 ($38,408,001–$59,672,173)	$120,717,047 ($95,789,339–$148,939,896)
India	$12,930,073 ($9,674,489–$16,499,072)	$63,422,693 ($47,576,673–$80,430,630)
World	$763,980,979 ($676,048,731–$857,982,275)	$1,918,318,955 ($1,700,574,632–$2,142,268,992)

* Income losses based on per capita gross national income (GNI) (Atlas method); CI, confidence interval.
†Income losses based on purchasing power parity–adjusted per capita GNI.

Table 6. Global annual cystic echinococcosis–associated livestock production losses

Category	Economic losses (95% CI) (US $)*
Liver condemnation†	$141,605,195 ($101,011,553–$183,422,465)
Decreased carcass weight†	$241,525,979 ($100,335,764–$518,035,773)
Decreased hide value‡	$34,871,148 ($23,965,776 – $46,162,828)
Decreased milk production§	$378,722,717 ($279,048,143–$495,682,356)
Decreased fecundity§	$453,141,617 ($278,287,046–$671,424,319)
Overall cost (no correction factor)	$1,249,866,660 ($942,356,157–$1,622,045,957)
Overall cost (adjusted for underreporting)	$2,190,132,464 ($1,572,373,055–$2,951,409,989)

*CI, confidence interval.
†Sheep, goats, cattle, camels, pigs.
‡Sheep, cattle.
§Sheep, goats, cattle.

CE is greater than estimated losses from multiple members of the tropical disease cluster, CE continues to be excluded from funding associated with conditions related to low socioeconomic status. This exclusion best illustrated by evaluating research and training funding provided by the United Nations Children's Fund (UNICEF)/United Nations Development Programme (UNCP)/World Bank/WHO-supported Special Programme for Research and Training in Tropical Diseases (TDR). If funding for CE were placed on the same scale as TDR-supported diseases, based on estimated DALYs lost, CE should receive approximately US $1,200,000 annually (Figure 2) (*38*). For now, however, CE continues to be widely underappreciated by most international agencies. These findings emphasize the need for CE to be taken seriously as a global public health condition, regardless of its economic implications. What makes this disease exceptional, however, is that it is not only a substantial human health problem but also has a considerable economic effect on the livestock industries of some of the most socioeconomically fragile countries.

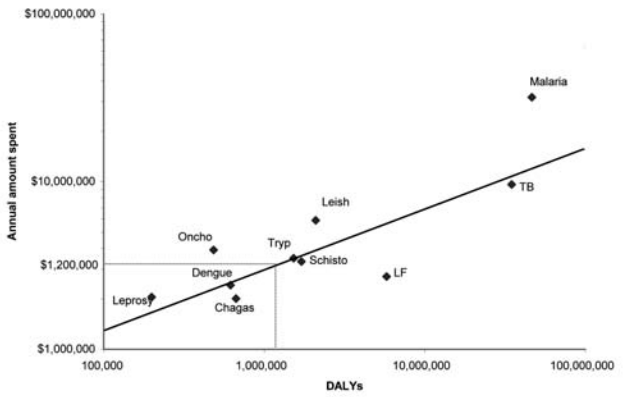

Figure 2. Annual budget (in US $) for diseases included in the United Nations Children's Fund/UNDP/World Bank/World Health Organization-supported Special Programme for Research and Training in Tropical Diseases (TDR) compared to their estimated global disability-associated life years (DALYs). The thinner lines indicate estimated DALYs lost because of cystic echinococcosis (CE) and the recommended funding level based on the TDR 2004-2005 approved program budget (Oncho, onchocerciasis; Tryp, trypanosomiasis; Schisto, schistosomiasis; Leish, leishmaniasis; LF, lymphatic filariasis; TB, tuberculosis). This figure does not take into account the substantial regional variability in both the estimates of DALYs lost and the annual budget for the diseases illustrated.

In addition to reporting the estimated global burden of CE, this study has shown the need for more accurate reporting of infected humans and livestock. Very few country-specific estimations of the true incidence of CE in humans have been made and no studies, to the authors' knowledge, that estimate its true prevalence in livestock (*16*,*17*). Presentation of the substantial economic losses for both the public health and agricultural sectors will also, we hope, encourage countries and international organizations to more closely examine potential control programs and cost-sharing methods between the 2 affected sectors (*10*).

The values presented in this paper are not definitive but instead estimates of the severity of the global situation from human- and livestock-associated CE. Considerable sums of money have been invested in the investigation and control of such parasitic conditions as lymphatic filariasis and onchocerciasis. Although these conditions can result in severe human disease, unlike CE they do not have severe secondary economic implications, such as massive livestock production losses (*39*,*40*). In addition, regional control programs that have been implemented and recommended thus far for CE, based on combinations of dog deworming, stray dog culling, sheep and goat vaccination, and education programs, have been shown to be very cost effective (*10*,*27*). CE is, therefore, a worthy condition for research and control program implementation, with substantial anticipated return on invested funding.

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Rotavirus and Severe Childhood Diarrhea

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Studies published between 1986 and 1999 indicated that rotavirus causes ≈22% (range 17%–28%) of childhood diarrhea hospitalizations. From 2000 to 2004, this proportion increased to 39% (range 29%–45%). Application of this proportion to the recent World Health Organization estimates of diarrhea-related childhood deaths gave an estimated 611,000 (range 454,000–705,000) rotavirus-related deaths.

Rotavirus is the leading cause of diarrhea hospitalization among children worldwide (*1*). In 2003, we published an estimate of rotavirus-related deaths worldwide based on a review of the literature published from 1986 through 1999 on deaths caused by diarrhea and rotavirus hospitalizations in children (*2*). This review indicated that rotavirus accounted for ≈22% of hospitalizations for childhood diarrhea. By applying this fraction to an estimate of 2.1 million annual deaths from diarrhea, we calculated that rotavirus causes 440,000 annual deaths in children <5 years of age worldwide. This estimate was ≈50% of the 1985 estimate of 873,000 rotavirus deaths per year (*3*), and the decrease in estimated rotavirus-related deaths paralleled the decrease in deaths from diarrhea of all causes from an estimated 4.6 million deaths in 1982 to 1.6–2.5 million deaths in 2000 (*4*–*6*).

Recent studies suggest that as global deaths from childhood diarrhea decreased during the past 2 decades, the proportion of diarrhea hospitalizations attributable to rotavirus may have increased. For example, prospective, sentinel hospital–based surveillance of rotavirus disease in 9 Asian countries demonstrated a median rotavirus detection of 45% among children hospitalized with diarrhea (*7*), a figure that was considerably greater than the detection rates in previous studies from the same countries. Similarly, a more extensive study of 5,768 children hospitalized from 1998 through 2000 in 6 centers in Vietnam identified rotavirus in 56% of patients (*8*), a proportion that was more than twice the 21% detection rate reported among children hospitalized with diarrhea in a hospital in Hanoi, Vietnam, from 1981 to 1984 (*9*).

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To systematically evaluate whether these recent reports are isolated observations or reflect a changing trend in the etiology of childhood diarrhea hospitalizations, we reviewed studies of rotavirus detection among children hospitalized with diarrhea published from 2000 through 2004 and compared the data with those of the previous review of studies published from 1986 through 1999.

The Study

Similar to the approach used in our previous review, we performed a computer search of the scientific literature (in English and other languages) published from January 2000 through June 2004 using the words rotavirus and the truncated stem rota-. We restricted the analysis to studies that met the following criteria: 1) were initiated after 1993; 2) were conducted for at least 1 full calendar year; and 3) examined rotavirus among at least 100 children <5 years of age hospitalized with diarrhea.

For each study, we determined the proportion of cases positive for rotavirus among children hospitalized with diarrhea. We plotted this proportion against the per capita gross national product (GNP) for the country in which the study was conducted. We then classified countries by per capita GNP into World Bank income groups (low, <US $756; low-middle, US $756–$2,995; high-middle, US $2,996–$9,265; and high, >US $9,265) (*10*), and calculated the median (interquartile range [IQR]) proportion of diarrhea hospitalizations attributable to rotavirus for each income group.

We next calculated an overall median detection rate by taking a weighted average of the median detection rates for each of the income groups. The weights assigned to each income group corresponded to the proportion of deaths from childhood diarrhea among countries in that income group as determined on the basis of our previous analysis (*2*): 85% in low-income countries, 13% in low-middle–income countries, 2% in high-middle–income countries, and <1% in high-income countries. To estimate deaths from rotavirus disease among children, we multiplied the overall median detection rate of rotavirus among children hospitalized with diarrhea by a recent World Health Organization estimate of deaths from diarrhea among children worldwide (*5*).

We abstracted information from 41 studies that met all the inclusion criteria (online Appendix Table, available from http://www.cdc.gov/ncidod/EID/vol12no02/05-0006_app.htm). Unlike the previous review of studies conducted for the period 1986–1999, in which the proportion of diarrhea-related hospitalizations attributable to rotavirus showed a distinct increasing trend with increasing income level, we found that the median detection rates increased only slightly with increasing income level (Figure 1). The median detection rate for rotavirus among children hospi-

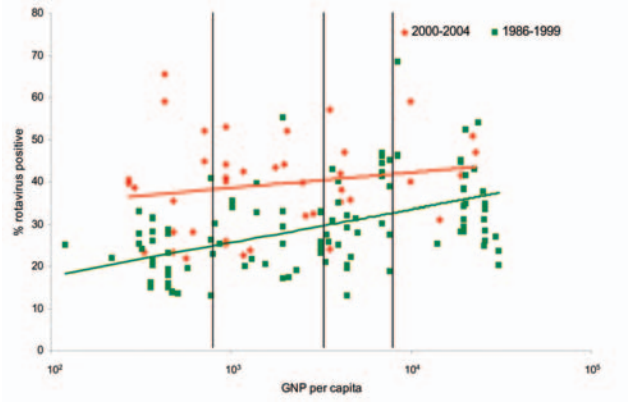

Figure 1. Percentage of severe diarrhea cases attributable to rotavirus for countries in different World Bank income groups, by per capita gross national product (GNP), for studies published in 1986–1999 and 2000–2004. GNP is in US dollars. Upper line, trend for 2000–2004; lower line, trend for 1986–1999.

Table. Percentage of diarrhea hospitalizations attributable to rotavirus for countries in different World Bank income groups, 1986–1999 and 2000–2004

	Median % (interquartile range) of diarrhea-associated hospitalizations due to rotavirus	
Income group	1986–1999	2000–2004
Low	20 (16–27)	39 (28–45)
Low middle	25 (20–33)	40 (32–43)
High middle	31 (25–42)	38 (35–45)
High	34 (28–38)	44 (40–50)
Total*	21 (17–28)	39 (29–45)

*The overall median was calculated by taking a weighted average of the median rotavirus detection rate for each income group. The weights applied to each group corresponded to that group's proportion of global diarrheal deaths: 85% for low-income countries, 13% for low-middle–income countries, 2% for high-middle–income countries, and <1% for high-income countries.

talized with diarrhea was 39% in studies conducted in low-income countries, 40% for low-middle–income countries, 38% for high-middle–income countries, and 44% for high-income countries, for an overall weighted median estimate of 39% (Table).

If we multiply the greater median rotavirus detection rate of 39% (IQR 29%–45%) from this analysis by 1,566,000 recently estimated childhood diarrhea deaths (*5*), we find that rotavirus causes ≈611,000 childhood deaths (IQR 454,000–705,000). More than 80% of all rotavirus-related deaths were estimated to occur in low-income countries of south Asia and sub-Saharan Africa (Figure 2).

Conclusions

Compared with results from studies published from 1986 to 1999, the proportion of diarrhea hospitalizations attributable to rotavirus appears to have increased between 2000 and 2004. This phenomenon likely reflects a relatively slower rate of decrease in hospitalizations for rotavirus compared with other causes of severe childhood diarrhea. This finding could be accounted for by several factors. First, interventions to improve hygiene and sanitation are likely to have a greater impact on diarrhea caused by bacterial and parasitic agents, which are transmitted primarily through contaminated food or water, unlike rotavirus, which is often spread from person-to-person. This hypothesis is supported by data from the United States (*11*) and Mexico (*12*), which showed that as diarrhea-related childhood deaths decreased dramatically in both countries; the decrease was greatest during the summer months when diarrheal diseases caused by bacteria are more prevalent. In both countries, diarrhea-related deaths in recent years have exhibited peaks only in the winter when rotavirus infections are common. Second, oral hydration therapy to replace loss of body fluids, which many regard as a major factor responsible for the decrease in diarrhea deaths (*13*), is often more difficult to successfully administer in children with severe vomiting (*14*), a common manifestation

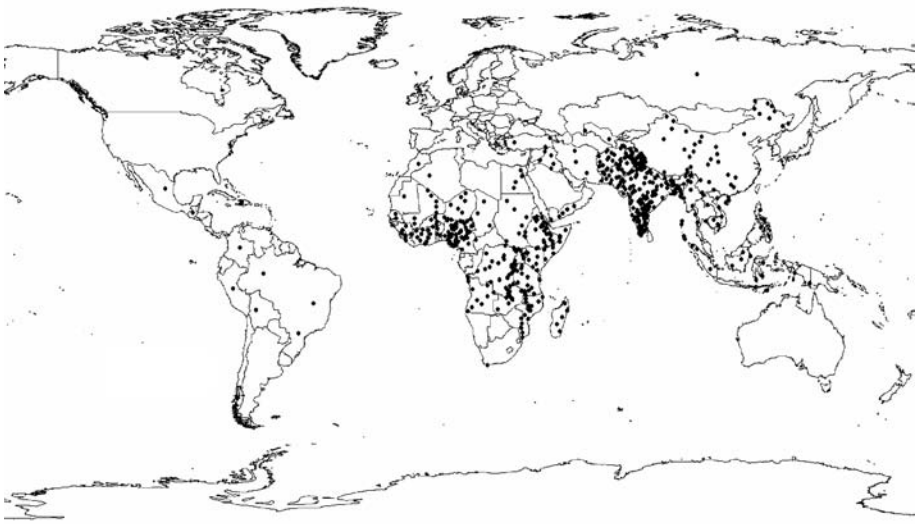

Figure 2. Estimated global distribution of rotavirus-related deaths. Each dot represents 1,000 rotavirus-related deaths.

of rotavirus disease. Third, unlike antimicrobial therapies that are effective against some bacterial and parasitic agents, no specific treatment for rotavirus infection is available.

We have derived preliminary updated estimates of rotavirus-related childhood deaths on the basis of the findings of our review. Because we wanted to assess the most recent trends in rotavirus incidence, we examined a relatively limited number of studies published in the last 5 years, particularly from upper-middle– and high-income countries. However, these 2 income groups account for only a small fraction (<5%) of all deaths from rotavirus disease, and the 28 studies available from low- and low-middle–income countries allowed for a reasonably robust analysis. Nevertheless, our findings should be updated as new data on rotavirus hospitalizations and updated estimates of childhood diarrhea deaths become available. In 2002, the World Health Organization published a generic protocol for hospital-based surveillance of rotavirus (*15*), and studies using this protocol are currently being conducted or planned in >30 countries in Asia, Africa, the Middle East, and Latin America. Data from these and other studies, particularly from countries such as India and China, which account for a large fraction of global rotavirus deaths, should be used to update our estimate of rotavirus-related deaths and further refine it to develop country-specific figures. These data, together with information on effects and costs of rotavirus disease, will allow policymakers to assess the magnitude of the problem of rotavirus and the value of new vaccines that may soon be available.

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Arcobacter butzleri: Underestimated Enteropathogen

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Molecular methods applied to 2,855 strains of *Campylobacter*-like organisms received from a surveillance network of *Campylobacter* infections in France identified 29 *Arcobacter butzleri* infections. This species ranks fourth for *Campylobacteraceae* isolation and appears to have the same pathogenic potential as the other species in the genus.

Kiehlbauch et al. (*1*) originally described the species *Arcobacter butzleri*, previously named *Campylobacter butzleri* (*1*), after studying aerotolerant *Campylobacter* strains from human and veterinary sources. The genus *Arcobacter* was created in 1992 (*2*).

A. butzleri is found in environmental samples, and untreated water appears to be a potential source of infection. In industrialized countries, the most important source of human contamination may be food. Indeed, *A. butzleri* has been isolated in different breeding animals and is present in a great variety of retail meats, including chicken, beef, pork, and lamb, with a high prevalence in poultry (*3*).

Although the prevalence of this bacterium in animals and food specimens is well documented, including the first publication by Kiehlbauch et al. that implied this newly described species could be an important human pathogen, only a few reports of human infections are found, and most of them were published before 1995. In the Far East, Taylor et al. formally identified *A. butzleri* in 2.4% of diarrheal stool samples collected from Thai children (*4*), and more recently, 2 bacteremia cases were reported in Taiwan and Hong Kong in patients with an underlying disease (liver cirrhosis and gangrenous appendicitis, respectively) (*5*,*6*). In a South African study, 15 *A. butzleri* were identified among 3,877 *Campylobacteraceae* strains isolated from children's diarrheic stools (*7*). Reports from Europe are scarce: an outbreak of recurrent abdominal cramps in 10 patients in Italy (*8*), bacteremia in a newborn in the United Kingdom (*9*), and 2 cases of severe diarrhea in Germany (*10*). The recent publication of Vandenberg et al. from Belgium used an *Arcobacter*-selective medium for stool specimens and found that *A. butzleri* ranked fourth among *Campylobacter* spp. and *Campylobacter*-like organisms (*11*), which stimulated interest to revisit the role of *Arcobacter* spp. as an agent of enteric infection.

In this study, our goal was to investigate the prevalence of *A. butzleri* with a different approach and to describe the clinical features of *A. butzleri* infection. We used molecular methods to identify *Campylobacter*-related organisms collected from a network of clinical laboratories that do not use specific *Arcobacter*-selective medium. The results nevertheless showed that this bacterium ranks fourth among these organisms.

The Study

The French surveillance network of human *Campylobacter* infections is composed of laboratories selected on a voluntary basis (*12*). The laboratories are randomly located throughout France; 93 are hospital laboratories, and 338 are private laboratories. They send their clinical *Campylobacter* isolates to the National Reference Center with a completed information sheet concerning the patient and epidemiologic data. The study period was from July 2002 to December 2003, and 2,855 *Campylobacter*-like strains were studied. In addition to standard phenotypic identification, specific polymerase chain reaction (PCR) assays were carried out to identify *C. jejuni*, *C. coli*, or *C. fetus* (*13*); other isolates were identified by comparing 2 sequences of 400 bp located at both extremities of a 1,100-bp fragment of the beginning of the 16S rRNA gene amplified with primers F2-16S-CHPEC (ATCCTGGCTCAGAGTGAACG) and R2-16S-CHPEC (AAGGGCCATGATGACTTGAC) with those of DNA databases by using the BLAST program (http://www.ncbi.nlm.nih.gov/blast/). An identification at the species level was regarded as correct when ≥99% identity was seen with only 1 species. The species distribution from stool samples was as follows: 2,114 *C. jejuni* (78.9%), 486 *C. coli* (18.1%), 36 *C. fetus* (1.3%), 27 *A. butzleri* (1.0%), 8 *C. lari* (0.3%), 4 *C. upsaliensis* (0.1%), 2 *C. hyointestinalis*, and 1 *Helicobacter canadensis*. From 177 nonstool samples, we detected 2 *A. butzleri*.

Almost the entire 16S rRNA gene, 1,450 bp, was sequenced for 9 *A. butzleri* to study its homogeneity because only 5 sequences are in the databank (accession nos. AF314538, AY621116, L14626, U34386, U34388). The intraspecies variability was only 0.4% (6/1,430 bp) among the 14 *A. butzleri* sequences. Comparison of these sequences with those of 4 *A. cryaerophilus* (AY314755, L14624, U34387, U25805), 1 *A. skirrowii* (L14625), and 1 *A. nitrofigilis* (L14627) showed that interspecies variability concerned 14 bp out of 1,430. The *Arcobacter* sequences differed considerably from those of

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Campylobacter spp., with only 86.2% identity with *C. coli* (AY621115) and 85.6% identity with *C. fetus* (AY621110), 2 species that can be phenotypically confounded with *A. butzleri*.

Twenty-seven of the 29 *A. butzleri* strains were isolated from feces of patients with gastroenteritis, whereas 1 strain was isolated from peritonitis pus and another from a blood culture specimen. The 27 *Arcobacter* strains from stools were isolated in 23 different laboratories. Most of them (n = 15) used Campylosel (bioMérieux, Marcy l'Etoile, France) incubated at 37°C for 2 to 3 days. Six used Karmali agar incubated at 37°C for 3 to 4 days. The last 2 used Butzler medium and the filtration method, respectively. None of these infections was part of an outbreak. Of the 16 patients with travel information available, only 1 indicated a recent trip, to Turkey. An associated pathogenic organism was described in 1 of 15 cases, but the nature of the associated organism is unknown. Seventeen of the 29 patients were hospitalized. Their age range was 1–89 years, with an average of 54 years. Seventeen patients (59%) were male. Detailed clinical information was obtained for 19 patients and is shown in the Table. Eighteen of 19 patients had diarrhea, including the patient with bacteremia; 11 of 15 had abdominal pain; 4 of 15 had bloody stools, including the patient with *A. butzleri* bacteremia; 2 of 14 patients vomited; and 5 of 17 patients had fever. Acute renal failure developed in 2 patients, 1 associated with pyelonephritis. Concerning the 17 other cases for which information was available, severe clinical symptoms (anorexia, weight loss, asthenia) were described for 10 patients.

The duration of symptoms without treatment was variable, from 2 days to several weeks. Antimicrobial drug therapy was administered in 5 cases, and infection was eradicated a few days later (Table). In all cases, the strains were susceptible to the antimicrobial agent used (amoxicillin, ofloxacin, ciprofloxacin [twice], amoxicillin-clavulanate).

Conclusions

During an 18-month period, 2,855 strains of *Campylobacter*-like organisms were identified at the species level by using phenotypic and molecular tools. Molecular methods permitted the identification of 29 *A. butzleri*, i.e., 1% of the strains studied and also 1% of the 2,678 isolates from stools, which makes this species the fourth most frequently isolated *Campylobacteraceae* in human clinical samples in France, before *C. lari* and *C. upsaliensis* but after *C. jejuni*, *C. coli*, and *C. fetus*. Our results agree with those of Vandenberg et al. (*11*) who also found *A. butzleri* in fourth position for *Campylobacter*-like isolates from stool specimens. However, their results were obtained in a different context: only 1 laboratory was involved for 8 years, and a specific *Arcobacter*-selective medium was used. A study conducted in Denmark by Engberg et al. (*14*), also to estimate the prevalence of

Table. Demographic and clinical characteristics of 19 patients infected with *Arcobacter butzleri*

Patient*	Age (y)	Sex	Diarrhea	Abdominal pain	Blood in stool	Vomiting	Fever	General symptoms†	Duration of symptoms and disease course
1	88	F	+	+	–		+	+	Death linked to associated neoplasia
2	75	M	+	+	–	–	–	+	4 days. Acute renal failure (treated with amoxicillin)
4	89	F	+	–	–	–	–	+	6 weeks. Favorable progress after treatment with ofloxacin
7	76	M	+	+	+	–	–	–	5 days (treated with ciprofloxacin)
8	2	M	+	–	–	+	–	+	2 days
10	69	M	+	+		–	–	+	
11	4	M	+	+	–	–	+	+	Several recurrences in 3 months
12	72	F	+	–	+	–	–	–	3 weeks
15	89	M	+		–			–	
18	53	M	+	+	–	–	–	+	Irritable bowel syndrome
19	6	M	+	+	+	+	+	+	10 days
20	30	F	–	+	–	–	–	–	
21	68	M	+	+	+	–	+	+	
23	77	M	+				–	+	Several days. Acute renal failure (treated with ciprofloxacin)
25	1	F	+				+	+	3 weeks
26	41	M	+	–	–	–	–	+	Several days (treated with amoxicillin-clavulanate)
27	44	F	+	+	–	–	–	–	
28	70	F	+						
29	83	F	+	+	–	–	–	+	Several days

*All samples were from stool, except from patient 12, whose sample was blood.
†Anorexia, asthenia, weight loss.

Campylobacter spp. and related bacteria in human fecal samples, did not produce the same results; only 1 *A. butzleri* was isolated out of 1,376 samples, and no *C. lari* or *C. upsaliensis* was recovered. The differences between these 3 studies could be explained by differences in geographic distribution of the species, but the difference in isolation methods (selective agar in the Belgian study) or in identification methods (molecular methods in our study) is likely the critical point. While not optimal, *Campylobacter*-selective agars, especially Campylosel, appear to allow the growth of *A. butzleri*. For the other media, a longer incubation period seems necessary. The role of *A. butzleri* in enteric infections is not definitively proven, but its involvement is likely. In at least 14 of 15 cases, no other enteropathogen was detected. In 1 case, a bacteremia with an enteric infection was found. These data are similar to those observed with *C. jejuni* infection. Isolation of *A. butzleri* is more frequent than well-known enteropathogenic *Campylobacter* spp., e.g., *C. upsaliensis* and *C. lari*, when adequate identification is carried out. The prevalence of *A. butzleri* may be underestimated because of false identification as *C. coli* or *C. fetus*. Indeed nearly all of the strains are able to grow at 25°C, like *C. fetus*, but are resistant to cephalothin like *C. coli*. We found that nearly all of the strains were resistant to nalidixic acid (4 had an intermediary resistance) and susceptible to ciprofloxacin with the disk diffusion method. The choice of a simple method for testing these drugs allows it to be used routinely in clinical laboratories to differentiate such strains phenotypically.

Nevertheless, molecular biology is a powerful tool for diagnosis. We used a 16S rRNA gene sequencing as a global approach for strains that were not *C. jejuni*, *C. coli*, or *C. fetus*. We confirmed that the intraspecies variability of *A. butzleri* 16S rRNA gene sequences is low (0.4%), lower than that found for *C. coli* (1.5%) or *C. lari* (2.5%) (*15*), and that interspecies variability is high. This approach, which is expensive and difficult to implement, is not accessible to a routine laboratory, but simple phenotypic tests can be applied to detect these microorganisms. The combination of phenotypic tests and selective agar medium will determine the real incidence of *A. butzleri* infection, which will add evidence that *A. butzleri* is an etiologic agent of diarrhea. A determination of the prevalence of *A. butzleri* in normal stool will also help establish the prevalence of this organism in diarrheal stool.

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Dr Prouzet-Mauléon is an engineer at the National Reference Center for Campylobacters and Helicobacters. She is responsible for the analyses and development of molecular methods applied to the diagnosis and characterization of *Campylobacteraceae*.

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Evaluation of a Direct, Rapid Immunohistochemical Test for Rabies Diagnosis

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A direct rapid immunohistochemical test (dRIT) was evaluated under field and laboratory conditions to detect rabies virus antigen in frozen and glycerol-preserved field brain samples from northwestern Tanzania. Compared to the direct fluorescent antibody test, the traditional standard in rabies diagnosis, the dRIT was 100% sensitive and specific.

In much of the developing world, rabies surveillance and diagnosis in domestic and wild animals are severely constrained. High ambient temperatures hinder the collection and preservation of fresh specimens. The use of the direct fluorescent-antibody assay (DFA), the traditional standard in rabies diagnosis (*1*,*2*), is limited by the costs of acquiring and maintaining a fluorescent microscope. Difficulties in obtaining diagnostic results from field material have led to widespread underreporting of disease.

