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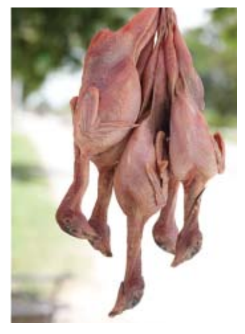

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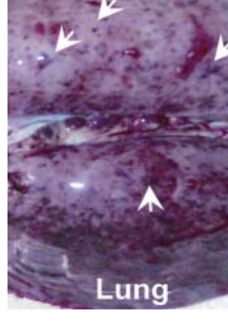

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Framework for Leadership and Training of Biosafety Level 4 Laboratory Workers

James W. Le Duc, Kevin Anderson, Marshall E. Bloom, James E. Estep, Heinz Feldmann, Joan B. Geisbert, Thomas W. Geisbert, Lisa Hensley, Michael Holbrook, Peter B. Jahrling, Thomas G. Ksiazek, George Korch, Jean Patterson, John P. Skvorak, and Hana Weingartl

Construction of several new Biosafety Level 4 (BSL-4) laboratories and expansion of existing operations have created an increased international demand for well-trained staff and facility leaders. Directors of most North American BSL-4 laboratories met and agreed upon a framework for leadership and training of biocontainment research and operations staff. They agreed on essential preparation and training that includes theoretical consideration of biocontainment principles, practical hands-on training, and mentored on-the-job experiences relevant to positional responsibilities as essential preparation before a person's independent access to a BSL-4 facility. They also agreed that the BSL-4 laboratory director is the key person most responsible for ensuring that staff members are appropriately prepared for BSL-4 operations. Although standardized certification of training does not formally exist, the directors agreed that facility-specific, time-limited documentation to recognize specific skills and experiences of trained persons is needed.

The threat of terrorists using as weapons the most deadly pathogens known, coupled with the recognition that virtually every year a new infectious disease is discovered, has led the US government to expand the number of Biosafety Level 4 (BSL-4) laboratories, also known as maximum containment laboratories (MCLs), to perform work essential for promoting public health and to ensure bioterrorism preparedness. A few such laboratories have been in existence for decades, primarily in Australia, Russia, South Africa, the United Kingdom, and the United States; these have had, in most instances, an exceptional history of safety in handling these most dangerous pathogens. However, construction of new facilities, including 2 national laboratories on academic campuses in the United States, and the expansion of existing US facilities, has resulted in public concern and Congressional inquiries regarding the safety of these laboratories and the qualifications of those persons working in them (*1,2*).

Historically, a small, close-knit group of dedicated scientists have made their careers working in this highly specialized BSL-4 environment. Laboratory directors have generally had hands-on involvement in research activities undertaken in their facilities, have personally trained those with whom they worked, and have been careful to restrict access to those not well suited for containment-laboratory endeavors. The current expansion of BSL-4 laboratories, within the United States and in several other countries, has resulted in a demand for experienced workers, which presents a unique challenge to currently established processes for BSL-4 training (*3*). Given the global demand for BSL-4 laboratory workers to staff the expanded number of facilities, there is a clear international need for more structured and transparent BSL-4 training processes to establish and verify standards for the next generation of containment-laboratory scientists (*4*). Development of rigorous standards for BSL-4 laboratory training will instill confidence in the public, policy makers, and security officials that the expanded international network of BSL-4 laboratories will continue to be operated safely and will pose no risk to scientific staff, local communities, surrounding environment,

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and host nations. Clarification and coordination of training standards will help to develop a cadre of highly qualified biocontainment workers and will result in a series of robust BSL-4 laboratory programs that will enable scientists to develop measures to deal with existing threat agents and to cope with new diseases that emerge (*5*).

In response to this challenge, the BSL-4 laboratory directors from most existing North American laboratories and those currently under construction met to develop a framework of standards and norms necessary for training future MCL scientists and support staff. The results of those deliberations are summarized below and offered as a model for the global BSL-4 laboratory community.

MCL Management Structure

Although the institutional director (e.g., director of the Centers for Disease Control and Prevention, commander of the US Army Medical Research Institute of Infectious Diseases, or dean at an academic campus) has ultimate responsibility for the BSL-4 facility housed within his or her institution, it is the BSL-4 laboratory director that senior leadership relies upon to ensure that this unique facility operates safely and securely. The BSL-4 program director oversees all personnel working in the containment laboratory, ensures proper training and qualifications for the work to be undertaken, ensures that all regulatory requirements are addressed, and is responsible for maintaining a safe and efficient work environment. The BSL-4 laboratory director often also serves as the lead scientist for investigations involving highly pathogenic organisms, sets priorities and coordinates activities within the facility, and frequently serves as the technical spokesperson for the program. The BSL-4 laboratory director grants final approval for personnel to operate independently in the BSL-4 laboratory. His or her efforts are complemented by those of the institutional biosafety officer or manager, who plays an important role as an independent advisor to the institutional director to ensure the safe and secure operations of the program, and the building engineer, who manages the complex mechanical infrastructure necessary to enable safe handling of highly dangerous pathogens. The BSL-4 laboratory director works in close partnership with these professionals to ensure smooth operation of the MCL. Each person has distinct responsibilities and, in most instances, a parallel reporting chain that ensures that problems are brought to the level of the institutional director or the academic dean for resolution.

Preparation of a Person for BSL-4 Work

Persons seeking access to a BSL-4 laboratory come from many different backgrounds, but they must all share common traits of having an aptitude for work with infectious agents and an appreciation of the need for careful adherence to safety standards and protocols. Medical examinations, security checks, and clearances are required before a person can handle select agents; some laboratories require vaccinations before a person can begin work with an infectious agent (licensed vaccines are not widely available for most BSL-4 pathogens.) There is a need to remain flexible in the selection of persons for BSL-4 training, recognizing that some persons rapidly acquire the skills needed to work safely in the BSL-4 laboratory, while others may never gain complete confidence of the MCL director and will always be required to work in partnership with a more experienced person. Prior work at a BSL-3 laboratory is generally considered a strong asset but is not an absolute requirement before being cleared to work in a BSL-4 laboratory. What is essential is that the person must be properly trained in the techniques he or she will be using in the BSL-4 laboratory.

In addition to core scientific staff, there is an ongoing need for BSL-4 laboratory support personnel to service equipment, maintain the building, conduct inspections, and assist in specific technical activities such as the care and use of laboratory animals. These persons also require specialized training and approval to operate independently.

Elements of Training

Formal training in preparation for work in a BSL-4 laboratory should consist of 3 elements: didactic or classroom-style theoretical preparation, one-on-one practical training in the facility, and mentored on-the-job training (Figure). Theoretical training helps laboratory workers develop an understanding of the underpinnings of biocontainment operations and the laboratory systems that support these operations. Hands-on practical training includes a comprehensive orientation to the specific facility in which the person will work to include a complete review and documented understanding of all standard operating procedures; orientation to engineering aspects of the facility; overview of all safety procedures, including alarms and emergency operations; and an introduction to the care and use of a protective suit or glove box. The institutional biosafety officer and building engineer typically assist in providing this orientation, some of which may be augmented by training videos.

BSL-4 laboratory orientation training assumes that the person has already mastered all procedures for safe and secure handling of infectious agents at the BSL-2 and ideally BSL-3 levels. This training generally involves individualized orientation within the facility provided by an experienced staff member or dedicated training officer. It may begin while the laboratory is shut down for annual recertification and maintenance or while it is operational. Training would involve use of entrances simply designed to demonstrate how one enters and exits the suite, general orientation on the use of air hoses, working within biologic

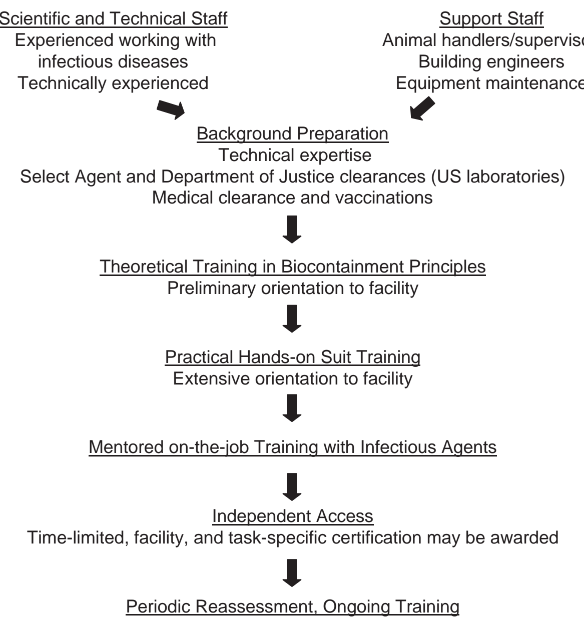

Figure. Framework for maximum containment laboratory training.

safety cabinets or glove boxes, storage and record keeping of pathogens, clean-up and decontamination following procedures or spills, solid and liquid waste management, use of autoclaves and other specialized equipment, communications with others inside and outside of the BSL-4 facility, and other general procedures.

Finally, the person under consideration is assigned a dedicated mentor and is introduced to working with live pathogens in the BSL-4 laboratory under the mentor's close supervision. This stage of training is basically open ended; the length of time and number of entries into the facility will vary greatly depending upon the skills of the person and his or her ability to master all procedures necessary for independent work. Final decision of when a person is allowed independent access is subjective and based on an assessment by the mentor and laboratory director; it is usually discussed only after the person has had extensive experience working in the facility. The time required to gain full independent access may also vary depending upon the kind of work that person will be undertaking. For example, persons not likely to be directly handling infectious material, such as safety officers, building engineers, or maintenance staff, may be offered limited independent access sooner than a person who will be handling live pathogens routinely. Partial or limited access may also be granted to a person for independent access only during normal duty hours. Laboratory procedures that involve animals or sharp instruments (e.g., needles, syringes, postmortem procedures) represent the greatest risk and consequently require special training and experience; these procedures should be mastered at lower containment levels before a person is permitted to undertake these activities under BSL-4 conditions. Most standard operating procedures for animal manipulation require that at least 2 persons be present, regardless of their experience level. Some laboratories require a final oral or written examination before granting a person independent access, which may be administered by the safety officer. However, the ultimate decision as to who is allowed independent access to the BSL-4 laboratory is made by the BSL-4 laboratory director.

A typical mentor will be an experienced person who has earned full unrestricted access to the laboratory and has the clear confidence of the laboratory director. Although there are no set time or formal educational requirements to become a mentor, mentors should have extensive practical experience working under BSL-4 laboratory conditions.

All laboratories should have developed a process for reevaluation of all persons working in the BSL-4 laboratory to ensure that their knowledge and skills remain current. This process may be an annual refresher course or periodic formal or informal review and training and may be augmented by orientation sessions as new equipment is introduced into the facility. Ensuring that senior program staff members are regularly present in the laboratory is important for maintaining consistent safety, security, and scientific standards.

Need for Certification of Training

The need to document that a person has completed appropriate training has been discussed extensively. It is evident that a tacit internal certification exists in BSL-4 facilities currently operating and this takes the form of approval to work independently. This certification may be more formally captured in a specific document or may be a checklist signed by the approval authority. A more broadly applicable documentation system could provide evidence of consistency in training, demonstrate recognized capabilities with certain tasks such as for animal handlers, and provide a mechanism to gauge the number of persons working in the field.

At present, those working in BSL-4 laboratories in the United States need security clearance and approval to handle select agents, must have completed the extensive training program described above, must have medical examinations, and must be known by the program director. Each BSL-4 laboratory is, however, unique and every program director should demand that all persons entering their facility be well prepared and knowledgeable of all safety and security procedures required of that facility. Although standardized documentation of training does not formally exist, there would be merit in developing an internationally agreed-upon facility-specific, time-limited document to recognize the specific skills and experiences of a per-

son. Such documentation would have the added benefit of facilitating collaborations and personnel exchanges among BSL-4 laboratories.

Conclusions

Directors of most North American existing and proposed BSL-4 laboratories agreed upon a framework for training of BSL-4 laboratory staff, including scientific, technical, and support personnel such as animal handlers, building engineers, and maintenance workers. Independent access to the BSL-4 facility would be granted at the discretion of the BSL-4 program director after successful completion of training in the theory of biocontainment principles, practical hands-on suit training, and extensive supervised work with infectious agents under the tutelage of a well-experienced mentor. Periodic reassessment of skills and ongoing refresher training would be a routine aspect of continuing education for all BSL-4 staff. The need for documentation of training that would be time-limited and specific for a given facility was also discussed. Such formal documentation could facilitate collaborations and personnel exchange between BSL-4 facilities and help to better certify the national BSL-4 workforce. The framework proposed could serve as a model for BSL-4 workforce development globally.

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Dr Le Duc is deputy director of the Galveston National Laboratory at the University of Texas Medical Branch in Galveston, Texas. His research interests include the epidemiology of infectious diseases, especially vector-borne virus diseases, and global health.

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Antimicrobial Drug–Selection Markers for *Burkholderia pseudomallei* and *B. mallei*

Herbert P. Schweizer and Sharon J. Peacock

Genetic research into the select agents *Burkholderia pseudomallei* and *B. mallei* is currently hampered by a paucity of approved antimicrobial drug–selection markers. The strict regulations imposed on researchers in the United States but not in other parts of the world lead to discrepancies in practice, hinder distribution of genetically modified strains, and impede progress in the field. Deliberation and decisions regarding alternative selection markers (antimicrobial and nonantimicrobial drugs) by the international community, regulatory authorities, and funding agencies are needed.

Antimicrobial drug–selection markers are essential tools for the bacterial geneticist. Gene deletions created by the insertion of a region of DNA carrying an antimicrobial drug resistance cassette enable geneticists to select mutants by using bacterial agar into which the relevant drug has been incorporated. Choice of an antimicrobial drug–selection marker for a given bacterial species is based on several factors: natural bacterial resistance, markers already available that would work in the organism of interest, and the choice of antimicrobial drugs used to treat natural disease caused by the pathogen.

Select agents are biological agents and toxins that have the potential to pose a severe threat to human health, animal or plant health, or animal or plant products. A recent increase in the number of persons and agencies undertaking research on select agents adds complexity to the use of antimicrobial drug–selection markers. First, there are specific regulations relating to select agents. Second, few genetic tools are available for those select agent organisms that have received limited attention. Third, some species are naturally resistant to a range of antimicrobial drug groups, limiting the choice of drugs for patient care and the possible markers available for experimental studies.

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Restricted Use of Drug-Selection Markers with Select Agents

In the United States, the acquisition, possession, and use of select agent bacteria are governed by the following Federal Register publications: 42 Code of Federal Regulations (CFR) Parts 72 and 73 for pathogens listed by the Department of Health and Human Services (HHS) and 7 CFR Part 331 and 9 CFR Part 121 for plant and animal pathogens listed by the United States Department of Agriculture (USDA). Some of the agents, including *Burkholderia pseudomallei* and *B. mallei*, are on both the HHS and the USDA lists. Restrictions and regulations include the application of antimicrobial drug–selection markers for genetic manipulation of these bacteria. A person or entity may not conduct a restricted experiment with a select agent unless approved by the appropriate entities. Restricted experiments pertaining to use of selection markers are defined in 42 CFR Part 73, §73.13, section (b)(1), and the National Institutes of Health (NIH) Recombinant DNA Guidelines (Section III-A-1-a) as follows: experiments utilizing recombinant DNA that involve the deliberate transfer of a drug resistance trait to microorganisms that are not known to acquire the trait naturally, if such acquisition could compromise the use of the drug to control disease agents in humans, veterinary medicine, or agriculture. The NIH Office of Biotechnology Activities is responsible for these guidelines. Issues concerning transfer of drug resistance traits to select agents are brought to the Intragovernmental Select

Agents and Toxins Technical Advisory Committee for assessment. The final decision is made by HHS, USDA, or both, depending on the agent.

Current policy is that persons or entities must apply for approval for use of even those drugs (antimicrobial and others) for which it has clearly been established that such use will not compromise their ability to control a particular disease agent in humans, veterinary medicine, or agriculture. Even if approval for use of these markers is granted to a number of institutions or persons, such approval does not automatically mean that their use is unrestricted and, therefore, exempt from restrictions under this policy.