Consequently, the true public health impact of rabies has been greatly underestimated (*3*–*5*), and political commitment for its control has been lacking. Moreover, the absence of a confirmatory test can result in the inappropriate management of animal bite injuries, with human deaths a potential consequence of delays in rabies postexposure prophylaxis (PEP) and unnecessary administration of PEP. The latter is a particular concern, given the scarcity and costs of human rabies vaccines and immunoglobulin in many parts of the world.

A rapid immunohistochemical test (RIT) to detect rabies virus (RABV) antigen has been developed in the Rabies Section of the Centers for Disease Control and Prevention (CDC) by incorporating various components of existing immunoperoxidase techniques (*6*). Like the DFA, the RIT is performed on brain touch impressions, but the product of the reaction can be observed by light microscopy, and RABV antigen appears as magenta inclusions against a blue neuronal background. The test recognizes all genotype 1 variants of RABV examined to date and all representative lyssaviruses. Modifications of a former indirect test have led to a direct test (dRIT) that uses a cocktail of highly concentrated and purified biotinylated anti-nucleocapsid monoclonal antibodies produced in vitro in a direct staining approach and allows a diagnosis to be made in <1 hour. For the routine diagnosis of rabies, glycerol saline is a convenient preservative in situations in which refrigeration or freezing facilities are not promptly available (*7*).

We report findings of a preliminary study to evaluate the dRIT, comparing results of the dRIT carried out under field conditions in Tanzania with the dRIT and DFA performed at CDC. The objectives were to validate the dRIT as a field test for rabies surveillance and evaluate the dRIT on glycerol-preserved field samples.

The Study

Brain stem samples from various animal species were obtained from December 2002 to September 2004 as a result of rabies surveillance operations established in the Mara, Mwanza, and Shinyanga regions of northwestern Tanzania. Some archived glycerolated specimens were also analyzed. Samples were collected by inserting a drinking-straw through the occipital foramen, according to World Health Organization recommendations (*7*) or by opening the skull.

Some specimens were frozen (–20°C). Other samples inside straws were placed into a solution of phosphate-buffered 50% glycerol and stored either at +4°C or at –20°C or kept at room temperature (25°C ± 5°C) for up to 4 months before refrigeration or freezing.

Samples were allocated to 4 groups, according to the method of preservation and whether the samples were tested in the field and at the CDC laboratory or at CDC only (Table 1). Group A samples were kept in glycerol solution for ≤15 months and washed in phosphate-buffered saline (PBS) before testing by dRIT in the field. They were then stored at –20°C for ≤5 months and retransferred into fresh glycerol for shipment. At CDC, the samples were kept in glycerol for ≤2 months and rewashed in PBS before retesting by both dRIT and DFA or DFA only. Group B samples were stored frozen for ≤6 months, processed by dRIT in the field, and placed into glycerol solution for shipment to CDC, where they were stored for 2 months before being washed in PBS and retested. Group C samples were preserved in glycerol solution for ≤60 months, shipped, and processed at CDC by dRIT and DFA without previous testing in the field. These samples were washed in PBS just before testing. Group D samples were stored at –20°C in

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Table 1. Methods of sample preservation and number of samples processed*

Preservation	No. washes in PBS	No. samples tested		
		dRIT field	dRIT CDC	DFA test CDC
Group A. glycerol saline/frozen/glycerol saline	2	44	39	44
Group B. frozen/glycerol saline	1	10	10	10
Group C. glycerol saline	1	0	89	89
Group D. frozen	0	0	16	16

*dRIT, direct rapid immunohistochemical test; PBS, phosphate-buffered saline; DFA, direct fluorescent-antibody assay; CDC, Centers for Disease Control and Prevention.

the field for 2 to 24 months, shipped frozen, and tested at CDC by dRIT and DFA

A qualitative assessment of the samples was made before testing. Five specimens at a time were stained by dRIT at ambient temperature as described below. Touch impressions were made on glass microscope slides as described (*8*). The slides were air-dried, fixed in 10% buffered formalin for 10 min, dip-rinsed in wash buffer PBS with 1% Tween 80 (TPBS), immersed in 3% hydrogen peroxide for 10 min, and dip-rinsed in fresh TPBS. After dipping, the excess buffer was shaken from the slides and blotted from the edges surrounding the impression. This treatment was repeated after each rinsing step. The slides were incubated in a humidity chamber (a cover on a moistened paper towel on an even surface) with the MAb cocktail for 10 min, dip-rinsed in TPBS, incubated with streptavidin-peroxidase complex (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD, USA) for 10 minutes and dipped in TPBS. A 3-amino-9-ethylcarbazole (AEC) stock solution was prepared by dissolving one 20-mg tablet AEC (Sigma-Aldrich Corp, St Louis, MO, USA) in 4 mL N,N-dimethylformamide (Fisher Scientific International, Inc., Pittsburgh, PA, USA) and stored at 4°C. A working dilution was prepared by adding 1 mL AEC stock solution to 14 mL 0.1 mol/L acetate buffer (Polyscientific, Bay Shore, NY, USA) and 0.15 mL 3% hydrogen peroxide. The slides were incubated with the AEC peroxidase substrate for 10 min and dip-rinsed in distilled water. They were then counterstained with Gill's formulation #2 hematoxylin (Fisher Scientific International) diluted 1:2 with distilled water for 2 min and dip-rinsed in distilled water. Finally, they were mounted with a water-soluble mounting medium (BioMeda Corp., Foster City, CA, USA) and examined by light microscopy (Leica Microsystems AG, Wetzlar, Germany) in Tanzania and Axioplan 2 (Carl Zeiss AG, Göttingen, Germany) at CDC at magnifications of ×200 to ×400. The same operator performed the dRIT in the field and at CDC. However, at CDC, identification numbers unknown to the operator were assigned. The DFA (FITC Anti-Rabies Monoclonal Globulin, Fujerebio Diagnostic Inc., Malvern, PA, USA) was performed in a blind manner by another operator as described (*8*) and read by fluoresent microscopy (Axioplan 2).

Confidence intervals for the sensitivity and specificity were calculated by using the exact binomial distribution (S-Plus, Insightful Corp., Seattle, WA, USA). Of 159 total samples tested, 59 specimens (37.1%) were positive for RABV antigen, and 100 (62.9%) were negative by dRIT, with 100% agreement between the tests, whether dRIT was performed in field conditions only, both in field and laboratory conditions, or in laboratory conditions only. Assuming that the DFA was 100% sensitive and specific, the dRIT was 100% sensitive (95% confidence interval [CI] 93.9%–100.0%) and 100% specific (95% CI 96.3%–100.0%). Table 2 shows the distribution of positive samples in the various animal species.

Table 2. Number of Tanzanian brain samples processed by dRIT and DFA for different animal species*

Species	No. brains examined†
Domestic dog	73 (39)
Domestic cat	7 (3)
Cow	8 (7)
Goat	6 (5)
Livestock‡	1 (1)
Aardwolf (Proteles cristatus)	1
African civet (Civettictis civetta)	2
Banded mongoose (*Mungos mungo*)	2
Slender mongoose (Herpestes sanguineus)	3
Dwarf mongoose (*Helogale parvula*)	2
White-tailed mongoose (*Ichneumia albicauda*)	8 (1)
Mongoose‡	2
Black-backed jackal (*Canis mesomelas*)	3
Bat-eared fox (Otocyon megalotis)	8
Black-backed jackal/bat-eared fox‡	2 (1)
Cheetah (Acinonyx jubatus)	3
Small-spotted genet (*Genetta genetta*)	7 (1)
Lion (Panthera leo)	6
Serval (Felis serval)	1
Spotted hyena (*Crocuta crocuta*)	12 (1)
Striped hyena (*Hyaena hyaena*)	1
Zorilla (Ictonyx striatus)	1
Total domestic	95 (55)
Total wildlife	64 (4)
Total	159 (59)

*dRIT, direct immunohistochemical test; DFA, direct fluorescent-antibody assay.
†The number of rabies-positive samples is shown in brackets.
‡Species not definitively identified.

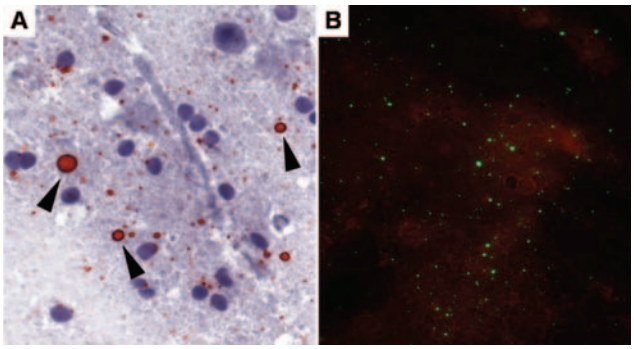

Figure 1. Touch impression of a rabies-positive Tanzanian domestic dog brain preserved in 50% glycerol saline solution for 15 months before testing by direct rapid immunohistochemical test (dRIT) and retested by direct fluorescent-antibody assay (DFA) after 5 months. A) Brain stained by dRIT: rabies virus antigen appears as magenta inclusions (arrowheads) against the blue neuronal hematoxylin counterstain. Magnification, ×630. B) Immunofluorescent apple-green viral inclusions in the same brain processed by DFA. Magnification, ×200.

The sensitivities of the dRIT and DFA were comparable, regardless of the method of preservation. We have no evidence that storage times affected positivity because 34 (77.2%) of 44 samples stored in glycerol solution remained positive for up to 10 months before being tested in the field and retested at CDC after an interval of up to 6 months. Furthermore, RABV antigen was successfully detected in the sample that had been preserved in glycerol for the longest duration (15 months) before dRIT in the field, stored frozen for 3 months before shipment to CDC, and kept in glycerol for 2 months before being retested (Figure 1). Similarly, viral inclusions were detected in a sample stored frozen for 24 months, although the antigen distribution was sparse with both tests. Our data do not provide any unequivocal conclusions on test sensitivity with samples preserved in glycerol solution for >15 months because results from all 15 archived brains were negative. For these samples, the presence of antigen at the time of collection cannot be excluded.

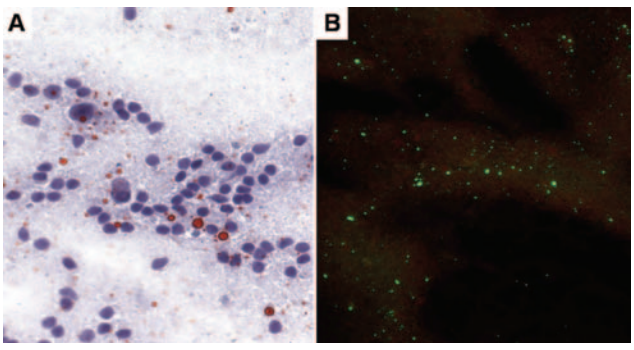

Figure 2. Touch impression of a deteriorated glycerolated brain from a Tanzanian spotted hyena (*Crocuta crocuta*) with rabies. A) Brain processed by direct rapid immunohistochemical test (dRIT). Magnification, ×400. B) DFA staining procedure on the same brain. Magnification, ×200.

Four of 10 (40.0%) deteriorated specimens were positive (Figure 2). Among the 6 brains with negative results, only 1 was suspected of containing rabies. The negative finding might have been caused by inadequate preservation, since the sample had been stored in glycerol solution at ambient temperature for up to 4 months before being refrigerated.

Conclusions

The dRIT showed a sensitivity and specificity equivalent to those of the DFA. The test is simple, requires no specialized equipment or infrastructure, and can be successfully performed on samples preserved in glycerol solution for 15 months or frozen for 24 months and in variable conditions of preservation. These qualities make it ideal for testing under field conditions and in developing countries. Although further laboratory and field evaluations are required, our results are promising and highlight the potential value of the dRIT for countries with limited diagnostic resources. First, this technique could greatly enhance epidemiologic surveillance in remote areas where rabies incidence data are difficult to obtain. Second, the test could improve the ability to respond to outbreaks with effective management decisions. Third, it could be extremely valuable in guiding decisions regarding rational use of rabies PEP.

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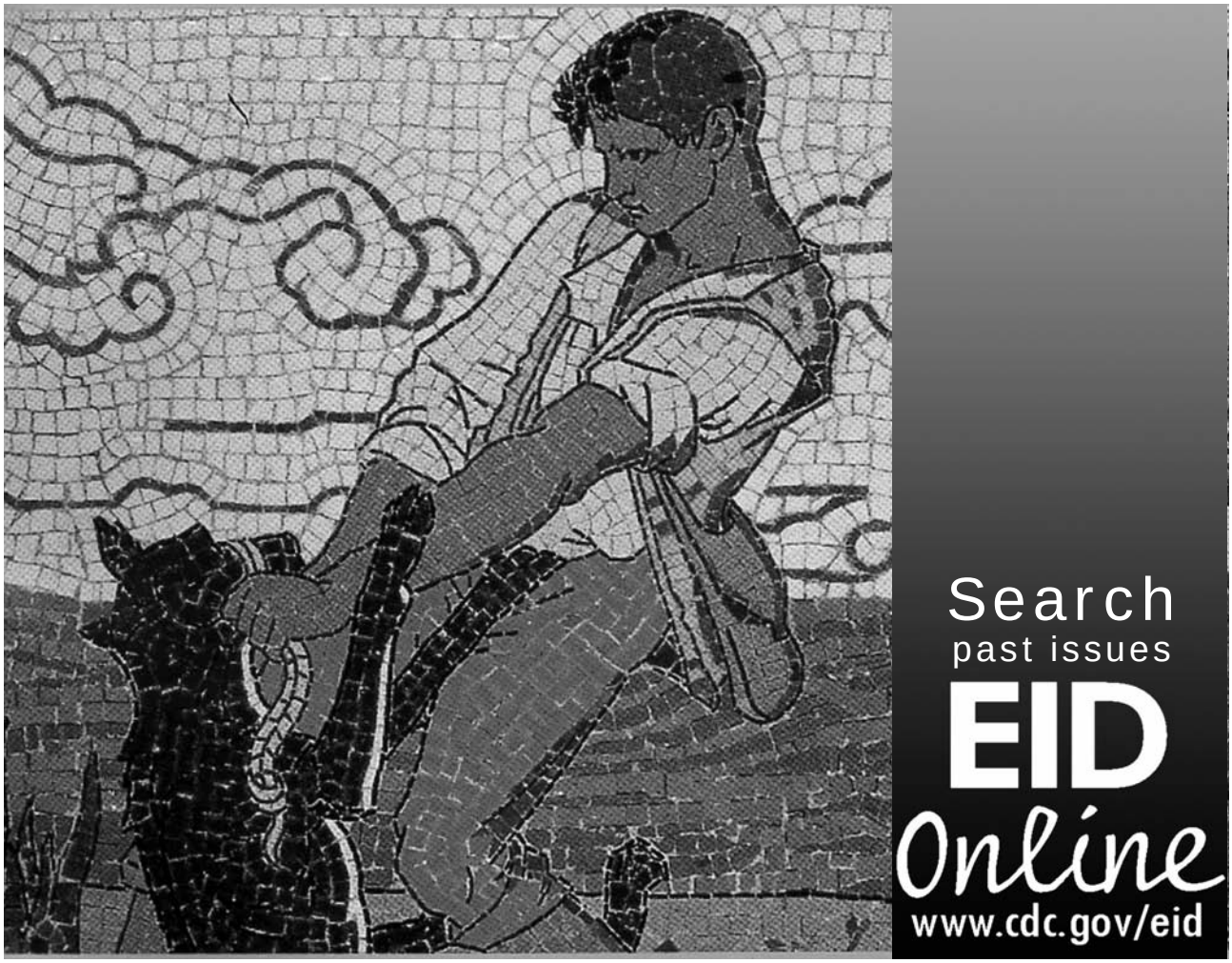

Introductions of West Nile Virus Strains to Mexico

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Complete genome sequencing of 22 West Nile virus isolates suggested 2 independent introductions into Mexico. A previously identified mouse-attenuated glycosylation variant was introduced into southern Mexico through the southeastern United States, while a common US genotype appears to have been introduced incrementally into northern Mexico through the southwestern United States.

West Nile virus (WNV), a mosquitoborne flavivirus for which birds serve as reservoir and amplification hosts, was introduced into New York in 1999 (*1*) and spread across the United States to California by 2003 (*2*). By 2002, serosurveys demonstrated WNV circulation in ≥6 eastern Mexican states and along its northern border with the United States (*3–5*). This pattern of WNV appearance in Mexico suggested a southwesterly spread across the United States and into northeastern Mexico through Texas. However, in the spring of 2003, the first WNV isolate found in Mexico was obtained from a raven in the southeastern state of Tabasco (*3*). If WNV reached southern Mexico by incremental spread through northern Mexico from Texas, the index isolate would have been expected sooner and in a northern Mexican state. Phylogenetic analyses showed the raven isolate to be unexpectedly divergent from contemporary Texas strains, but exact relationships and a route of entry could not be determined by using premembrane and envelope glycoprotein (prM-E) sequences (*3*).

The divergence between the southern Mexican raven and Texas isolates suggested that WNV arrived in southern Mexico by an alternate route, perhaps the Caribbean. After its spread throughout the northeastern United States, WNV appeared abruptly in Florida in 2001, appearing to bypass several mid-Atlantic states. This pattern could be explained by spread of migratory birds (*6*); the Atlantic coast flyway overlaps both New York and Florida, while the Mississippi Valley flyway overlaps both Louisiana and the Yucatan Peninsula of Mexico. Longitudinal avian serosurveys that began in 2000 showed WNV seropositivity in at least 3 migratory and 2 resident bird species captured in the Yucatan Peninsula from 2002 to 2003 (*7*). Thus, migratory birds may have carried WNV from the southeastern United States into Mexico, either directly or through the Caribbean. Serosurveys have suggested WNV circulation among birds in various Caribbean islands since 2002 (*8–10*).

The possibility of a third WNV introduction into Mexico at the California border must also be considered. A 2003 horse isolate from the northern Mexican state of Nuevo Leon was closely related to Texas isolates from 2002 (*11*), based on its prM-E sequence. We do not know whether WNV reached California from Texas and the Midwest by crossing the Rocky Mountains or by traveling first into northern Mexico and subsequently spreading north from Baja California. The latter route is suggested by the geographic link with the first detection of WNV in southeastern California (*2*).

The reported incidence of human West Nile encephalitis is much greater on the US (California) than on the Mexican (Baja California and Sonora) side of the common border. Possible explanations for this discrepancy include differences in disease surveillance and reporting. Another possibility is that the WNV strains circulating in Mexico are attenuated compared to US strains, and the identification of a murine-attenuated glycosylation variant in Tabasco State (*12*) is consistent with this hypothesis.

The Study

To investigate possible routes of WNV entry into Mexico from the United States, 9 isolates from Mexico (all strains available) and 13 strains isolated in the United States from hypothetical points of introduction into Mexico (2 from Florida, 2 from Louisiana, 3 from Arizona, and 6 from California) were compared. Isolates from several northern Mexican states, 1 from Sonora, 1 from Tamaulipas, and 7 from Baja California, were obtained from a variety of birds and from a horse (Table, Figure 1) by injection of Vero cells. RNA was extracted from first or second Vero cell passages by using the QIAamp Viral RNA Mini-kit (Qiagen Inc, Valencia, CA, USA). Reverse transcription–polymerase chain reactions (RT-PCRs) were performed to amplify the complete WNV genome in 6 overlapping amplicons by using primers described previously (*12*). Amplicons were purified from agarose gels by using the QIAquick gel-extraction kit (Qiagen), and both strands were sequenced directly by using the PCR primers

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Table. West Nile virus isolates included in the phylogenetic analyses*

GenBank No.	Strain	Year	Host	Location†
AB185914	(NY)I	1999	Not reported	New York
AB185915	(NY)II	1999	Not reported	New York
AB185916	(NY)III	1999	Not reported	New York
AB185917	(NY)IV	1999	Not reported	New York
AF196835	NY '99	1999	Flamingo	New York
AF202541	NY	1999	Human	New York
AF206518	2741	1999	*Culex pipiens*	Connecticut
AF260967	NY99-eqhs	1999	Equine	New York
AF260968	Eg101	1951	Human	Egypt
AF260969	RO97-50	1996	*Cx. pipiens*	Romania
AF317203	VLG-4	1999	Human	Russia
AF404753	crow265	2000	Crow	Maryland
AF404754	MQ5488	2000	*Cx. Pipiens*	New Jersey
AF404755	grouse3282	2000	Grouse	New York
AF404756	crow3356	2000	Crow	New York
AF404757	Italy equine	1998	Equine	Italy
AF481864	IS-98 STD	1998	Store	Israel
AF533540	US Hum. 1	2001	Human	New York
AY185911	V1151	2002	Mosquito	Texas
AY262283	Kenya3829	1998	Mosquito	Kenya
AY268132	PaAn001	2000	Equine	France
AY268133	PaH001	1997	Human	Tunisia
AY277252	27889	2003	Human	Russia
AY278441	Ast99-901	1999	Human	Russia
AY278442	Vlg00 27924	2000	Human	Russia
AY289214	TVP 8533	2002	Human	Beaumont, Texas
AY490240	Chin-01	2003	Not reported	China
AY660002	TM171-03	2003	Raven	Tabasco
AY701412	96-111	1996	Equine	Morocco
AY701413	04.05	2003	Equine	Morocco
AY712945	Bird 1153	2003	Bird	Harris Co., Texas
AY712946	Bird 1171	2003	Bird	Harris Co., Texas
AY712947	Bird 1461	2003	Bird	Harris Co., Texas
AY712948	v4369	2003	Mosquito	Harris Co., Texas
AY842931	385-99	1999	Not reported	New York
D00246	Kunjin MRM61C	1960	*Culex* spp.	Australia
M12294	Uganda WNFCG	1937	Human	Uganda
DQ080070	**TVP 9115**	**2003**	**Grackle**	**Sonora, Mexico**
DQ080069	**TVP 9117**	**2003**	**Horse**	**Tamaulipas**
DQ080068	**TVP 9218**	**2003**	**Blue heron**	**Baja California, Mexico**
DQ080067	**TVP 9219**	**2003**	**Green heron**	**Baja California, Mexico**
DQ080066	**TVP 9220**	**2003**	**Cormorant**	**Baja California, Mexico**
DQ080065	**TVP 9221**	**2003**	**Grackle**	**Baja California, Mexico**
DQ080064	**TVP 9222**	**2003**	**Coot**	**Baja California, Mexico**
DQ080063	**TVP 9223**	**2003**	**Pigeon**	**Baja California, Mexico**
DQ080060	**Cc**	**2004**	**Raven**	**Baja California, Mexico**
DQ080072	**FL232**	**2001**	**Catbird**	**Palm Beach Co., Florida**
DQ080071	**FL234**	**2002**	**Equine**	**Sumter Co., Florida**
DQ080062	**TWN 165**	**2002**	**Mosquito**	**Iberia Co., Louisiana**
DQ080061	**TWN 496**	**2004**	**Northern cardinal**	**Iberia Co., Louisiana**
DQ080051	**AZ-03-1623**	**2003**	***Cx. tarsalis***	**Cohise Co., Arizona**
DQ080052	**Az-03-1681**	**2003**	***Cx. tarsalis***	**Maricopoa Co., Arizona**
DQ080053	**Az-03-1799**	**2003**	***Cx. tarsalis***	**Apache Co., Arizona**
DQ080054	**CA-03 GRLA**	**2003**	***Cx. quinquefasciatus***	**Los Angeles, California**
DQ080055	**CA-03 IMPR 102**	**2003**	***Cx. tarsalis***	**Imperial Valley, California**
DQ080056	**CA-03 IMPR 1075**	**2003**	***Cx. tarsalis***	**Imperial Valley, California**
DQ080057	**CA-03S0333081**	**2003**	**Crow**	**Arcadia, California**
DQ080058	**CA-03S0334814**	**2003**	**Crow**	**Arcadia, California**
DQ080059	**CA-04 04-7168**	**2003**	**Yellow-billed magpie**	**Sacramento, California**

*Newly sequenced strains are printed in **bold text**.
†Locality and state (Mexico and United States) or country of isolation.

Figure 1. Map showing hypothetical routes of West Nile virus introduction into Mexico. Circles indicate locations of isolates in the Florida-Louisiana-Tabasco 2001–2003 clade (Figure 2). Stars indicate locations of isolates in the California-Arizona-northern Mexico clade.

and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied BioSystems, Foster City, CA, USA) with a 3100 Genetic Analyzer (Applied Biosystems).

Complete genomic sequences excluding the 5′ and 3′ terminal 20 nucleotides (representing primers incorporated into amplicons) were aligned with all homologous WNV sequences from the GenBank library by using ClustalW. Sequences were analyzed by using maximum parsimony and neighbor-joining programs implemented in the PAUP 4.0 software package (*13*) as well as Bayesian analysis using MRBAYES v3.0 (*14*) with 100,000 generations, a general time-reversible model with empirically estimated base frequencies, and either a codon position-specific (for the open reading frame alone) or a gamma distribution of substitution rates among nucleotide sites.

All phylogenetic trees placed the North American WNV isolates into monophyletic groups with strong bootstrap and Bayesian support values; the tree generated using the Bayesian analyses is presented in Figure 2. The Tabasco 2003 isolate grouped with 3 strains from 2001 and 2002 in Florida and Louisiana and more distantly with a New York isolate from 2000, with strong Bayesian probability and bootstrap support; inclusion of the New York grouse strain was weakly supported (bootstrap and Bayesian probability values <80%). In contrast, a 2004 Louisiana isolate and other recent strains from Texas were positioned basally to the large clade containing all northern Mexico, California, and Arizona isolates. This California/Arizona/northern Mexico group was highly conserved, with ≤0.5% nucleotide and 0.04% amino acid sequence divergence. The 2003 Tabasco strain was phylogenetically distinct from all other Mexico isolates, which grouped with the California and Arizona isolates. Surprisingly, despite the greater geographic distances between Tamaulipas and Baja California/Sonora, compared to the distance between Tamaulipas and Texas, the Tamaulipas WNV strains grouped more closely with strains from Baja California and Sonora than with those from Texas.

Compared to the Tabasco strain, the other Mexican isolates differed by 0.55%–0.66% nucleotide sequence divergence across the genome. The gene with the most sequence divergence was prM, with 0.72%–1.4% divergence from the Tabasco strain. However, the 5′ untranslated region was more variable with 3.0%–4.6% divergence. The most conserved gene was NS2B, with 0.0%–0.24% divergence from the Tabasco strain. The E gene, often used for phylogenetic analyses, had 0.46%–0.66% sequence divergence.

Of the Mexican WNV isolates, only the 2003 Tabasco raven isolate had the E-156 Pro residue, which ablates the N-linked glycosylation site found in most North American strains. In addition, 2 other WNV isolates (GenBank accession nos. AY490240 and AF260968) share this E-156 Pro residue despite their geographic diversity (China and Egypt, respectively) and their placement in different lineages. Although the paraphyletic nature of this Pro mutation suggests that it could be selected either during laboratory isolation or passage, its identification in the low-passage Tabasco isolate may indicate its presence in nature.

Conclusions

Our data support the hypothesis that WNV entered Mexico through at least 2 independent introductions. The introduction detected by the first virus isolation in May 2003 from a raven in Tabasco State probably occurred from a migratory bird that flew southward from the southeastern United States to the Gulf of Mexico or the Caribbean and bypassed northern Mexico. The isolation and sequencing of WNV isolates from islands in the Caribbean may shed further light on how WNV reached southern Mexico. However, the extreme genetic conservation of North American WNV strains may preclude identifying the exact routes of introduction. Independently, other WNV strains probably spread incrementally from the southwestern United States into northern Mexico. Both northward and southward movements of WNV between northern Mexico and California or Arizona may also occur.

Available WNV strains from Mexico indicate that the murine-attenuated, E-156 glycosylation-negative variant identified in Tabasco state may be limited in its distribution to southern Mexico, while the glycosylated variant typical of US strains is widespread in northern Mexico. However, our sampling was limited and may also be biased because many WNV isolates were from sick or dying animals; the attenuated E-156 Pro residue phenotype

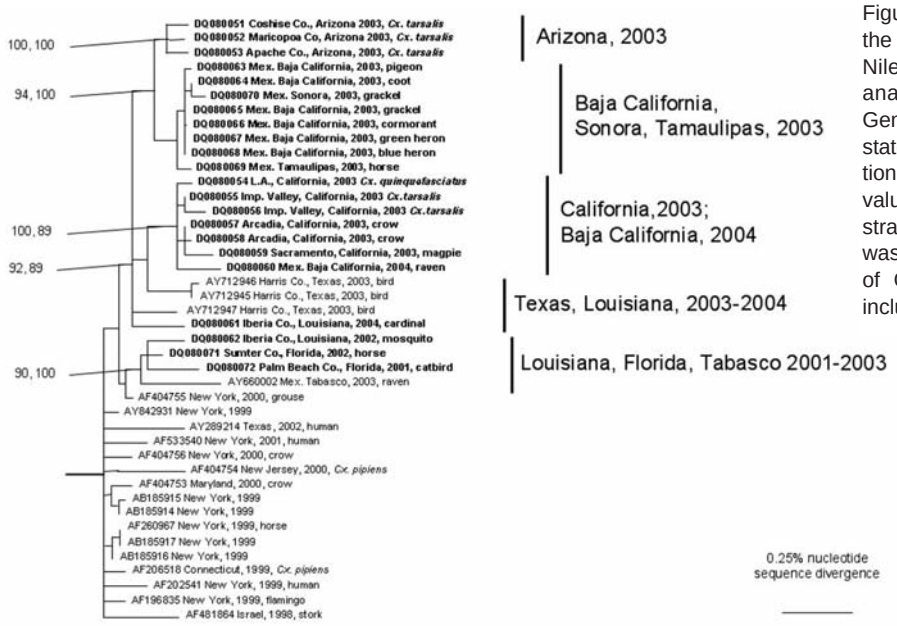

Figure 2. Phylogenetic tree generated from the complete open reading frame of West Nile virus sequences using a Bayesian analysis. Virus strains are labeled by GenBank accession number followed by the state and/or country, year, and host of isolation. Numbers indicate Bayesian probability values followed by neighbor-joining bootstrap values for groups to the right. The tree was rooted by using an outgroup comprised of Old World strains of West Nile virus, including a lineage 2 strain (Table).

could be undersampled because relatively benign infections are rarely identified.

The epidemiology of WNV-associated disease in Mexico is puzzling. According to the Centers for Disease Control and Prevention, 2,470 human cases of WNV infection were confirmed during 2004 in the United States, with >80% of these from areas of California and Arizona bordering the northern Mexico states of Baja California and Sonora where many of our viral isolations were made. In contrast, only 7 human cases of WNV have been confirmed in Mexico. The cases occurred in the border states of Chihuahua (n = 4), Sonora (n = 1), and Nuevo Leon (n = 1) in 2003, and Sonora (n = 1) in 2004 (*15*). Our results of extremely low sequence divergence between the southwestern United States and northern Mexican WNV isolates indicate that this epidemiologic discrepancy is unlikely to be explained by genetic and phenotypic differences among WNV strains. The possibility that WNV circulating in Mexico has an attenuated phenotype was suggested by the murine-attenuating mutation in the Tabasco raven isolate (*12*). However, none of our northern Mexico isolates have the E-156-P attenuating mutation, and all appear extremely closely related to isolates made in southwestern areas of the United States with a high disease incidence.

Another possible explanation for the low incidence of WNV disease in Mexico is resistance in the Mexican human population, possibly because cross-protective immunity from other flavivirus infections such as dengue and St. Louis encephalitis viruses. Although St. Louis encephalitis virus is common in some areas of the continental United States, including California, dengue virus infections are rare; only 157 cases of dengue were reported in the northern states of Mexico in 2004: 25 in Sonora; 21 in Nuevo Leon; 88 in Tamaulipas; 3 in Coahuila; 0 in Chihuahua; and 0 in Baja California (http://www.dgepi.salud.gob.mx/boletin/2004/sem52). Of all Mexican states, Baja California and Sonora adjacent to the US border have the lowest incidence of flaviviral infections. Human flavivirus serosurveys should be conducted in northern Mexico to further evaluate the possibility of cross-protective flavivirus immunity. Newer approaches to detect and identify flaviviral disease are also needed in Mexico to more accurately assess the impact of WNV.

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Surveillance for Prion Disease in Cervids, Germany

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An active survey on transmissible spongiform encephalopathies was performed from 2002 to 2005 on 4,255 roe deer, 1,445 red deer, and 1,604 fallow deer in Germany. All cervids tested negative. This survey has been the largest in European wildlife and provides no evidence of prion diseases in free-living German cervids.

Germany has one of the largest deer populations in Europe (*1*), and ≈19,000 tons of venison are consumed in Germany each year (*2*,*3*). In light of the increasing number of scrapie cases, presence of bovine spongiform encephalopathy (BSE) in Germany (*4*), and increasing prevalence of chronic wasting disease (CWD) in North America, concern exists that transmissible spongiform encephalopathies (TSE) could also affect German wildlife, especially cervids. TSE could be transmitted to German cervids through importation of infected cervids; by their sharing habitats with other infected animals (scrapie from sheep); by exposure to BSE-contaminated meat, bone meal, or milk powder; or by exposure to other European cervids (spontaneous form of TSE). Until now, TSE has not been shown to exist in European deer. However, little surveillance has taken place (*1*). In Germany, only passive surveillance on suspected deer and a TSE survey that focused exclusively on Bavarian cervids have been conducted (*5*). However, risk for human exposure cannot be excluded until sufficient surveillance has been performed (*6*).

The Study

The objective of our study was to determine possible TSE occurrence in cervids from Germany from 2002 to 2005. Our target species were the 3 main cervid species, roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus elaphus*), and fallow deer (*Dama dama*). Since TSE in young animals is unlikely (*7*), only adult animals (age >18 months) were studied. Information on distribution of age groups was obtained from local hunting authorities. The target population of our 3-year study was ≈3,492,000 roe deer, 181,000 red deer, and 157,000 fallow deer (Table 1). The population size was estimated by assuming that the annual hunting bag (number of animals killed each year) represents approximately one third of the population, that age distributions in the hunting bag correspond to those of the deer population, and that the annual population sizes before hunting did not change during the study period. These assumptions correspond to management regulations for hunting (*2*). During the 2002–2003 hunting season, 1,117511 roe deer, 60,407 red deer, and 52,240 fallow deer were killed in Germany (*2*). On the basis of these data, the hunting bags of cervids >18 months for the 3-year study period were estimated at 2,095,000 roe deer, 109,000 red deer, and 94,000 fallow deer (Table 1).

The target region was all of Germany; the 323 administrative districts of Germany were our sampling areas (Figure). Within these districts, samples were taken from different hunting areas to ensure including as many local deer populations as possible. However, the hunting areas are not necessarily identical to home ranges of deer populations. As CWD in North America tends to occur focally, this strategy was chosen to ensure that potential foci would not be missed.

Several attributes may be associated with greater probability of TSE (*8*). Accordingly, we defined 2 risk categories for each species, an increased risk and a normal risk category. Animals were considered to be at increased risk for TSE if ≥1 of the following conditions applied: 1) BSE incidence in cattle (also relating to their parentage) in the district was higher than the average BSE incidence in Germany, 2) scrapie occurred in sheep in the district, or 3) fallow deer or red deer were distributed in districts with high density. If the disease were endemic, this method would be more likely to detect it in these areas. Moreover, special sampling efforts were directed toward animals that showed cachexia and central nervous system disorders and animals that were found dead.

Because we expected low prevalence or lack of TSE in our target population, we expected to find any positive animals only in the increased risk category. We wanted to ensure that our sample sizes were adequate to detect TSE even at a prevalence of 0.5% for cervids at increased risk and 1% for cervids at normal risk with 95% confidence. The respective sample sizes were calculated by using the approximation formula of the hypergeometric distribution (*9*). We than stratified the sample by hunting bag of each district.

Data analyses were performed on the basis of the hunting bag as well as on the estimated population of cervids >18 months of age in the 3-year study period (Table 1). For

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Table 1. Minimum prevalence levels evaluated for German cervids tested for TSE, 2002–2005*

Species	Increased risk†			Normal risk‡			Total§		
	No. tested (MPL)	HB	EPS	No. tested (MPL)	HB	EPS	No. tested	HB	EPS
Roe deer	1,959 (0.15%)	822,000	1,370,000	1,684 (0.18%)	1,273,000	2,122,000	3,643	2,095,000	3,492,000
Red deer	1,110 (0.27%)	84,000	140,000	297 (1.00%)	25,000	42,000	1,407	109,000	181,000
Fallow deer	1,097 (0.27%)	76,000	127,000	293 (1.02%)	18,000	30,000	1,390	94,000	157,000

*TSE, transmissible spongiform encephalopathy; MPL, minimum prevalence level (upper limit of the percentage of positives in the population, given no positives found in the sample); HB, hunting bag (cervids >18 months estimated for the 3 study years); EPS, estimated population size (cervids >18 months in the 3 study years).
†Animals were considered at increased risk if ≥1 risk factor applied.
‡Animals were considered at normal risk if no risk factors applied.
§Data analysis was possible for 6,440 animals; information on risk factors was lacking in 616 cases.

each sample, we retrospectively calculated the minimum prevalence level (MPL). MPL is an upper limit to the percentage of positives in the population if no positives are found in the sample (*9*). It represents the detection threshold below which the survey cannot detect a TSE infection at the 95% confidence level. Data from captive cervids were regarded as 1 sample and analyzed separately.

Foresters, hunters, and game farmers submitted the heads of 7,056 free-living and 248 captive deer that had been hunted, found dead, or suspected of having disease. Samples from free-living deer were obtained from 280 (87%) of the 323 German districts. Samples from captive deer originated from 43 of ≈6,000 German deer farms and from 12 of 16 federal states. Data from collected deer included species, age (estimated on tooth patterns), sex, location of kill, and health status (Table 2). Brain stem (obex region) and medial retropharyngeal lymph nodes were tested for TSE by Platelia BSE enzyme-linked immunosorbent assay (Bio-Rad Laboratories GmbH, Munich, Germany) according to manufacturer's instructions (*1*,*11*). Recombinant bovine prion protein was the positive control.

Protease-resistant prion protein (PrP^{res}) was not detected in any samples from free-living roe deer (n = 4,250), red deer (n = 1,416), or fallow deer (n = 1,390). Regarding the different risk categories, data analysis was possible for 6,440 animals (Table 1). MPLs for the 3-year populations differed by no more than 0.001% from those calculated for the hunting bags. More than 200 samples came from animals with suspected disease or animals found dead. All 248 captive cervids were negative for TSE (Table 2). Because of the small sample size, MPLs of 1.39% for fallow deer (n = 214) and 9.81% for red deer (n = 29) were accordingly high.

Conclusions

This study represents the largest surveillance program on TSE in European wildlife. Eighty-seven percent of all German districts were covered by our investigation (Figure). Recent data show that CWD prevalence in mule deer varied, depending on whether samples had been collected in biologically relevant units or in administrative

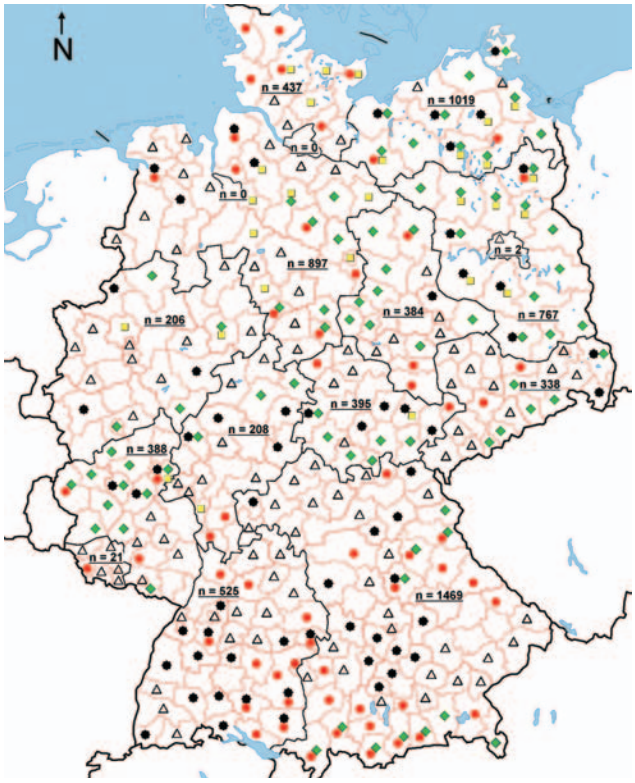

Figure. Distribution of free-ranging roe deer, red deer, and fallow deer tested for transmissible spongiform encephalopathies that shows the risk for each district where samples were obtained. △, samples originating from a district without any risk attributes; ●, samples originating from a district where BSE incidence in cattle was higher than average BSE incidence in Germany; ✱, samples originating from a district with occurrence of scrapie in domestic sheep; ◆, samples from red deer originating from a district with high red deer density; ■, fallow deer samples originating from a district with high fallow deer density; n, number of samples from each federal state. Samples came from 14 (88%) of the 16 federal states (2 missing states are 2 major cities with almost no deer population) and from 280 (87%) of the 323 German administrative districts.

Table 2. Free-living German cervids tested for transmissible spongiform encephalopathies, 2002–2005

Characteristic	Roe deer	Red deer	Fallow deer	Total
No. animals tested	4,250	1,416	1,390	7,056
Sex				
Female	3,137	1,257	1,246	5,640
Male*	502	148	144	794
Unknown	611	11	0	622
Age (y)				
<2	279	127	154	560
2–3	2,196	743	841	3,780
4–6	890	383	329	1,602
>6	273	154	66	493
Unknown	612	9	0	621
Increased risk category	1,959	1,110	1,097	4,166
BSE risk†	1,409	334	236	1,979
Scrapie risk‡	693	215	443	1,351
Fallow deer high density§	–	–	1,035	1,035
Red deer high density§	–	1,030	–	1,030
Clinical suspects¶	55	9	5	69
Found dead	123	16	3	142
Normal risk category#	1,684	297	293	2,274
No BSE risk	2,234	1,073	1,154	4,461
No scrapie risk	2,950	1,192	947	5,089
Fallow deer low density	–	–	355	355
Red deer low density	–	377	–	377
Cervids with unknown risk**	607	9	0	616

*Relatively few male animals were tested. According to a recent report, male cervids appear to be at higher risk for chronic wasting disease than sympatric females (*10*).
†Animal came from a district where bovine spongiform encephalopathy (BSE) incidence in cattle was higher than the average BSE incidence in Germany (true for all target species).
‡Animal came from a district with occurrence of scrapie in sheep (true for all target species).
§Fallow deer distributed in districts with high fallow deer density or red deer distributed in districts with high red deer density.
¶Cachexia and central nervous system disorders.
#An animal was allocated to the normal risk category if none of the above risk factors applied.
**Animals (n = 616) with unknown risk attributes were not included in risk analysis.

jurisdictions (*10*). For logistic reasons, our sampling areas did not always completely cover biologically relevant geographic units. However, we ensured that within each district, samples were derived from different hunting areas so that potential TSE foci would not be missed.

Moreover, our findings were below the targeted minimum prevalence levels of 0.5% for cervids at increased risk (Table 1). The overall CWD prevalence observed in free-living cervids in disease-endemic areas of Colorado and Wyoming is ≈5% for mule deer (*Odocoileus hemionus*), 2% for white-tailed deer (*O. virginianus*), and <1% for elk (*Cervus elaphus nelsoni*) (*7*). In Wisconsin, where CWD only occurs focally, an overall prevalence of 0.62% was detected in free-living white-tailed deer (*12*). In our study, we reached lower detection limits.

If CWD or any other TSE were present in our target population in Germany at a minimum prevalence of 0.15% to 1.02%, depending on the species and risk category analyzed, we should have detected ≥1 infected animal with a 95% probability (*8*). As no PrP^{res} was detected, our study does not indicate that TSE is present in free-living cervids in Germany. Even if TSE occurs in German cervids, it is not widely distributed. The negative results seen with captive cervids in our study are of limited significance, since the sample size was small. Here, more risk analysis is required.

Apart from surveillance, more experimental research on transmission of TSE is required; we have not resolved whether European deer species are susceptible to CWD or other TSE (*1*). As with all prion diseases, a species barrier seems to exist for CWD (*1*); moose (*Alces alces*) and caribou (*Rangifer tarandus*) have not been found naturally infected with disease, even in CWD-endemic areas (*1*,*13*). A higher risk for CWD can be assumed for red deer since they belong to the same species as elk (*1*). BSE was only observed in different species from the families *Bovidae* and *Felidae* in zoos (*14*) but not in members of the family *Cervidae*, even though they were most likely also exposed to BSE-contaminated food (*6*). Our study indicates that TSE is unlikely to exist in free-living cervids from Germany and that the risk for TSE transmission to humans from eating venison is low.

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Elizabeth S. Williams (1951–2004), an essential person involved in establishing the field of CWD research, was killed last year in a traffic accident. The Department of Wildlife Diseases, Institute for Zoo and Wildlife Research, Berlin, is indebted to Professor Williams, and we wish to dedicate this article to her memory.

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Verocytotoxin-producing *Escherichia coli*, Japan, 1999–2004

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In 1999, an infectious disease prevention law was enacted in Japan that affected the nationwide infectious surveillance system. A total of 19,304 laboratory-confirmed verocytotoxin-producing *Escherichia coli* cases were reported through 2004. The annual incidence was 2.74/100,000 population; its fluctuation over time and space was associated with climate, socioeconomic, and population factors.

Triggered by 2 major outbreaks of verocytotoxin-producing *Escherichia coli* (VTEC) in Japan (*1*,*2*), the nationwide surveillance system of the National Institute of Infectious Diseases (NIID) was reengineered in April 1999 by enacting a new infectious disease prevention law to better ascertain the state of laboratory-confirmed VTEC cases across the nation. In this study, we used these nationwide, population-based surveillance data to determine the infectious status of VTEC and to explore factors that affect the incidence of VTEC.

The Study

Since the new surveillance system under the new law began, all laboratory-confirmed VTEC cases are reported and counted in Japan. Under this system, stool samples or rectal swabs are obtained from patients when the clinician suspects hemorrhagic enterocolitis due to pathogenic *E. coli* based on clinical symptoms such as hemorrhagic colitis. These specimens are sent to laboratories at the hospital, private companies, national institutions in each prefecture, or the NIID. To maintain high levels of sensitivity and specificity to detect VTEC, the protocol and training in these laboratories fall under the guidance of the NIID. At these laboratories, the specimens are cultured on specific media such as CHROMagar O157 (Kanto Co. Ltd., Tokyo, Japan) or cefixime-tellurite sorbitol MacConkey agar (Oxoid, Unipath Ltd., Hampshire, UK); specific antibodies against each serotype of *E. coli* are used (*3*,*4*). If the existence of pathogenic *E. coli* is confirmed, the ability to produce verocytotoxin from isolates is investigated by using reversed passive latex agglutination or a multiplex polymerase chain reaction assay (*3*,*5*,*6*).

If the production of verocytotoxin is confirmed by the laboratory, the case is considered symptomatic VTEC. Persons associated with the initial case (e.g., family members) may be further examined for VTEC at the doctor's discretion. When a doctor diagnoses either symptomatic or asymptomatic VTEC infection, he or she has to report this event to the local health center immediately and manage the cases to prevent further spread of the disease. The number of VTEC cases in Japan is totaled for each prefecture weekly. In this study, we used this surveillance data reported from April 1999 to October 2004 (287 weeks), which were retrieved from the Infectious Agents Surveillance Report published by NIID. Climate variables, which were summarized weekly, were retrieved from meteorologic agencies in the capitals of the 47 prefectures. Considering the incubation period between infection and reporting a diagnosis of VTEC, we used the climatic conditions from the 2-week period before each case was reported. We also used annual socioeconomic data for each of the 47 prefectures (*7*), including the following information: population density, percentage of children (≤15 years of age), percentage of elderly (≥65 years of age), average number of persons in the household, number of livestock (beef cattle, dairy cattle, hogs, and chickens) per person in the prefecture, and average income. All statistical analyses were performed by using Stata 8.0 software (Stata Corp. LP, College Station, TX, USA).

Conclusions

Nationwide, 19,304 cases of VTEC were reported during the study. The annual incidence was 2.74 per 100,000. The highest number that occurred in a prefecture was 63 VTEC cases per 1,000,000 during 1 week in a single prefecture. More than 16 VTEC cases were observed in 10% of 13,489 weeks (287 weeks × 47 prefectures), and no cases were reported in 57.6% of 13,489 weeks. Age distribution of patients indicates that the number of VTEC cases was highest in children <5 years of age and fewer cases were reported in older age groups. A total of 65 outbreaks, defined as >11 laboratory-confirmed VTEC cases in a certain time frame and area, were reported during the study. The biggest outbreak occurred during September 2003 in Kanagawa prefecture and included 252 symptomatic and 197 asymptomatic cases of VTEC.

The change in VTEC cases over time is shown in Figure 1, on which the average air temperature (>25°C) during each week of the summer season is overlaid. Though the annual incidence showed no clear tendency to increase or decrease during this study, a marked seasonal oscillation pattern with peaks centered in July and August was shown.

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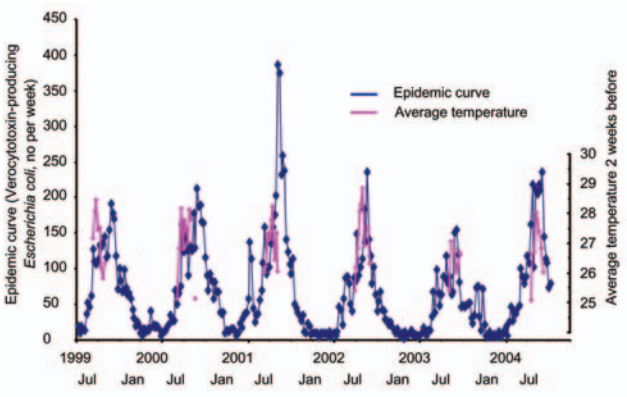

Figure 1. The annual oscillation of verocytotoxin-producing *Escherichia coli* (VTEC) cases during the study period. In addition to the VTEC cases, the average air temperature (>25°C) during each week of the summer season is overlaid in the graph.

The geographic distribution of VTEC cases per 100,000 per year in each of the 47 prefectures indicated that a relatively higher incidence of VTEC was clustered in western sections of several Japanese prefectures and northeastern sections of 2 Japanese prefectures (Figure 2). The 4 prefectures with the highest annual incidences were rural areas: Saga (9.2/100,000), Ishikawa (7.9/100,000), Akita (5.8/100,000), and Iwate (5.8/100,000). Conversely, the prefectures with the lowest incidences were near urban areas: Yamanashi (1.3/100,000), Ibaraki (1.1/100,000), Niigata (0.9/100,000), and Shizuoka (1.4/100,000).

The association of climate and socioeconomic factors with the fluctuation of VTEC cases was estimated by using multiple regression analyses (Table). Within the climate variables, average air temperature of the day, wind speed, and the number of sunny days were significantly associated with the incidence of VTEC cases per 100,000 per week

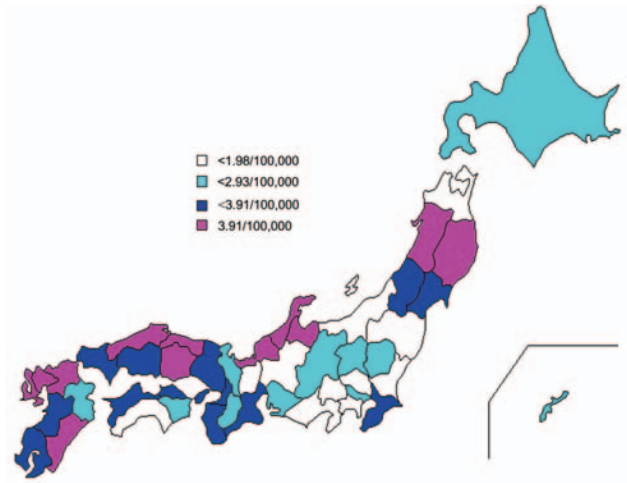

Figure 2. Average number of VTEC cases per 100,000 population per year in each of 47 prefectures from 1999 to 2004, Japan.

per prefecture. By adjusting for these 3 climate variables as well as calendar months, associations between 7 socioeconomic variables and VTEC incidence/100,000 population per week per prefecture were analyzed. Results indicated that the following population-related factors were strong risk factors for VTEC incidence: a higher percentage of elderly people in the prefecture, higher population density, higher number of persons in a household of the prefecture, and higher percentage of children. The following socioeconomic factors in the prefecture showed a positive association with VTEC incidence: lower average income in the prefecture and greater number of beef cattle per person. On the other hand, the number of chickens per person was negatively associated with VTEC incidence. Moreover, this multiple regression model showed that these population, socioeconomic, and climate factors could statistically explain 31% of the variability of VTEC incidence.

We cannot determine a causal relationship because of the nature of the ecologic study that we used in this research. However, the results imply that higher beef cattle density, higher population density, and more persons per household might increase the risk of developing VTEC infection.

Because our surveillance data were collected from different regions of Japan, we compared them on the assumption that 1) people seek care with the same frequency in all regions when they are ill, 2) doctors request stool specimens with the same frequency in all regions, and 3) laboratories test for VTEC with the same standards in all

Table. Climate and socioeconomic variables associated with the number of cases of verocytotoxin-producing *Escherichia coli* * by multiple linear regression†

Climate‡ and socioeconomic§ variable	*t*	p value
Average air temperature of the day (ºC)	9.72	<0.001
Wind speed (m/s)	4.69	<0.001
No. sunny days	–1.91	Not significant
Average no. persons in a household¶	6.30	<0.001
Population density	8.61	<0.001
% children (≤15 years of age)	2.69	0.007
% elderly (≥65 years of age)	20.70	<0.001
Average income#	–10.43	<0.001
Beef cattle/population**	2.71	0.007
Chicken/population	–3.36	0.001

*5,580 of 13,489 weeks (287 weeks × 47 prefectures) or 41.4 % of the weeks were included in the analysis as no cases of *E. coli* were reported during 7,909 weeks (58.6%).
†R^2 = 0.31: calculated based on the multiple linear regression model using the 7 socioeconomic variables, 3 climate variables, and calendar months.
‡Data for the 2-week period before the week *E. coli* was reported were used to approximate the period between infection and diagnosis.
§Annual data in each prefecture were used.
¶Correlation between average no. persons in a household and population density was –0.4.
#Correlation between average income and population density, average no. persons in a household, and percentage of elderly was 0.9, –0.4, and –0.6, respectively.
**Beef cattle/population had a strong correlation with hog/population.

regions. Thus, some degree of observation bias may exist even under control of the law, which is a limitation of this study. In addition, the number of cases includes not only symptomatic but also asymptomatic VTEC, which may also raise the incidence rate in the Japanese surveillance system.