Special Considerations for *B. pseudomallei* and *B. mallei*

B. pseudomallei and *B. mallei* are 2 closely related select agents that cause melioidosis and glanders, respectively. *B. mallei* causes natural glanders, a rare disease of equids (*1*), although it can also cause rare infections in humans (*2*). By contrast, the disease caused by *B. pseudomallei,* human melioidosis, is endemic to much of Southeast Asia, northern Australia, and other parts of the Tropics around the world (including Central America and South America) and causes thousands of cases each year (*3*,*4*). These 2 organisms are intrinsically resistant to many antimicrobial drugs, including first-, second-, and third-generation cephalosporins; penicillins; and polymyxin B (*3*). *B. pseudomallei* is naturally resistant to gentamicin, but *B. mallei* is susceptible because of deletion of the genes encoding the AmrAB-OprA efflux pump (*5*). These 2 species are usually susceptible to ceftazidime, the carbapenems, amoxicillin-clavulanate, piperacillin-tazobactam, doxycycline, and trimethoprim-sulfamethoxazole (TMP-SMX) (*6*). First-line treatment for acute human melioidosis is intravenous ceftazidime or a carbapenem for at least 10–14 days, followed by oral TMP-SMX with or without doxycycline for 12–20 weeks (*3*,*6*). For patients who cannot tolerate first-line therapy or for whom this therapy is contraindicated (e.g., children and pregnant women), the choice of oral therapy is amoxicillin-clavulanate. The choice of treatment for human glanders is uncertain because of the rarity of this disease, but clinical experts suggest that treatment should be the same as for *B. pseudomallei*. Acquired resistance to carbapenem drugs has not been reported, and the rate of acquired resistance to ceftazidime and amoxicillin-clavulanate is low (<0.2%) (*6*). Acquired resistance to doxycycline is 2% and to TMP-SMX is geographically variable (2.5% in Australia compared with 13%–16% in northeast Thailand) (*6*).

Approved Selection Markers for *B. pseudomallei* and *B. mallei*

The markers approved for use with *B. pseudomallei* and *B. mallei* in the United States are kanamycin, gentamicin, zeocin, and polymyxin B (for *B. pseudomallei*), and kanamycin, zeocin, and polymyxin B (for *B. mallei*). Because both species are naturally resistant to polymyxin B, this drug is therefore of little use for the genetic manipulation of these bacteria. *B. pseudomallei* is almost always naturally resistant to gentamicin and other aminoglycosides, although ≈1 in 1,000 isolates cultured from patients with cases of melioidosis at a large hospital in northeast Thailand, where >250 cases are seen each year, were found to be susceptible. *B. mallei* is naturally susceptible to gentamicin, but this marker is prohibited for use with this species because it could potentially be used to treat infection. However, gentamicin can be used as a marker in *B. pseudomallei* if the strain used is naturally susceptible. During 1990–2005, the Wellcome Unit in Thailand identified 4 such strains (708a, 2188a, 2188b, and 3799a) in 3 patients with melioidosis (*7*). One potential drawback is that these strains are poorly characterized. For example, whether these strains are representative of the bacterial population as a whole is not clear.

To answer this question, the Wellcome Unit undertook sequence typing and determined that strain 708a is sequence type (ST) 23, strains 2188a and 2188b are ST47, and strain 3799a is ST154. Because all 3 STs have been previously identified, and several gentamicin-resistant strains have been identified for each of the 3 clones, the susceptible strains are not rare in population genetic terms. Recent unpublished observations from our laboratories indicate that the gentamicin susceptibility in strains 708a, 2188b, and 3799 results from a deletion (708a) or lack of expression (2188b and 3799a) of the *amrAB-oprA* efflux pump operon. These results suggest that 708a may be a natural candidate for genetic manipulation experiments that use gentamicin, spectinomycin, streptomycin (*8*), and nourseothricin (*9*), and validate the use of laboratory-constructed Δ(*amrAB-oprA*) mutants in such experiments.

Kanamycin and zeocin can be used for genetic manipulation of *B. pseudomallei* and *B. mallei*, especially when driven from constitutive promoters; but even then, high concentrations of antimicrobial drugs are required (*10*). The recent development of site-specific recombinase systems for use in *Burkholderia* spp. enables in vivo excision and recycling of selection markers, thus expanding the use of the few precious markers currently approved for genetic manipulation of these bacteria (*10*).

Approval has also been granted for testing of some nonantimicrobial drug–selection markers such as tellurite and triclosan. Unpublished work from our laboratories showed that tellurite resistance conferred by *kilA-telAB* (*11*) may be a useful marker for *B. pseudomallei* (MIC ≈1 μg/mL) and *B. mallei* (MIC <0.5 μg/mL). Similarly, FabL-mediated triclosan resistance (*12*) may be useful in *B. mallei* (MIC = 5 μg/mL) but not in *B. pseudomallei* (MIC >64 μg/mL).

Restricted Selection Markers

Consideration of currently restricted selection markers highlights several possible candidates. Until relatively recently, chloramphenicol was used in Thailand for the oral phase of melioidosis treatment, but this use ceased after a clinical trial showed it to be unnecessary (*13*). Rare exceptions to this exist, an example of which is a patient with neurologic involvement who is infected with an organism that is resistant to TMP-SMX or who cannot tolerate this drug. Chloramphenicol penetrates well into the brain; amoxicillin-clavulanate does not. Neurologic involvement occurs infrequently (1.5% of cases in Thailand; *14*), and the chance of neurologic disease with a strain that is resistant to TMP-SMX developing in a patient is low. Use of chloramphenicol for postexposure prophylaxis has not been reported, and its considerable side effects make it a drug of last choice. If this marker were to be allowed, it should never be used in a strain resistant to TMP-SMX. Other potential markers may encode resistance to members of the fluoroquinolone group. These drugs have poor activity in vitro (*6*) and are not recommended for the treatment of melioidosis because of their poor clinical efficacy. A comparison of ciprofloxacin and azithromycin for 12 weeks versus TMP-SMX and doxycycline for 20 weeks demonstrated relapse rates of 22% and 3%, respectively (*15*). The relative contribution of differences in treatment duration to the rates of relapse is not known. However, treatment of 57 adult melioidosis patients with ciprofloxacin or ofloxacin for a median of 15 weeks was associated with an unacceptably high failure rate of 29% (*16*). Therefore, because they are clinically of little use, we reasoned that some of the recently discovered enzyme-mediated fluoroquinolone resistance determinants, e.g., *qnrA* and *aac(6')-Ib* conferring resistance to ciprofloxacin and norfloxacin (*17*), may be useful for genetic manipulation in *Burkholderia* spp. However, in exploratory experiments they did not confer sufficient levels of norfloxacin resistance to *B. thailandensis* to be of genetic utility (K.-H. Choi and H.P. Schweizer, unpub. data).

International Discrepancies

In addition to being limited in range, permissible markers are also subject to considerable international discrepancies in practice and in regulations. For example, although TMP-SMX and doxycycline are first-line drugs for treatment of melioidosis in disease-endemic regions, researchers in other parts of the world consistently use trimethoprim and tetracycline (which leads to cross-resistance with doxycycline) as markers. Use of markers that are prohibited in the United States but not elsewhere leads to several problems. First, mutant strains that are resistant to either agent cannot be imported to and used in the United States, which limits scope for collaborations and sharing of strains. Second, US publications describing select agent research are monitored, and investigators using nonapproved markers risk mandated destruction of their mutant collections containing such markers. Furthermore, this discrepancy encourages US researchers to enlist colleagues abroad to advance their research, although such research cannot be financed by NIH. A possible argument for use of trimethoprim is that natural resistance is seen at a relatively high frequency (at least in isolates from Thailand), and so its use as a marker may be permissible according to the regulatory guidelines. Furthermore, handling of these organisms in a Biosafety Level 3 facility while wearing protective clothing limits exposure risk for workers. However, we consider it inappropriate to create resistance to an antimicrobial drug that is a first-line treatment for melioidosis and that is the agent of choice for postexposure prophylaxis after a laboratory accident in which a worker has had substantial accidental exposure to *B. pseudomallei* or *B. mallei* (*18*).

Conclusions

The list of approved drugs, antimicrobial and nonantimicrobial, and their respective selection markers is evolving, but its evolution has not been very transparent because no listing of officially approved drugs and allowed selection markers is publicly available. Unlike all other bacterial select agents, no approved attenuated strains of *B. pseudomallei* and *B. mallei* are currently available. Strains of select agents can be excluded from the regulations if the request is accompanied by data showing that the strain is no longer virulent or that the strain is attenuated. Antimicrobial drug–resistance markers, including those used in human and veterinary medicine, can be introduced into excluded strains after approval by local institutional biosafety committees. To our knowledge, no exclusions had been requested of any strain of *B. pseudomallei* and *B. mallei* at the time of the writing of this article. Recent initiatives from the NIH Institute of Allergy and Infectious Diseases regarding funding of research for development of nonantimicrobial drug–selection markers may alleviate some of the problems raised in this article, but not all. Failure to address these issues in a timely manner may compromise genetic research with *B. pseudomallei* and *B. mallei*, cause loss of interest by existing researchers, and contribute to failure to recruit new persons with expertise into the field. We propose that the international community, regulatory authorities, and funding agencies should meet and make timely and conclusive decisions to resolve these problems.

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Molecular Epidemiology of Methicillin-Resistant *Staphylococcus aureus*, Rural Southwestern Alaska[1]

Michael Z. David, Karen M. Rudolph, Thomas W. Hennessy, Susan Boyle-Vavra, and Robert S. Daum

USA300 is the dominant strain responsible for community-associated (CA) methicillin-resistant *Staphylococcus aureus* (MRSA) infections in most of the United States. We examined isolates from outbreaks of MRSA skin infections in rural southwestern Alaska in 1996 and 2000 (retrospective collection) and from the hospital serving this region in 2004–2006 (prospective collection). Among 36 retrospective collection isolates, 92% carried Panton-Valentine leukocidin (PVL) genes; all carried staphylococcal chromosomal cassette *mec* (SCC*mec*) type IV. None belonged to clonal complex (CC) 8, the CC associated with USA300; 57% were sequence type (ST) 1, and 26% were ST30; 61% were clindamycin resistant. In the prospective collection, 42 isolates were PVL+ and carried SCC*mec* type IV; 83.3% were ST1, 9.5% were ST30, and 7.1% were ST8. Among 120 prospective isolates, 57.5% were clindamycin resistant. CA-MRSA epidemiology in southwestern Alaska differs from that in the lower 48 states; ST8 strains were rarely identified and clindamycin resistance was common.

Staphylococcus aureus is a common cause of skin and soft tissue infections (SSTIs), endovascular infections, pneumonia, septic arthritis, endocarditis, osteomyelitis, foreign-body infections, and sepsis (*1*). In the United States, epidemic infection with community-associated (CA) methicillin-resistant *Staphylococcus aureus* (MRSA) is occurring, with many reports of MRSA infections among persons without traditional healthcare-associated MRSA risk factors (*2–4*). As a result, the epidemiology of CA-MRSA has become complex (*5*).

Novel MRSA isolates that are less likely to be resistant to antimicrobial drugs other than β-lactams have been identified in association with epidemic CA-MRSA infections. These CA-MRSA strains are commonly susceptible to drugs such as clindamycin, gentamicin, tetracyclines, and rifampin. Moreover, the genes encoding the pore-forming, bicomponent cytotoxin, Panton-Valentine leukocidin (PVL), are nearly universally present in novel CA-MRSA strains. However, evidence from animal studies has been contradictory in assessing the importance of PVL in the virulence of these isolates (*6,7*).

In addition to the PVL genes, strains that cause CA-MRSA infections typically carry staphylococcal chromosomal cassette *mec* (SCC*mec*) types IV and V, small genetic resistance elements that are presumably mobile. A single CA-MRSA genetic background, USA300 (defined by pulsed-field gel electrophoresis), corresponding to sequence type (ST) 8 by multilocus sequence typing (MLST), has become predominant among CA-MRSA isolates in many centers in the United States (*8–10*). The reason for the dominance of USA300 is not clear.

Other MRSA strains that are broadly susceptible to non–β-lactams and have PVL genes and SCC*mec* IV or V have predominated among CA-MRSA strains collected in various regions of the world (*11*). In an area of rural Alaska, SSTIs caused by CA-MRSA isolates have been a public health concern since 1996 (*12–14*). We explored the molecular diversity of strains causing CA-MRSA in this region and

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investigated the hypothesis that a transition to the dominance of USA300 had also occurred in this region, similar to that documented elsewhere in the United States (*15*).

Methods

The study was reviewed and approved by the Institutional Review Boards of the Centers for Disease Control and Prevention (CDC) (Atlanta, GA, USA) and the Alaska Area Native Health Services. The study protocol and draft manuscript were reviewed and approved by the Yukon Kuskokwim Health Corporation (YKHC).

Two collections of MRSA isolates were available for study. They were obtained from the same region of southwestern Alaska. Southwestern Alaska is a roadless area populated principally by Alaska Natives, the descendants of the indigenous people of Alaska. The regional commercial center is Bethel (population 5,700), which is served by the 50-bed Yukon Kuskokwim Delta Regional Hospital (YKDRH) (*13,14*). This hospital is the only one serving the 25,000 persons in the YKHC, which is a tribal health corporation that operates a comprehensive system of care in the region for Alaska Natives, including hospital and village-based clinics. Most residents have a subsistence lifestyle, and many homes lack running water. Primary healthcare delivery occurs at village health clinics staffed by community health aides who provide acute and preventive services. Travel between villages is principally by airplane, boat, or snowmobile; no roads connect these regions to the rest of Alaska. The clinical microbiology laboratory at YKDRH serves the hospital and all outpatient clinics in the surrounding region.

Retrospective Collection

This collection included 36 MRSA strains randomly selected from those obtained during 2 SSTI outbreaks. The 1996 outbreak occurred in 1 village (*12*). In the second outbreak in 2000, MRSA isolates were collected from infections, usually SSTIs, among residents of 29 villages, from wooden surfaces of steam baths in 1 village and from cultures obtained to assess nasal colonization (*14*).

Prospective Collection

We conducted laboratory-based surveillance for MRSA from January 2004 through January 2006 at the YKDRH in Bethel. During the study period, 85% of all *S. aureus* isolates at YKDRH were identified as MRSA (L. Pruitt, pers. comm., January 2008). The first 5 unique-patient MRSA isolates each month were collected by the YKDRH laboratory and sent to the Arctic Investigations Program at CDC for storage and processing. Information accompanying each isolate included basic demographics, location of clinical services, and date and anatomic site of isolation. Isolates were identified at the YKDRH clinical microbiology laboratory. Antimicrobial drug susceptibilities were determined by the Vitek automated system (bioMérieux Vitek, Inc., Durham, NC, USA) at the University of Chicago. Isolates were stratified by patient age and clindamycin susceptibility. Age strata were then compared for differences in the percentage of isolates susceptible to clindamycin by χ^2 test using Stata SE version 9.2 (StataCorp, College Station, TX, USA).

Genetic Testing

MLST was performed as described (*16*) at the University of Chicago. Sequence types were grouped into clonal complexes (CCs) when they were genetically related; e.g., ST8 belongs to CC8. CCs were assigned by using the eBURST algorithm as described (*17*). The presence of *mecA* was assessed and the SCC*mec* type of each strain was determined by using criteria previously described (*18*). Presence of *lukF-PV* and *lukS-PV* encoding the PVL toxin was assessed by PCR as described (*19*). Isolates that had these genes were PVL+.

Results

Thirty-six isolates were available from the retrospective collection. Six were from the 1996 investigation outbreak collected from January 6, 1997, through January 6, 1998, and 30 from the 2000 outbreak collected from April 17, 2000, through September 20, 2000. Of the 2000 outbreak isolates, 3 were collected from steam bath bench surfaces, 12 from nasal survey cultures, and 15 from material obtained from SSTIs. Of 36 isolates in the retrospective collection, 33 (92%) were PVL+ (*14*).

SCC*mec* and MLST typing were performed on the retrospective collection isolates. All carried SCC*mec* type IV. ST1 was the most common MLST genotype, accounting for 20 (57%) of 36 isolates; ST30 accounted for 9 (26%) of 36 and ST59 for 1 (2.8%) of 36. When isolates were grouped in clonal complexes, 22 (63%) belonged to CC1, 11 (32%) to CC30, and 2 (6%) to CC59. None were ST8 or belonged to CC8, the CC most closely associated with USA300 (Table 1).

Only 14 (39%) of 36 MRSA retrospective collection isolates were susceptible to clindamycin when clindamycin single-agent testing and D-test results were taken into account; 31% were susceptible to erythromycin. Susceptibility to gentamicin, ciprofloxacin, rifampin, vancomycin, and trimethoprim-sulfamethoxazole was nearly universal (Table 1).

Of the 120 MRSA patient isolates available from the prospective collection, 117 were from patients whose sex was known; 61 (50.8%) were from males. Most patients were 13–49 years of age. Among patients with known venue of care, 106 (90.6%) of 117 were outpatients, 59 (50.4%) of whom received care in the emergency department. All

Table 1. Selected characteristics of MRSA isolates from southwestern Alaska, 1997, 2000, and 2004–2006*

Characteristic	Retrospective collection, no. (%)	Prospective collection, no. (%)
Drug susceptibility	n = 36	n = 120
Ciprofloxacin	36 (100)	112 (93.3)
Clindamycin, total†	14 (39)	51 (42.5)
Single-agent testing	33 (92)	51 (42.5)
D-test negative	3/22 (14)	NA
Erythromycin	11 (31)	48 (40.0)
Gentamicin	35 (97)	115 (95.8)
Rifampin	36 (100)	117 (97.5)
Vancomycin	36 (100)	120 (100)
Trimethoprim-sulfamethoxazole	35 (97)	120 (100)
Resistance to ≥2 non–β-lactam antimicrobial drug classes	21 (58)	69 (58)
PVL genes present	n = 36	n = 42
Yes	33 (92)	42 (100)
No	3 (8)	0
SCC*mec* type IV	36 (100)	42 (100)
MLST CC type		
CC1	22 (63)	35 (83)
ST1	20 (57)	35 (83)
ST1slv‡	1 (3)	0
ST474	1 (3)	0
CC8	0	3 (7)
ST8	0	3 (7)
CC30	11 (32)	4 (10)
ST30	9 (26)	4 (10)
ST484	1 (3)	0
ST535	1 (3)	0
CC59	2 (6)	0
ST59	1 (3)	0
ST59slv‡	1 (3)	0

*MRSA, methicillin-resistant *Staphylococcus aureus*; NA, not applicable; PVL, Panton-Valentine leukocidin; SCC*mec*, staphylococcal chromosomal cassette *mec*; MLST, multilocus sequence typing; CC, clonal complex; ST, sequence type.
†Indicates clindamycin susceptibility by single-agent testing and negative D-test results. D-tests for inducible clindamycin resistance were not indicated for any isolates from the prospective collection and were performed for 22 isolates in the retrospective collection.
‡slv, single-locus variant by MLST testing.

but 1 of the 11 inpatients had their isolate obtained within 72 hours of admission, i.e., their infections had onset in the community. Most (90.0%) isolates were from material obtained from SSTIs. Other sites of isolation included blood, urine, respiratory tract, and bone (Table 2).

A random sample of one third of the prospective collection isolates (42/120) spanning the entire collection interval were assayed for PVL toxin genes, SCC*mec*, and MLST type. All 42 isolates tested were PVL+, and all carried the SCC*mec* type IV element. Most MRSA isolates in the prospective collection were ST1 (35/42, 83.3%), which likely represent USA400. Other types included ST30 (4/42, 9.5%) and ST8 (3/42, 7.1%). ST8 isolates likely represent USA300 (Table 1).

Antimicrobial drug susceptibility data were available for all 120 isolates in the prospective collection; 51 (42.5%) of 120 were susceptible to erythromycin and clindamycin. There was no discordance in erythromycin and clindamycin susceptibility; therefore, no isolate underwent the D-zone test. Nearly all isolates in the prospective collection were susceptible to ciprofloxacin (93.3%), gentamicin (95.8%), and rifampin (97.5%). All isolates were susceptible to trimethoprim-sulfamethoxazole and vancomycin. More than half (57.5%) of the isolates were resistant to ≥2 classes of non–β-lactam antimicrobial drugs; most of these were accounted for by isolates resistant to erythromycin and clindamycin (Table 1).

We examined the susceptibility to clindamycin of MRSA isolates from the prospective collection stratified by patient age groups (0–2, 3–12, 13–20, 21–49, ≥50 years of age). There were no significant differences or trends in the rate of clindamycin resistance among different age strata ($p = 0.47$).

Discussion

CA-MRSA isolates from southwestern rural Alaska differed in important ways from isolates collected in other parts of the United States. These isolates almost universally belonged to CC1, with a minor representation in CC30, CC59, and CC8. Elsewhere in the United States, USA300 (belonging to CC8) has become the most common cause of community-associated SSTIs at medical centers in Atlanta,

Table 2. Demographic and clinical characteristics for 120 patients infected with MRSA isolates in the prospective collection, southwestern Alaska, 2004–2006*

Characteristic	No. (%)
Sex	
Male	61 (50.8)
Female	56 (46.7)
Unknown	3 (2.5)
Age, y	
>1–2	11 (9.2)
3–12	14 (11.7)
13–20	22 (18.3)
21–49	53 (44.2)
≥50	18 (15.0)
Unknown	2 (0.2)
Location of care	
Inpatient	11 (9.2)
Outpatient	106 (88.4)
Emergency department	59 (49.2)
Other outpatient	47 (39.2)
Unknown	3 (2.5)
Place of onset by 72-h rule†	
Community	114 (95.0)
Hospital	1 (0.8)
Unknown	5 (3.3)
Site of isolation	
Blood	3 (2.5)
Respiratory tract	1 (0.8)
Skin/wound	108 (90.0)
Urine	2 (1.7)
Bone/joint	1 (0.8)
Other	5 (4.2)

*MRSA, methicillin-resistant *Staphylococcus aureus.*
†MRSA isolates were considered community onset if they were obtained from a patient in the outpatient setting or from a hospitalized patient within 72 h of admission; only 1 isolate was obtained from a patient who was considered to have had onset of the infection in the hospital.

Baltimore, San Francisco, Houston, Chicago, and Los Angeles (*2–4,8,9*) and among adults with SSTIs who came to emergency departments in 17 cities (*8*). In contrast, ST8 (corresponding to USA300) was an infrequent genotype among MRSA isolates from rural Alaska in 2004–2006 and was absent among isolates from 2 outbreaks in separate villages in 1996 and 2000. Despite these differences in genetic background among the CA-MRSA isolates, nearly all isolates tested were PVL+ and all carried SCC*mec* IV.

The molecular epidemiology of CA-MRSA infection in Alaska underscores the worldwide geographic diversity of novel CA-MRSA genetic backgrounds identified in the past decade. Isolates containing the PVL genes and either SCC*mec* IV or SCC*mec* V that lack resistance to most non–β-lactam antimicrobial drugs have been identified in 6 continents. Examples include ST5 in France and Switzerland; ST80 in Belgium, Croatia, Denmark, England, Finland, Germany, Greece, the Netherlands, Norway, Romania, Scotland, Slovenia, and Sweden; and ST22 in Germany and the Netherlands (*11*).

The 3 most common genetic background types in rural Alaska (ST1, ST59, and ST30) have been reported from studies of MRSA in communities elsewhere. ST1, corresponding to pulsed-field gel electrophoresis type USA400, was the strain type responsible for the deaths of 4 children reported in the midwestern United States (*20*) and the type that predominated in Chicago (*10*) and other regions in the late 1990s. The prototype strain is MW2, the genome of which has been sequenced (*21*). ST1 has become a rare cause of SSTIs in Chicago, Texas, and California, and among SSTI patients at emergency departments in 17 US cities (*8,10*).

In Taiwan, ST59 is predominant among strains that are PVL+ and carry SCC*mec* IV or V_T (*18*). USA1000, which is also an ST59 strain, circulates among persons with no known exposure to the healthcare system (*22*). Some genetic diversity has been noted among ST59 strains as shown by variation in staphylococcal protein A (*spa*) typing (*11,23–25*). ST59 strains were also isolated at a decreasing frequency in 1997–2001 from patients in a California jail (*15*), and in Western Europe and Singapore (*11*). We found 1 ST59 isolate and 1 single-locus variant of ST59 in the retrospective collection from the 1996 and 2000 outbreaks but found none in the prospective collection.

The evolutionary history of ST30 MRSA strains is complex; the acquisition of the SCC*mec* element and the PVL genes has likely occurred in this genetic background on several occasions. Phage type 80/81 strains of *S. aureus*, virulent nosocomial pathogens in the 1950s and 1960s, shared this ST background (*26*). By examining the pattern of resistance-gene carriage in various MRSA genetic backgrounds, Diep et al. proposed an evolutionary relationship among ST30 strains, suggesting that an MSSA ST30 strain sequentially added to its genome phage-encoded PVL toxin genes and the SCC*mec* IV element (*27*). However, a strain of ST30 MRSA isolated in 1991 from Wisconsin lacked the PVL genes but carried SCC*mec* IV (*25*), which suggested that the sequence of events hypothesized by Diep et al. does not universally describe the evolution of these strains (*27*). Among 5 ST30 MRSA isolates collected in Japan in 1979–1985, 3 were PVL+ and all carried the SCC*mec* type I element (*28*). ST30 isolates reported from various regions commonly carry PVL genes and the SCC*mec* IV element but can differ in *spa* type, which suggests a continued and complex evolutionary trajectory for this prevalent sequence type (*8,11,18,24,29–32*).

The difference in PVL+, SCC*mec* IV strain types of MRSA in rural Alaska compared with those in the lower 48 states suggests that Alaska may represent an earlier part of the epidemic curve of CA-MRSA. For example, there was a shift from USA400 to USA300 as the predominant clindamycin-susceptible, PVL+, SCC*mec* IV-containing MRSA strain in Chicago after 2000 (*10*). The predomi-

nance of ST1 strains in southwestern Alaska may reflect geographic isolation of this region or improved fitness of the strain in the rural Alaskan environment. The clinical spectrum of these community-onset cases is similar to MRSA disease elsewhere with a predominance of SSTIs and few associated instances of bacteremia or other invasive illnesses. This disease spectrum is also similar to that of earlier reports of infections caused by MRSA from this region (*13,14*). O'Hara et al., using phylogenetic analyses of the *lukSF-PV* sequences coding PVL toxin in a sample of international clinical MRSA isolates, recently hypothesized that USA300 emerged after a CC8 MRSA strain acquired the PVL genes from the preexisting, virulent MW2 strain (*33*). If this event was the genesis of USA300, this event may have occurred in the lower 48 states, and USA300 had not spread to southwestern Alaska, where USA400 strains still predominated in early 2006.

All MRSA isolates we tested carried SCC*mec* type IV. So-called healthcare-associated MRSA isolates typically carry SCC*mec* types II or III, lack PVL genes, tend to be resistant to a greater number of non–β-lactam antimicrobial drugs, and were predominant among strains isolated from cases of hospital infections in the United States before 2000 (*5*). Such healthcare-associated MRSA isolates were absent from our isolate collections, even from the prospective collection, which was a random sample of MRSA isolates that included inpatients in the region served by the hospital laboratory for 2 years. In contrast, at the University of Chicago in 2004–2005, 8.6% of MRSA isolates from pediatric infections and 51.7% from adult infections carried SCC*mec* II (*5*).

The PVL+, SCC*mec* IV–bearing strains of MRSA from Alaska that we studied showed a high percentage of clindamycin resistance (57.5%). In contrast, strains of MRSA that cause community-onset skin infections elsewhere in the United States are commonly susceptible to clindamycin (*2–4,8*), although exceptions have been documented (*34*), most recently in San Francisco among men who have sex with men infected by USA300 strains (*35*). Isolates from Alaska also had a relatively low percentage of erythromycin resistance, which reflects the predominance of the ST1 background. Erythromycin-resistant MRSA strains likely have the *erm* gene, which confers inducible or constitutive resistance to clindamycin, although there are other molecular mechanisms for clindamycin resistance. Surprisingly, among the prospective isolate collection, every isolate resistant to erythromycin was also resistant to clindamycin by single-agent testing, an observation suggesting that the presumably *erm*-mediated phenotype became constitutive more often. Compared with antimicrobial drug susceptibilities among MRSA identified in Alaska in 2000, clindamycin resistance remained high but decreased slightly from 61% to 57.5%, ciprofloxacin resistance increased from 0% to 7%, and susceptibilities to other antimicrobial drugs remained similar in the prospective collection. In contrast to the situation elsewhere in much of the United States, in southwestern Alaska, clindamycin should be avoided as a first-line agent for treatment of community-onset SSTIs.

Our study was limited because the isolates we examined were from patients in 1 region and the number available in the retrospective collection was not large. The prospective collection was obtained from 1 clinic system and its community hospital, which may not be representative of other regions of Alaska. Furthermore, few clinical data were available regarding patients from whom these isolates were obtained. Our data suggest that further research is needed to clarify the enigma of nearly simultaneous emergence and high prevalence of MRSA strains with PVL toxin genes and SCC*mec* type IV elements in different predominant genotype backgrounds in different regions of the world.

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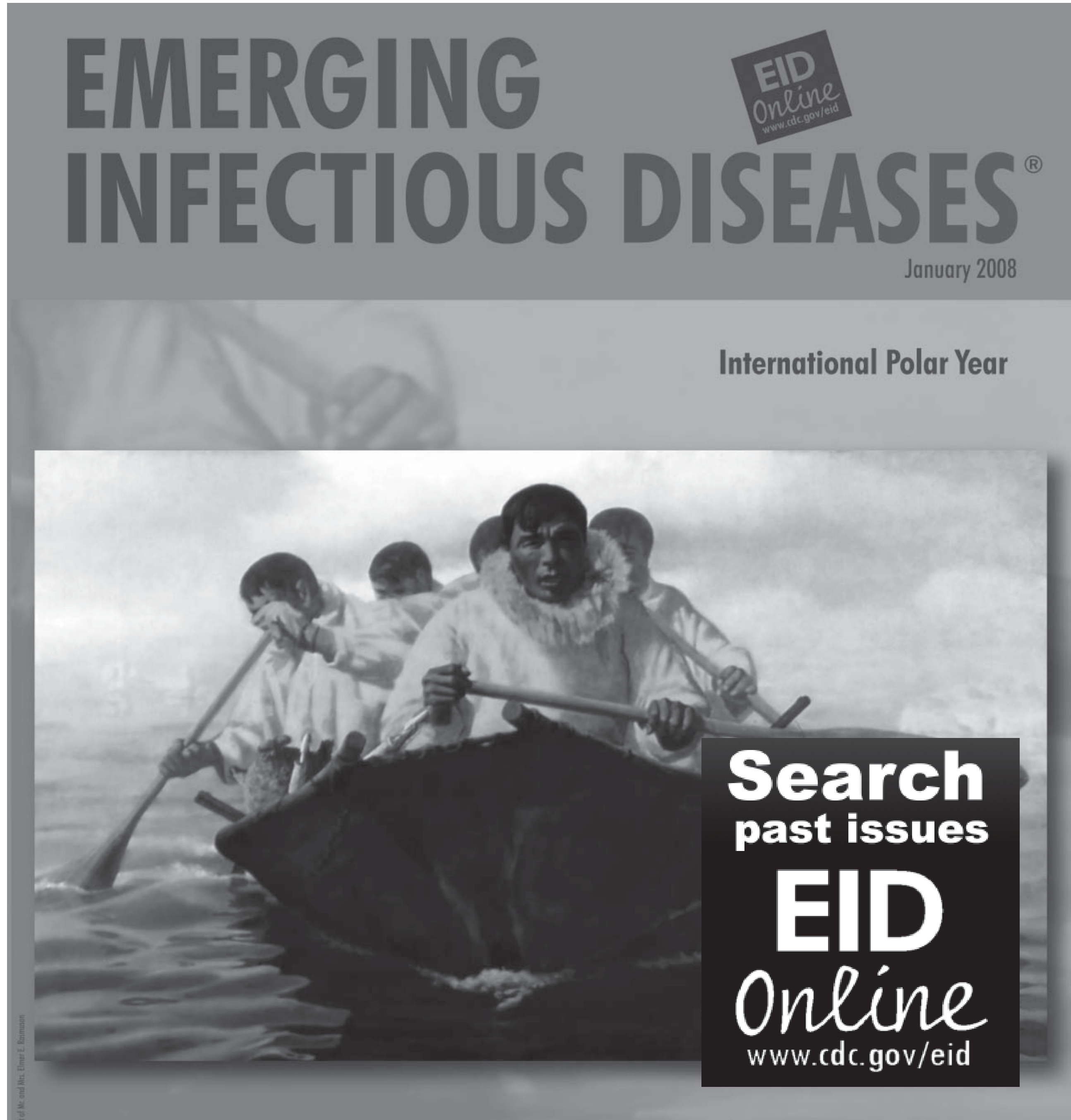

Multidrug- and Extensively Drug-Resistant Tuberculosis, Germany

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We evaluated risk factors and treatment outcomes associated with multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis (TB) in Germany in 2004–2006. In 177 (4%) of 4,557 culture-positive TB cases, *Mycobacterium tuberculosis* isolates were identified as MDR TB; an additional 7 (0.15%) met criteria for XDR TB. Of these 184 patients, 148 (80%) were born in countries of the former Soviet Union. In patients with XDR TB, hospitalization was longer (mean ± SD 202 ± 130 vs. 123 ± 81 days; p = 0.015) and resistance to all first-line drugs was more frequent (36% vs. 86%; p = 0.013) than in patients with MDR TB. Seventy-four (40%) of these 184 patients received treatment with linezolid. Treatment success rates ranged from 59% for the entire cohort (59% for MDR TB and 57% for XDR TB) to 87% for those with a definitive outcome (n = 125; 89% for MDR TB and 80% for XDR TB). Extensive drug susceptibility testing and availability of second- and third-line drugs under inpatient management conditions permit relatively high treatment success rates in MDR and XDR TB.

Tuberculosis is among the leading causes of death worldwide. The World Health Organization (WHO) estimates that 32% of the world population is infected with *Mycobacterium tuberculosis*, the causative agent of TB (*1*). There were an estimated 9.2 million new TB cases and 1.7 million deaths from TB in 2006 (*2*).