In conclusion, we showed a high annual incidence of VTEC of 2.74 per 100,000 that was associated with climate, socioeconomic, and population factors. However, because this was an ecologic study, further longitudinal studies are necessary to address these complicated associations.

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Dr Sakuma is a PhD candidate at the Jikei University School of Medicine, Tokyo, and a pediatrician at the Jikei University hospital. Her research focuses on the study of trace elements in cord blood and association of a child's development, behavior, and health condition with the distribution of trace elements.

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Waterborne Toxoplasmosis, Brazil, from Field to Gene

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Water was the suspected vehicle of *Toxoplasma gondii* dissemination in a toxoplasmosis outbreak in Brazil. A case-control study and geographic mapping of cases were performed. *T. gondii* was isolated directly from the implicated water and genotyped as SAG 2 type I.

Water has been considered an important vehicle for disseminating human toxoplasmosis in outbreaks (*1*,*2*) and in endemic settings in Brazil (*3*). We investigated a large toxoplasmosis outbreak in which the exposure to known sources of *Toxoplasma gondii* infection was assessed. We found that unfiltered, municipally treated water was the epidemiologically implicated source of infection for this outbreak. Isolation, polymerase chain reaction (PCR) detection, and genotyping of *T. gondii* from the implicated water source were demonstrated.

The Study

In November 2001, in Santa Isabel do Ivai, (southern state of Paraná), a local physician requested serologic tests to diagnose dengue, mononucleosis, cytomegalovirus infection, hepatitis, and toxoplasmosis in 2 persons in whom fever, headache, and myalgias had developed. Positive results were obtained for anti–*T. gondii*–immunoglobulin M (IgM) and IgG only. Through the end of 2001, 294 similar cases, which were serologically confirmed as toxoplasmosis, were reported to health authorities in the same area.

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The outbreak peaked between November 2001 and January 2002 (Figure 1). Symptoms were reported by 155 persons; the main symptoms were headache (n = 135), fever (n = 128), malaise (n = 128), myalgia (n = 124), lymphadenitis (n = 117), anorexia (n = 107), arthralgia (n = 95), night sweats (n = 83), vomiting (n = 60), and rash (n = 11). The duration and magnitude of the epidemic curve could have been influenced by the intensity of media reporting at specific times, which led to people seeking toxoplasmosis testing.

Case-patients were located by active contacting of and passive reporting from local physicians, and media campaigns (television, radio, and newspapers). A matched case-control study was conducted from January 15 to February 2, 2002. Acute cases were defined by standard serologic criteria (*4*) and were selected from a list of volunteers. A total of 2,884 of 6,771 persons living in the urban area of the city volunteered to be serologically tested. A total of 426 (11.5%) persons had anti–*T. gondii* IgM and IgG antibodies; 1,255 (51%) were positive only for IgG antibodies. Of 426 persons who had anti–*T. gondii* IgM and IgG antibodies, 176 met the case definition; of these, 156 (89%) participated in the case-control study. Sex and age matched controls (±5 years, n=220) were selected from the same group of volunteer who were asymptomatic and seronegative for *T. gondii*.

Serum samples from case-patients and controls were tested for anti–*T. gondii* IgM and IgG antibodies by the Central Laboratory of the Paraná State by using 3 different commercially available enzyme-linked immunosorbent assays (ELISAs) because it was not possible for a single vendor to provide the number of required kits. Fifty percent of the case serum samples (78 samples of 156 participants) were randomly retested in a toxoplasmosis

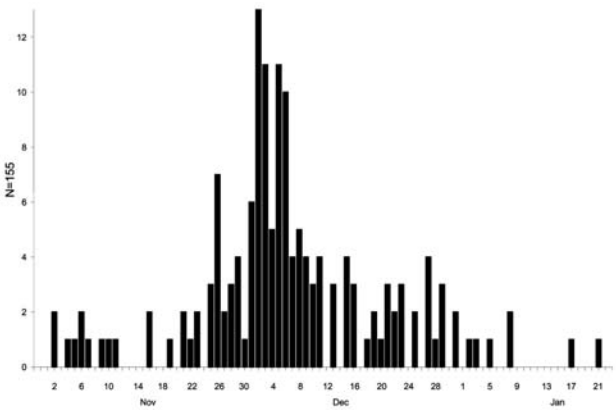

Figure 1. Epidemic plot of the 155 cases registered from November 2001 to January 2002. The dates of the initial symptoms are known only for the 155 individuals among 156 who participated in the case control study.

[1]These authors contributed equally to this study.

serology reference laboratory, Laboratory of Protozoology at the Tropical Medicine Institute of São Paulo. Five (6.4%) IgM- and IgG-positive serum samples, tested previously with 1 of the commercial kits, showed very low IgG avidity when tested by this laboratory. All the other serum sample test results were confirmed by testing conducted in this laboratory.

Of the 156 participants, 138 (88%) lived in the area served by reservoir A and 17 individuals lived in area served by reservoir B (Figure 2); 1 person had a private well. Table 1 shows the univariate analysis results and Table 2 shows the multivariate analysis results. Case-patients were significantly more likely than controls to drink water supplied by municipal reservoir A than reservoir B, as well as to eat commercial ice cream than not. The 4 case-patients that reported not drinking water from reservoir A, however, reported eating ice cream. The frequency of eating ice cream among the persons who drank water from the reservoir A was 32%.

The environmental investigation included mapping the city water supply system which is served by 2 municipal tank reservoirs (reservoir A and reservoir B) that both receive water from underground, protected deep wells. Both reservoirs are tanks with 150,000 L storage capacity. Case distribution showed a clustering in the central area served by reservoir A (Figure 2).

Because the environmental investigations and the case-control study started in parallel on January 9, 2002, and the outbreak had peaked (Figure 1), the chances of detecting parasites in the municipally distributed water were theoretically low. To increase the chances of detecting the parasite in water, household tanks that had water that had been distributed during the outbreak peak were identified. These tanks could be investigated in municipal schools that stopped water use due to summer vacations from December 17, 2001, to the end of January 2002. Despite the risk from eating ice cream (Table 2), no ice cream made during the outbreak period was available for laboratory testing. The ice cream was prepared locally in small batches with water from reservoir A.

We identified 4 schools that had water in their household tanks that had been distributed by reservoir A during the peak of the outbreak. Approximately 4,650 L of water collected from these tanks was filtered through 56 fluoropore membrane filters (Millipore Billerica, MA, USA). We retrieved 19 liters of water concentrated to 60 mL by centrifugation (600 × *g* 30 min 4°C). The membrane filters were divided into 3 equal sets. One set remained in Brazil (Universidade Estadual do Norte Fluminense Darcy Ribeiro) for bioassays in *T. gondii*–seronegative chickens and further genotyping. One set was sent to the US Department of Agriculture for bioassays in *T. gondii*–seronegative pigs and cats, and 1 was sent to the Centers for Disease Control and Prevention for PCR analysis. Chickens and pigs were fed with membrane filters and their serum samples tested by ELISA and or modified agglutination test (*5*) until seroconversion. The seropositive animal organs were examined for *T. gondii* (*6*). Control animals were fed with noncontaminated membrane filters. Water samples from the 4 schools' household tanks were positive for *T. gondii* by at least 1 assay method. Parasites were found in the lungs of mice injected with brain and heart tissue of seropositive chickens. Cats fed pig tissues shed *T. gondii* oocysts after 4–5 days. Oocysts from cat feces were injected into mice, which died of acute toxoplasmosis. Viable *T. gondii* was recovered in mice after subpassage as verified by optical microscopy. The nested amplification of SAG 2 followed by restriction fragment length polymorphism identified type I *T. gondii* from chickens and pigs (*7*).

DNA extraction from fluoropore membranes was performed with the FastDNA extraction method (Qbiogene, Irvine, CA, USA), by using a procedure previously published (*8*), and PCR was performed on extracted DNA by using primers Toxo B22 and B23 (*9*). PCR from DNA extracted directly from the fluoropore membranes was tested blindly by 2 persons on 3 aliquots extracted individually from each membrane filter. The correct size fragment of 115 bp from B1 *T. gondii* gene was amplified from each DNA aliquot extracted from membranes used to process water from 3 of the implicated tanks.

Conclusions

Our investigation determined that this toxoplasmosis outbreak was associated with consumption of contaminated

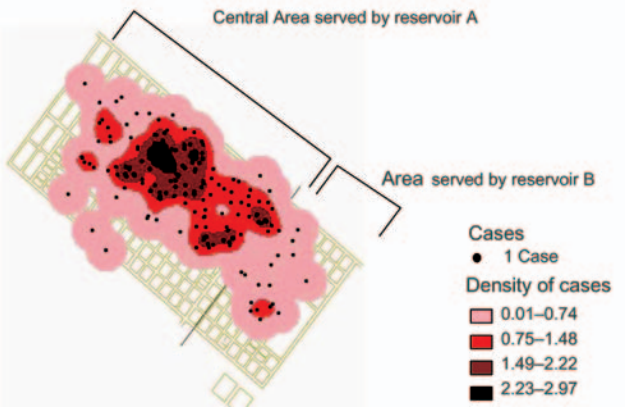

Figure 2. Spatial distribution in km^2 of the 176 cases that met the case definition. The number of cases is higher in the central area than in the periphery. The reservoir tanks served 2 different parts of the city as depicted by the letters A and B. Water samples from reservoir B, which was considered not implicated in the outbreak, were not investigated; during the water sample collection period (January 9–18), there were no identified household tanks served by reservoir B that had stored water that had been distributed during the outbreak peak.

Table 1. Univariate analysis showing risk factors for *Toxoplasma gondii* infection for statistically significant factors (positive results only), N=376

Characteristic	No. persons*	Case	Control	Matched odds ratio	p value	95% confidence interval
Water exposure						
Drank water exclusively from municipal tank reservoir				3.73	0.016	1.27–10.93
A	350	152	198			
B	26	4	22			
Household tank				2.16	0.006	1.24–4.01
No	95	28	67			
Yes	281	128	153			
Drank >10 cups water per day				2.07	0.004	1.24–3.61
No	270	97	173			
Yes	106	59	47			
Drank beverages made with unfiltered water				2.25	0.044	1.02–5.50
No	34	20	14			
Yes	342	136	206			
Food exposure						
Ate undercooked meat in past 30 days				2.71	0.027	1.11–7.34
No	345	136	209			
Yes	31	20	11			
Ate commercial ice cream				3.43	0.000	2.08–5.67
No	188	51	137			
Yes	188	105	83			
Ate bacon				1.89	0.009	1.15–3.02
No	228	82	146			
Yes	148	74	74			
Ate lamb				1.85	0.043	1.02–3.51
No	316	122	194			
Yes	60	34	26			
Ate in restaurants in the past 30 days				1.71	0.028	1.06–2.96
No	277	105	172			
Yes	99	51	48			

*Case-patients ranged from 1 to 72 years of age (median = 28); 79 (51%) were male; 6 (3.8%) were pregnant woman.

water, or ice cream prepared with contaminated water, during the outbreak peak. The main factor leading to contamination of reservoir A was the vulnerability to infiltration due to its precarious state of conservation. We propose that reservoir A was contaminated with *T. gondii* oocysts because 1) a female cat living in the reservoir A area delivered 3 kittens in early October 2001; 2) the kittens lived on the top of the tank reservoir; and 3) the kittens were most likely weaned by the first week of November. However, it was not possible to confirm *T. gondii* in the kittens because we were not able to catch them. The reservoir shelter roof tiles were removed and not replaced until the end of heavy summer rains. From November 4 to December 12, the daily rainfall varied from 27 mm to 72 mm. Reservoir A, constructed in the 1940s, had cracks that were unprotected from rain water, which were likely contaminated with cat feces. These factors could have been enhanced by the lack of filtration and flocculation processes as part of the water treatment. Additionally, the level of chlorination used to treat water in municipal systems is inadequate to eliminate *T gondii* oocysts (*10*).

Of the 408 case-patients examined for ophthalmologic conditions through February of 2002 who were *Toxoplasma* IgM and IgG positive, 10% had ocular lesions; however, only 4.4% had necrotizing retinal lesions (*11*). The frequency of symptoms observed in this study

Table 2. Risk for *Toxoplasma gondii* infection shown as odds ratios estimated with conditional backward elimination logistic regression, N=376

Variable	Odds ratio	Wald confidence limits		p value*
		Lower	Upper	
Drinking water from reservoir A	4.55	2.01	5.49	0.001
Drinking >10 glasses of water per day	3.29	1.46	4.46	0.001
Having household water storage tank	1.81	0.99	3.33	0.054
Eating commercial ice cream	4.55	2.01	5.49	0.001

*Significant ($p \leq 0.001$, rounded).

may be associated with the dose and virulence of organisms ingested since parasites of genotype I, which are of high virulence (*12*,*13*), were isolated from the water implicated in the outbreak. These data are consistent with other studies also showing SAG-2 type I parasites isolated from the environment from different geographic areas in Brazil (*14*), including in the outbreak area (*15*). Demonstration of the parasite in the outbreak implicated water was decisive in the closing of reservoir A and the construction of a new municipal reservoir.

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Instructions for Infectious Disease Authors

Dispatches

Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and a brief biographical sketch of first author—both authors if only 2. Dispatches are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Chlamydophila psittaci in Fulmars, the Faroe Islands

Björn Herrmann,* Heléna Persson,† Jens-Kjeld Jensen,‡ Høgni Debes Joensen,§ Markus Klint,* and Björn Olsen¶#

Chlamydophila psittaci was detected in 10% of 431 fulmars examined from the Faroe Islands. Analysis of *ompA* showed a sequence almost identical to that of the type strain. The origin of *C. psittaci* outbreaks in fulmars is discussed. Despite a high level of exposure, the risk for transmission of *C. psittaci* to humans is low.

During the winter of 1929–1930, widespread epidemics of chlamydophilosis (psittacosis) occurred in Europe and the United States, and the causative agent was isolated from humans and affected birds (*1*). Presumably the epidemics originated in Argentina (*2*), and the disease was exported by shipments of pet birds. From the Faroe Islands (Figure), 174 cases of human chlamydophilosis were reported from 1930 to 1938 (*3*). The human death rate was 20%; it was especially high (80%) in pregnant women. Experimental work confirmed that "psittacosis virus" was contracted by humans when juvenile fulmars (*Fulmarus glacialis*) were caught and prepared for cooking (*4*). After the outbreaks in the 1930s, hunting fulmars for human consumption was prohibited until 1954, and data on chlamydophilosis have been scarce. In this study, our aim was to determine the current prevalence of *Chlamydophila psittaci* in fulmars and to relate it to available information on chlamydophilosis in humans in the Faroes.

The Study

Cloacal swab samples were collected in September 1999 from 431 nonflying, juvenile fulmars. Samples were stored in sucrose–phosphate-buffered saline (PBS) in dry ice and transported to Sweden for analysis.

DNA was extracted from 400 µL of each sample (High Pure PCR Template Preparation, Roche, Branchburg, NJ, USA) and eluted in 100 µL of buffer. *C. psittaci* was detected by using 23S rRNA-based quantitative polymerase chain reaction (PCR) (*5*). A positive control was used for each round of DNA extraction and PCR. To monitor contamination, 1 negative control of PBS was included for every 5 samples in each run of DNA extraction and the ensuing PCR.

To characterize *Chlamydophila* cases, a 1,101-bp PCR fragment of *ompA* was sequenced. A nested PCR was used; the outer primers were FOMPF1 5′-GAAATCGGCATTATTRTTTGCC-3′ and FOMPR2 5′-CCAGTGATTGACCATTTGTCA-3′. Initial amplification was performed by using 0.2 µmol/L of each primer, 200 µmol/L deoxynucleoside triphosphates, 2 U Taq DNA polymerase (Qiagen, Hilden, Germany) in 1.5 mmol/L $MgCl_2$ and 5 µL template DNA. Thermal conditions included 40 cycles of 20 s at 95°C, 60 s at 52°C, and 80 s at 72°C. In a second amplification, the primers FOMPF2 5′-TACGGGTTCCGCTCTCTC-3′ and FOMPR1 5′-CATTTGTCAGCGTCGATTAACG-3′ were used as in the first step. For sequencing, the inner primers and the primers CpsF2 5′-YGTAGGTGCACGYGGAG-3′ and 201FAG 5′-GGAGCIGARTTCCAATACGCTCAITC-3′ were used together with a BigDye terminator labeled sequencing kit (Applied Biosystems (Foster City, CA, USA).

We found that 10% of 431 juvenile fulmars were infected with *Chlamydophila* spp. Detection rates ranged from 7% to 21% in different collection areas. Since the juvenile birds were caught on the sea surface near their nesting cliffs before they were fledged, the different detection rates suggest that the prevalence of infection varies among colonies. This hypothesis is supported by the fact that ringing of fulmars shows their lifestyle is local, that is, they do not travel over long distances. The reported *C. psittaci* prevalence in other studies of wild birds varies widely, from 0% to 74% (*6,7*). This wide range is partly attributable to small and selected study populations and to the use of insensitive techniques or methods that measure exposure rather than prevalence.

Adult birds often have nonsymptomatic infections, while young birds frequently have acute disease. These descriptions are consistent with those from epidemics in the Faroes, where humans contracted chlamydophilosis only when handling juvenile fulmars, not when catching adult birds (*3*).

Analysis of *ompA* from 29 representative specimens from the *Chlamydophila*-positive birds showed no variation and showed sequences almost identical (T471C mutation in the variable domain 2, GenBank accession no. AM050561) to that of the prototype strain 6BC for *C. psittaci*. This finding is in contrast to our previous study of seabirds, in which we found a *C. psittaci* strain closely related to *C. abortus* (*8,9*). To our knowledge, further sequence data on *Chlamydophila* infections in seabirds have not been reported.

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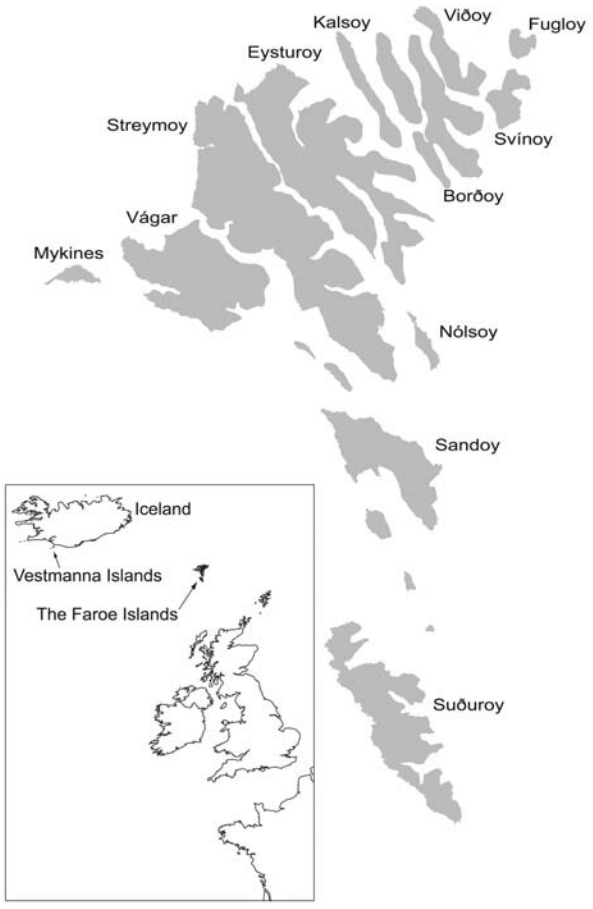

Figure. Map of the northern Atlantic showing the Faroe Islands and surrounding areas. Source: Faroese Museum of Natural History. Adapted by Janus Hansen.

Conclusions

Our finding of a detectable infection rate of 10% is representative of juvenile fulmars; however, in adult fulmars the rate is probably lower, since they may develop immunity and are less exposed to dust contaminated by *C. psittaci* in nests. Serotyping and genotyping of *C. psittaci* strains are based on the major outer membrane protein coded by *ompA*, and at least 6 types are described. Type A is considered to have psittacine birds as natural hosts, and types B and D are associated with pigeons and turkeys, respectively; the natural hosts for types C, E, and F are unknown. The *ompA* sequence in infected fulmars is almost identical to the 6BC isolate, a strain isolated 64 years ago in a parakeet (*10*). Knowledge of the correlation between *C. psittaci* types and wild bird species as natural hosts, however, is limited. The question is further complicated by the fact that birds are excellent vectors and may contract *C. psittaci* when they feed on the detritus of infected animals of all kinds. Our *ompA* data may support the speculation that fulmars in the Faroes acquired *C. psittaci* from infected and dead parrots thrown overboard during shipment from Argentina to Europe in 1930 (*11*). The first human case in the Faroes appeared that year on the southernmost island of Suduroy, and during the years 1933–1938 severe outbreaks occurred on Sandoy and other islands (Figure) (*3*). In Iceland, the first human chlamydophilosis cases linked to fulmars were reported on the Vestmanna Islands in 1939. Six cases were reported; all occurred after birds had been prepared for human consumption (H. Briem, pers. comm.) This finding further supports the hypothesis that *C. psittaci* was spread from ships by psittacine birds to fulmars in the northern Atlantic and then was gradually introduced to more distant areas.

Catching young fulmars in the Faroes was prohibited in 1938. Only sporadic episodes of chlamydophilosis were observed after the new legislation. Since 1954, when the ban on taking fulmars was lifted, and until 2003, the chief medical officer has reported 48 cases. Apart from an outbreak of 8 cases in 1972, the annual number of cases has ranged from 0 to 3, none fatal. On average, 2.2 cases per 100,000 inhabitants have been reported, which is much higher than figures from other countries where chlamydophilosis is a reportable disease. Also, awareness of chlamydophilosis is probably considerably higher in the Faroes than in the other countries, a fact that may lead to higher detection rates. What proportion of human chlamydophilosis cases in the Faroes is associated with fulmars is unknown, but since 50,000–100,000 juvenile fulmars are prepared for human consumption each year, and *C. psittaci* prevalence is 10%, up to 10,000 potential human exposures to *C. psittaci* occur yearly. The risk of humans acquiring symptomatic *C. psittaci* infection from fulmars is thus very low. This conclusion agrees with our previous finding that bird ringers were antibody-negative for *C. psittaci*, despite high exposure to birds (*12*). A contrasting report of a human chlamydophilosis outbreak associated with wild birds in Australia has recently been published. Clinical, laboratory and epidemiologic data indicate disease episodes related to bird contact and lawn mowing, but the source of infection could not be identified (*13*).

Nevertheless, unanswered questions remain. Why have more chlamydophilosis cases not been noted after the taking of fulmars was resumed in 1954? Why did the annual incidence decrease from 42 per 100,000 inhabitants during the epidemic period in the 1930s to 2.2 after 1954? If a *C. psittaci* infection was introduced among fulmars in 1930 as a result of shipping parrots to Europe, an initially high attack rate could have been expected. Subsequently, adaptation between bacteria and host may have led to less symptomatic infections and lower shedding of bacteria. The ensuing decrease in human exposure to *C. psittaci* may have resulted in a falling incidence of disease. An alternative explanation for the low incidence of chlamydophilosis

in recent years may be a general improvement in public health. By analogy with the eye disease trachoma, caused by *Chlamydia trachomatis*, the incidence of chlamydophiliosis would be expected to decrease as hygienic conditions improved. However, human chlamydophilosis is a zoonosis, and the impact of improved public health is not clear. A third explanation for the lower disease rates could be changes in the methods used to prepare caught fulmars. No systematic investigation has been made of changes in handling procedures, and identifying any specific modification that might have led to major reductions in exposure to infected birds is difficult. Thus, this issue is still unresolved.

C. psittaci is rarely an agent of community-acquired pneumonia (*14*), and in outbreaks related to pet birds the disease may be mild rather than severe (*15*). Consequently, investigation for chlamydophilosis in humans should only be considered when clinical and epidemiologic data indicate such a diagnosis.

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Sequencing and Staphylococci Identification

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The emerging clinical importance of staphylococcal infections prompted us to establish a reference database for partial RNA polymerase B (*rpoB*; nucleotides 1444–1928) gene sequences from type strains of all staphylococcal species and subspecies. This database correctly identified 55 clinical staphylococcal isolates; all were correctly identified at the species level. At the subspecies level, *rpoB* misidentified only 2 isolates.

The emerging clinical importance of *Staphylococcus aureus* and coagulase-negative staphylococci (*1*) in connection with the expanding number of staphylococcal subspecies described requires accurate identification to the subspecies level. Currently, the genus *Staphylococcus* is divided into 36 species and 21 subspecies. Staphylococcal subspecies not included in the databases of commercial identification systems, as well as phenotypic variants (e.g., small-colony variants), are often misidentified (*2*).

We recently described the usefulness of genotypic identification of staphylococcal subspecies by using partial 16S rDNA sequences in comparison with phenotypic tests (*3*). However, the partial 16S rDNA sequences used were not discriminative enough to differentiate all staphylococcal subspecies. When searching for a molecular target for discrimination of staphylococci, several genes have been evaluated, e.g., heat shock protein 60 (*hsp60*) (*4*), superoxide dismutase A (*sodA*) (*5*), and RNA polymerase B (*rpoB*) (*6*). However, these studies concentrated only on a limited number of staphylococcal species. Therefore, a complete reference database of partial *rpoB* gene sequences from type strains (n = 47) and other culture collection strains, including all validly described staphylococcal subspecies, was created for this study. This reference database was then evaluated with clinical isolates. Results were compared with those previously obtained by 16S rDNA sequencing and conventional phenotypic tests.

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The Study

We analyzed 82 type and other culture collection strains encompassing all validly described staphylococcal species (n = 38) and subspecies (n = 21; according to the current List of Bacterial Names with Standing in Nomenclature, updated May 14, 2005) (*7*). Two strains of the recently proposed candidate species *S. pettenkoferi* (*8*) were added to complete the *rpoB* sequence reference database. Using this database, we analyzed 55 clinical staphylococcal isolates collected from human (n = 52) and animal (*S. intermedius*, n = 2; *S. felis*, n = 1) specimens; 6 of the human isolates exhibited the small-colony variant (SCV) phenotype.

This strain collection was previously analyzed by the API ID 32 Staph and VITEK 2 systems (both obtained from bioMérieux, Marcy l'Etoile, France), partial 16S rDNA sequencing, chemotaxonomy, and riboprinting to determine species designation (*3*). The thermal cycling condition to amplify the partial *rpoB* gene (899 bp) was 35 cycles of denaturation at 94°C for 45 s (300 s for the first cycle), annealing (60 s at 52°C), and extension (90 s at 72°C, 600 s for the last cycle). The *Staphylococcus*-specific primers used for amplification and sequencing of *rpoB* are shown in Table 1. Sequencing reactions were performed in a total volume of 10 μL containing 0.5 μL premix (ABI Prism BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit, Applied Biosystems, Darmstadt, Germany), 1.8 μL 400 mmol/L Tris-HCl, 10 mmol/L $MgCl_2$, 10 pmol sequencing primer, and 2 μL polymerase chain reaction product. The sequencing products were purified by using the Centri-Sep Spin Columns (Princeton Separations, Adelphia, NJ, USA) and analyzed with the ABI Prism 3100 Avant Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions. For further analysis, nucleotides 1444–1928 (corresponding to *S. aureus rpoB* gene positions of the GenBank accession no. X64172) of the *rpoB* gene were used. The sequences were analyzed by using Ridom TraceEditPro

Table 1. Primers used for amplification and partial sequencing of the partial staphylococcal RNA polymerase B (*rpoB*) gene

Primer	Application	Primer sequence (5′→3′)	Annealing temperature (°C)	Reference
Staph rpoB 1418f*	Amplification and sequencing	CAA TTC ATG GAC CAA GC	52	Modified from 7
Staph rpoB 3554r	Amplification	CCG TCC CAA GTC ATG AAA C	52	7
Staph rpoB 1975r*	Sequencing	GCI ACI TGI TCC ATA CCT GT	52	Modified from 7
Staph rpoB 1876r*†	Sequencing	GAG TCA TCI TTY TCT AAG AAT GG	52	This study

*Primers are numbered from the 3′ end of the primer on the forward strand of *Staphylococcus aureus* (GenBank accession no. X64172).
†Primer was used for sequencing when primer Staph rpoB 1975r did not work.

Table 2. Identification of 55 clinical staphylococcal isolates by using RNA polymerase B (*rpoB*) gene sequencing

Strain	*rpoB* gene (% similarity*)	Definitive identification†
M01	*Staphylococcus arlettae* (100.0)	*S. arlettae*
M02	*S. aureus* subsp. *aureus* (100.0)	*S. aureus* subsp. *aureus*
M03	*S. aureus* subsp. *aureus* (100.0)	*S. aureus* subsp. *aureus*
M04	*S. aureus* subsp. *aureus* (99.8)	*S. aureus* subsp. *aureus*
M05‡	*S. aureus* subsp. *aureus* (99.8)	*S. aureus* subsp. *aureus*
M06	*S. aureus* subsp. *aureus* (100.0)	*S. aureus* subsp. *aureus*
M07‡	*S. aureus* subsp. *aureus* (100.0)	*S. aureus* subsp. *aureus*
M08	*S. haemolyticus* (94.0)	*S. haemolyticus*
M09	*S. epidermidis* (100.0)	*S. epidermidis*
M10	*S. capitis* subsp. *capitis* (100.0)	*S. capitis* subsp. *capitis*
M11	*S. epidermidis* (100.0)	*S. epidermidis*
M12‡	*S. epidermidis* (100.0)	*S. epidermidis*
M13‡	*S. capitis* subsp. *capitis* (99.8)	*S. capitis* subsp. *capitis*
M14	*S. caprae* (99.8)	*S. caprae*
M15	*S. caprae* (99.8)	*S. caprae*
M16	*S. chromogenes* (100.0)	*S. chromogenes*
M17	*S. cohnii* subsp. *cohnii* (99.8)	*S. cohnii* subsp. *cohnii*
M18	*S. cohnii* subsp. *cohnii* (99.8)	*S. cohnii* subsp. *cohnii*
M20	*S. saprophyticus* subsp. *saprophyticus* (100.0)	*S. saprophyticus* subsp. *bovis*
M21	*S. epidermidis* (99.0)	*S. epidermidis*
M22	*S. epidermidis* (100.0)	*S. epidermidis*
M23‡	*S. epidermidis* (100.0)	*S. epidermidis*
M24	*S. epidermidis* (100.0)	*S. epidermidis*
M25‡	*S. epidermidis* (100.0)	*S. epidermidis*
M26	*S. equorum* subsp. *equorum* (100.0); *S. equorum* subsp. *linens* (100.0)	*S. equorum*; subspecies not known
M27	*S. felis* (99.8)	*S. felis*
M28	*S. haemolyticus* (100.0)	*S. haemolyticus*
M29	*S. haemolyticus* (99.8)	*S. haemolyticus*
M30	*S. epidermidis* (100.0)	*S. epidermidis*
M31	*S. epidermidis* (100.0)	*S. epidermidis*
M32	*S. hyicus* (100.0)	*S. hyicus*
M33	*S. intermedius* (100.0)	*S. intermedius*
M34	*S. intermedius* (100.0)	*S. intermedius*
M35	*S. intermedius* (100.0)	*S. intermedius*
M36	*S. xylosus* (100.0)	*S. xylosus*
M37	*S. lugdunensis* (100.0)	*S. lugdunensis*
M38	*S. lugdunensis* (100.0)	*S. lugdunensis*
M39	*S. saprophyticus* subsp. *saprophyticus* (100.0)	*S. saprophyticus* subsp. *bovis*
M40	*S. aureus* subsp. *aureus* (100.0)	*S. aureus* subsp. *aureus*
M41	*S. schleiferi* subsp. *schleiferi* (100.0)	*S. schleiferi* subsp. *schleiferi*
M42	*S. schleiferi* subsp. *schleiferi* (100.0)	*S. schleiferi* subsp. *schleiferi*
M43	*S. sciuri* subsp. *sciuri* (99.8)	*S. sciuri* subsp. *sciuri*
M44	*S. sciuri* subsp. *sciuri* (99.8)	*S. sciuri* subsp. *sciuri*
M45	*S. sciuri* subsp. *sciuri* (100.0)	*S. sciuri* subsp. *sciuri*
M46	*S. simulans* (100.0)	*S. simulans*
M47	*S. hominis* subsp. *novobiosepticus* (99.6)	*S. hominis* subsp. *novobiosepticus*
M48	*S. felis* (99.8)	*S. felis*
M49	*S. felis* (99.8)	*S. felis*
M50	*S. warneri* (95.9)	*S. warneri*
M51	*S. warneri* (95.3)	*S. warneri*
M52	*S. warneri* (96.0)	*S. warneri*
M53	*S. equorum* subsp. *equorum* (99.8); *S. equorum* subsp. *linens* (99.8)	*S. equorum*; subspecies not known
M54	*S. xylosus* (99.0)	*S. xylosus*
M55	*S. xylosus* (97.1)	*S. xylosus*
M56	*S. xylosus* (98.6)	*S. xylosus*

*Similarity in comparison with the reference database.
†By phenotypic and genotypic methods as previously published (*4*).
‡Isolate exhibiting the small colony variant phenotype.

version 1.0 software (Ridom GmbH, Würzburg, Germany). Staphylococcal partial *rpoB* reference sequences determined in this study were deposited in GenBank under accession nos. DQ120729–DQ120752.

Partial *rpoB* sequences were determined for 82 culture collection strains and 55 clinical isolates. All staphylococcal type strains were distinguishable by *rpoB*; the only exception was the *S. equorum* subspecies that shared the same sequence (online Appendix Figure, available from http://www.cdc.gov/ncidod/EID/vol12no02/05-0962_G.htm). The mean pairwise distance of all type and other culture collection strains exhibiting a unique *rpoB* sequence (n = 68) was 13.7% (range 0%–21.4%) and the standard deviation was 3.3%. When assuming a normal distribution for the distances and choosing a reporting criterion ≥94.0%, the similarity for a distinct species correlates with a statistical error probability of 1.0% (*9*).

The definitive identification of 55 clinical isolates and the *rpoB* gene sequence similarity search results are shown in Table 2. At the species level, the correct species designation for all 55 clinical isolates was made by *rpoB* sequence similarity search (sequence similarity ≥94.0%). Of 21 clinical isolates belonging to species currently divided into subspecies, 17 isolates were correctly identified to the subspecies level. Subspecies identification for isolates M26 and M53 was unsuccessful by *rpoB* or partial 16S rDNA sequencing, riboprinting, and chemotaxonomy (data not shown). Only isolates M20 and M39 were misidentified by *rpoB* sequencing as *S. saprophyticus* subsp. *saprophyticus* instead of subsp. *bovis*.

Conclusions

Our previous study demonstrated the superiority of sequence-based methods over phenotypic approaches using the API ID 32 Staph and VITEK 2 systems (*3*). The advantage of a sequence-based method became most evident when differentiating isolates with the SCV phenotype, in which the API ID 32 Staph and VITEK 2 systems misidentified 2 and 4 isolates, respectively. When both sequence-based approaches used were compared, *rpoB* sequencing was superior to partial 16S rDNA identification. Although the 16S rDNA procedure differentiated 50 (90.9%) of all tested clinical isolates at species level, *rpoB* identified 100%. Therefore, if an unknown organism needs to be identified, 16S rDNA sequencing is the method of choice because of the availability of universal primers (*10*). However, if the genus is already known, the *rpoB* method should be used.

Compared with other published molecular probes, *rpoB* showed the highest discriminatory power, e.g., *hsp60* and *sodA* sequencing did not differentiate subspecies of *S. carnosus*, *S. cohnii*, *S. hominis*, *S. schleiferi*, or *S. succinus* (*4*,*5*). In a previous study, *rpoB* sequence-based identification of *Staphylococcus* species has been reported (*6*). However, a limited number of taxa were included, and the primers used were not appropriate to detect all staphylococcal subspecies.

Sequencing of *rpoB* was also used to identify other bacterial species (*11*,*12*). A higher discrimination with *rpoB* sequencing compared with 16S rDNA sequencing has been demonstrated for the genera *Corynebacterium* (*13*) and *Bacillus* (*14*). DNA sequencing is a rapid alternative to biochemical and other phenotypic procedures for the differentiation of bacterial pathogens because of its decreased costs and increased automation (*15*). Thus, *rpoB* is a useful molecular target for differentiating staphylococcal isolates to the species and subspecies level.

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Salmonella-associated Deaths, Sweden, 1997–2003

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We examined excess deaths after infection with *Salmonella* in a registry-based matched cohort study of 25,060 persons infected abroad and 5,139 infected within Sweden. The domestically infected have an increased standardized mortality ratio, whereas those who acquired Salmonella infection abroad had no excess risk of death.

We were interested in studying deaths attributable to *Salmonella* infection. To avoid the problem of misreporting and underreporting when using death certificates, we linked all cases of salmonellosis (with information on country of infection) reported in Sweden to the national civil register on reported deaths during the years 1997–2003. The primary objective was to investigate whether patients with a diagnosis of *Salmonella* infection have a death rate from all causes that differs from that of the general population. If so, the second objective was to determine whether this general mortality rate in previous cases of salmonellosis could be used as a surrogate for *Salmonella*-related death rates. We would in this case expect that any elevated death rate in the *Salmonella* cohort would be highest near time of infection and then gradually diminish and approach the general death rate in the population. Since the patients with domestic cases and patients who contracted the infection abroad may be 2 fundamentally different groups, we analyzed these 2 groups separately.

The Study

From 1997 through 2003, a total of 30,438 cases were reported to the Swedish Institute for Infectious Disease Control (SMI) of which 25,060 were stated to have been infected abroad. For 239, country of infection was unknown, and they were excluded from the analysis. The median age for the domestically infected salmonellosis patients was 36 years (interquartile range 20–56 years). For persons infected abroad, median age was 40 years (interquartile range 25–56 years).

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For general surveillance purposes, SMI receives a file every week from the Swedish National Tax Board with all registered deaths that occurred during the preceding week. This file does not contain any information on cause of death, only the personal identification number and date of death. These data were used to identify *Salmonella*-infected patients who had died after receiving that diagnosis.

For every *Salmonella*-infected patient, whether they died or recovered, follow-up time was counted from the date of onset of illness. Risk time was accumulated until time of death or August 1, 2004. Sex-specific and age group–specific death rates were obtained from Statistics Sweden and were used to calculate the number of expected deaths in the *Salmonella*-infected cohort. The observed number of deaths was divided by the expected number of deaths to produce a standardized mortality ratio (SMR). Poisson regression was used to investigate changes in SMR over time for different strata (expected cases explanatory variable). Exact confidence intervals were calculated, assuming that the number of deaths in each stratum was Poisson distributed.

For the group of persons infected within Sweden (n = 5,139), SMR was increased during every period after onset, falling from 5.6 during the first month to 1.4 after >1 year (Table 1). Within each stratum for time after infection in this group, homogeneity in the results was investigated by calculating separate SMRs for the age groups ≤14 years, 15–64 years, and ≥65 years (Table 2). For all time strata, the SMR was approximately equal in all age groups. However, <1 month after infection, SMR = 11.2 (95% confidence interval [CI] 4.1–21.8) for the age group 15–64 years and 4.7 (95% CI 2.6–7.4) for the ≥65 age group. This difference is not significant because of the small sample, but the assumption that the older age group is responsible for the elevated SMR is highly unlikely.

For persons who had acquired their *Salmonella* infection abroad (n = 25,060), we found no increased deaths compared to the general population; SMR = 0.6 (95% CI 0.2–1.2) <1 month after onset, 1.3 (95% CI 0.8–2.0) after 1–3 months, 0.6 (95% CI 0.4–0.8) after 4–12 months, and again 0.6 (95% CI 0.5–0.7) after >1 year had passed since the acute infection (Table 1). Instead, a significantly lower mortality ratio is evident in this subgroup, compared to that for the Swedish general population, for every period after 3 months have passed since onset of illness. We also calculated SMRs for different age groups separately for the imported salmonellosis cases, but no obvious differences were found between age groups.

Among the isolates that were subtyped, *S.* Enteritidis and *S.* Typhimurium dominated in both of the groups. *S.* Dublin and *S.* Wirchow, which sometimes are believed to

Table 1. Standardized mortality ratios (SMRs) among 30,199 Swedish patients with reported cases of salmonellosis acquired domestically and abroad, 1997–2003*

Time after infection (mo)	Infected domestically (n = 5,139)			Infected abroad (n = 25,060)		
	Obs	Exp	SMR (95% CI)†	Obs	Exp	SMR (95% CI)
<1	21	3.8	5.6 (3.4–8.2)	4	7.0	0.6 (0.2–1.2)
1–3	34	7.2	4.7 (3.3–6.5)	19	14.2	1.3 (0.8–2.0)
4–12	55	30.3	1.8 (1.4–2.3)	36	64.3	0.6 (0.4–0.8)
>12	146	107.3	1.4 (1.1–1.6)	215	341.2	0.6 (0.5–0.7)

*Obs, observed number of deaths; Exp, expected number of deaths; CI, confidence interval.
†p<0.0001

be more pathogenic than others, constituted together ≈1% of the isolates among persons infected within Sweden and 2.6% among persons infected abroad.

Conclusions

Undoubtedly, not all deaths identified by linkage to the civil registration system occurred as a result of *Salmonella* infection, but the finding of a high SMR near time of infection and a steady decrease over time nevertheless indicates that salmonellosis is a contributing factor to the increased risk of death in this group of patients. Studies have shown that of all persons with salmonellosis only a small proportion seek medical care and thus have their case end up in the surveillance statistics (*1*,*2*). However, patients with a severe infection, as well as patients with a travel history before disease onset, are more likely to seek care, receive a diagnosis, and be included in the registry, compared to the average salmonellosis patient (*2*). These 2 patient groups differ greatly with respect to disease severity. Generally, a surveillance system will miss the milder domestic *Salmonella* cases, whereas it will tend to pick up travel-associated cases regardless of severity. A generalization of our results would be the following: SMRs for domestic cases are more representative for severe cases in the population, while the SMRs for travel-associated cases are probably more representative for the milder or moderate cases of salmonellosis.

A Danish study used an approach like ours and found that 3.1% of persons infected with salmonellae were dead within 1 year of infection (*3*). In this study, 0.56% of patients (2.1% with domestic cases and 0.24% with imported cases) were found to have died within the same period. That domestic cases had a more severe prognosis could be 1 explanation for this discrepancy. In Denmark, domestic cases constitute ≈86% of all salmonellosis cases (*4*), whereas in Sweden only ≈17% of cases are reported to be domestic. Death certificates or hospital charts have been used in other studies to measure salmonellosis deaths (*5*–*7*), but none of these studies have thoroughly analyzed the interval from diagnosis to death, used any other population group for comparing death rates, or stratified cases according to presumed country of infection.

In the present analysis, we have not adjusted our results for coexisting illnesses. The assumption that our domestic cases represent a more vulnerable subpopulation is born out by the fact that the SMR for this group remains significantly >1.0 even 1 year after the acute salmonellosis episode.

The most plausible explanation for finding completely different SMRs for persons infected domestically and for those infected abroad is a "healthy traveler effect" (*8*). The least healthy persons in any age group are not those who are traveling abroad.

Future studies on deaths due to salmonellosis should take this healthy traveler effect into consideration and, for domestic cases, also consider the inherent bias of any national surveillance system to include more severe cases.

Table 2. Standardized mortality ratios (SMRs) by age group among 5,139 domestically infected *Salmonella* patients, Sweden, 1997–2003*

Time after infection (mo)	Age group (y)	Obs	Exp	SMR (95% CI)
<1	≤14	0	0.1	0 (0–59.6)
	15–64	6	0.5	11.2 (4.1–21.8)
	≥65	15	3.2	4.7 (2.6–7.4)
1–3	≤14	0	0.1	0 (0–30.3)
	15–64	6	1.1	5.6 (2.1–10.9)
	≥65	28	6.0	4.7 (3.1–6.5)
4–12	≤14	0	0.4	0 (0–7.3)
	15–64	11	4.7	2.4 (1.2–3.9)
	≥65	44	25.3	1.7 (1.3–2.3)
>12	≤14	1	1.0	1.0 (0.03–3.8)
	15–64	27	16.9	1.6 (1.1–2.2)
	≥65	118	89.4	1.3 (1.1–1.6)

*Obs, observed number of deaths; Exp, expected number of deaths; CI, confidence interval.

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Diagnosing *Capnocytophaga canimorsus* Infections

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We reviewed clinical and epidemiologic features of 56 human *Capnocytophaga canimorsus* isolates submitted during a 32-year period to California's Microbial Diseases Laboratory for identification. An increasing number of isolates identified as *C. canimorsus* have been submitted since 1990. Many laboratories still have difficulty correctly identifying this species.

Dogs are the most common household pets in the United States. Estimates predict that 50% of all Americans will be bitten in their lifetime by an animal (*1*) and that ≈1 million dog bites will occur annually (*2*). *Capnocytophaga canimorsus* is the main human pathogen associated with dog bites; this organism causes septicemia, meningitis, endocarditis, and rare ocular infections (*3*,*4*). Persons at increased risk of developing *C. canimorsus* infections include patients who have undergone a splenectomy and those who abuse alcohol. We describe a series of 56 isolates submitted to California's Microbial Diseases Laboratory (MDL) in a 32-year period with laboratory and epidemiologic factors associated with these infections.

The Study

MDL is California's reference laboratory for detecting and identifying bacterial, parasitic, and fungal infections of public health importance. Cultures submitted to MDL come from >500 clinical laboratories through a network of 39 county or city public health laboratories. Isolates for identification are forwarded to MDL by public health laboratories with standardized forms that include information on clinical condition or suspected disease, date of onset, a brief case history, antimicrobial therapy, origin of specimen, and laboratory results. In this manner, clinical information and patient demographics were obtained and analyzed for human cases of *C. canimorsus* infections identified from 1972 to 2004.

Confirmatory testing by our laboratory includes a combination of conventional and molecular techniques involving biochemical tests, fatty acid methyl ester analysis, and 16S rRNA gene sequencing. These procedures have been described in detail elsewhere (*5*). Morphologically, *Capnocytophaga* spp. appear as gram-negative medium-to-long rods with tapered or spindle-shaped ends. The major phenotypic characteristics of *C. canimorsus* include positive test results for oxidase, catalase, arginine dihydrolase, and *o*-nitrophenyl-β-D-galactopyranoside and negative reactions for urease, nitrates, and indole. Fermentation of glucose, lactose, and maltose is often observed but not of raffinose and inulin. Growth is often enhanced by the addition of rabbit serum and incubation in a carbon dioxide–enriched environment.

Sixty *C. canimorsus* isolates were forwarded to MDL for identification or confirmation during the 32-year period (1972–2004), 56 from humans and 4 from animals. The average number of clinical strains submitted per year was 1.75; the highest number was recorded in 1998 (n = 8). The average number of isolates from human cases forwarded to MDL increased from 1990 to 2004, when 2.2–2.5 strains were submitted each year, roughly a 4-fold increase over that observed in the 1970s (Figure).

The characteristics of these 56 patients are listed in the Table. The average age was 57.5 years (range 4 months to 99 years); 70% of patients were >50 years of age. Male patients represented 57% of cases. All strains were recovered from adult patients except for 2 blood isolates recovered from 2 infants. One of these infant cases was previously described (*6*). More than 60% of patients from whom *C. canimorsus* was recovered initially had sepsis, a combination of septicemia and meningitis, or a fever of unknown origin. The most commonly reported symptoms associated with these conditions were fever (85%), diarrhea or abdominal pain (21%), vomiting (18%), headache (18%), confusion (12%), and myalgia or malaise (<10%). Disseminated intravascular coagulation (DIC) or septic shock developed in 7 patients (13%) during hospitalization. For 6 patients (11%), the admitting diagnosis was cellulitis; in each instance, *C. canimorsus* was recovered from the blood but not from wounds. In 55 (98%) of 56 cases, *C. canimorsus* was believed to have caused the clinical syndrome. The singular exception was a 19-year-old woman with acute pharyngitis; both *C. canimorsus* and group A streptococcus were isolated from her oropharynx.

Among patients with known risk factors associated with *C. canimorsus* infection, 3 patients were asplenic; no patients with a history of alcohol abuse were identified, although complete medical information was not always available. Other co-existing conditions in these patients included chronic obstructive pulmonary disease, diabetes mellitus, cirrhosis, Grave's disease, hemosiderosis, Hodgkin lymphoma, and ovarian cancer. For 27 patients, records on animal exposure were available. In 21 (78%), a recent history of a dog bite or close contact with dogs or

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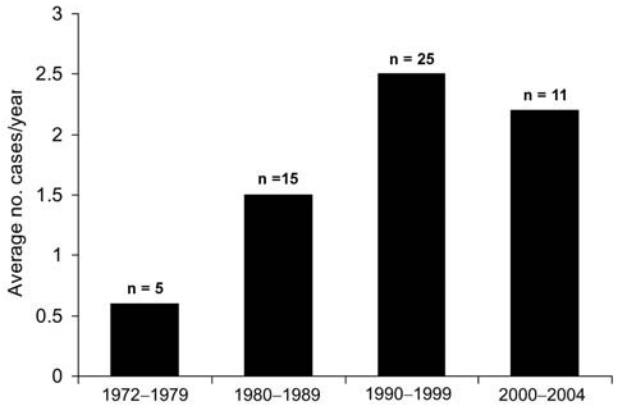

Figure. *Capnocytophaga canimorsus* cases (1972–2004); numbers above bars indicate total human cases during the indicated period.

cats was noted (Table). The median time from a dog bite to onset of symptoms was 3 days (range 1–10 days).

Complete or partial medical records were available for 30 cases in which the outcome of infection was recorded. The case-fatality ratio was 33%. Five of the 6 patients with culture-confirmed meningitis survived. Patient 6, a 56-year-old truck driver with meningitis, overwhelming sepsis, and DIC, died, as did all other persons with *C. canimorsus*–associated DIC.

Only one third (32%) of all isolates forwarded to MDL were submitted with the correct species identification. Many strains were received as either an unidentified gram-negative rod or "identification unknown" (55%). In some instances (≈13%), strains were submitted with incorrect identifications, such as *Streptobacillus* spp., anaerobes, *Legionella* spp., or *Haemophilus* spp. Microbiologists continue to have difficulty correctly identifying this organism. From 1998 to the present, only 5 (28%) of 18 *C. canimorsus* strains were correctly identified to genus and species, a slightly lower percentage from that observed for the entire study period.

During the 32-year period, the techniques and methods used by MDL to identify *C. canimorsus* evolved. Recently, 2 cultures received by MDL were nonviable when isolation techniques were attempted from submitted blood culture bottles (1 submitted as a gram-negative rod, the other as *Streptobacillus* sp.). In both instances, however, *C. canimorsus* was identified as the etiologic agent by polymerase chain reaction (PCR) amplification and 16S rRNA gene sequencing. This sequence-based approach is proving increasingly useful for identifying slow-growing, fastidious bacteria, and it can readily differentiate *C. canimorsus* and the phenotypically similar *C. cynodegmi* (*7*).

Conclusions

Our report describes the single largest series of *C. canimorsus* isolates (N = 56) reported in the medical literature and includes cases of infection from before the species was described (*8*) and before the first case report by Bobo and Newton was published (*9*). *C. canimorsus* isolates have been forwarded to MDL with increasing frequency since 1990 (Figure). The increased frequency associated with *C. canimorsus* may be related to several underlying factors, including more pet (dog, cat) owners, greater opportunities for animal bites (*1*,*2*), and enhanced laboratory techniques to recover this agent from clinical material. However, the accurate identification of this life-threatening pathogen continues to be elusive. The ability of commercial bacterial identification systems to accurately identify these organisms is largely unstudied. Clinical laboratories should consider *C. canimorsus* in patients with bacterial sepsis and a recent history of a dog bite or animal exposure and with the laboratory observation of fastidious, oxidase- and catalase-positive, gram-negative rods with fusiform shape.

The clinical characteristics and demographics of 55 *C. canimorsus*–infected persons closely resembled those described in several other studies or reviews (*3*,*4*,*10*). Most

Table. Clinical data on persons infected with *Capnocytophaga canimorsus*

Characteristic	No. positive (%)
Admitting diagnosis (N = 56)*	
Sepsis	18 (32)
Fever of unknown origin	7 (13)
Meningitis	7(13)
Cellulitis	6 (11)
Septic shock	5 (9)
Respiratory tract infections	4 (7)
Phlebitis	1 (2)
Endocarditis	1 (2)
Urosepsis	1 (2)
Septic knee	1 (2)
Diverticulitis	1 (2)
Meningococcemia	1 (2)
Unknown	3 (5)
Sources of isolates (N = 56)	
Blood	49 (88)
Cerebrospinal fluid	4 (7)
Blood and cerebrospinal fluid	2 (4)
Respiratory tract	1 (2)
Animal exposure (n = 27)*	
Dog bite	17 (63)
Close animal contact	3 (11)
Cat scratch	1 (4)
No known exposure	6 (22)
Outcome (n = 30)*	
Survived	20 (67)
Died	10 (33)

*Number of cases for which medical information was available.

patients were men >50 years of age and had either recently been bitten by a dog or had prolonged contact with dogs. Septic shock and DIC carried a poor prognosis. The observed case-fatality ratio (33%) was comparable to that (30%–31%) found in 2 other surveys (*3*,*4*). Five (83%) of 6 patients with laboratory-confirmed meningitis survived their systemic infections. LeMoal et al. (*11*) recently summarized the literature on case reports of *C. canimorsus* meningitis and found a low death ratio (5%) associated with 19 central nervous system infections; our report supports those observations.

A known risk factor for disseminated *C. canimorsus* infection is asplenia, although this condition could only be demonstrated in 3 (10%) of 31 patients for whom partial or complete medical histories were available. No cases of alcoholism were identified in this series, although several previous series have identified 18%–24% of infected patients with alcoholism as a predisposing factor (*3*,*4*). However, a limitation of the current study was our inability to obtain medical histories on a sizeable number of patients, despite repeated attempts. Lack of such information may considerably bias the data presented.

C. canimorsus is a fastidious organism, often difficult to isolate and identify. Identification of isolates may require an extended incubation period (days), delaying laboratory reports and indirectly affecting therapy options and treatment. Many laboratories were unable to presumptively identify *C. canimorsus* isolates, commonly reporting these strains as either gram-negative rods or fastidious gram-negative bacilli. Reasons for mislabeling may include lack of familiarity with the organism, lack of appropriate biochemical tests, or use of commercial identification systems not designed for identifying fastidious microorganisms. These facts, coupled with the low correct identification rate (32%) provided by laboratories in 3 decades of study, suggest that the frequency of *C. canimorsus* infections in the general population may be underestimated, especially if all such generically identified isolates are not forwarded to reference or public health laboratories for definitive identification. Our most recent 2 cases in this study were eventually identified by 16S rRNA gene sequencing and were inadequately or incorrectly identified as other microorganisms by the original submitting laboratories. These cases would have been missed without a molecular approach. Since the case-fatality ratio associated with this infection has remained unchanged, new approaches need to be developed to provide a more rapid and specific diagnosis of this zoonotic pathogen. Such approaches could include 16S rRNA gene sequencing or PCR assays targeting species-specific genes.