Drug resistance to isoniazid and rifampin, the 2 most potent first-line drugs for the treatment of TB (the definition for MDR), is increasing globally (*3*,*4*). Surveillance data indicate MDR TB is an emerging global problem, especially in countries of the former Soviet Union (FSU), Israel, and areas of the People's Republic of China (*5*–*7*). Since active TB will develop in only a proportion of persons infected with *M. tuberculosis* directly after primary infection, the prevalence of MDR TB may still be underestimated. Furthermore, strains of *M. tuberculosis* that are resistant to second-line drugs are also emerging. In vitro drug resistance of *M. tuberculosis* to any fluoroquinolone and to at least one of the injectable drugs (capreomycin, kanamycin, or amikacin), in addition to isoniazid and rifampin resistance, is defined as XDR TB (*8*,*9*). Strains of XDR TB have now been isolated from patients in >45 nations worldwide, and they are associated with worse treatment outcomes than strains of MDR TB (*8*,*10*,*11*). Strains of XDR TB are increasingly seen in HIV-seropositive persons with TB in southern Africa, where these strains are passed by person-to person contact. XDR TB has become a serious problem for the health administrations in this region (*12*). In contrast, infections with XDR TB strains are rarely seen in Western Europe, mainly among the population of pretreated migrants from countries of the FSU (*13*).

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Although the incidence of TB is steadily declining in Germany, numbers of cases with MDR TB strains are increasing. In 2006, of 3,501 TB cases in Germany for which resistance data were available, 78 (2.2%) were MDR TB (*14*); these cases mainly occurred among immigrants from countries with high prevalence of MDR TB (*14*,*15*).

TB surveillance data for Germany are reported annually by a national disease surveillance center, the Robert Koch Institute (*14*). However, data on MDR TB are only reported for in vitro first-line drug resistance against isoniazid, rifampin, ethambutol, pyrazinamide, and the injectable agent streptomycin. To ascertain risk factors associated with MDR and XDR TB and to evaluate treatment outcome in relation to level of drug resistance and level of care, we performed a retrospective survey among the network of hospitals participating in the Tuberculosis Network European Trials group (TBNET); these hospitals specialize in treating TB in Germany.

Materials and Methods

Clinical outcomes (available from the original clinical records) were evaluated by attending physicians at hospitals specialized in the care of patients with TB in Germany; they completed a standard questionnaire for all patients with culture-confirmed isoniazid and rifampin drug-resistant *M. tuberculosis* hospitalized from January 1, 2004, through December 31, 2006. The survey included information on the patients' age, gender, country of origin, HIV-seropositivity status, history of previous treatment, *M. tuberculosis* drug-resistance profile, treatment duration, and treatment outcome. Drug-susceptibility testing (DST) for first-line anti-TB drugs was performed by quality-assured laboratories. Isolates with resistances to anti-TB drugs were (re-)tested at one of the WHO's Supranational Reference Laboratories in Borstel or Gauting (*16*). DST for second-line drugs (ethionamide, amikacin, capreomycin, p-aminosalicylic acid, cycloserine, kanamycin) or third-line drugs (linezolid) were exclusively performed in 1 of the 2 reference centers. The BACTEC MGIT 960 (Becton Dickinson Diagnostic Systems, Sparks, MD, USA) was used for DST of first-line drugs and BACTEC MGIT 960 or the proportion method on Lowenstein–Jensen medium, or both, was used for DST of second- and third-line drugs. XDR TB was defined as resistance to isoniazid and rifampin (MDR TB definition), a fluoroquinolone, and at least one of the injectable anti-TB drugs capreomycin, kanamycin, or amikacin (*17*). MDR TB cases with isolates resistant to all first-line drugs were defined as those resistant to isoniazid, rifampin, ethambutol, streptomycin and, when tested, pyrazinamide.

According to Laserson criteria, a patient was defined as "cured" when he or she had completed treatment according to the country protocol and had been consistently culture-negative (with at least 5 results) for the final 12 months of treatment; "treatment completed" when he or she had completed treatment according to the country protocol but did not meet the definition for cure or treatment failure or bacteriologic results were missing (i.e., <5 cultures were performed in the final 12 months of therapy) (*18*). Outcomes were compared by using the χ^2 test or Fisher exact test (categorical variables) in cases achieving a final outcome (different from default, transferred out, or still on treatment), and by using the Kaplan-Meier curve where appropriate. Logistic regression analysis was performed. The following variables were included in the statistical analysis: country, gender, HIV seropositivity, immigrant status, previous TB treatment for >30 days, DST results (ethambutol, pyrazinamide, streptomycin, any fluoroquinolone, any injectable second-line drug), and resistance to all second-line drugs. A patient was considered HIV positive, when results of the HIV-antibody ELISA (once) and at least 1 confirmatory test (Western blot or nucleic acid amplification technique) were positive.

Results

Among 4,557 culture-confirmed TB cases at 27 participating hospitals (representing 37% of all culture-confirmed cases in Germany in the 3-year period 2004–2006), 184 (4%) *M. tuberculosis* isolates were in vitro resistant at least to isoniazid and rifampin. They MDR TB isolates represented 65% of all MDR and XDR TB cases diagnosed in Germany in the study period (*14*,*19*,*20*). Of these cases, 177 were MDR TB, and 7 were XDR TB.

Of the 184 study patients, 174 (95%) had *M. tuberculosis* isolates resistant to streptomycin, 119 (65%) to ethambutol, 103 (56%) to rifabutin, 79 (43%) to pyrazinamide, 23 (13%) to amikacin, 20 (11%) to a fluoroquinolone, 19 (10%) to capreomycin, 36 (19%) to ethionamide, 15 (8%) to para-aminosalicylic acid, 9 (5%) to cycloserine, 3 (2%) to kanamycin, and 1 (<1%) to linezolid. Demographic and clinical characteristics are described in Table 1 and the online Appendix Table (available from www.cdc.gov/EID/content/14/11/1700-appT.htm).

Forty-five (24%) patients with MDR TB strains were female (median age 28 years), and 139 (76%) were male (median age 39 years). HIV testing was performed for 142 (80%) of 177 patients with MDR TB and 4 (57%) of 7 patients with XDR TB. Seven patients with MDR TB (4.9%) and no patient with XDR TB tested positive for HIV-1. Notably, 148 (80.4%) of 184 patients with MDR TB were immigrants from the FSU (online Appendix Figure, available from www.cdc.gov/EID/content/14/11/1700-appG.htm).

Ninety-four (53%) patients with MDR TB and 6 (86%) patients with XDR TB had previously received anti-TB treatment for >1 month ($p = 0.08$). Of the 100 previous-

Table 1. Demographic and clinical characteristics of 184 patients with MDR TB and XDR TB, Germany*

Variables	MDR TB, n = 177	XDR TB, n = 7	p value	95% CI
Male gender, no. (%)	133 (75.1)	6 (85.7)	0.54	–0.37 to 0.17
Age, y, mean ± SD	37.7 ± 15.4	42.4 ± 11.9	0.42	–16.33 to 6.93
Country of birth, no. (%)				
Former Soviet Union	142 (80.2)	6 (85.7)	0.74	–0.32 to 0.22
Germany	11 (6.2)	–	–	–
Others	24 (13.6)	1 (14.3)	0.93	–0.27 to 0.25
HIV positive, no. (%)	7/142 (4.9)	0	0.54	0.01 to 0.08
Kind of TB, no. (%)				
Pulmonary TB	162 (91.5)	6 (85.7)	0.59	–0.2 to 0.32
Extrapulmonary TB	5 (2.9)	–	–	–
Pulmonary and extrapulmonary TB	10 (5.6)	1 (14.3)	0.29	–0.34 to 0.16
Days in hospital, mean ± SD	123.3 ± 81	202 ± 130	0.015†	–141.8 to –15.53
Previous TB treatment, no. (%)	94 (53)	6 (86)	0.08	–0.59 to 0.06
Resistance to all first-line drugs, no. (%)	64 (36)	6 (85.7)	0.008	–0.76 to –0.21
Resistance to fluoroquinolones, no. (%)	13/162 (8)	7 (100)	<0.001†	–0.96 to –0.87
Resistance to injectable second-line drugs, no. (%)	21/164 (12.8)	7 (100)	<0.001†	–0.92 to –0.83
Linezolid treatment, no. (%)	69 (39)	5 (71.4)	0.09	–0.66 to 0.02
Treatment outcome, no. (%)				
Cured	79 (44.6)	3 (42.8)	0.91	–0.35 to 0.39
Completed	26 (14.7)	1 (14.3)	1	–0.26 to 0.26
Successful treatment (cured + completed)	105 (59.3)	4 (57.1)	0.91	–0.35 to 0.39
Died	14 (7.9)	1 (14.3)	0.4	–0.32 to 0.18
Failure	1 (0.6)	–	–	–
Treatment failure (death or failure)	15 (8.4)	1 (14.3)	0.57	–0.32 to 0.2
Default	1 (0.6)	–	–	–
Transferred out	25 (14.1)	–	–	–
Uncertain outcome (default + transferred out)	26 (14.7)	–	–	–
Still on treatment	31 (17.5)	2 (28.6)	0.45	–0.44 to 0.22
Duration of therapy from beginning MDR treatment, mo, mean ± SD	18 ± 9	20 ± 5	0.56	–8.78 to 4.78
Sputum smear conversion, no. (%)	98 (55.4)	5 (71.4)	0.4	–0.5 to 0.18
Culture conversion, no. (%)	132 (74.6)	5 (71.4)	0.85	–0.31 to 0.37
Sputum smear conversion, d, mean ± SD	69.4 ± 76	129.8 ± 129.2	0.09	–132 to 11.2
Culture conversion, d, mean ± SD	81.3 ± 74.6	141 ± 99.7	0.08	–127.6 to 8.2

*MDR, multidrug-resistant; TB, tuberculosis; XDR, extensively drug-resistant; CI, confidence interval.
†Significant result (p<0.05).

ly treated patients, 89% were immigrants from the FSU, 6% from other countries, and 5% were born in Germany. Only 1 of the 7 patients with XDR TB had previously received directly observed treatment. Strains from patients with XDR TB had a significantly higher probability to be resistant to all first-line drugs (isoniazid, rifampin, pyrazinamide, ethambutol) (6/7, 85.7% vs. 64/177, 36%; $p = 0.08$) than strains from other patients with MDR TB. The median time from the onset of treatment to conversion of smear microscopy and culture to negative results was 88 days (mean ± SD 129.8 ± 129.2 days) and 117 days (mean ± SD 141 ± 99.7 days), respectively, with XDR TB vs. 53.5 days (mean ± SD 69.4 ± 76.1 days) and 61.5 days (mean ± SD 81.3 ± 74.6 days), respectively, with MDR TB.

Of 177 patients with MDR TB, 14 (7.9%) died, one's treatment failed (0.6%), 105 (59.3%) were treated successfully (6/105 underwent surgery), 31 (17.5%) were still receiving treatment, and 26 (14.7%) were lost to follow-up. Of 7 patients with XDR TB, 4 (57.1%) were treated successfully (1/6 underwent surgery), 2 (28.6%) were still receiving treatment, and 1 (14.3%) died.

The overall treatment success including all patients was 59.2% (59.3% for patients with MDR TB and 57.1% for patients with XDR TB). After the 26 patients lost to follow-up were removed from the analysis, the treatment success rate increased to 69% (69.5% for patients with MDR TB and 57.1% for patients with XDR TB). When we also removed the 33 patients still receiving treatment, the treatment success rate increased to 87.2% (87.5% for patients with MDR TB and 80% for patients with XDR TB). Patients with XDR TB were less likely to achieve sputum-smear and culture conversion (5/7, 71.4% vs. 142/177, 80.2%; $p = 0.63$) and required a longer duration of hospitalization (mean ± SD 202 ± 130 vs. 123.3 ± 81.0 days, $p = 0.015$) than patients with MDR TB. Logistic regression analysis of the association of treatment failure (death or failure) with potential covariates was performed; no statistical significant odds ratio was obtained on either

the univariate or multivariate analysis; a negative prognosis related to several variables could be seen, but sample size might have influenced the statistical results (Table 2). Treatment outcomes were compared between patients who were never treated and those who were previously treated with anti-TB drugs; no statistically significant difference was evident between the 2 groups (Table 3).

Seventy-four (40.2%) of the 184 study patients were treated with linezolid. Fifty-eight (78.4%) of them were born in the FSU, and 44 (59.5%) had received previous treatment. Two (2.7%) were HIV seropositive. *M. tuberculosis* isolates of patients receiving linezolid treatment were more frequently resistant to pyrazinamide (49/74, 66.2% vs. 30/110, 27.3%; p<0.001), capreomycin (16/74, 21.6% vs. 3/110, 2.7%; p<0.001), amikacin (15/74, 20.8% vs. 8/110, 7.3; p = 0.009), fluoroquinolones (14/74, 18.9% vs. 6/110, 5.5%; p = 0.004) and cycloserine (6/74, 8.1% vs. 3/110, 2.7%; p = 0.16). Patients with XDR TB were more frequently treated with linezolid (5/7, 71.4% vs. 69/177, 38.9%; p = 0.12) than other patients with MDR TB. In the group of patients with linezolid treatment, the median time to sputum-smear conversion (XDR TB: 134 days vs. 44 days; MDR TB: 57 days vs. 36.5 days; log rank p = 0.0213) and to culture conversion (XDR TB: 160 days vs. 105 days; MDR TB: 68 days vs. 59 days; log rank p = 0.0023) was longer than in the group of patients not receiving linezolid (Figure). However, the duration of hospitalization was comparable in both groups (mean ± SD 135.4 ± 84.1 days with linezolid vs.120.5 ± 84.2 days without linezolid; p = 0.241) as was the case-fatality rate (p = 0.28). Different outcomes (e.g., successful treatment) were identified between those treated with linezolid versus those without linezolid (Table 4). Adverse effects ascribed to linezolid were observed in 25 (33.8%) of 74 cases (35% in cases with MDR-and 20% in cases with XDR TB). Linezolid was interrupted in 19 (76%) of 25 cases and not reintroduced in 11 (58%) of 19 cases. Severe anemia appeared in 14 (56%) of 25 cases.

Sixty-four (36.2%) of 177 MDR TB patients showed resistance to pyrazinamide, ethambutol, or both. These patients were less likely to achieve sputum-smear and culture conversion (49 [77%] of 64 vs. 93 [82%] of 113; p = 0.36) and were more frequently treated with linezolid (38 [60%] of 64 vs. 31 [27%] of 113; p = 0.00003). Thirty (47%) of them were successfully treated, 19 (30%) were still receiving treatment, one's treatment failed (2%), 10 (16%) were lost to follow-up, and 4 died (6%). Of 7 patients with XDR TB, 6 (86%) harbored strains that were resistant to pyrazinamide and ethambutol. Three (50%) of them achieved successful treatment outcome, 2 (33%) were still receiving treatment, and 1 (16.7%) died. These patients with XDR TB required longer hospitalization than those with MDR TB with resistance to pyrazinamide and ethambutol (mean ± SD 210.7 ± 140.1 vs. 132.5 ± 92.8 days; p = 0.063).

Discussion

We present the results of our national survey on clinical parameters associated with MDR and XDR TB in a Western European country. Of the patients hospitalized with MDR or XDR TB in Germany who were included in this survey, 53% were treated previously against TB, and nearly 90% of them had immigrated from FSU countries. Relatively high treatment success rates were achieved with conventional medical treatment, intensified medical care, including long-term inpatient treatment, directly observed therapy, and use of third-line anti-TB drugs. Less then 6% of patients with MDR TB required a surgical intervention.

In the German observational cohort, the proportion of MDR TB among all TB cases was 4%. Strains of *M. tuberculosis* in 7 (3.8%) of 184 patients with MDR TB met the case definition for XDR TB, an infection now recognized as a global problem (*10*). Alarming reports on the spread of XDR TB among HIV-seropositive persons have been published recently for Kwa Zulu Natal, South Africa (*12*). While HIV coinfection was not a risk factor for XDR TB in our cohort, XDR TB was related to previous treatment mismanagement including the lack of directly observed therapy in FSU countries.

Patients with XDR TB have a higher risk for death and treatment failure than those with MDR TB (*21*,*22*). In infections with MDR TB, drug resistance to additional first-line

Table 2. Logistic regression analysis of the association of treatment failure (death and failure) with potential explanatory factors*

Variables	Crude OR (95% CI)	Adjusted OR (95% CI)
Male gender	4.46 (0.56–35.5)	5.8 (0.61–56.6)
Age, y	1.05 (1.01–1.09)	1.06 (1.01–1.1)
Immigrant status	1.18 (0.13–10.2)	0.7 (0.07–6.6)
HIV seropositivity	5 (0.76–32.6)	2.5 (0.28–22.1)
Previous anti-TB treatment >30 d	0.7 (0.26–2.2)	0.4 (0.11–1.3)
Streptomycin resistance	1.35 (0.15–11.4)	1.18 (0.13–10.69)
Ethambutol resistance	1.74 (0.52–5.7)	0.99 (0.29–3.3)
Pyrazinamide resistance	1.65 (0.57–4.7)	1.08 (0.35–3.3)
Fluoroquinolone resistance	1.67 (0.32–8.6)	0.86 (0.09–7.7)
Resistance to injectable second-line drugs	1.16 (0.3–4.5)	1.28 (0.31–5.2)
Resistance to all second-line drugs	1.18 (0.34–3.9)	1.35 (0.37–4.8)

*OR, odds ratio; CI, confidence interval; TB, tuberculosis.

Table 3. TB treatment outcomes in study patients not previously treated for TB compared with those treated previously for TB*

Treatment outcomes	No. (%) patients not treated previously, n = 84	No. (%) patients treated previously, n = 100	p value	95% CI
Cured	40 (47.6)	42 (42)	0.49	–0.09 to 0.19
Completed	8 (9.5)	19 (19)	0.05	–0.19 to –0.001
Successful treatment (cured + completed)	48 (57)	61 (61)	0.58	–0.18 to 0.1
Died	7 (8.3)	8 (8)	1	–0.07 to 0.07
Failure	1 (1.19)	–	–	–
Treatment failure (death or failure)	8 (9)	8 (8)	0.8	–0.07 to 0.09
Default	–	1 (1)	–	–
Transferred out	13 (15.5)	12 (12)	0.55	–0.06 to 0.12
Uncertain outcome (default + transferred out)	13 (15.5)	13 (13)	0.69	–0.08 to 0.12
Still receiving treatment	15 (17.9)	18 (18)	0.85	–0.12 to 0.1

*TB, tuberculosis; CI, confidence interval.

drugs other than isoniazid and rifampin has recently been shown to be a predictor of negative treatment outcomes (*13*). Resistance to fluoroquinolones and injectable second-line drugs (capreomycin in particular) also contributes to increased risk for treatment failure and death in these cases (*23*,*24*). XDR TB–defining drugs are those considered essential to achieve successful outcomes in MDR TB cases (*9*,*17*,*24*,*25*). While rapid direct sensitivity testing of *M. tuberculosis* for all cases with a suspicion of multidrug resistance is highly important, this technology is currently not available in many geographic areas with a high incidence of MDR TB.

Our findings support the observation that treatment success in cases with MDR TB is dependent on the number of drugs the strain is resistant to and the previous treatment history. The probability to observe any TB drug resistance or MDR TB has been shown to be 4-fold and 10-fold higher when patients have received TB treatment in the past (*8*,*26*,*27*).

As expected, patients infected with strains of XDR TB and MDR TB resistant to all first-line drugs were more likely to have a poor treatment outcome than patients infected with other strains of multidrug-resistant *M. tuberculosis*. Patients with XDR TB required longer hospitalization and were less likely to achieve sputum-smear and culture conversion, although the latter result was not statistically significant.

More than 40% of patients in this cohort received off-label treatment against MDR or XDR TB with the oxazolidinone linezolid (*28*). In vitro and pharmacogenetic data suggest that oxazolidinones could be useful in management of mycobacterial infection, including MDR TB (*29*–*32*). However, clinical experience with the use of linezolid in the management of mycobacterial infections has been mainly restricted to case reports in nontuberculous mycobacterial diseases (*33*–*35*) and to a few case reports on patients with MDR TB (*28*,*36*,*37*). Cases of 24 patients with mycobacterial infections who were treated with linezolid were recently reviewed (*38*). Sterilization of mycobacterial cultures or resolution of symptoms was achieved in 15 (62.5%) of the 24 cases, although serious adverse events were observed in up to 75% of patients.

In this study, the description of 74 patients who were treated with linezolid against MDR or XDR TB in routine clinical practice substantially adds to the knowledge of the efficacy and tolerability of this drug. Drug toxicity from linezolid occurred in more than one third of patients and lead to treatment discontinuation in 76% of them. Patients who were treated with linezolid had a much higher level of drug resistance than those who were not treated with this drug, and they had a longer time to sputum-smear and culture conversion. Nevertheless, patients who were treated with a linezolid-containing regimen experienced a sustained culture conversion rate of almost 80%. Despite the fact that patients who were treated with linezolid had a much higher level of drug resistance, the mortality rate was comparable to that of patients with fewer drug resistances who were not treated with linezolid. Drug resistance to linezolid in cases never treated previously (occasionally reported) (*39*) was extremely low in this cohort (1/184 patients with MDR TB). These data suggest that a linezolid-containing combination treatment might be an effective option against MDR or XDR TB. However, the high frequency of adverse

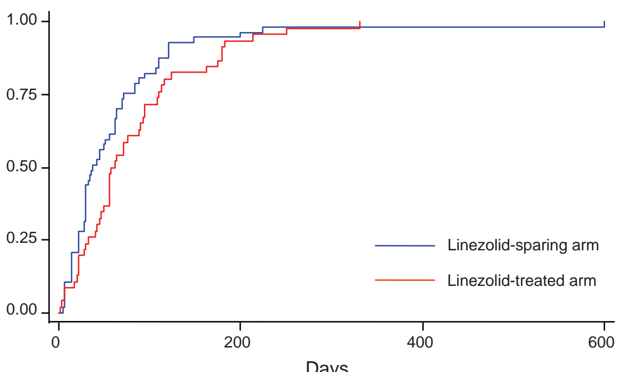

Figure. Kaplan-Meier plot showing the time to sputum smear conversion according to treatment received (linezolid-containing regimen, n = 74, vs. linezolid-sparing regimen, n = 110) in Germany (log-rank test 0.0924). The proportion of case-patients reaching conversion is shown along the vertical axis.

Table 4. TB treatment outcomes in study patients treated with linezolid compared with those not treated with linezolid*

Treatment outcomes	No. (%) patients treated with linezolid, n = 74	No. (%) patients not treated with linezolid, n = 110	p value	95% CI
Cured	21 (28.4)	61 (55.5)	0.0003†	–0.41 to –0.13
Completed	12 (16.2)	15 (13.6)	0.56	–0.07 to 0.13
Successful treatment (cured + completed)	33 (44.6)	76 (69.1)	0.0007†	–0.39 to –0.1
Died	8 (10.8)	7 (6.4)	0.31	–0.04 to 0.12
Failure	1 (1.4)	–	–	–
Treatment failure (death or failure)	9 (12.2)	7 (6.4)	0.15	–0.02 to 0.14
Default	–	1 (0.9)	–	–
Transferred out	10 (13.5)	15 (13.6)	0.98	–0.1 to 0.09
Uncertain outcome (default + transferred out)	10 (13.5)	16 (14.5)	0.84	–0.11 to 0.09
Still on treatment	22 (29.7)	11 (10)	0.0009†	0.07 to 0.3

*TB, tuberculosis; CI, confidence interval.
†Significant result ($p<0.05$).

effects to linezolid warrants extreme caution when this drug is used for a prolonged period. Further investigations are needed to determine the best duration and dosage of linezolid treatment if the drug is to be routinely used as a life-saving therapy in cases of MDR or XDR TB.

In this cohort, most patients with MDR TB for whom complete treatment data were available were treated for a 24-month period with a combination treatment of 4 or 5 effective drugs. Long-term inpatient care (mean ± SD 202 ± 130 days for XDR TB and 123.3 ± 81.0 days for MDR TB) and availability of all third-line drugs was necessary to achieve an overall treatment success rate of 59% (overall sample) to 87% (excluding patients still receiving treatment and lost to follow-up) in the German TBNET hospitals. The results are consistent with those of previous studies showing overall treatment success rates in MDR TB of 54% (*13*) and 62% (*40*).

The study has several limitations. Fourteen percent of patients were lost to follow-up by their hospital physicians. Their clinical outcome is uncertain. Complete data on previous treatment regimens were not available for most patients with recurrent TB who immigrated from FSU countries. Additional factors, including variability of provider treatment practices in the patients' native countries and existence of additional co-existing conditions, may have confounded the results of our analysis. The proportion of patients with strains of *M. tuberculosis* with more advanced drug resistance was higher among the 27 participating hospitals of the German TBNET than other hospitals in Germany, which are not specialized in TB treatment. Data for 35% of patients with MDR TB who were identified in Germany during the time of the survey were not available for this study as their cases were not diagnosed and treated in a hospital participating in this survey, which could have resulted in a selection bias. Nevertheless, the large and representative sample size, the availability of treatment outcomes, and the quality of laboratory data (all XDR TB–defining drugs tested and drug susceptibility tests controlled for quality) strengthen the results of this study.

In conclusion, cases of MDR and XDR TB in Germany appear to be largely restricted to immigrants from FSU countries. Previous treatment mismanagement is the probable cause of *M. tuberculosis* drug-resistance selection in most of these patients. Off-label treatment with linezolid is frequently used to treat advanced cases of MDR and XDR TB in Germany, despite high rates of adverse effects and paucity of clinical evidence for safety, tolerability, and efficacy of this medication. Relatively high sustained culture conversion rates can still be achieved in advanced cases of MDR and XDR TB; this requires high level, labor-intensive, and costly case management, including quality-controlled drug-susceptibility testing for all second-line drugs, long-term inpatient care, directly observed therapy, and availability of all third-line drugs. However, these resources are currently not available for patients with MDR or XDR TB in many other places outside Western Europe.

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Mixture for Controlling Insecticide-Resistant Malaria Vectors

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The spread of resistance to pyrethroids in the major Afrotropical malaria vectors *Anopheles gambiae* s.s. necessitates the development of new strategies to control resistant mosquito populations. To test the efficacy of nets treated with repellent and insecticide against susceptible and insecticide-resistant *An. gambiae* mosquito populations, we impregnated mosquito bed nets with an insect repellent mixed with a low dose of organophosphorous insecticide and tested them in a rice-growing area near Bobo-Dioulasso, Burkina Faso. During the first 2 weeks posttreatment, the mixture was as effective as deltamethrin alone and was more effective at killing *An. gambiae* that carried knockdown resistance (*kdr*) or insensitive acetylcholinesterase resistance (*Ace1^R*) genes. The mixture seemed to not kill more susceptible genotypes for the *kdr* or *Ace1^R* alleles. Mixing repellents and organophosphates on bed nets could be used to control insecticide-resistant malaria vectors if residual activity of the mixture is extended and safety is verified.

Pyrethroids are the only class of insecticides that are recommended by the World Health Organization (WHO) and the Centers for Disease Control and Prevention for net impregnation to control malaria transmission (*1*,*2*). Unfortunately, malaria vector resistance to pyrethroids is becoming widespread across Africa. Pyrethroid resistance mechanisms can be divided into 2 groups: metabolic (alterations in the expression levels of activities of detoxification enzymes) and target site (nonsilent point mutations within structural receptor genes, e.g., knockdown resistance [*kdr*] mutations) (*3*). Whether the spread of resistance genes will pose a serious threat to vector control programs that are based uniquely on pyrethroid use is uncertain. Some studies have shown that *kdr* resistance does not decrease the level of protection conferred by insecticide-treated nets (ITNs) (*4*) and that ITNs do not induce *kdr* selection (*5*). Conversely, more recent studies have reported a fitness advantage for *kdr*-resistant phenotypes (*6*) and decreased efficacy of ITNs in an area of pyrethroid resistance in Benin (*7*). The need for alternative insecticidal molecules is becoming increasingly clear; however, fewer novel active ingredients are available and the timeframe needed to satisfy the regulatory requirements of public health formulations is exceedingly long (*8*). Thus, the only option for managing insecticide resistance in malaria vectors is optimal use of existing compounds. Two such tactics have already been explored: 1) the alternating use of different classes of insecticides by rotation of active ingredients and mosaic treatments (*9*,*10*), and 2) the use of insecticide mixtures (*10*,*11*).

We tested the ability of existing ingredients, a mixture of insect repellents and nonpyrethroid insecticides, to achieve vector death and excito-repellency (irritancy when mosquito contacts net and repellent activity in air) (*12*). The rationale behind this concept is that nonpyrethroid compounds can mimic the original features of pyrethroids, i.e., lethality and irritancy. Laboratory results showed that a combination of propoxur and diethyl-3-methylbenzamide (DEET) induced irritancy, knockdown, and death rates as

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high as those from deltamethrin against a susceptible strain of *Aedes aegypti* and significantly higher death rates against a pyrethroid-resistant strain of this mosquito. Such findings were mostly explained by a strong synergistic interaction (in terms of death and knockdown effect) between DEET and propoxur (*12*). Similar synergism was also observed in a test tunnel apparatus when DEET or another insect repellent (hydroxyethyl isobutyl piperidine carboxylate [also known as icaridin or KBR 3023]) was mixed with an organophosphate (OP; pirimiphos methyl [PM]) on mosquito nets baited with guinea pigs (*13*). These studies constitute a first step toward the development of an alternative strategy based on insect repellents for malaria vector control in areas of pyrethroid resistance.

We present results of the first evaluation of this new strategy under field conditions. The objective of the trial was to compare the efficacy of mosquito nets impregnated with mixtures of DEET+PM or KBR 3023+PM (repellent and insecticide–treated nets [RITNs]) with bed nets treated with a standard formulation of a pyrethroid (deltamethrin). The field trial was carried out in an area where *Anopheles gambiae* s.s. populations are either susceptible or resistant to pyrethroids, OPs, and carbamates, depending on the season. Seasonal influence results from temporal fluctuations in the relative frequency of the 2 molecular forms of *An. gambiae*, Mopti (M) and Savannah (S), which in this area of Burkina Faso carry insecticide-resistant alleles at contrasting frequencies (*14*,*15*). In particular, the S form carries the *kdr* and the insensitive acetylcholinesterase resistance ($Ace1^R$) alleles at high frequency, whereas these alleles are carried at much lower frequency in the M form. This article describes the response of vector populations to the lethal effect of the formulations tested. Moreover, we investigated whether RITNs could select for the insecticide-resistance genes.

Methods

Study Area

The field trial was carried out during May–June and September–October 2006 in the village identified in this study as VK7, in the valley of the Kou River, near Bobo-Dioulasso, in southwestern Burkina Faso. The area is used by farmers for large-scale cultivation of rice. Throughout most of the year, rice paddies provide extensive sites for mosquito breeding, particularly of the molecular M form of *An. gambiae* s.s. Conversely, the molecular S form of this malaria vector appears mainly during the wet season, because these mosquitoes breed mostly in puddles created by rains and in other rain-dependent larval habitats.

Insecticidal and Repellent Formulations

An OP insecticide and 2 insect-repellent formulations were evaluated as mixtures impregnated on mosquito nets. For our OP, we used Pirigrain 250 (Compagnie Générale des Insecticides, Cergy Pontoise, France), an emulsifiable concentrate formulation containing 25% PM. Our repellents were KBR 3023 and DEET. KBR 3023 was formulated as a liquid concentrate containing 25% of active ingredient. DEET was also formulated as a liquid concentrate containing 30% of active ingredient. The 2 repellent formulations are designed and distributed for application on skin by Osler (Melun, France). Deltamethrin was our pyrethroid of choice because it is one of the 2 standard pyrethroids used for net impregnation with permethrin. The water-dispersible tablets of deltamethrin were safe according WHO risk assessment and have undergone the WHO Pesticide Evaluation Scheme (*16*). For this trial, we used a standard suspension concentrate at 20% deltamethrin (Kothrin; Bayer Crop Science, Monheim am Rhein, Germany), which is routinely used to impregnate bed nets. No toxic or repellent chemicals other than those mentioned above were declared in the formulations tested.

Mosquito Nets and Treatments

We used nets made of 100-denier polyester with a mesh size of 156 threads/square inch. To simulate the conditions of bed-net wear and tear that can be encountered in the field, 6 holes, 4 × 4 cm each, were cut on the sides and ends of each net. Three groups of nets were created: 1) nets impregnated with the repellent DEET or KBR 3023 at a dose of 10 g/m² and the insecticide PM at a dose of 150 mg/m², 2) positive-control nets dipped into standard pyrethroid deltamethrin at a dose of 25 mg/m², and 3) negative-control nets not treated.

Experimental Huts, Volunteer Participants, and Mosquito Collections

The treated nets were set inside 4 experimental huts, according to the design and procedures described by Darriet et al. (*17*) and N'Guessan et al. (*18*). The 3.5 × 2 × 2 m huts were built with local materials and designed with 4 entry baffles that enabled mosquitoes to fly into the hut but then hindered their escape from the hut. This design enabled us to account for most mosquitoes. A veranda trap made of polyethylene sheeting and mesh screening (2 m long × 1.5 m wide × 1.5 m high) projected from the back wall of each hut. Movement of mosquitoes between the huts and the verandas was unimpeded during the night. Each hut rested on a concrete base surrounded by a water-filled moat to prevent entry of ants that would otherwise eat mosquitoes knocked down on the floor of the hut.

Local adult male volunteers were recruited to sleep on mats under the nets. They provided informed consent before enrollment. They received malaria chemoprophylaxis and medical surveillance during and 3 weeks after

the trial. The Institut de Recherche pour le Développement and Burkina Faso national ethical committees formally approved the ethics of the protocol.