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Novel Dengue Virus Type 1 from Travelers to Yap State, Micronesia

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Dengue virus type 1(DENV-1), which was responsible for the dengue fever outbreak in Yap State, Micronesia, in 2004, was isolated from serum samples of 4 dengue patients in Japan. Genome sequencing demonstrated that this virus belonged to genotype IV and had a 29-nucleotide deletion in the 3′ noncoding region.

Dengue virus (DENV) is a mosquitoborne flaviviruses; there are 4 serotypes, DENV-1, -2, -3 and -4. DENV has been found in ≥100 countries and 2.5 billion people live in areas where dengue is endemic. Fifty to one hundred million cases of dengue infection are estimated to occur annually (*1*). In Japan, outbreaks of dengue fever occurred in Nagasaki, Hiroshima, Kobe, and Osaka from 1942 to 1945, but none thereafter (*2*). However, ≈50 imported dengue cases occur annually in Japan.

The DENV genome is a single-stranded positive-sense RNA of ≈11,000 nucleotides (nt) that encodes 3 structural proteins (capsid, membrane, and envelope) and 7 nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (*3*). Surrounding the open reading frame (ORF) are 5′ and 3′ noncoding regions (NCRs) that form RNA secondary structures (*4*). These regions are ≈100 and ≈400 nt, respectively (*5*).

Dengue fever developed in 9 Japanese patients in 2004 after they returned from Yap state. We report the genetic characterization of RNA from DENV-1 isolates from these patients.

The Study

Yap is the westernmost state of the Federated States of Micronesia and composed of 4 major islands. Yap has a total area of 102 km^2 and a population of 11,241 (2000 census). The climate is moderate and fairly constant. The mean annual temperature is 27°C. Relative humidity ranges from 65% to 100% (annual mean 83%). Rainfall averages 120 inches a year and is seasonal.

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In 1995, a dengue epidemic caused by DENV-4 occurred in Yap state (*6*), but no dengue outbreaks have since been reported. However, the Yap Epinet Team reported a dengue outbreak caused by DENV-1 in Yap state that began in the last week of May 2004. A total of 658 reported dengue fever cases (defined by the World Health Organization) occurred as of December 29, 2004. No deaths or dengue hemorrhagic fever/dengue shock syndrome cases were reported (*7*).

Fever, headache, and diarrhea developed in 7 Japanese adults who visited Yap after their return to Japan in August 2004. DENV infection was serologically confirmed in 5 patients (patients 1–5) by an immunoglobulin M (IgM) capture enzyme-linked immunosorbent assay (ELISA) (Focus Diagnostics Inc., Herndon, VA, USA) and an IgG ELISA (PANBIO Ltd., Brisbane, Queensland, Australia) at the National Institute of Infectious Diseases in Tokyo, Japan. Of these 5 patients, 4 had a primary DENV infection and 1 had a primary dengue infection and a secondary flavivirus infection. DENV infection was serologically confirmed in the sixth patient at another institute. The seventh patient did not visit a medical facility but had symptoms of dengue fever. In addition, 2 other Japanese patients who traveled to Yap in September 2004 were diagnosed with dengue (data not shown).

Four virus isolates (NIID04-27, -31, -41, and -47) were obtained from serum samples from patients 1–4, respectively. Two hundred microliters of serum samples diluted 1:40 was injected onto C6/36 cells in minimal essential medium supplemented with 2% fetal calf serum. The cells were incubated at 28°C for 7 days and culture supernatant fluids were collected. DENV isolates were used for analysis without any further passage.

Complete nucleotide sequencing of RNA of NIID04-27, -31, and -47 and partial sequencing of NIID04-41 were performed. Viral RNA was extracted by using a High Pure RNA extraction kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions, transcribed to cDNA, and amplified by polymerase chain reaction, as described previously (*8*). The cDNA was purified and sequenced by using the ABI PRISM 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Seventeen pairs of primers were designed based on the DENV-1 NIID02-20 sequence (GenBank accession no. AB178040) and used in the analyses (*9*).

The nucleotide sequences of the viral isolates were compared with published complete sequences of DENV-1 (Table 1). Sequence alignment and analysis were performed by using ATGC analysis programs (version 4.02; Genetyx Corp., Tokyo, Japan). Phylogenetic analyses of nucleotide sequences were conducted with ClustalX software version 1.83 (ftp://ftp-igbmc.u-strasbg.fr/pub/ClusterW/). A phylogenetic tree was reconstructed for

Table 1. Dengue virus (DENV) strains used in the study

Virus	Strain	Origin	Year isolated	GenBank accession no.
DENV-1	NIID04-27	Yap Island	2004	AB204803
DENV-1	NIID03-41	Republic of Seychelles	2003	AB195673
DENV-1	FGA/89	French Guiana	1989	AF226687
DENV-1	BR/90	Brazil	1990	AF226685
DENV-1	BR/97-111	Brazil	1997	AF311956
DENV-1	BR/01-MR	Brazil	2001	AF513110
DENV-1	Abidjan	Côte d'Ivoire	1998	AF298807
DENV-1	Mochizuki	Japan	1943	AB074760
DENV-1	S275/90	Singapore	1990	M87512
DENV-1	16007	Thailand	1964	AF180817
DENV-1	GZ/80	China	1980	AF350498
DENV-1	A88	Indonesia	1988	AB074761
DENV-1	Cambodia	Cambodia	1998	AF309641
DENV-1	Djibouti	Ethiopia	1998	AF298808
DENV-1	West Pac 74	Nauru	1974	U88535
DENV-1	98901530	Indonesia	1998	AB189121
DENV-1	98901518	Indonesia	1998	AB189120
DENV-1	259par00	Paraguay	2000	AF514883
DENV-1	295arg00	Argentina	2000	AF514885
DENV-1	ARG9920	Argentina	1999	AY277664
DENV-1	NIID02-20	Thailand	2002	AB178040
DENV-1	99-36-1HuNIID	Paraguay	1999	AB111065
DENV-1	01-27-1HuNIID	The Philippines	2001	–
DENV-1	01-32-1HuNIID	The Philippines	2001	–
DENV-1	01-36-1HuNIID	Singapore, Malaysia	2001	AB111067
DENV-1	01-42-1HuNIID	Thailand, Cambodia	2001	AB111069
DENV-1	01-44-1HuNIID	Tahiti	2001	AB111070
DENV-1	01-54-1HuNIID	India	2001	–
DENV-1	01-54b-1HuNIID	India	2001	–
DENV-1	01-61-1HuNIID	Cambodia	2001	AB111071
DENV-1	01-65-1HuNIID	Thailand	2001	AB111072
DENV-1	01-66-1HuNIID	Thailand	2001	–
DENV-2	DENtype2-TB16i	Indonesia	2004	AY858036
DENV-3	DENtype3-TB55i	Indonesia	2004	AY858048
DENV-4	DENtype4-8976/95	Indonesia	2004	AY762085

aligned nucleotide sequences by using the neighbor-joining method. Bootstrap reassembling analysis of 1,000 replicates was used to assess confidence values for virus groupings. The phylogenetic tree was constructed by using Treeview software version 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.htm).

The full-length RNA genomes of NIID04-27, -31, and -47 were 10,706 nt. A previous study reported that the full-length RNA genome of DENV-1 was 10,735 nt (*8*). The differences in the genome sequence between NIID04-27 and the other 2 isolates (NIID04-31 and -47) were subtle; identities with NIID04-31 and -47 were 99.94% and 99.92%, respectively. The results suggest that these 3 isolates belong to the same strain. Therefore, we used NIID04-27 as a representative isolate for further analysis.

To characterize the molecular structure of the genome, the complete NIID04-27 nucleotide sequence was compared with those of other DENV-1 strains available in GenBank (Table 1). NIID04-27 shared sequence identity ranging from 90.9% to 96.9% (Table 2) with 12 other DENV-1 strains. With respect to the alignment of full-length genomes, some alterations were found in the 3′ NCR. These alterations included a deletion of 29 nt starting at the 13th position from the ORF termination codon (Figure 1). The same deletion in the 3′ NCR was found in the viral genome amplified directly from the serum sample from patient 1 and was also observed in NIID04-31, -41, and -47.

To further analyze the genetic variation in the 3′ NCR of DENV-1, we analyzed the sequence of 24 other DENV-1 strains. Only the NIID03-41 strain, which was isolated in our laboratory from a patient returning from the Republic of Seychelles, had a 17-nt deletion in the 3′ NCR (Figure 1). The complete genomes of the 25 DENV-1 strains analyzed showed high levels of nucleotide sequence identity in the 3′ NCR, except for a small region of 50 nt immediately after the ORF, which is the hypervariable region. The nucleotide sequence identities in the 3′ NCR between NIID04-27 and 12 other DENV-1 strains ranged from 89.3% to 92.5% (Table 2).

Table 2. Pairwise comparisons of full-length genome and 3′ noncoding region sequences of dengue virus type 1 (DENV-1) strains*

Virus strain	% identify of nucleotide												
	NIID 04-27	FGA/89	BR/90	BR/97 -111	Abidjan	Mochizuki	S275/90	16007	GZ/80	A88	Cambodia	Djibouti	West Pac74
	Full-length genome												
NIID04-27		**91.5**	**91.5**	**91.3**	**90.9**	**93.4**	**91.9**	**93.1**	**92.3**	**96.9**	**91.7**	**91.7**	**95.6**
FGA/89	**89.5**		98.3	97.9	94.5	93.7	93.4	93.5	92.5	92.3	92.0	92.1	93.3
BR/90	**89.5**	99.4		98.8	94.5	93.9	93.7	93.7	92.7	92.3	92.2	92.0	92.9
BR/97-111	**89.3**	98.7	99.4		94.4	93.7	93.5	93.5	92.5	92.2	92.1	91.8	92.8
Abidjan	**89.7**	94.0	93.8	94.2		92.9	92.8	92.9	92.1	92.0	91.7	91.6	92.3
Mochizuki	**91.2**	94.8	94.7	94.9	93.4		95.0	95.4	96.1	94.6	95.3	95.2	95.1
S275/90	**91.2**	91.7	91.5	91.9	96.6	94.4		93.8	96.1	93.0	96.4	95.2	93.7
16007	**90.8**	96.2	95.9	95.7	93.2	97.4	93.4		94.2	94.2	93.7	93.5	94.7
GZ/80	**90.2**	94.1	93.8	94.4	93.6	97.4	94.4	95.7		93.3	96.7	98.0	93.9
A88	**92.5**	93.8	93.6	93.8	92.3	96.8	92.9	96.8	95.5		92.7	92.7	97.2
Cambodia	**89.9**	92.1	92.1	92.1	96.4	94.4	96.9	93.6	95.1	92.8		95.9	93.4
Djibouti	**89.5**	92.1	91.9	91.9	95.3	95.3	96.9	94.0	95.5	93.2	97.6		93.3
WestPac74	**91.4**	95.1	94.9	95.1	93.8	93.8	93.2	97.2	95.1	97.2	93.0	93.4	
	3′ noncoding region												

*The percentage nucleotide sequence identities of the complete genomes are shown in the upper right half of the table. The percentage nucleotide sequence identities of the 3′ noncoding region of the genomes are shown in the lower left half of the table. The percentage sequence homologies between NIID04-27 and each of 12 other DENV-1 strains are shown in **boldface**.

To understand the genetic relationships and evolution of DENV-1 strains, we also performed phylogenetic analysis of the fully sequenced DENV-1 strains that included NIID04-27 (Figure 2). NIID 04-27 belonged to genotype IV along with A88, 98901518, 98901530, NIID03-41 and West Pac74 .This cluster was called the Pacific group in a previous report (*10*). NIID04-27 and NIID03-41 are the first DENV-1 strains to have deletions in 3′ NCR.

Conclusions

We have genetically characterized DENV-1 isolate NIID04-27 by determining its complete nucleotide sequence and comparing the sequence with most of the available DENV-1 full-length sequences. Sequence heterogeneity in the 3′ NCR of the genus *Flavivirus* has been reported for tickborne encephalitis virus, Japanese encephalitis virus, DENV-2, and DENV-4 (*11–13*). For example, DENV-2 isolated in Texas, Peru, Venezuela, Mexico, and Puerto Rico had a 10-nt deletion starting at the 19th nucleotide position from the ORF termination codon (*13*).

The terminus of the 3′ NCR has a conserved sequence and secondary structure. The functions of the 3′ NCR of flaviviruses have not been fully determined. The 3′ NCR in flaviviruses affects RNA replication but does not affect viral translation (*14*,*15*). Introduction of a 30-nt deletion starting at the 212th position from the ORF termination codon in the 3′ NCR of DENV-4 reduced the ability of the virus to propagate in vivo and in vitro (*16*).

We have identified a 29-nt deletion in the 3′ NCR of DENV-1 isolated from a dengue patient returning to Japan from Yap. Isolates from 3 other patients infected in the same outbreak also had the same deletion. The DENV-1 strain with a 29-nt deletion in the 3′ NCR was responsible for the dengue epidemic in Yap in 2004. The biologic characteristics induced by this deletion should be further analyzed.

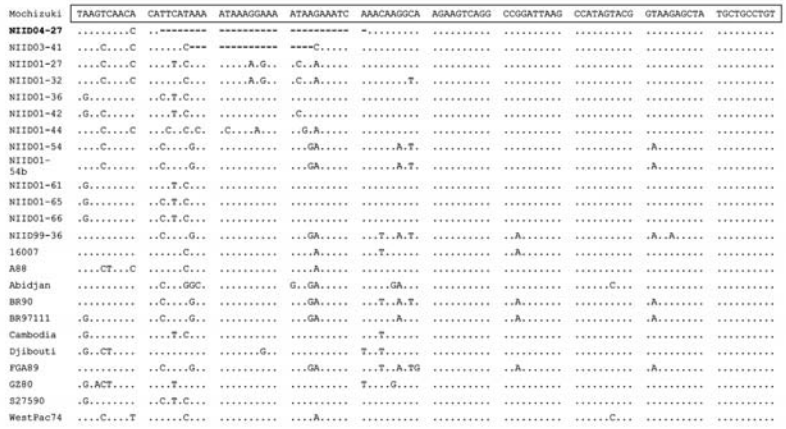

Figure 1. Nucleotide sequence alignment of the variable region in the 3′ noncoding region of dengue virus type 1 strains, including NIID04-27 sequenced in the present study. The Mochizuki strain was used as the consensus sequence, and the sequence of 100 nucleotides immediately downstream of the open reading frame termination codon is shown at the top. Solid dots indicate nucleotides identical to the consensus sequence and hyphens indicate deletions.

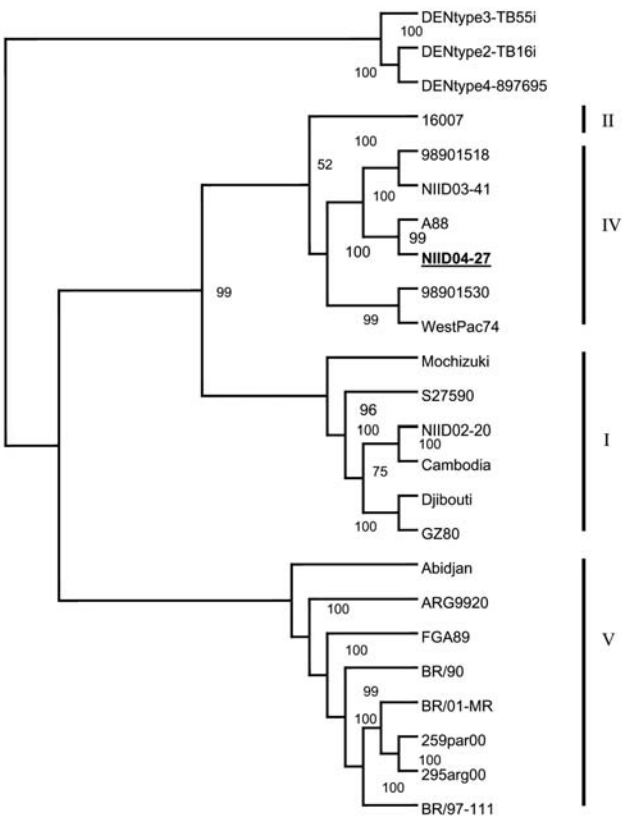

Figure 2. Phylogenetic tree based on the full-length genome sequence of 21 available dengue virus (DENV) type 1 strains and DENV-2, -3, and -4. The multiple sequence alignments were obtained with ClustalX, and the tree was constructed by the neighbor-joining method. The percentage of successful bootstrap replicates is indicated at the nodes. The NIID04-27 strain is indicated in boldface. Genotypes I, II, IV, and V correspond to DENV-1 genotypes as defined by Goncalvez et al. (*10*).

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Human Metapneumovirus, Peru

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We retrospectively studied 420 pharyngeal swab specimens collected from Peruvian and Argentinean patients with influenzalike illness in 2002 and 2003 for evidence of human metapneumovirus (HMPV). Twelve specimens (2.3%) were positive by multiple assays. Six specimens yielded HMPV isolates. Four of the 6 isolates were of the uncommon B1 genotype.

Human metapneumovirus (HMPV) has been detected in patients with acute respiratory infection in North America, South America, Europe, Asia, the Middle East, Africa, and Oceania (*1–7*). Capitalizing on a preexisting US Department of Defense influenza surveillance system (*8*), we sought to detect and genotype HMPV in Latin American patients in whom influenzalike illness developed.

The Study

Research was conducted on culture specimens collected from patients with influenzalike illness in Argentina and Peru under a US Department of Defense Global Emerging Infections System (GEIS) influenza surveillance program. Influenzalike illness is defined as fever (temperature >38°C) and cough or sore throat for <72 h. Under the GEIS influenza surveillance system (*8*), US and international sites collect posterior pharyngeal swabs for virus culture from patients who meet the influenzalike illness case definition.

Specimens were labeled with a unique specimen number and stored in cryovial boxes at –70°C until thawed for reverse transcription–polymerase chain reaction (RT-PCR) study. The specimens were linked by a unique laboratory number to an electronic database with patient's sex, age, collection date, city, and state.

After thawing to room temperature, the 420 swab specimens were screened with a 1-step RT-PCR procedure, with the F2 primer set. Briefly, RNA from each respiratory specimen was extracted with the QIAamp Viral RNA MiniKit (Qiagen, Valencia, CA, USA). The 1-step RT-PCR specimen screen was performed in a 100-μL reaction mix containing 11 μL RNA, 0.4 μmol/L forward primer, 0.2 μmol/L reverse primer, 0.163 mmol/L deoxynucleoside triphosphates, 100 U Moloney murine leukemia virus–reverse transcriptase, 10 U RNAse inhibitor, and 2.5 U DNA polymerase in 1× DNA polymerase buffer (*PfuTurbo*, Stratagene, La Jolla, CA, USA). Amplification conditions consisted of 1 h at 42°C; 5 min at 94°C; 34 cycles of 30 s at 94°C, 30 s at 52°C, and 1 min at 72°C; and a final extension at 72°C for 10 min. PCR products were analyzed by electrophoresis (BioRad, Hercules, CA, USA) in a 1.2% (wt/vol) agarose gel stained with ethidium bromide.

Screened specimens that gave bands within 200 bp of the expected 347-bp product were further tested with a 2-step RT-PCR with F1-, F2-, and N-gene primer sets. The 2-step RT-PCR was performed by using the RETROscript Kit (Ambion, Austin, TX, USA) with heat denaturation of RNA. PCR products were analyzed by gel electrophoresis. Specimens were designated RT-PCR–positive if the confirmatory N-gene primer set and at least 1 of the confirmatory F-gene primer sets yielded a band within 50 bp of the expected size (primers available from the corresponding author) (*9*).

Both 1- and 2-step RT-PCR procedures were adapted from previous reports (*9–12*). With every specimen batch, a known HMPV-positive and HMPV-negative sample was tested in parallel to validate the run.

RT-PCR–positive specimens were further studied with shell-vial cell culture for viable HMPV. A shell vial containing a near confluent monolayer of LLC-MK2 cells (Diagnostic Hybrids, Inc., Athens, OH, USA) was injected with 100 μL specimen and 900 μL HMPV growth media (1× minimum essential medium with L-glutamine and Earle salts, 0.1% bovine albumin, 1× HEPES, 0.001% porcine pancreatic trypsin, 0.4505 mol/L D-glucose, 10,000 U penicillin, 10 mg streptomycin, and 50 μg amphotericin), centrifuged for 1 h at 37°C and 2,800 rpm (1,500 × *g*), followed by a 37°C incubation with 5% CO_2. The cell monolayers were microscopically examined weekly for cytopathic effect (CPE) and contamination. Shell vials were incubated 3–4 weeks or until cell disruption occurred. Infected cell supernatant media were harvested each week upon cell media replacement. From an aliquot of the infected media, RNA was extracted and subsequent RT-PCR was performed by the HMPV F2-gene 1-step protocol.

Sequencing was performed on the RT-PCR–positive specimens by using G_{univ} primer set (available from the corresponding author), adapted to amplify an 800- to

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1,000-bp region. Products were subsequently electrophoresed across a 1.0% agarose gel stained with ethidium bromide. RT-PCR–positive products were purified with QIAquick PCR Purification/Gel Extraction Kits (Qiagen). Strands of the amplicons were sequenced by automated sequencing with the G_{univ} primers. Big Dye Terminator Kit v3.1 (Applied BioSystems, Foster City, CA, USA) was used in sequencing reactions. Samples were run on a 3730xl DNA Analyzer (Applied BioSystems).

Alignments of partial amino acid sequences of the HMPV G protein were generated with the ClustalW software (National Center for Biotechnology Information, Bethesda, MD, USA). Prototypic sequences of different types (A and B) and subtypes (A1, A2, B1, and B2) from the Netherlands and Canada were included in the alignments. Phylogenetic analysis was performed by the neighbor-joining method by using MEGA 2 (University of Pittsburgh, Pittsburgh, PA, USA).

Specimen laboratory results were studied for demographic and temporal predictors of RT-PCR positivity by using standard categoric data techniques. Age group cut points were selected based on age quartiles. Exact binomial 95% confidence intervals (CIs) were calculated around prevalence statistics. Similarly, 95% CIs around odds ratios were calculated by using logistic regression. Analyses were performed by using SAS software version 9.1 (SAS Institute, Inc., Cary, NC, USA).

Conclusions

We studied 420 posterior pharyngeal swab specimens collected from January 2002 to November 2003 (Table 1). Because of differences in clinic focus, the distribution of influenzalike illness differed by site; children made up higher proportions in each country (median age 11 years, range <1–89 years, Table 2). Overall, 51% of the 302 specimens for which patient's sex was known were from male patients. Most influenzalike illness specimens were obtained during the coldest months (July through September, data not shown).

Twelve (2.9%) of 420 specimens were considered HMPV RT-PCR–positive (Table 2). All 12 positive specimens were cultured on LLC-MK2 cells. Six of the 12 specimens grew HMPV, and none of them showed evidence of viral CPE before 7 days. The nonviability of the 6 remaining positive specimens was likely due to the 4 freeze-thaw cycles that occurred before LLC-MK2 cell culturing or possibly the degradation of HMPV RNA within the specimens during transport and storage.

All 6 of the specimens that yielded an HMPV isolate in cell culture were successfully sequenced and were used to develop a phylogenetic tree (Figure) (*13*). Sequencing was not attempted until ≈2 years after specimen collection. This delay in sequencing and multiple freeze-thaw cycles may explain our inability to amplify and sequence G-gene product from the other 6 positive specimens. Sequence data were compared to previously sequenced HMPV isolates, showing a high prevalence of genotype B, with 4 isolates (Peru2-2002, Peru3-2003, Peru4-2003, and Peru5-2003) of the B1 subtype and 2 isolates (Peru1-2002 and Peru6-2003) of the B2 subtype. The high prevalence of genotype B isolates could be due to our methodologic approach and requires validation through other studies of similar Peruvian specimens.

Our data suggest that HMPV is circulating in Peru. Consistent with results of other studies, the prevalence of

Table 1. Prevalence and OR of RT-PCR positivity for HMPV by risk factor*

Risk factor	n	% RT-PCR–positive (95% CI)	OR (95% CI)
Age group (y)†			0.9 (0.8–0.99)
<7	151	6 (2.8–11)	7.2 (1–319.4)
7–20	152	1.3 (0.2–4.7)	1.5 (0.1–90.5)
>20	115	0.9 (0–4.8)	Reference
Unknown	2		
Sex			
Male	154	4.6 (1.9–9.1)	2.3 (0.5–14)
Female	148	2 (0.4–5.8)	Reference
Unknown	118		
Site			
Peru	388	2.8 (1.4–5)	Reference
Argentina	32	3.1 (0.1–16.2)	1.1 (0–8.1)
Season			
Autumn‡	106	6.6 (2.7–13.1)	4.3 (1.3–13.8)
Others	307	1.6 (0.5–3.8)	Reference
Unknown	7		

*OR, odds ratio, RT-PCR, reverse transcription–polymerase chain reaction; HMPV, human metapneumovirus; CI, confidence interval.
†Age as a continuous variable.
‡Autumn in the Southern Hemisphere was considered to be from March 22 to June 21, per Centro de Divulgacao Cientifica e Cultural, São Paulo University, Brazil.

Table 2. Human metapneumovirus (HMPV)–positive samples by F- and N-gene primers

Case	Date collected	City, Country	Age (y)	F2 1-step (347 ± 200 bp)	F2 2-step (137 ± 50 bp)	N 2-step (212 ± 50 bp)	LLC-MK2 culture result
SA1131	6/02	Chanchamayo, Peru	9	+	+	+	No growth*
SA1066	6/02	Cuzco, Peru	2	+	+	+	No growth
SA1071	6/02	Cuzco, Peru	4	+	+	+	HMPV Peru2-2002
SA1226	10/02	Buenos Aires, Argentina	3	+	+	+	No growth†
SA1156	10/02	Cuzco, Peru	4	+	+	+	HMPV Peru1-2002
SA1385	4/03	Iquitos, Peru	5	+	+	+	No growth
SA3156	6/03	Iquitos, Peru	7	+	+	+	HMPV Peru3-2003
SA3157	6/03	Iquitos, Peru	4	+	+	+	HMPV Peru4-2003
SA3158	6/03	Iquitos, Peru	3	+	+	+	HMPV Peru5-2003
SA1532	8/03	Cuzco, Peru	0.75	+	+	+	No growth
SA1568	9/03	Cuzco, Peru	38	+	+	+	No growth
SA1606	10/03	Cuzco, Peru	0.75	+	+	+	HMPV Peru6-2003

*Bacteria contamination; specimen required filtering.
†Mold contamination; specimen required filtering.

HMPV infection in this research was low among patients with influenzalike illness and more common among younger children (6% in children <7 years of age, Table 1) (*1*). In our study, HMPV was more often detected in male patients and from April to June.