At 6:00 PM, before the start of the tests, the volunteers removed spiders and other mosquito predators. They then slept from 8:00 PM to 5:00 AM, at which time they closed the entry baffles; lowered the curtain separating the sleeping room from the veranda-trap; and collected all mosquitoes, dead and alive, from the room, bed net, and veranda. Female mosquitoes were scored by location as dead or alive, fed or unfed; species was identified according to morphologic characteristics. To minimize bias related to mosquito attractiveness of each volunteer and spatial variation in mosquito densities, the volunteers and bed nets were rotated between huts each day. The trial was run twice, each time for 27 nights over 4 weeks. The first trial was conducted during the dry season (May 5 to June 3), when mainly the molecular M form of *An. gambiae* is present in the village; the second, during the rainy season (September 18 to October 14), when the S form predominates.

Molecular Analyses

To determine the presence and relative frequency of the molecular M and S forms of *An. gambiae* s.s., we extracted genomic DNA from field-collected mosquitoes and amplified it by PCR according to the method of Favia et al. (*19*). The methods of Martinez-Torrez et al. (*20*) and Weill et al. (*21*) were used for molecular detection of the *kdr* and $Ace1^R$ alleles, respectively, in individual mosquitoes collected, alive or dead, from the control hut. Genotypes between live and dead mosquitoes were differentiated by using the exact test of Goudet et al. (*22*) and the software GENEPOP (*23*).

Statistical Analysis

The effect of each treatment relative to the control was expressed in terms of the overall mosquito mortality rate ([no. immediately dead + no. dead after 24 hours]/overall no.). We considered mortality rate to have the most significant epidemiologic effect. For statistical purposes, we fitted a logistic regression model, assuming a binomial error distribution with regression parameters calculated by maximum likelihood with the software GLIM v.4 (*24*); we used the number of dead mosquitoes (y) as response variable, and the total number (n) of mosquitoes collected in the hut as binomial denominator. The proportion of dead mosquitoes ($p = y/n$) was related to time (in days) posttreatment, insecticidal treatment, and season. The statistical significance of main effects and interaction terms in the model was tested with F-tests by analysis of deviance, which involved looking at the change in deviance caused by the removal of each term from the maximal model after having allowed for overdispersion in the data by calculating a variance heterogeneity coefficient with the Williams algorithm (*25,26*). Median effective times (ET_{50}) were calculated with the minimal model that better fits the data. Confidence limits for ET_{50} were calculated by using the Fieller theorem (*25,26*).

Results

Vector Population and Insecticide Resistance

Molecular analysis showed a marked seasonal change in molecular form composition and insecticide resistance status (Table 1). During the dry season trial, the molecular S form accounted for 5% of the *An. gambiae* s.s. population, whereas during the rainy season it represented 85%. Accordingly, the *kdr* allele, which confers resistance to pyrethroids, was found at a frequency of 8% in the *An. gambiae* s.s. sample during the dry season trial and at 88% at the end of the rainy season. Similarly, the frequency of the $Ace1^R$ allele, which confers resistance to OPs and carbamates, increased from 1% at the end of the dry season to 40% during the rainy season. The change in frequency of the insecticide resistance genes reflects the fact that these genes are carried at high frequency only in the molecular S form of *An. gambiae*.

Efficacy of Repellent-plus-OP Mixtures versus Deltamethrin

The analysis of deviance showed that the 3-way interaction term between time, treatment, and season was statistically significant ($F_{n,m} = 4.705$; $p = 0.01$), which indicates that the decrease in lethal effect over time was significantly different for treatments and between seasons. Hence, the minimal adequate model was that with a different curve relating the decrease in deaths with days posttreatment for each combination of treatments and seasons (Figures 1, 2). Accordingly, the estimates of the regression parameters for the 6 logistic curves are shown in Table 2, together with the inferred effective times in days posttreatment.

During the dry season trial, lethality of the PM+ KBR 3023 mixture lasted longer than Kothrin over the first 15 days posttreatment ($ET_{90}^{PM + KBR} = 11.1$ days vs. $ET_{90}^{Kothrin}$

Table 1. Frequency of molecular forms and alleles in *Anopheles gambiae* mosquitos, southwestern Burkina Faso*

Season	S form frequency/no. tested	*kdr* frequency/no. tested	$Ace1^R$ frequency/no. tested
May–June (dry season)	0.05/43	0.08/41	0.01/40
September–October (rainy season)	0.85/49	0.88/48	0.40/49

*Mosquito samples were randomly taken from a control (untreated) hut; S form, Savannah form; *kdr*, knockdown resistance allele; $Ace1^R$, insensitive acetylcholinesterase resistance allele.

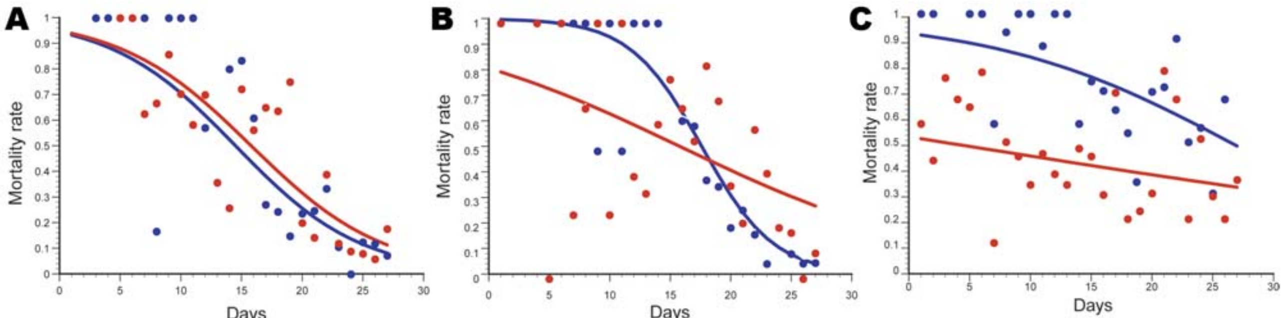

Figure 1. Comparative efficacy of repellent and insecticide–treated nets during dry (blue) and rainy (red) seasons. A) PM+DEET–treated nets; B) PM+KBR–treated nets; C) Kothrin–treated nets in each of 2 seasons. PM, pirimiphos methyl; DEET, diethyl-3-methylbenzamide; KBR, hydroxyethyl isobutyl piperidine carboxylate; Kothrin, 20% deltamethrin (Bayer Crop Science, Monheim am Rhein, Germany).

= 4.9 days; Figure 2, panel A; Table 2). The PM+DEET mixture was as efficacious as Kothrin over only ≈4 days (Figure 2, panel A). The efficacy of the nets impregnated with the 2 mixtures decreased faster ($b^{PM + KBR} = -0.337 \pm 0.032$; $b^{PM + DEET} = -0.194 \pm 0.039$) than those impregnated with Kothrin ($b^{Kothrin} = -0.099 \pm 0.048$) (Figure 2, panel A). This explains the shorter median effective time of the 2 mixtures.

The trend was different for the rainy season trial, because the lethality of nets impregnated with Kothrin was consistently lower than that of the dry season trial (Figure 1, panel C), in view of the change in molecular form composition and insecticide resistance status of the vector population (Table 1). Efficacies of the PM+DEET and PM+KBR 3023 were significantly higher than those for the deltamethrin formulation against the *An. gambiae* population carrying high frequencies of the *kdr* and *Ace1*^{R} genes. Indeed, Kothrin never induced a mortality rate >55% throughout the course of the trial, whereas the PM+DEET and PM+KBR 3023 mixtures killed ≥90% of the exposed mosquitoes until ≈4 and ≈7 days posttreatment, respectively (Table 2; Figure 2, panel B).

Model estimates of the PM+DEET mixture did not differ between the 2 trials (Student *t* test $t_a = 0.248$, p = 0.8; $t_b = 0.101$, p = 0.92) (Figure 1, panel A), which indicates that the response in mortality rate over time was the same across seasons. Conversely, the PM+KBR 3023 efficacy changed significantly across the 2 trials ($t_a = 3.34$, p<0.01; $t_b = 2.01$, p<0.05); induced deaths were lower during the rainy season shortly after impregnation of the nets, but the decrease in efficacy over time was subsequently slower (Figure 2, panel A). Similarly, a significant difference in efficacy between the 2 seasons was observed for Kothrin ($t_a = 2.55$, p<0.05; $t_b = 4.06$, p<0.005); lethality was much lower during the rainy season than during the dry season; lethality of the nets, however, was always higher during the dry season trial up until the end of the 27-day replicate trials, despite a slower decrease in efficacy over time for the rainy season trial (Figure 1, panel C).

Effect of Treatments on Insecticide-Resistance Genotypes

A total of 192 *An. gambiae* females were genotyped for molecular form status, *kdr* and *Ace1*^{R} genes. Because of severe restrictions in gene flow between the M and S molecular forms (*27,28*), which led to marked differences in their resistance status (*15*), we chose to investigate the selection pressure of our 3 treatments against only the S molecular form of *An. gambiae* (88% of the total population collected during the rainy season, Table 1). The results of *kdr* genotyping of 152 specimens are shown in Table 3. The gene was in Hardy-Weinberg equilibrium (p = 1). No evidence of a significantly higher frequency of *kdr* allele was found in those that survived the 3 treatments. No S/S (susceptible homozygote) or R/S (susceptible heterozygoye) mosquito survived with the Kothrin treatments in contrast with PM+DEET and PM+KBR treatments, but susceptible genotypes were too rare to conclude about the effect of treatments on *kdr* selection pressure. The results of the *Ace1*^{R} genotyping of the 153 S form of *An. gambiae* are shown in Table 3. We observed a heterozygote excess for *Ace1*^{R} gene ($\chi^2 = 85.3$, degrees of freedom = 8, p<0.001). No evidence of a significantly higher frequency of *Ace1*^{R} allele was found in those that survived the 3 treatments.

Discussion

Our results demonstrated that a mixture of an OP (PM) and an insect repellent (either DEET or KBR 3023) on mosquito nets in an area of insecticide resistance near Bobo-Dioulasso, Burkina Faso, was as lethal as the pyrethroid deltamethrin alone for a few days against susceptible *An. gambiae* s.s. mosquitoes. However, the efficacy of each mixture was substantially higher than that of deltamethrin against a multilocus-resistant *An. gambiae* population of mosquitoes (mainly composed of the molecular S form) carrying 2 resistance genes for pyrethroids and OPs/carbamates (*kdr* and *Ace1*^{R}, respectively), at moderate to high frequency. The efficacy of the mixtures was due to strong synergism between the 2 active ingredients, as

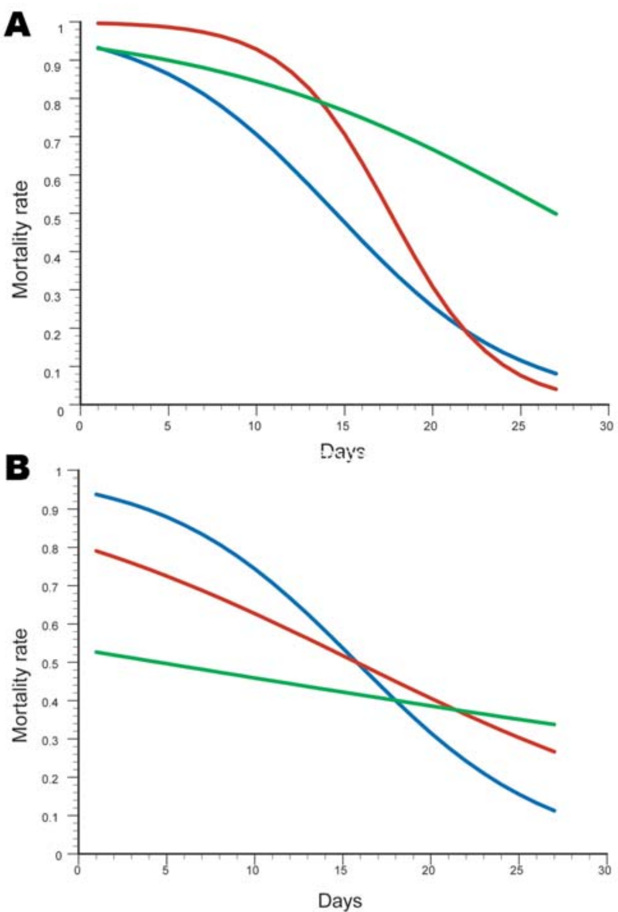

Figure 2. Comparative efficacy of repellent and insecticide–treated nets during A) first trial in dry season and B) second trial in rainy season. Blue lines indicate PM+DEET–treated nets; red lines indicate PM+KBR–treated nets, and green lines indicate Kothrin–treated nets. Curves drawn according to logistic plane regression of equation parameters, which are shown in Table 2. PM, pirimiphos methyl; DEET, diethyl-3-methylbenzamide; KBR, hydroxyethyl isobutyl piperidine carboxylate; Kothrin, 20% deltamethrin (Bayer Crop Science, Monheim am Rhein, Germany).

demonstrated in another study (C. Pennetier et al., unpub. data). Some OPs like chlorpyriphos methyl (*11*) PM (*29*) and the carbamate carbosulfan (*29,30*) were also recently tested on nets to verify their efficacy in terms of induced deaths against pyrethroid-resistant populations of *An. gambiae* mosquitoes and were found to be as lethal as deltamethrin, lambda cyalothrin, or permethrin. The major constraint to the use of OPs or carbamates on bed nets is their higher toxicity for humans (*9*,*31*) and the possibility that they might induce selection pressure for resistance mechanisms other than *kdr*, such *Ace1*^{R} (*32*). In view of these results, the concept of mixing an insect repellent with an OP offers a potential alternative to the use of pyrethroids on mosquito nets.

Mixtures of insect repellents and OPs have several advantages. First, the addition of a repellent enables use of lower OP dosages. The recommended dose of PM to achieve an ≈100% mortality rate is 1,000 mg/m² (*29,33*), 6-fold the dosage that we used in our mixtures. The possibility of using insecticides with different modes of action at lower dosages than either ingredient used alone was also observed in previous studies with OP/pyrethroid mixtures (*10*,*11*). Second, the behavioral effects of pyrethroids on mosquito nets, such as irritancy (which inhibits blood feeding), that confer personal protection to the sleeper under the net are restored by the presence of the repellent in RITNs. Previous laboratory studies on repellent-plus-OP mixtures have shown that the mixtures have the same irritant effect as pyrethroids (*12*) and that they induce protection against blood feeding (*13*). Our field trial confirmed the excito-repellency of the repellent-plus-OP mixtures (C. Pennetier et al., unpub. data). Third, we could not detect statistically significant differences in the frequency of 2 important insecticide-resistance genes, *kdr* and *Ace1*^{R}, among mosquitoes that survived or died after exposure to RITNs. This finding indicates that PM+DEET and PM+KBR 3023 would not select for the *Ace1*^{R} allele. Unfortunately, the high *kdr* frequency among *An. gambiae* mosquitoes did not allow us to conclude anything about the effect of RITNs on *kdr* selection pressure. RITNs should now be evaluated in an area where *kdr* allelic frequency among *An. gambiae* is moderate. Nevertheless, RITNs appear to be a promising tool for controlling malaria vectors in areas of insecticide resistance.

Our results show that mosquito deaths in response to treated nets changed between seasons, depending on the combination of repellent and insecticide used. The response to the PM+DEET mixture did not change with the resistance status of the *An. gambiae* mosquitoes, whereas the efficacy of PM+KBR 3023 decreased significantly at the end of the rainy season but lasted comparatively longer than during the dry season trial. This difference may result from a difference in mode of action of the 2 insect repellents used and their interaction with the insecticide PM. Indeed, PM is an acetylcholinesterase inhibitor, and DEET has recently been shown to exert a neurotoxic effect through alteration of neuronal function and synaptic transmission (*34*). Indeed, through elevation of intracellular calcium concentration and inhibition of the acetylcholinesterase, DEET increases the release of acetylcholine in the synaptic cleft (*34*). That led to us to hypothesize a synergism between the OP and DEET resulting from the implication of presynaptic muscarinic receptors involved in the negative-feedback regulation process (*35*), which thereby modulate acetylcholine release. Because the exact mode of action of KBR 3023 is not yet known, it is probably premature to propose an explication for why its efficacy changed in response to changes in the resistance status of the vector population.

Table 2. Regression parameters (standard errors) and median and 90% effective duration of effectiveness of antimalarial vector treatments*

Treatment	a	b	ET_{50}, d (95% CI)	ET_{90}, d
First trial				
PM+DEET	2.814 (±0.737)	–0.194 (±0.039)	14.5 (11.1–16.4)	3.2
PM+KBR	5.932 (±0.628)	–0.337 (±0.032)	17.6 (16.8–18.2)	11.1
Kothrin	2.693 (±0.656)	–0.100 (±0.033)	26.8 (22.7–42.2)	4.9
Second trial				
PM+DEET	2.907 (±0.520)	–0.184 (±0.030)	15.8 (13.8–17.7)	3.9
PM+KBR	1.424 (±0.657)	–0.090 (±0.036)	15.9 (5.4–22.7)	–8.6
Kothrin	0.136 (±0.320)	–0.030 (±0.019)	4.5 (0–13.9)	–68.1

*First trial run in dry season (May and June); second trial run in rainy season (September and October). a, intercept; b, slope of curve; ET_{50} and ET_{90}, median and 90%, respectively, effective time of the minimal adequate regression model fitted to the experimental hut data; CI, confidence interval; PM, pirimiphos methyl; DEET, diethyl-3-methylbenzamide; KBR, hydroxyethyl isobutyl piperidine carboxylate; Kothrin, 20% deltamethrin (Bayer Crop Science, Monheim am Rhein, Germany).

Use of RITNs in community-based vector control programs is not yet practical because of the short persistence of the lethal effect induced by the repellent-plus-OP mixture (1–2 weeks, depending on season and combination). This effect presumably results from the high vapor pressure of the repellents, which act mainly in the vapor phase and hence do not persist long enough on the net at higher than threshold concentrations. Of note, the residual killing effect activity of RITNs in the field is much lower than that found in our previous laboratory study (*13*), probably the result of different storage conditions. In the laboratory, nets were stored in aluminium paper, which may have slowed evaporation of the active ingredient; in the present study, RITNs stayed all the day in experimental huts. However, long-lasting formulations, such as resins, microcapsules, and cyclodextrins, might increase the persistence of the mixture on the net. We suggest that industry has a vital role to play in the development of such formulations. We are currently testing a microencapsulated formulation of DEET+PM; preliminary results are encouraging (data not shown).

Another factor preventing the immediate application of RITNs in the field is the lack of knowledge of the toxic properties of repellant-plus-OP mixtures. Despite the fact that the 2 repellents and PM are reported as safe products (*36–40*), little is known about the interaction of repellents with OPs. We used an acetylcholinesterase inhibitor with DEET, but none of our compounds was applied on the skin. The contact between the user and the active ingredients on the bed net surface would be limited compared with a skin application, and the DEET concentration we used on nets was >3-fold lower than that recommended (30% of DEET active ingredient in commercial lotions). Nevertheless, because a mixture of chemicals must be considered as a new chemical, assessing the risk of using repellent plus OP at the operational doses used to impregnate bed nets is crucial.

In summary, application of low doses of an OP plus insect repellents as mixtures on mosquito nets was as much or more lethal shortly after application than application of the pyrethroid deltamethrin against the malaria vector *An. gambiae* in an area of resistance to multiple insecticides. The recent concept of combining repellents with insecticides is still limited by the short residual effect of the treatments and the lack of toxicologic knowledge. However, this combination appears to be a potential tool warranting further development for the control of vectors and management of insecticide resistance in malaria-endemic areas.

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Table 3. Comparative frequencies of 2 resistance genes between mosquitoes after exposure to treated nets*

	kdr frequency/no. tested				$Ace1^R$ frequency/no. tested			
Treatment	Surviving mosquitoes	Dead mosquitoes	p value	Total no. tested	Surviving mosquitoes	Dead mosquitoes	p value	Total no. tested
PM+DEET	0.93/22	0.98/27	0.32	49	0.50/23	0.45/28	0.51	51
PM+KBR	0.89/23	0.96/28	0.22	51	0.43/23	0.46/26	0.74	49
Kothrin	1.00/33	0.95/19	0.13	52	0.44/34	0.39/19	0.49	53

**kdr*, knockdown resistance allele; $Ace1^R$, insensitive acetylcholinesterase resistance allele; PM, pirimiphos methyl; DEET, diethyl-3-methylbenzamide; KBR, hydroxyethyl isobutyl piperidine carboxylate; Kothrin, 20% deltamethrin (Bayer Crop Science, Monheim am Rhein, Germany).

identifying alternative chemicals or new strategies to maintain the effectiveness of impregnated materials used in vector control programs.

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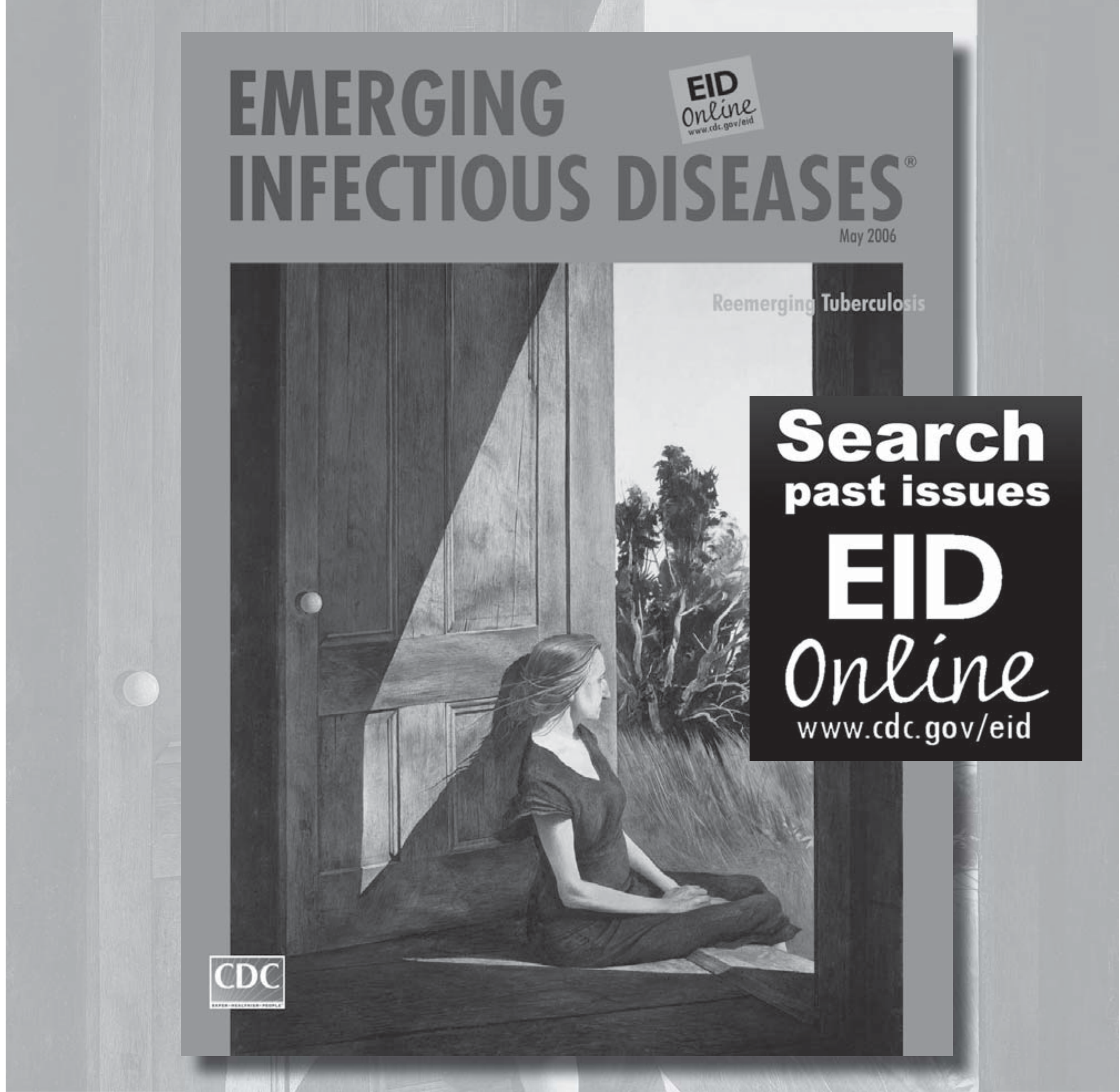

Multidrug-Resistant Tuberculosis Outbreak among US-bound Hmong Refugees, Thailand, 2005

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In January 2005, tuberculosis (TB), including multidrug-resistant TB (MDR TB), was reported among Hmong refugees who were living in or had recently immigrated to the United States from a camp in Thailand. We investigated TB and drug resistance, enhanced TB screenings, and expanded treatment capacity in the camp. In February 2005, 272 patients with TB (24 MDR TB) remained in the camp. Among 17 MDR TB patients interviewed, 13 were found to be linked socially. Of 23 MDR TB isolates genotyped, 20 were similar according to 3 molecular typing methods. Before enhanced screening was implemented, 46 TB cases (6 MDR TB) were diagnosed in the United States among 9,455 resettled refugees. After enhanced screening had begun, only 4 TB cases (1 MDR TB), were found among 5,705 resettled refugees. An MDR TB outbreak among US-bound refugees led to importation of disease; enhanced pre-immigration TB screening and treatment decreased subsequent importation.

Globally, 9 million new cases of tuberculosis (TB) were reported in 2004 (*1*), ≈4.3% of which were multidrug-resistant TB (MDR TB) (*2*). MDR TB, defined as infection with *Mycobacterium tuberculosis* resistant to at least isoniazid and rifampin, complicates TB control efforts because it requires prolonged treatment with drugs that are less potent, more costly, and more toxic than traditional isoniazid- and rifampin-based regimens (*3*,*4*). These factors challenge implementation of effective TB control programs, particularly in developing countries.

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Currently, 56% of TB cases in the United States occur in foreign-born persons (*5*), and rates are highest among recently arrived immigrants (*6*,*7*). Refugee populations are particularly vulnerable to TB and drug-resistant TB (*8*–*12*). Annually, 50,000–70,000 refugees immigrate to the United States (*13*); before entry, they must undergo standard pre-immigration screening for TB. Despite screening, however, refugee populations have exhibited high TB incidence rates shortly after arrival in the United States (*14*–*16*). One contributor to high postarrival TB rates is the low sensitivity of the current pre-immigration TB screening algorithm, which has been estimated to identify <35% of all TB cases (*17*).

In December 2003, the US Department of State announced a refugee resettlement program for ≈16,000 Hmong refugees from Laos, who had been living in a temporary camp for displaced persons in Lopburi, Thailand, for >10 years. The first refugees arrived in the United States in June 2004; by January 2005, ≈10,000 had immigrated to the United States. Initial reports, after health assessments of newly arrived refugees, identified 37 TB cases, 4 of which were MDR TB (*18*). Simultaneously, cases of MDR TB were confirmed in Thailand in refugees awaiting resettlement. Immigration to the United States was temporarily halted in January 2005, while the US Centers for Disease Control and Prevention (CDC) and international partners investigated the factors that led to emergence and dissemination of TB, including MDR TB, among these refugees. Results from the investigation guided implementation of enhanced TB screening and treatment for the ≈6,000 refugees remaining in the camp in Thailand.

Materials and Methods

This investigation was deemed an urgent public health response. Under the federal regulation for the protection of human research participants, Code of Federal Regulations Title 45, part 46, this investigation was determined by CDC to not be human subject research.

Case Definition and Case Detection

Cases were defined by positive sputum smears or cultures or by a physician's decision to initiate TB treatment in the context of radiographic abnormalities and clinical features consistent with TB. From April 2004 through January 2005, pre-immigration TB screening in the camp detected TB cases among the refugees. The TB screening algorithm used initially consisted of a medical history, a physical examination, and, for applicants ≥15 years of age, a chest radiograph. Persons whose clinical or radiographic findings suggested TB disease submitted 3 sputum specimens for acid-fast bacilli smear microscopy. Limited laboratory capacity was available, and mycobacterial culture was performed on sputum samples that were smear positive for acid-fast bacilli. Refugees with smear-positive results were allowed to travel to the United States after they had begun anti-TB treatment and had smear-negative results for 3 follow-up sputum specimens. In July 2004, after a site visit by CDC and because of concerns about potential high prevalence of TB, including drug-resistant TB, the pre-immigration screening algorithm was expanded to include mycobacterial culture and drug-susceptibility testing for both smear-negative and smear-positive specimens. From February 2005 (after the TB outbreak was detected) through April 2007, suspected TB cases were also identified through contact tracing.

Patient Interviews

Hmong interpreters interviewed TB patients in the camp, using a 24-item questionnaire about history of previous TB diagnosis and treatment. When asked about previous TB treatment, participants were shown anti-TB medications and asked if they had ever taken any of the displayed pills in the past. Patient responses were stratified by drug-susceptibility testing results. To assess the possibility of recent MDR TB transmission in the camp, MDR TB patients were asked additional questions regarding social links to other known MDR TB patients. A strong link was defined as sharing a household or having contact with another MDR TB patient at least 1×/week. A weak link was defined as having contact with another MDR TB patient <1×/week.

M. tuberculosis Genotyping

Available *M .tuberculosis* isolates were genotyped by spoligotyping (*19*,*20*), mycobacterial interspersed repetitive units (MIRUs) (*21*,*22*), and IS*6110* restriction fragment length polymorphism (RFLP) analysis (*23*). A cluster was defined as ≥2 *M. tuberculosis* isolates that had identical spoligotyping and MIRU results and for which RFLP results were identical or differed by only 1 band. To assess whether the proportion of MDR TB isolates that clustered was greater than that among pansusceptible isolates, we used the Fisher exact test with a significance level of 0.05.

Mapping Patient Households

To assess geographic clustering of cases, we used global positioning system (GPS) technology to map TB patient households in the camp according to drug-susceptibility testing results. GPS data were analyzed with a spatial scan statistic that uses a varying-sized cylinder to encapsulate cases within the radius of the cylinders and calculates a p value and log likelihood ratio to determine the statistical significance of any clusters that may be detected (*24*).

Tuberculin Skin Testing

Refugees received tuberculin skin tests (TSTs) to evaluate latent TB infection. Induration ≥5 mm was considered a positive test result (*25*). To assess recent transmission in the camp, we summarized and compared TST results for 3 categories of contacts: 1) a housemate or family member of a TB patient with at least 1 sputum smear-positive result for acid-fast bacilli; 2) a housemate or family member of a TB patient with only sputum smear-negative results; or 3) not a housemate or family member of a TB patient. Using those who were not a housemate or family member of a TB patient as the referent group, we calculated prevalence ratios and 95% confidence intervals (CIs) for each exposure group.

Results

From April 2004 through January 2005, TB was diagnosed for 272 of the 15,455 refugees screened (Table 1). All 272 persons with a TB diagnosis were tested for HIV infection; only 1 was infected. Sputum-smear acid-fast bacilli results were available for 247 TB patients; 34 (13.8%) were positive. Culture results were available for 242 TB patients; 57 (23.6%) patients had positive culture results for *M. tuberculosis*.

Drug-susceptibility testing found that 24 (42.1%) isolates were pansusceptible, 24 (42.1%) were MDR TB, and 9 (15.8%) were resistant to ≥1 anti-TB medications but were not MDR TB. Drug resistance was found in 9 patterns, 4 of which were MDR TB. Several additional resistance patterns were noted among the MDR TB isolates: streptomycin (n = 4); streptomycin and ethambutol (n = 15); streptomycin and pyrazinamide (n = 2); streptomycin, ethambutol, and pyrazinamide (n = 3). Of the 24 MDR TB patients, 15 (62.5%) had positive sputum smear results.

Table 1. Demographic and disease characteristics among Hmong refugees with tuberculosis, Thailand, February 2005*

Characteristic	No. (%)
Total	272 (100)
Sex	
F	112 (41.2)
M	160 (58.8)
Age, y	
<15	21 (7.7)
15–64	153 (56.3)
≥65	98 (36.0)
Culture results	
Positive	57 (21.0)
Negative	185 (68.0)
Unknown	30 (11.0)
DST results	
MDR TB	24 (8.8)
Other patterns	9 (3.3)
Pansusceptible	24 (8.8)
Smear results	
Ever positive	34 (12.5)
Always negative	213 (78.3)
Unknown	25 (9.2)

*DST, *Mycobacterium tuberculosis* drug-susceptibility test results; MDR TB, multidrug-resistant tuberculosis.

Patient Interviews

Of 272 TB patients, 241 (88.6%) were interviewed. Treatment for TB before pre-immigration screenings began in April 2004 was reported by 15 (6.2%); none had received directly observed therapy (DOT). Of the 15 previously treated patients, 3 (20.0%) had a current diagnosis of MDR TB, 1 (6.7%) had TB with isoniazid resistance only, 1 (7.0%) had TB that was pansusceptible, and 10 (66.7%) had received clinical diagnoses without culture confirmation. All 3 MDR TB patients who had a history of previous treatment had received their treatment during the 3 years before their current diagnosis.

After pre-immigration TB screenings started, some camp residents visited healthcare providers outside the camp to seek treatment for conditions that might have precluded their passing the pre-immigration medical examination. Chest radiograph screenings were obtained outside the camp by 39 (16.2%) patients during the period between when medical screening began at the camp and when their own pre-immigration screening was scheduled. Of these, 32 (82.1%) took TB medications during this time; only 9 (28.1%) reported that they were told by a doctor or nurse that they had TB. Among the remaining 23 patients not reporting a diagnosis of TB, 1 (4.3%) had MDR TB, 1 (4.3%) had streptomycin-resistant TB, 2 (8.7%) had pansusceptible TB, and the remaining 19 (82.6%) were diagnosed clinically without culture confirmation.