Of the 12 HMPV RT-PCR–positive patients, 9 had clinical reports available for review. Three children from a small Peruvian Amazon village whose specimens were collected within 3 days of each other were infected with HMPV from the B1 subtype. Among these 3 children, the youngest (3 and 4 years of age) were the most debilitated and had the highest maximum oral temperature (39.8°C and 39.6°C). Among the remaining 6 HMPV-positive patients, 1 had pneumonia and 1 was hospitalized. These data show a higher likelihood (odds ratio 4.3, 95% CI 1.3–13.8) of detecting HMPV from patients with influenzalike illness during the Southern Hemisphere's autumn (March to June) (Table 1).

HMPV genotypes B1 and B2 were detected (Figure). Four of the 6 isolates belonged to genotype B1, which had been uncommon in Europe, Canada, and South Africa (*7,13,15*). These results represent some of first genotype data from HMPV isolates collected in Peru.

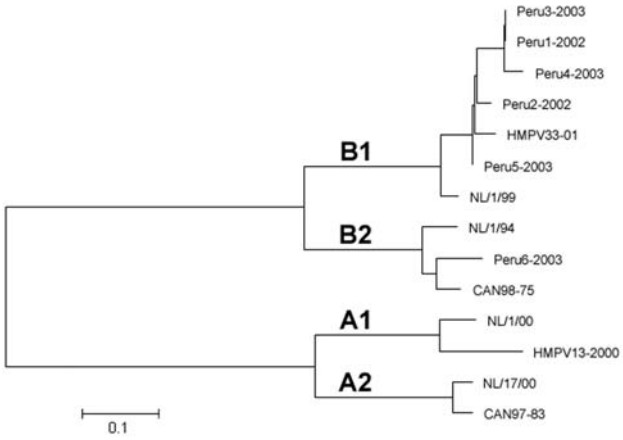

Figure. G-gene phylogenetic relationships of 6 human metapneumovirus (HMPV) isolates detected in South America during 2002 and 2003 compared to prototypic HMPV isolates from the Netherlands: NL/1/00, NL/17/00, NL/1/99, NL/1/94 (accession nos. AF371337, AY296021, AY525843, and AY296040, respectively) and from Canada: Can97-83, HMPV-13-00, CAN98-75, and HMPV-33-01 (accession nos. AY485253, AY485232, AY485245, and AY485242, respectively). Classification of genotypes was made according to previous reports (*13,14*).

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Fluoroquinolone-resistant *Salmonella* sp. in Carcasses

To the Editor: Fluoroquinolone (FQ)-resistant *Salmonella* has been isolated from patients in Taiwan (*1–7*). Recently, a report further indicated that several patients were infected with *Salmonella enterica* serovar Schwarzengrund with high-level FQ resistance (*1*). *S.* Schwarzengrund has never been isolated from food animals in Taiwan.

We report the isolation of FQ-resistant strains from pork and broiler carcasses sampled from 2000 to 2003: 27 in 2000, 3 in 2001, 4 in 2002, and 2 in 2003. These isolates made up 18.85% of the 191 *Salmonella* strains obtained from pork and broiler carcasses in the study period. Of these isolates, 16 FQ-resistant *S.* Schwarzengrund strains were further analyzed to elucidate the possible mechanism of FQ resistance. Ciprofloxacin MIC levels in these isolates ranged from 4 to 16 μg/mL, and all had high-level nalidixic acid resistance (≥1,024 μg/mL). All of the 16 investigated strains displayed mutations possibly associated with high-level FQ resistance. The mutation sites included 2 sites (Ser83Phe and Asp87Gly) in the quinolone resistance–determining region (QRDR) of *gyrA*, 2 sites (Thr57Ser and Ser80Arg) in the QRDR of *parC*, and 1 site (Ser458Pro) in the QRDR of *parE*, respectively. Four strains had mutations in the QRDR of *gyrA* and *parC* only but not in the QRDR of *parE* (Table).

In conclusion, high-level FQ resistance was detected in *S.* Schwarzengrund isolated from pork and chicken in Taiwan. Specific mutation sites of *gyrA*, *parC*, and *parE* were associated with high-level FQ resistance in all the isolates investigated. Our results warrant further investigation of the public health consequences of FQ use in food animals in Taiwan.

Table. Characteristics of ciprofloxacin-resistant *Salmonella enterica* serovar Schwarzengrund strains from carcasses*†

Strain no.	Origin*	Year isolated	Antimicrobial drug resistance profile	Quinolone MICs (μg/mL)				Substitutions in QRDR‡		
				NAL	FLU	ENR	CIP	*gyrA*	*parC*	*parE*
A5	B, M	2000	CmSxtTc	1,024	512	32	8	Ser83Phe; Asp87Gly	Thr57Ser; Ser80Arg	Ser458Pro
A16	P, E	2000	ApCmNSxtTc	2,048	512	32	8	Ser83Phe; Asp87Gly	Thr57Ser; Ser80Arg	Ser458Pro
A17	P, E	2000	ApCmNSxtTc	2,048	512	32	16	Ser83Phe; Asp87Gly	Thr57Ser; Ser80Arg	Ser458Pro
A18	P, E	2000	ApCmNSxtTc	2,048	512	32	16	Ser83Phe; Asp87Gly	Thr57Ser; Ser80Arg	Ser458Pro
A19	P, E	2000	ApCmCnNSxtTc	1,024	512	32	8	Ser83Phe; Asp87Gly	Thr57Ser; Ser80Arg	Ser458Pro
A20	P, E	2000	ApCmNSxtTc	2,048	512	32	8	Ser83Phe; Asp87Gly	Thr57Ser; Ser80Arg	Ser458Pro
A29	B, S	2000	CmNSxtTc	1,024	512	32	8	Ser83Phe; Asp87Gly	Thr57Ser; Ser80Arg	Ser458Pro
A36	B, S	2000	ApCmSxtTc	1,024	512	32	8	Ser83Phe; Asp87Gly	Thr57Ser; Ser80Arg	Ser458Pro
A41	P, S	2000	ApCmCnNSxtTc	1,024	512	32	16	Ser83Phe; Asp87Gly	Thr57Ser; Ser80Arg	Ser458Pro
A45	P, S	2000	ApCmNSxtTc	1,024	512	32	16	Ser83Phe; Asp87Gly	Thr57Ser; Ser80Arg	
A51	P, S	2000	ApCmCnNSxtTc	1,024	512	16	4	Ser83Phe; Asp87Gly	Thr57Ser; Ser80Arg	
A56	B, M	2000	ApCmCnNSxtTc	2,048	512	64	16	Ser83Phe; Asp87Gly	Thr57Ser; Ser80Arg	
A61	P, S	2000	CmSxtTc	1,024	512	32	8	Ser83Phe; Asp87Gly	Thr57Ser;Ser 80Arg	Ser458Pro
A62	P, S	2000	ApCmCnSxtTc	2,048	512	64	16	Ser83Phe; Asp87Gly	Thr57Ser; Ser80Arg	
B16	P, E	2001	ApCmCnCroTc	2,048	512	32	16	Ser83Phe; Asp87Gly	Thr57Ser; Ser80Arg	Ser458Pro
B73	P, N	2003	ApCmCnNSxtTc	2,048	512	32	16	Ser83Phe; Asp87Gly	Thr57Ser; Ser80Arg	Ser458Pro

*QRDR, quinolone resistance–determining region; B, broiler; M, middle Taiwan; P, pork; E, east Taiwan; S, south Taiwan; N, north Taiwan.
†Antimicrobial agents are ampicillin (Ap), chloramphenicol (Cm), ciprofloxacin (CIP), enrofloxacin (ENR), flumequine (FLU), gentamicin (Cn), ceftriaxone (Cro), nalidixic acid (NAL),neomycin (N), trimethoprim/sulfamethoxazole (Sxt), and tetracycline (Tc).
‡No *gyrB* substitutions were detected.

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Cocirculation of Dengue Serotypes, Delhi, India, 2003

To the Editor: Delhi, in the northern part of India, has had outbreaks of dengue caused by various dengue virus types in 1967, 1970, 1982, 1988, and 1996 (*1*–*5*). In 1988, for the first time, a few cases of dengue hemorrhagic fever (DHF) were seen (*4*). Subsequently, we reported the largest outbreak of DHF/dengue shock syndrome (DSS) in Delhi in 1996 and confirmed dengue virus type 2 as the etiologic agent (*5*).

We report the results of virologic testing of samples received at the All India Institute of Medical Sciences from patients with suspected dengue fever or denguelike illness from Delhi and its adjoining areas during a 2003 outbreak of dengue. According to the World Health Organization (*6*), 2,185 laboratory-confirmed cases were reported during this outbreak.

Of the blood samples received by the virology laboratory, 42 were received on ice from patients with acute denguelike illness. Serum was separated aseptically and stored at –70°C. The standard method of virus cultivation, which used the C6/36 clone of the *Aedes albopictus* cell line, was followed with some modifications (*7*). On days 5 and 10, harvested cells were tested by an indirect immunofluorescence assay (IFA) using monoclonal antibodies to dengue virus types 1–4 (provided by the Centers for Disease Control and Prevention, Atlanta, Georgia, USA, during the 1996 outbreak). If IFA results were negative for dengue viruses on first passage, a second passage was made, and cells were again harvested on days 5 and 10 for IFA. The 4 dengue virus types (obtained from the National Institute of Virology, Pune, India) were included as positive controls, and uninfected C6/36 cells were kept as negative controls.

Dengue virus could be isolated in C6/36 cells from 8 (19%) of 42 samples processed for virus isolation (Table). Of the 8 isolates, two each were identified as dengue virus types 1 and 2, three as type 3, and one as type 4. All but one isolate were from patients with uncomplicated dengue fever. One dengue type 2 isolate was obtained from a 7-year-old boy with secondary dengue infection and DHF/DSS. The ages of culture-positive patients ranged from 5 to 62 years, with a median of 22 years. These patients were equally distributed between children (<12 years) and adults. The male-to-female ratio for these 8 patients was 5:3. The duration of fever at the time of viral isolation was 1–5 days, with a median of 3 days.

All previous outbreaks in Delhi have occurred during the monsoon (rainy) season between August and November and subsided with the onset of winter. We recently reported the results of serologic testing during the 2003 outbreak, which also occurred from September to November, with a peak in mid-October 2003 (*8*). This outbreak was

Table. Culture-positive dengue patients*

Age (y)/sex	Dengue type isolated	Secondary infection (anti–dengue IgG antibodies + by ELISA)	Duration of fever (d)
9/M	DENV-1	Yes	4
25/F	DENV-3	No	4
7/M	DENV-2	Yes	5
7/F	DENV-4	No	1
40/F	DENV-1	Yes	3
62/M	DENV-2	Yes	3
39/M	DENV-3	Yes	2
5/M	DENV-3	No	3

*ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G.

milder than the 1996 outbreak, with less illness and death; most patients had uncomplicated dengue fever, and only a few had DHF/DSS. Of the 874 serum samples that we tested, 456 (52.3%) were positive for dengue-specific immunoglobulin M antibodies by enzyme-linked immunosorbent assay (Panbio, Sinnamon Park, Queensland, Australia), and more than one third of these were from patients in the 21- to 30-year age group (*8*).

Dengue virus types 1, 2, and 3 have all been isolated during previous dengue outbreaks in Delhi, but a particular type has always predominated. During the 1996 outbreak of DHF/DSS, we had 26 isolates of dengue virus type 2, but only 1 isolate was identified as dengue type 1 (*5*). However, we subsequently showed that dengue virus type 1 continued to circulate during the postepidemic period and became the predominant strain (*9*). Dengue virus type 3 has recently reemerged in South Asia, including north India (*10*). We now report this culture-confirmed outbreak of dengue from Delhi, during which the simultaneous transmission of all 4 dengue virus types has been demonstrated for the first time in India, with no particular type predominating. This finding suggests that dengue is now truly endemic in this region.

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Borrelia burgdorferi and *Anaplasma phagocytophilum* Coinfection

To the Editor: In central Europe, *Anaplasma phagocytophilum* and *Borrelia burgdorferi* are transmitted by the hard tick *Ixodes ricinus* (*1*). Acute human granulocytic ehrlichiosis (HGE) caused by *A. phagocytophilum* has rarely been documented in Europe (*2*). Typical symptoms include fever, headache, myalgia, leukopenia, thrombocytopenia, and abnormal liver function test results. The serologic prevalence ranges from 1.9% to 14% in Germany (*1*), while clinically apparent infections of HGE have not been reported.

Acute Lyme borreliosis in Europe is associated with erythema migrans (*3*), recognized in up to 90% of patients (*4*). Erythema migrans may be accompanied by systemic symptoms such as fever, fatigue, myalgia, arthralgia, headache, or stiff neck (*3,4*). In southern Germany, an incidence of 111 per 100,000 inhabitants has been reported (*4*).

A 60-year-old woman from northern Germany was admitted with temperature of ≤40°C, headache, myalgia, and generalized weakness that had begun 6 days earlier. She had noticed an erythema migrans on her right thigh 4 days before she sought treatment. At admission, a tender, 5 × 8 cm rash and a central papule were seen, but without central clearing. The clinical examination was otherwise normal. Three weeks earlier she had been on a trekking tour in Austria and Slovenia but had not been aware of any tick bites.

The leukocyte count was 3,030/μL (normal 4,000–9,000), with 65% neutrophils, 24% lymphocytes, 10% monocytes, and 1% lymphoid cells. The following results were observed: platelets 127,000/μL (normal 150,000–450,000), aspartate aminotransferase

108 U/L (normal <31), alanine aminotransferase 154 U/L (normal <34), gamma-glutamyl transferase 98 U/L (normal <38), lactate dehydrogenase 317 U/L (normal <247), alkaline phosphatase 314 U/L (normal <237), direct bilirubin 4.7 µmol/L (normal <3.4), C-reactive protein 132 mg/L (normal <5), and neopterin 30 nmol/L (normal <10). All other routine laboratory parameters were normal.

May-Grünwald-Giemsa (Fluke, Neu Ulm, Germany)–stained whole-blood smears did not show *Anaplasma* initially and during follow-up. On admission serum antibody tests were negative for *A. phagocytophilum*, *B. burgdorferi*, hepatitis A, B, and C, human herpes virus 6, herpes simplex virus 1 and 2, Epstein-Barr virus, cytomegalovirus, and tick-borne encephalitis virus. Because Lyme borreliosis and possible HGE were suspected, the patient was treated with oral doxycycline 200 mg once daily for 3 weeks. Within 4 days after initiation of treatment, the patient recovered completely; thrombocytes and leukocytes had normalized. Liver enzyme levels were still elevated but had normalized at a follow-up examination 28 days later.

Four days after the initial examination, results for *Borrelia*-specific immunoglobulin M (IgM) antibodies were positive, while results for IgG antibodies remained negative (Table). Four weeks after the onset of symptoms, a test for *A. phagocytophilum*–specific IgM antibodies was positive and IgG was negative thereafter (Table). An initial EDTA blood sample that was stored frozen and examined retrospectively as well as follow-up EDTA blood samples were negative for *A. phagocytophilum* in a polymerase chain reaction (PCR) assay.

One year after initial examination, results for *Borrelia*-specific IgM antibodies were positive and results for *A. phagocytophilum*-specific antibodies were negative (Table). Although HGE has not been reported in Germany, a coinfection with *B. burgdorferi* and *A. phagocytophilum* should be considered in patients with erythema migrans and atypical changes for Lyme borreliosis such as fever, leukopenia, thrombocytopenia, and elevated liver function test results.

The patient had traveled to an area where both tickborne pathogens, *A. phagocytophilum* and *B. burgdorferi*, were endemic. Erythema migrans and antibody follow up suggested Lyme borreliosis. High fever, leukopenia, thrombocytopenia, and elevated liver enzyme levels indicated HGE. *Anaplasma* PCR was negative, possibly because blood samples were tested retrospectively after 3 months of storage at –20°C. However, a commercially available indirect fluorescent antibody assay was able to demonstrate seroconversion of HGE-specific IgM antibodies 1 month after the initial onset of symptoms. According to manufacturer's information, specificity ranged from 97.5% to 100%; sensitivity was 71.4% at 60 days after *A. phagocytophilum* infection. *A. phagocytophilum* IgG antibodies were not detected during follow-up, likely because of prompt treatment with doxycycline.

Wormser et al. (*5*) suggested that *Borrelia*-specific antibodies might indicate false-positive results in patients with HGE infection. Our case, however, meets criteria of a newly acquired infection with *B. burgdorferi* sensu lato, with an erythema migrans and seroconversion of *Borrelia*-specific IgM antibodies.

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Table. Results of serologic tests at diagnosis and during follow-up*

Time (d) after onset of symptoms	*Anaplasma (Ehrlichia) phagocytophilum* (IFA)†		*Borrelia burgdorferi* (ELISA)‡		*Borrelia burgdorferi* (Immunoblot)§	
	IgM	IgG	IgM	IgG	IgM	IgG
6	Negative (<1:20)	Negative (<1:32)	Negative	Negative	Negative	Negative
10	ND	ND	Positive	Negative	ND	ND
28	Positive (1:40)	Negative (1:32)	Positive	Negative	Positive	Negative
107	Positive (1:20)	Negative (<1:32)	Positive	Negative	Positive	Negative
380	Negative (<1:20)	Negative (<1:32)	Equivocal	Negative	Positive	Negative

*Symptoms (fever, headache, myalgia) started 6 days before presentation. IFA, immunofluorescent assay; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin; ND, not done.
†Genzyme Virotech, Germany. Positive titers: IgM ≥1:20, IgG ≥1:64.
‡Behring, Germany.
§In-house Immunoblot, Max von Pettenkofer-Institut, Munich, Germany.

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Corynebacterium pseudogenitalium Urinary Tract Infection

To the Editor: A 64-year-old man was admitted to the urology department of Cochin Hospital in Paris, France, for acute urinary retention. He had a history of recurrent urolithiasis and undocumented urinary tract symptoms. At admission, a urethral catheter was inserted, and a plain radiograph showed 2 bladder stones and milk of calcium calcifications. Three days later, he underwent extracorporeal shock wave lithotripsy treatment, and empiric antimicrobial drug therapy with intravenous ceftriaxone, 1 g/day for 3 days, was administered. Three consecutive urinalyses showed a pH of 9, crystals of struvite, hematuria, and 10^5 leukocytes/mL. Gram-positive rods with rounded ends and nonparallel sides, arranged in palisades or in V shapes, were observed, which suggested the presence of corynebacteria. Urine cultures were positive and yielded a pure culture of 10^5 CFU/mL of *Corynebacterium* spp.

The isolated strain showed slight growth after 24 or 48 h of incubation on horse blood agar medium but abundant growth on the same medium containing 1% Tween 80 under aerobic conditions (5% CO_2). Colonies were white, opaque, smooth, convex, and nonhemolytic. This lipid-requiring strain was catalase positive and strongly urease positive. Testing with the API-Coryne strip (bioMérieux, Marcy l'Etoile, France) showed that the strain was nitrate-reduction positive and produced acid from glucose, ribose, sucrose, and maltose. However, this strain, which was designated CCH052683, did not hydrolyze gelatin or esculin. It was identified as *Corynebacterium* group F1 (the corresponding numeric profile of the gallery API-Coryne was 3001325).

The strain was correctly identified to the species level as *Corynebacterium pseudogenitalium* by using polymerase chain reaction and sequencing 16 rRNA as previously described (*1*,*2*). Comparison of 785 nucleotides (546–1,331) gave a 16S rDNA similarity value of 99.9% between the sequences of the isolated strain and *C. pseudogenitalium* ATCC 33039/NCTC11860 (European Molecular Biology Laboratory accession no. X81872).

The strain was sensitive to penicillin, ampicillin, gentamicin, rifampin, vancomycin, teicoplanin, tetracycline, sulfamethoxazole, trimethoprim, fusidic acid, ciprofloxacin, and norfloxacin and resistant to erythromycin, lincomycin, and nitrofurantoin. Ceftriaxone was replaced by norfloxacin (400 mg twice a day) for 1 month. The patient improved and remained healthy 6 months after therapy.

Nondiphtheric corynebacteria are of increasing importance. They have been observed in human specimens, and many new taxa of coryneform bacteria have been described (*3*). Interest in their taxonomy is increasing, and molecular, phenotypic, and biochemical analyses have resulted in the reclassification of this genus (*3*). *C. pseudogenitalium* was described in 1979 by Furness et al. (*4*) for lipophilic corynebacteria isolated from urinary tract and was not considered a pathogen, in contrast to *C. genitalium*. However, these 2 species were not included in the official list of recognized species.

C. pseudogenitalium was divided into 5 types based on biochemical patterns, and strains of the type C-5 were differentiated from other types on the basis of urease production. The biochemical and physiologic characteristics of this C-5 type were similar of those of the coryneform group F-1 described by the Centers for Disease Control and Prevention (CDC). In 1995, a comprehensive study on lipophilic corynebacteria demonstrated by DNA-DNA hybridization the similarity between a reference strain of *C. pseudogenitalium* type C-5 and reference strains of the CDC coryneform group F-1 (*1*). The CDC group F-1 make up 2 genomic groups at the species level. As shown by 16S rDNA gene comparisons, isolate CCH0-52683 belongs to the genomic group, including a reference strain of *C. pseudogenitalium* type C-5 ATCC 33039 (CCUG 27540, sequence X81872) and a reference strain of CDC group F-1 (CDC G4330, sequence X81905) (Figure). The other genomic group of CDC group F-1 is represented by strain CDC G5911 (sequence X81904). The molecular genetic investigations identified our isolate as *C. pseudogenitalium* and placed it in 1 of the 2 genomic groups of CDC group F-1, which cannot be differentiated by biochemical tests (*1*).

The pathogenicity of this bacterium was associated with strong urease activity. This activity is similar to that of other urease-positive microorganisms, such as *C. urealyticum* and *Proteus* spp. (*5*,*6*), which infect the urinary tract. Unfortunately, the bladder stones were not analyzed after extracorporeal shock wave lithotripsy treatment. The *C. pseudogenitalium*

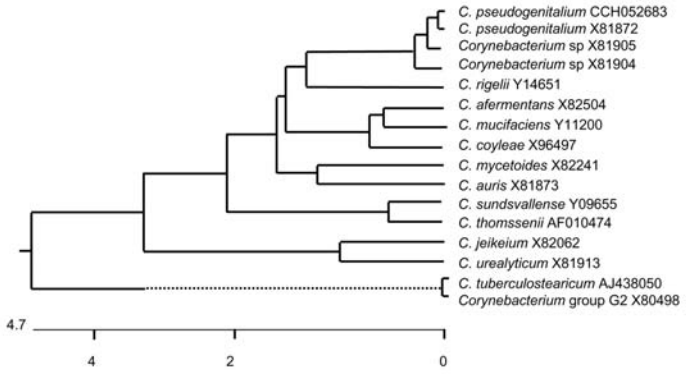

Figure. Unrooted tree showing phylogenetic relationships of *Corynebacterium pseudogenitalium* CCH052683 and other members of the genus *Corynebacterium*. The tree was constructed by using the DNAstar program (DNAstar Inc., Madison, WI, USA) (Clustal method) and based on a comparison of 785 (546–1,331) nucleotides. European Molecular Biology Laboratory sequence accession numbers are shown. The scale bar shows the percentage sequence divergence. Dotted line indicates a distant phylogenetic group for which the scale is not applicable.

isolate was sensitive to most antimicrobial drugs, particularly β-lactams, aminoglycosides, and quinolones. Thus, urinary tract infections caused by this species of bacteria respond more readily to treatment than those caused by multidrug-resistant *C. urealyticum* (*3*).

In conclusion, we show that *C. pseudogenitalium* (CDC coryneform group F-1) can cause urinary tract infection (*7*) and produce urease, and like *C. urealyticum*, cause stone formation in humans. Thus, urease-positive microorganisms isolated by urinalysis that shows urinary alkalinization and struvite and pyuria crystallization should be considered pathogenic. Our results also confirm the difficulty in phenotypic identification of these strains and the need to use a molecular approach to identify coryneform bacteria with clinical relevance.

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Puumala Virus RNA in Patient with Multiorgan Failure

To the Editor: The hantaviruses (genus *Hantavirus*, family *Bunyaviridae*) include human pathogens and occur worldwide (*1*). In Western and Central Europe, the predominant serotype is Puumala virus (PUUV), which causes epidemic nephropathy. We report the first Austrian patient with reverse transcription–polymerase chain reaction (RT-PCR)–confirmed PUUV infection and, to our knowledge, the first detection of PUUV-specific RNA in bone marrow.

On April 27, 2004, a previously healthy 52-year-old bus driver stopped his bus because of visual disturbance, dizziness, headache, and weakness in his legs; he then lost consciousness for a few minutes. He was seen at the neurology emergency service and subsequently admitted to the university hospital in Graz. He smoked tobacco, drank beer on the weekends, and cleaned his bus in the garage daily. The patient showed slight paresis of the right leg, nystagmus, cognitive deficit, and retrograde amnesia. Laboratory tests showed increases in (normal values are shown in parentheses) C-reactive protein (CRP) 40 mg/L (<9), creatine kinase (CK) 224 U/L (<170), lactate dehydrogenase (LDH) 244 U/L (<240), and myoglobin 416 ng/mL (<90). Cerebrospinal fluid showed elevated protein of 60 mg/dL (<45) but no other abnormalities. Results of computed tomographic scan of the brain and chest radiograph were normal. Because of increasing CRP (115 mg/L), empiric antimicrobial therapy with pipera-cillin/tazobactam was started. During an electroencephalogram on April 29, the patient deteriorated and was admitted to the intensive care unit for respiratory failure with a partial oxygen pressure of 40 mm Hg; he required intubation and

mechanical ventilation. A chest radiograph showed diffuse pulmonary infiltration and slight bilateral pleural effusion. Laboratory examination showed CRP 265 mg/L, CK 42,570 U/L, LDH 1,235 U/L, myoglobin >3,000 ng/mL, aspartate aminotransferase 368 U/L (<35), alanine aminotransferase 96 U/L (<45), γ-glutamyl transpeptidase 182 U/L (<55), erythrocytes 3.76×10^9/mL, leukocytes 9.09×10^6/mL, thrombocytopenia of 9.2×10^4 platelets/mL, and lymphoplasmacytoid cells on peripheral blood smear. Serum electrophoresis and immunofixation showed an increased γ-globulin fraction with oligoclonal immunoglobulin G (IgG) λ and IgGκ components. A bone marrow biopsy showed hypercellularity and 15% lymphoid cells with plasmocytoid features. Fluorescence-activated cell sorter testing showed 3% reactive B- and T-cell blasts but no signs of a malignant hematologic disease. Culture of bronchoalveolar lavage for bacteria and fungi was negative. Urinary antigen tests for *Legionella* spp. and pneumococci were negative. Serum antibody tests for *Leptospira* spp. were negative, but IgM against PUUV was detected by POC Puumala rapid test (Erilab Ltd, Kuopio, Finland) and recomLine Bunyavirus IgG/IgM test (Mikrogen, Martinsried, Germany). PUUV RNA was detectable in serum and in bone marrow by RT-PCR (*2*). PUUV was confirmed with a bootstrap probability of 99% on phylogenetic analysis (*2*). On May 1, status epilepticus developed and was treated with clonazepam. On May 2, renal function deteriorated and progressed to a maximum serum creatinine concentration of 4 mg/dL (0.6–1.3) and urea of 244 mg/dL (10–45), which required hemodialysis. CRP increased to 360 mg/L, and blood pressure decreased to 95/65 mm Hg. The patient received intensive supportive care including dopamine and norepinephrine. After improvement, the patient was extubated on May 9. Eight days later, fever (temperature up to 40°C), *Enterococcus faecalis* bacteremia, nosocomial pneumonia from methicillin-resistant *Staphylococcus aureus*, respiratory failure requiring mechanic ventilation, and renal failure developed in the patient. Despite antimicrobial drug therapy with linezolid, the patient died 19 days after reintubation.

In Austria, before this case, PUUV RNA had only been detected by RT-PCR in rodents (*2*). We report the first Austrian patient with RT-PCR–confirmed PUUV infection. Furthermore, PUUV-specific RNA had never been detected in bone marrow. In animal studies, PUUV induces production of proinflammatory cytokines, such as interleukin (IL)-6 and IL-10 (*3*). IL-6 constitutes a major growth factor for myeloma and plasma cells, induces immunoglobulin production, and is an active factor in B-cell differentiation (*4*,*5*). IL-10 is a differentiation factor for plasma cell formation and immunoglobulin secretion. Since we detected a clear increase of IL-6, IL-10, and tumor necrosis factor α (TNFα) during the acute phase of infection (IL-6 133.0 pg/dL, IL-10 218.0 U/mL, and TNFα 29.7 pg/mL), we assume that lymphoplasmacytoid cells in bone marrow and peripheral blood of our patient and his production of oligoclonal γ-globulins were due to PUUV-induced cytokine release. Epidemic nephropathy usually takes a benign course, but multiorgan failure with cerebral involvement developed in our patient. Whereas neurologic symptoms such as headache (97% of patients), blurred vision (40%), and vomiting (31%) are common in patients infected with PUUV, only a few cases have been reported with severe central nervous system involvement (i.e., meningitis, epileptiform seizures) (*6*,*7*). Our patient had visual disturbances, slight paresis of the right leg, nystagmus, cognitive deficit, retrograde amnesia, and status epilepticus. We want to draw attention to the severe course PUUV infections can rarely take. The presence of PUUV in bone marrow explains the marked hematologic changes with lymphoplasmacytoid cells in marrow and peripheral blood.

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Reptile-associated Salmonellosis in Man, Italy

To the Editor: Reptiles are reservoirs of a wide variety of *Salmonella* serotypes, including all S*almonella enterica* subspecies and *S. bongori*. In reptiles born in captivity or kept as pets, *S. enterica* subsp. *enterica* is frequently isolated (*1*). *Salmonella* strains are well adapted to reptiles, and they usually cause asymptomatic infections in such animals, while retaining pathogenicity for warm-blooded animals. For several years, reptiles have been recognized as a source of human salmonellosis. In North America, reptile-associated salmonellosis (RAS) has been reported, particularly in children, the elderly, or immunocompromised persons; severe and fatal infections are described occasionally (*2*). In contrast, only a limited amount of information on RAS is available in Europe. We report a case of RAS that occurred in an adult man in Italy.

A 32-year-old man had symptoms of enteritis. For 2 weeks, he had experienced intermittent watery diarrhea, mild fever, and abdominal pain. He was then treated with ciprofloxacin, and after 15 days of treatment, he recovered from enteritis. A stool sample, collected before treatment, underwent bacteriologic analysis, and *Salmonella* spp. were identified biochemically (api 20E, bioMérieux, Marcy l'Etoile, France) and by a polymerase chain reaction assay specific for the *invA* gene of *Salmonella* spp. (*3*). Since the man was a reptile owner, RAS, rather than a foodborne infection, was initially suspected. He owned several cold-blooded animals; all had been tested for *Salmonella* spp. (at least 3 times at 2- to 3-week intervals), and results were negative. Three weeks before the onset of enteric symptoms, he acquired a boa (*Boa imperator*) that was subjected to routine analysis for *Salmonella* spp. in our laboratories (*1*). *Salmonella* spp. were isolated from a cloacal swab of the snake. Subsequently, both the human and reptile *Salmonella* isolates were characterized as *S. enterica* serovar Paratyphi B. In addition, both strains were found to be d-tartrate–fermenting (dT+) biovars (*4*), susceptible to ampicillin, amoxicillin-clavulanic acid, cephalothin, ceftazidime, gentamicin, streptomycin, chloramphenicol, tetracycline, neomycin, nalidixic acid, norfloxacin, and ciprofloxacin and resistant to sulfamethoxazole and co-trimoxazole.

By pulsed-field gel electrophoresis analysis of DNA, the strains displayed the same pattern, which suggests a clonal origin (*4*). The isolates were also assayed for virulence-associated genes. The *Sop*E1 gene was detected in both isolates, and the *avr*A gene was not detected, which is consistent with an invasive pathovar of *S.* Paratyphi B (*4*). Conversely, the *spv*C, *pef*, and *sef* genes were not detected (*5*).

In recent years, a general increase in RAS detection has been observed, which may be the result of the increasing diffusion of reptiles as pets and a better awareness of RAS risk. In the United States, annual reports of RAS cases are published by the Centers for Diseases Control and Prevention (*2*). In Europe, studies on free-living and captive reptiles have shown a high prevalence of *Salmonella* spp. (*1*). Nevertheless, national surveillance systems for RAS do not exist, and epidemiologic data are incomplete.

Notably, since Sweden became a member of the European Union in 1995, and the import restriction rules for reptiles were removed, a marked increase in RAS was observed in that country (6). As the deregulation of the trade in reptiles is applied, in agreement with the European Union laws, a similar scenario may be projected in other European countries. As is the case for nontyphoid salmonellosis, RAS may be underestimated, especially if patients are not hospitalized. Although a few cases of RAS have been previously reported in children in Italy (*7,8*), this report provides the first description of RAS in adults. *S.* Paratyphi B dT+, also known as *S. enterica* serovar Java, has been isolated in reptiles and tropical fish and has been associated with epidemics of human salmonellosis acquired from food, such as goat milk or chicken (*9*). The evidence shows that salmonellosis by *S.* Paratyphi B dT+ apparently occurs more frequently in adults (*10*), while so-called exotic reptile strains seem to be more prone to causing salmonellosis in children (*7,8*), which has led to the proposition that *S.* Paratyphi B dT+ strains may be highly pathogenic. By screening virulence-associated genes, both our isolates were found to be *Sop*E1+ and *avr*A–, a pattern usually observed in the systemic pathovars of *S.* Paratyphi B (*4*) and associated with invasiveness, which suggests a high pathogenic potential. Accordingly, strict preventive sanitation measures should be adopted when handling reptiles (*2*), and reptiles should be always regarded as a potential source of pathogenic *Salmonella* strains for humans.

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Congenital Visceral Leishmaniasis

To the Editor: Visceral leishmaniasis (VL) is usually transmitted by phlebotomine sandflies. Nonvector transmission occasionally occurs through blood transfusions, contaminated needles of drug users, organ transplants, or laboratory infection (*1*). Only a few cases of congenital transmission have been reported. We describe a case of VL in a German infant, who never had been to a VL-endemic area. Most likely, the parasite was congenitally transmitted from the asymptomatic mother to her child.

A 9-month-old girl had a 4-week history of intermittent fever, recurrent upper respiratory tract infections, and failure to thrive. Physical examination showed a distressed infant with bilaterally enlarged cervical lymph nodes, hepatosplenomegaly, and a rectal temperature of 40°C. The following laboratory results were remarkable: hemoglobin 6.4 mg/dL, erythrocyte count 3.3 million/μL with 10.9% reticulocytes, platelet count 74,000/μL, and leukocyte count 4,300/μL (29.8% neutrophils, 62.3% lymphocytes, 7.4% monocytes, 0.5% basophils, and 0% eosinophils). Serum electrophoresis showed pronounced hypoalbuminemia and hypergammaglobulinemia. Abdominal sonography verified hepatosplenomegaly. Cultures from blood and other materials as well as additional investigations for a wide spectrum of infectious diseases, including HIV infection, were negative. Leukemia was suspected, and a bone marrow biopsy was performed. It showed enhanced myelo-, erythro-, and thrombopoesis with slight lymphopenia but no leukemic cells. However, *Leishmania* amastigotes were detected in bone marrow macrophages at a density of ≈1 to 2 parasitized macrophages per 400× oil immersion field, corresponding to a Chulay score of 1+ (*2*). Serology was positive for *Leishmania* spp. by indirect immunofluorescence antibody test, with cultured promastigotes of *L. donovani* used as antigen (immunoglobulin G [IgG] antibody titer 1:1,024). Specific antibodies against 14- and 16-kDa proteins of *L. infantum* promastigotes (Figure) were confirmed by immunoblot (*3*). Polymerase chain reaction (PCR) on scrapings of stained bone marrow slides amplified a *Leishmania* spp.–specific sequence of the internal transcribed spacer-1 gene (*4*), and subsequent *Hae*III-restriction fragment length polymorphism helped identify the species as *L. infantum* (Figure). Liposomal amphotericin B, at a daily dose of 4 mg per kg body weight, was given by infusion on 6 consecutive days and repeated on days 14 and 21. The therapy was well tolerated. Within 3 days, the fever subsided. The child recovered completely, and blood cell counts reached normal values 5 weeks after treatment was begun.

Since the child had never been outside Germany, vector transmission seemed highly improbable. The girl was born to a 26-year-old prima gravida, prima para, woman at 39 weeks' gestation by spontaneous labor; the infant's birth weight was 3,350 g, and she was 51 cm long. She showed normal development until the age of 8 months.

The mother had been healthy during pregnancy and had no history of serious disease; she did not show any pathologic findings at clinical investigation or in standard laboratory tests. However, *Leishmania* serologic tests conducted on blood samples from the mother showed positive results (IgG antibody titer 1:128 against promastigotes of *L. donovani*), and immunoblot analysis confirmed specific antibodies (Figure). During the last 15 years, she had spent holidays every year in Spain (Alicante) but had never been to a tropical country. She stayed in Spain during weeks 29–32 of her pregnancy. However, she could

not remember any episodes of fever. She was not addicted to drugs nor had she ever received any blood products. Microscopic and PCR examinations of the mother's blood (buffy coat) and breast milk were performed with negative results. Cultures of both specimens in NNN medium were also negative. Since she was asymptomatic, a bone marrow biopsy was unwarranted. Four months later, she became pregnant again. No abnormalities were noted during pregnancy, delivery, or development of the second child.

Although sandflies (*Phlebotomus mascittii* Grassi, 1908) were recently found for the first time at 3 different locations along the upper Rhine Valley in southwestern Germany (*5*), no evidence exists for autochthonous transmission of leishmaniasis in Germany. Congenital transmission from the infected but asymptomatic mother is the most probable scenario in our case. Since 1926, only 10 case reports of congenitally acquired VL have been published (reviewed in *6*). Most cases have been observed after the mother had VL during pregnancy. One previous report describes congenital transmission from an asymptomatic mother to her child (*6*). However, this rarity in reporting does not necessarily reflect the frequency of the event. In VL-endemic areas, cases of congenital VL cannot be distinguished from cases of infection by vector transmission during the first year of life. Congenital transmission may occur either through blood exchange from the mother to the child during labor or by transplacental infection during pregnancy. Which of the 2 transmission routes led to infection in our case is unclear. In the congenital cases reported to date, typical symptoms of the disease developed from 4 weeks to 18 months (mean 8.5 months) after birth. The incubation period after vector transmission is also highly variable (typically 2–6 months but varying from 10 days to >10 years [*1*]). All patients reported have been treated with pentavalent antimonial agents; this treatment is still widely used in VL-endemic areas, but it has considerable side effects, and resistance is increasing (*1*). Liposomal amphotericin B is the drug of choice for treating Mediterranean VL. An alternative, especially for low-income countries, is oral treatment with miltefosine.

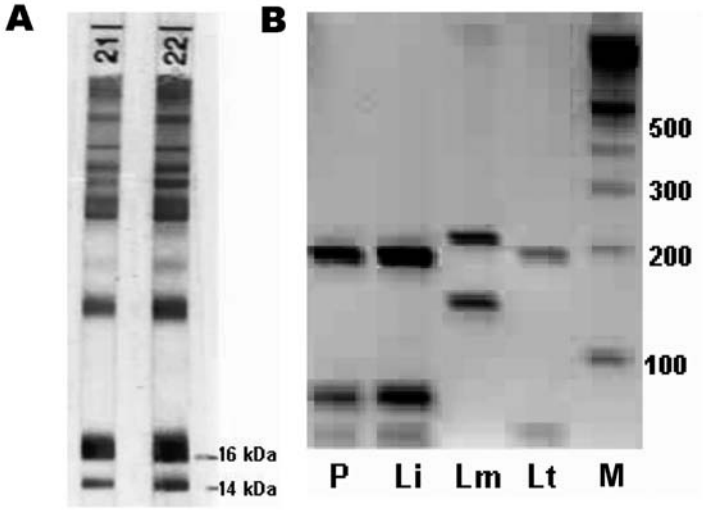

Figure. A) Immunoblot of the patient (strip no. 21) and the patient's mother (strip no. 22) showing specific antibodies against 14- and 16-kDa proteins of *Leishmania infantum*. B) restriction fragment length polymorphism patterns after *Hae*III digestion of the ribosomal internal transcribed spacer 1 polymerase chain reaction products. P, patient; Li, *L. infantum*; Lm, *L. major*; Lt, *L. tropica*; M, 100-bp ladder.

This report suggests that in infants with fever, splenomegaly, and pancytopenia, VL should be considered even if the patient has not been to an disease-endemic area. Congenital transmission is possible, not only as a consequence of VL during pregnancy but also by transmission from an asymptomatic mother to her child in utero or during labor.

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Dictionary of Parasitology

Peter J. Gosling

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The authors' intent in writing this dictionary is to provide a concise, clear, up-to-date, accurate use of terms to be used when communicating scientific information in the field of parasitology. This exhaustive text, with more than 11,500 entries, is at first read simply an alphabetized collection of names of organisms and terms associated with the science of parasitology. Upon closer reading, however, one spends more and more time going page by page either refreshing forgotten terminology, or learning new meaning for a particular term or disease. For the student of words, both newcomers to the field or seasoned hands, this book will provide useful information. Some concerns exist, such as continued use of outdated names, e.g., *Dipetalonema* for a number of filarial infections that have been correctly placed in the genus *Mansonella* for >20 years. There are also some gaps, such as the absence of an important genus of microsporidia, *Enterocytozoon*, but overall, readers will be able to find definitions for common and eclectic terms. The dictionary covers a wider range of terms than parasitology; some virology terms, such as Aino virus, are included, as are some far afield terms, including hundredweight, hydrogen half-cell, and zwitterions. These additions add to the level of interest as the reader leafs from page to page looking for familiar friends and making new acquaintances. Zwitterions, in case you have forgotten, are ions that carry both a positive and negative charge.

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Interdisciplinary Public Health Reasoning and Epidemic Modelling: The Case of Black Death

George Christakos, Ricardo A. Olea, Marc L. Serre, Hwa-Lung Yu, and Lin-Lin Wang

Springer, Berlin, Germany, 2005
ISBN: 3-540-25794-2
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Because public health officials increasingly rely on mathematical models to help them prevent and control diseases, this book is a very timely addition to the literature. The authors' overall theme is that generating accurate and useful (to public health officials) mathematical models of disease epidemiology and the impact of interventions requires a true interdisciplinary approach. They maintain that there is a need to incorporate knowledge and data from both physical and life sciences into such models. For example, the authors argue that information should be included on the clinical (life science) aspects of a disease (e.g., incubation period, efficiency of transmission), as well as on how the disease spreads geographically (physical science) over time (different communities could experience very different patterns of spread). They also note that the onus of improving models does not lie solely with the modelers. Users, particularly public health officials, are part of an interdisciplinary team. Consequently, users have to better acquaint themselves with what models can and cannot do (i.e., the production of mathematical "black boxes" is not entirely the fault of the modelers). The authors illustrate their themes by comprehensively examining the spread of the Black Death in the mid-1300s.

Many Emerging Infectious Diseases readers are likely to find this book overly technical, containing many mathematical formulas, mathematical notations, and complex graphs. However, a reader willing to ignore the potential intimidation of such material may find interesting discussions of modeling philosophy, such as the importance of including probability (i.e., uncertainty or "randomness") and the impact of space-time. For the latter, even the most ardently nonmathematical reader is likely to be fascinated by the maps in Chapter 5 that depict the spread of the Black Death. The data required to model (map out) the spread of disease over time and space require intensive "detective work," to which epidemiologists and public health officials can readily contribute. Readers interested in the background data related to the epidemiology of the Black Death will probably enjoy perusing the detailed, annotated data appendices.

This would be a fine addition to a technical library as a resource for persons who conduct sophisticated mathematical modeling. However, persons looking for a more general historic overview of the Black Death (how it spread and its consequences) would be advised to consider other works such as those by McNeill (*1*) or Cantor (*2*).

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Structural Biology of Bacterial Pathogenesis

Gabriel Waksman, Michael Caparon, and Scott Hultgren, editors

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Research into the pathogenesis of microbial infections has a long and fruitful history, rich with elucidation of mechanisms that have resulted in better treatments and new strategies for vaccine development. Stanley Falkow's investigations into the intimate relationships between bacteria and host cells followed his comment that, "The microbe is just trying to make a living." Structural Biology of Bacterial Pathogenesis, by Gabriel Waksman, Michael Caparon, and Scott Hultgren, is a state-of-the-art treatise describing the known molecular mechanisms by which bacterial pathogens actively probe, sense, and respond to their environment through 2-component systems and through sigma and anti-sigma factors. In addition, the authors describe in great detail the recognition of host receptors by pili and the pilus biogenesis by chaperon-user pathways. The chapter on the role of sortases on surface expression of surface proteins among gram-positive bacteria is comprehensive and well written. Four excellent chapters describe 6 secretion systems among bacterial pathogens and elucidate the specific mechanisms by which bacterial pathogens usurp intracellular mechanisms of the host cell.

This book is enjoyable to read, is extensively referenced, and has 52 superb structural models in full color, based in part on x-ray crystallography. Unfortunately, these plates are all in 1 section and require the reader to page back and forth from specific chapters to the "core color plates." This book is not a compendium of bacterial toxins and virulence factors but rather a selection of molecular mechanisms of host-parasite interaction. This is a unique book that will be a valuable asset for researchers in the field of pathogenesis, graduate students, faculty who teach microbial pathogenesis, biotech companies, and pharmaceutical companies involved in antimicrobial drug or vaccine development.

The sophisticated molecular mechanisms that pathogenic bacteria have developed through their evolution with the human host, as described in this book, are credible evidence that our adversaries, the microbes, are doing better than "just making a living."

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Archibald J. Motley, Jr. (1891–1981). **Nightlife (1943) (detail).** Oil on canvas (91.4 cm × 121.3 cm). The Art Institute of Chicago. Restricted gift of Mr. and Mrs. Marshall Field, Jack and Sandra Guthman, Ben W. Heineman, Ruth Horwich, Lewis and Susan Manilow, Beatrice C. Mayer, Charles A. Meyer, John B. Nichols, Mr. and Mrs. E. B. Smith, Jr.; James W. Alsdorf Memorial Fund; Goodman Endowment, 1992.89

Host-Pathogen-Venue Combinations and All That Jazz

Polyxeni Potter*

"To call yourself a New Yorker you must have been to Harlem at least once. Every up-to-date person knows Harlem, and knowing Harlem generally means that one has visited a night club or two," wrote novelist and editor Wallace Thurman, "….The music is good, the dancers are gay, and setting is conducive to joy" (*1*). In the 1920s, nightclubs, bars, and cabarets were much in vogue in most of the western world. In New York, many talented entertainers worked in these clubs, Duke Ellington's orchestra, Cab Calloway's band, Lena Horne, Adelaide Hall. Chicago became a jazz center with more than 100 clubs. "Midnight was like day," wrote poet Langston Hughes describing the city's South Side (*2*). The exotic, glamorous, intoxicated environment of these clubs, which dominated American entertainment for most of the 20th century, was a main source of inspiration to Chicago painter, Archibald Motley.

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Motley was born in New Orleans, Louisiana, but his family moved north when he was very young. His mother was a schoolteacher, his father a railroad man, operating a buffet car running on the Michigan Central. Even as a child he sketched scenes and people around him and knew that he wanted to be an artist. The elder Motley mentioned his son's ambitions to Frank W. Gunsaulus of the Armour Institute, a train patron, who paid the youth's first year's tuition at the Art Institute of Chicago. A receptive and eager student, Motley studied under accomplished painter Karl Buehr, who encouraged and advised him: "I want to tell you something, Mr. Motley. I don't want you to ever change your style of painting… please continue it, for my sake" (*3*).

At the Art Institute, Motley indulged his admiration of the old masters, particularly Dutch painter Frans Hals, and was exposed to the work of other American artists (George Bellows, John Sloan, Randall Davey). His graduation in 1918 coincided with the advent of Harlem Renaissance, a cultural movement encompassing the literary, musical,

visual, and performing arts and promoting celebration of African identity and heritage. Motley exhibited widely and received many prestigious awards, among them a John Simon Guggenheim Fellowship, which gave him the opportunity to live in Paris for a year. "It is remarkable and beautiful… the way the light travels on the pigmentation of the skin, how gradually the light changes from warm into cool in various faces…. I used to go to the Louvre and study, oh, I studied Delacroix, I studied all the old masters carefully. You know, what we call 'in' painting, the passages of tones" (*3*).

Motley was very productive in Paris. He completed 12 paintings, among them the celebrated Blues, inspired by the local nightclub scene. But he returned to Chicago to exhibit the work. "Artists feel that they're more readily recognized in Europe than they are here in America…. I am staying here in wonderful America. And I love Chicago" (*3*).

"I think that every picture should tell a story," Motley noted (*3*). His narrative paintings, like the work of Frans Hals, peered into the lives of the common people, whom he painted with enthusiasm. But while 17th-century Dutch masters ridiculed drunkenness and warned against the moral laxity of the tavern scene, he viewed social life with affection and offered a glittering rendition of people at play. The club scene with its "total experience" setting provided a perfect backdrop. Extravagantly decorated rooms filled with smoke and spirits called for people to dress up and step out, to escape the reality of postwar depression and social inequity and experience fantasy and luxury in an electrified, unreal environment (*4*). His empathetic portraits and earthy descriptions reflected both his own exuberant love of life and the nightclub scene's whole new view of celebrating: good food, music and dance, and the chance to see and be seen.

"When my grandmother found out that I was playing jazz music," said jazz composer Jelly Roll Morton, "she told me that I had disgraced the family and forbade me to live in the house" (*2*). The music played inside colorful, thickly populated nightclubs all over the United States and spreading around the world, cool jazz, red hot jazz, all manner of jazz, was not always viewed as art form. The music's irregular, sensuous tunes, mixing folk with blues, engaging new instruments, embracing regional sounds, evolved independently in many locations and created an incredible diversity of sounds and styles.

Nightlife, on this month's cover, one of Motley's most celebrated works, is a glimpse of the action at a dance hall in Chicago's Bronzeville neighborhood. Painted during World War II, the picture does not address the dire global circumstances. It focuses instead on a lighthearted moment of gaiety, inside a comfortable establishment, vibrant with the sounds of music, dancing, and conversation. A lively jazz band in the background guides the figures. Diagonal lines indicate sharp syncopated movement amidst free-flowing activity around the dance floor.

The stage is framed with bar paraphernalia, stools, and tables. But the scene is not about the venue. The artist is painting energy and motion, the group dynamic of a community, laughing, gesturing, mingling. The figures are bold but stylized, so the viewer is not distracted by individual features. Body language and overall carriage are harmonious and integrated, and the crowd is engaged and receptive.

Even as Motley focused on the moment's thrill inside a nightclub, he created a microcosm analogous to broader outside reality, an allegory of the world. The stylish crowd socializing and the jazz band orchestrating their movements mirror the group dynamics of microbial populations, swinging to nature's tune in niches they make for themselves. The spontaneity of jazz music and its adaptations to local culture over time around the globe parallel the emergence and export of new diseases from their seedbeds to audiences the world over. A glance at this issue's contents confirms the immense diversity of disease emergence over time and place, from birds with flu and *Helicobacter* infections to drug-resistant HIV strains, from Nipah virus to *Arcobacter*, from dengue to ameba-associated pneumonia. All neatly choreographed to music we cannot yet hear.

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

Look in the March issue for the following topics:

Relapsing Fever Perspectives and Possibilities for Reemergence

Cost-effectiveness of West Nile Virus Vaccination from Societal Perspective

Web-based Surveillance and *Salmonella* Distribution, 2000–2002

Bartonella in Pets: Impact on Human Health

Estimated Incident West Nile Virus Infections, 2003

Aspergillus ustus Infections among Transplant Recipients

Medication Sales as Syndromic Surveillance Tool, France

Personal Hygiene as Risk Factor for Methicillin-resistant *Staphylococcus aureus* Infection

Real-Time PCR and Capillary Electrophoresis To Identify Pathogen Genotypes

Self-medication: a Survey in 19 European Countries

New Spotted Fever Group Rickettsia, *Candidatus Rickettsia kelly*

Fluoroquinolone-resistant *Salmonella* Paratyphi A, India

West Nile Virus–associated Flaccid Paralysis Outcome

Highly Pathogenic Pantropic Variant of Canine Coronavirus

Complete list of articles in the March issue at http://www.cdc.gov/ncidod/eid/upcoming.htm

Upcoming Infectious Disease Activities

March 16–18, 2006
International Conference on Women and Infectious Diseases: Progress in Science and Action
Marriott Marquis Hotel
Atlanta, GA, USA
http://www.womenshealthconf.org

March 19–22, 2006
International Conference on Emerging Infectious Diseases 2006
Marriott Marquis Hotel
Atlanta, GA, USA
http://www.iceid.org

March 22–24, 2006
International Symposium on Emerging Zoonoses
Medical and Veterinary Partnerships To Address Global Challenges
Marriott Marquis Hotel
Atlanta, GA, USA
http://www.isezconference.org

May 19–23, 2006
Council of Science Editors 49th Annual Meeting
Hyatt Regency Tampa
Tampa, FL, USA
http://www.councilscienceeditors.org

June 17–22, 2006
Negative Strand Viruses 2006: Thirteenth International Conference on Negative Strand Viruses
Salamanca, Spain
Contact: 404-728-0564 or meeting@nsv2006.org
http://www.nsv2006.org

June 25–29, 2006
ISHAM 2006 (International Society for Human and Animal Mycology)
Palais des Congrès
Paris, France
Contact: 770-751-7332 or c.chase@imedex.com
http://www.imedex.com/calendars/infectiousdisease.htm

EMERGING INFECTIOUS DISEASES
A Peer-Reviewed Journal Tracking and Analyzing Disease Trends

Editorial Policy and Call for Articles

Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles see below and visit http://www.cdc.gov/eid/ncidod/ EID/instruct.htm.

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Manuscript Preparation. For word processing, use MS Word. Begin each of the following sections on a new page and in this order: title page, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, figure legends, appendixes, and figures. Each figure should be in a separate file.

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Types of Articles

Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch of first author. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch of first author—both authors if only 2. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research Studies. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary, and a brief biographical sketch of first author—both authors if only 2. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and brief biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., “The Study” and “Conclusions.” Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and a brief biographical sketch of first author—both authors if only 2. Dispatches are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no figures or tables.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Book Reviews. Short reviews (250–500 words) of recently published books on emerging disease issues are welcome. The name of the book, publisher, and number of pages should be included.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted on the journal Web page only, depending on the event date.)

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only. Summaries, which should contain 500–1,000 words, should focus on content rather than process and may provide illustrations, references, and links to full reports of conference activities.