Of the 24 MDR TB patients, 17 (70.8%) responded to the questions regarding social links to other MDR TB patients (Figure 1.) Among these, 9 (52.9%) reported having at least 1 strong link with another MDR TB patient, and 4 MDR TB patients (23.5%) reported having at least 2 strong links with another MDR TB patient. One patient who had sputum smear-positive TB (Figure 1, patient 11) was central to a social network that linked 13 (76.5%) patients. The 3 MDR TB patients that had been previously treated for TB (Figure 1, patients 1, 2, and 3,) were all directly linked to patient 11 and included in the 13-patient network.

TB Genotyping

Genotyping results were available for 46 (80.7%) of the 57 culture-confirmed cases. Of these, 30 (65.2%) belonged to 1 of 5 clusters (clusters A–E), which ranged from 2 to 20 matching isolates. The remaining 16 (34.8%) isolates were unique (Table 2). The largest culture, cluster C, had 20 cases, all of which were MDR TB; they represented 87.0% of the 23 MDR TB isolates with genotyping results. The cluster C spoligotype pattern was 000000000003771, and the MIRU pattern was 223325173533. Isolates in cluster C had a 21-band RFLP result. Of the MDR TB isolates that were not included in cluster C, 2 had spoligotype and RFLP results that matched those included in cluster C, but according to MIRU results, they differed at 1 locus. The third MDR TB isolate not included in cluster C differed according to both MIRU and RFLP results. MDR TB isolates were more likely than non–MDR TB isolates to cluster (Fisher exact p = 0.02).

Among the 17 MDR TB patients who responded to the questions regarding social links to other MDR TB patients (Figure 1), 15 (88.2%) were included in cluster C. Isolates from patients 5 and 12 matched cluster C according to spoligotype and RFLP results but differed at 1 locus according to MIRU results.

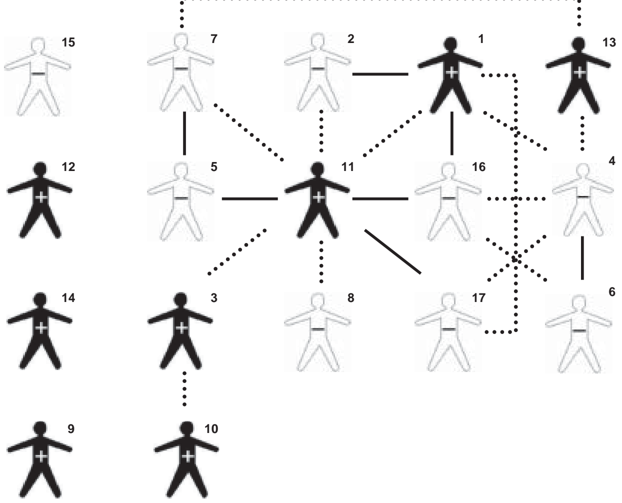

Figure 1. Social links between Hmong refugees with multidrug-resistant tuberculosis, Thailand, February 2005. Numerals indicate patients, in order of diagnosis. +, smear positive; –, smear negative; dotted lines, weak link; solid lines, strong link.

Table 2. *Mycobacterium tuberculosis* drug-susceptibility test results, by genotype, Hmong refugee tuberculosis patients, Thailand, February 2005*

Genotype cluster	Isolate results			
	Some resistance	MDR TB	Pansusceptible	Total
Unique isolates	5	3	8	16
A	0	0	3	3
B	0	0	2	2
C	0	20	0	20
D	0	0	2	2
E	1	0	2	3
Total	6	23	17	46

*MDR TB, multidrug-resistant tuberculosis.

As of 2007, genotyping results were available for 7 additional patients from this camp who had received a diagnosis of MDR TB after arrival in the United States (California). According to spoligotyping and MIRU results, isolates collected from 6 of these patients matched the cluster C strain. The seventh isolate had the same spoligotype result but differed at 1 locus according to MIRU.

Patient Household Maps

TB patients were widely distributed throughout the camp living quarters (≈0.5 km^2) (Figure 2). The spatial analysis showed 3 nonsignificant spatial clusters (p>0.05), which suggests no significant geographic clustering of TB patient households, either among all patients or among subsets with similar drug-susceptibility testing patterns.

TST Results

Of the 5,637 camp residents that had TSTs performed and results read, 1,624 (28.8%) had positive results. Among those who were family members or housemates of a camp patient with a sputum smear-positive TB diagnosis, 96 (44.0%) had positive TST results. These contacts were 1.6× (95% CI 1.4–1.9) as likely as the referent group to have a positive result. There was an increased risk (not statistically significant) for a positive result associated with being a family member or housemate of a patient with sputum smear–negative disease (relative risk = 1.1, 95% CI 1.0–1.2). After controlling for patients' sputum smear status, we found that household contacts of patients with MDR TB, pansusceptible TB, and TB with some drug resistance had the same risk for a positive TST result.

Modifications and Enhancements to Pre-immigration TB Screening and Treatment

After the investigation in February 2005, recommendations for pre-immigration TB screening and treatment for Hmong refugees in Thailand were again modified and enhanced (Table 3). These modifications required that all refugees ≥6 months of age be screened with chest radiography. Persons with suspected TB had 3 sputum specimens collected for smear microscopy, culture, and drug-susceptibility testing with rapid, liquid-based methods. All patients were required to show documentation of having received DOT for the duration of their TB treatment before they were permitted to travel to the United States. In addition, a TB culture laboratory was built within the camp, mechanisms were developed to import quality-assured second-line TB medications, and nurses and physicians in the camp received advanced training in TB diagnosis and treatment. Finally, although TSTs were performed for all camp residents during this investigation, the enhanced TB screening algorithm required that only those 6 months to 10 years of age receive TST.

After implementation of the final enhanced TB screening and treatment requirements, 97 additional TB cases, including 2 MDR TB, were diagnosed in Thailand, resulting in an overall total of 369 TB cases. As of April 2007, health departments in the United States reported 46 cases of TB, 6 of which were MDR TB, among 9,455 Hmong refugees who immigrated to the United States before implementation of enhanced screening (487 cases/100,000 persons). In

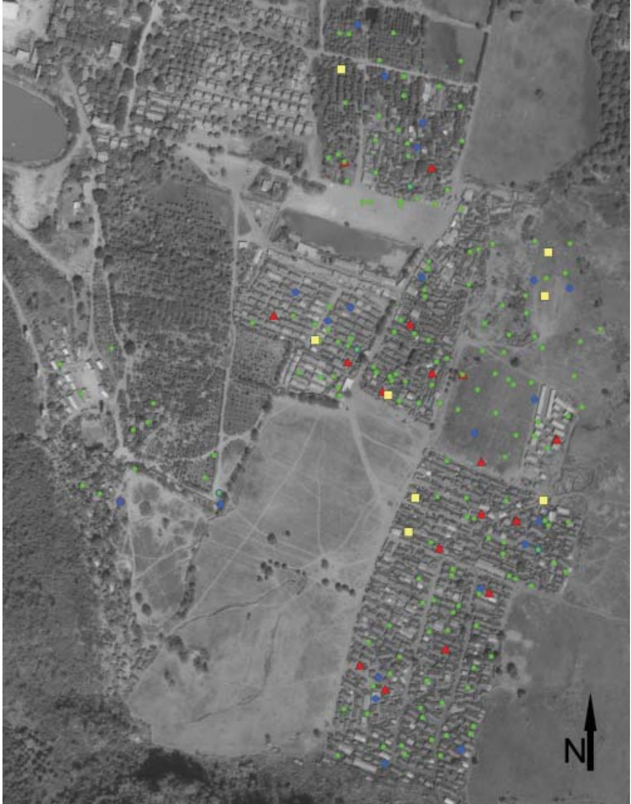

Figure 2. Locations of dwellings within camp for Hmong refugees with tuberculosis (TB), Thailand, February 2005. Symbols indicate dwellings of patients with the following types of TB: red triangles, multidrug-resistant; yellow squares, resistant to ≥1 anti-TB medications but not MDR TB; blue circles, pansusceptible; green circles, unknown drug-susceptibility testing results.

Table 3. Summary of tuberculosis screening algorithm components for Hmong refugees, by date, Thailand*

Date	Medical history	Physical examination	TST	CXR		Sputum smear	Culture	Drug-susceptibility testing
				1 view	2 views			
Apr–Jun 2004	Yes	Yes	No	Yes, for those ≥15 years of age	No	Yes, if CXR indicated possible TB	Yes, if sputum-smear positive for AFB	No
Jul 2004–Jan 2005	Yes	Yes	No	Yes, for those ≥15 years of age	No	Yes, if CXR indicated possible TB	Yes, regardless of sputum-smear status	Yes
Feb 2005–Apr 2007	Yes	Yes	Yes, for those 6 mo to 10 y of age	Yes, for those >10 years of age	Yes, for those 6 mo to 10 y of age	Yes, if CXR indicated possible TB	Yes, regardless of sputum-smear status	Yes

*TST, tuberculin skin test; CXR, chest radiograph; TB, tuberculosis; AFB, acid-fast bacilli.

contrast, 4 cases of TB, 1 of which was MDR TB, have been diagnosed in the United States among the 5,705 Hmong refugees who immigrated after implementation of enhanced screening (70 cases/100,000 persons). The proportion of cases diagnosed in the United States after the enhanced screening was significantly lower than the proportion diagnosed before the enhanced screening (Fisher exact test $p<0.001$).

Discussion

An outbreak of MDR TB occurred among a population in which TB rates were already elevated; as a result, TB (some MDR TB) was imported into the United States. Several lines of evidence support the conclusion that this was an outbreak. First, 13 (76.5%) of 17 MDR TB patients interviewed reported having had recent and regular exposure to another MDR TB patient. Although GPS did not demonstrate geographic clustering, lack of clustering is not unexpected because the camp was small and its population density was high, making social networks, rather than absolute physical distance between dwellings, the most important facilitator of TB transmission. Second, 20 (87.0%) of 23 MDR TB isolates were strains that matched by 3 different molecular subtyping methods. It is possible that 2 additional isolates (22 total) were part of the outbreak as well because they matched the outbreak strain according to spoligotyping and RFLP results and differed by only 1 locus according to MIRU; therefore, this difference may represent a change in genotype in the same clone over time (*26*). Third, elevated rates of tuberculin reactivity in household contacts of smear-positive patients suggest substantial recent transmission, not simply endemic disease. As rates of tuberculin reactivity were elevated in household contacts of all smear-positive patients, transmission of pansusceptible TB, TB with drug-resistance but not MDR TB, and MDR TB likely occurred in the camp before and during the initial resettlement of refugees to the United States in 2004. Rates of TST positivity were not presumed to be inflated by vaccination with *M. bovis* BCG, because there was no indication or documentation of BCG vaccination among this group of refugees.

Delayed recognition of TB transmission in the camp had several negative consequences: increased number and severity of cases among refugees and importation of TB, including MDR TB, into the United States. In addition, the costs incurred by the US government were substantial and resulted from halting immigration (e.g., cancellation of flights, emergency overseas investigations, and program enhancements), public health investigations in the United States and Thailand, and medical costs of diagnosis and treatment. Because half of the cases of MDR TB were sputum smear–negative for acid-fast bacilli, had sputum smear microscopy continued to be used as the only tool for initial screening, the outbreak of MDR TB would likely have gone undetected and many more MDR TB cases would have been imported into the United States.

Hmong refugees who had a positive TST result did not receive treatment for latent TB infection before immigrating to the United States because they were not contagious and thus were eligible to travel on commercial airplanes. No universally accepted standard therapy is available for latent TB infection with a suspected MDR TB strain. Rather, therapy for suspected latent infection from MDR TB is determined on a case-by-case basis in accordance with drug-susceptibility testing results from the putative source. After arrival in the United States, the receiving local health department jurisdictions decided how to evaluate, reevaluate, and treat patients with latent TB infection. Those who had no known contact with an MDR TB patient were treated with isoniazid; those who had had contact with an MDR TB patient, either overseas or in California, were treated with an MDR TB contact treatment regimen tailored to the source case isolate's susceptibility pattern. Most often, they were treated with fluoroquinolone and pyrazinamide because TB isolates from Hmong refugees were resistant to isoniazid, rifampin, and ethambutol. If a patient refused

medication for latent TB infection, that patient was closely clinically monitored for 2 years.

This outbreak led to major changes in public health practice for this refugee group and in future pre-immigration medical screening policies. Enhancements to pre-immigration TB screening (Table 3) contributed to a reduction in the number of imported TB cases. As a result, CDC is working with the US Department of State, panel physicians, the International Organization for Migration, and other organizations to implement similar enhancements to general pre-immigration TB screening guidelines. These new technical instructions are being implemented first in priority countries, as determined by immigration patterns and TB prevalence. Eventually, all refugees and immigrants entering the United States will be screened with a revised TB screening algorithm that includes mycobacterial culture and susceptibility testing. Since the end of 2007, applicants for US immigration who have been screened according to the new technical instructions have originated from Mexico, the Philippines, Nepal, and Thailand. CDC notifies US state and local health departments when panel physicians in a specific country begin implementing this revised algorithm. Ongoing US national TB surveillance will help determine the effect of this effort on reducing the number of foreign-born persons with TB living in the United States. In this outbreak investigation, 3 MDR TB patients were identified who had received treatment in the past 3 years at healthcare facilities outside the refugee camp; however, none reported having received DOT, the strategy recommended for reducing emergence of drug resistance (*27*). Our findings support the goals of the World Health Organization's second Global Plan to Stop TB, which includes refugees as a high-risk group requiring attention by TB control programs. This outbreak also highlights the need for US public health preparedness efforts to focus on containment of threats of emergent diseases, such as MDR TB, at their source (*28*).

To control TB and prevent MDR TB, multiple organizations—including government agencies, multilateral agencies, and nongovernment organizations—must work together to provide high-quality TB diagnosis and treatment consistent with international standards of care in both host and receiving countries (*29*). For low-incidence countries, such as the United States, investing in global TB control is a cost-effective strategy for reducing TB, domestically and globally (*30*,*31*).

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Dr Oeltmann is an epidemiologist in the Division of Tuberculosis Elimination, Centers for Disease Control and Prevention. His research interests include molecular epidemiology, MDR TB, TB/HIV, and examining the effectiveness of methods used during TB contact and outbreak investigations.

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Antimicrobial Drug Use and Resistance in Europe

Nienke van de Sande-Bruinsma, Hajo Grundmann, Didier Verloo, Edine Tiemersma, Jos Monen, Herman Goossens, Matus Ferech, and the European Antimicrobial Resistance Surveillance System and European Surveillance of Antimicrobial Consumption Project Groups[1]

CME ACTIVITY

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Learning Objectives

Upon completion of this activity, participants will be able to:

- Identify the classes of antimicrobial drugs most commonly used in Europe.
- Describe patterns of antimicrobial drug use across regions in Europe.
- Identify the most widely used antimicrobial drugs by country in Europe.
- List European countries that show the highest antimicrobial drug resistance proportions.
- Describe the association between antimicrobial drug use and the emergence of resistance.

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Our study confronts the use of antimicrobial agents in ambulatory care with the resistance trends of 2 major pathogens, *Streptococcus pneumoniae* and *Escherichia coli,* in 21 European countries in 2000–2005 and explores whether the notion that antimicrobial drug use determines resistance can be supported by surveillance data at national aggregation levels. The data obtained from the European Surveillance of Antimicrobial Consumption and the European Antimicrobial Resistance Surveillance System suggest that variation of consumption coincides with the occurrence of resistance at the country level. Linear regression analysis showed that the association between antimicrobial drug use and resistance was specific and robust for 2 of 3 compound pathogen combinations, stable over time, but not sensitive enough to explain all of the observed variations. Ecologic studies based on routine surveillance data indicate a relation between use and resistance and support interventions designed to reduce antimicrobial drug consumption at a national level in Europe.

For the past 60 years, antimicrobial chemotherapy has been the mainstay of medical intervention against infectious diseases caused by bacterial pathogens. The continuous decline of therapeutic effectiveness as a result of extensive use of antimicrobial chemotherapy has been long predicted and seems inescapable (*1*). Many surveillance efforts have over the last decade (1997–2007) drawn attention to this phenomenon (*2*–*5*). At the same time, the once-abundant supply of new and improved antimicrobial compounds has worn thin, as

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drug development becomes increasingly challenging and pharmaceutical companies invest in more lucrative markets (*6*). It is therefore critical to realize that antimicrobial drug effectiveness, widely accepted as a common good, cannot be taken for granted and that such substances are increasingly attaining the status of nonrenewable resources.

Our study confronts the population-adjusted use of antimicrobial agents in ambulatory care with the resistance trends of 3 compound pathogen combinations in 21 European countries over a period of 6 years (2000–2005). This initial study was made possible by combining data from the 2 most comprehensive European surveillance systems on antimicrobial drug consumption and resistance, the European Surveillance of Antimicrobial Consumption (ESAC) (*7*) and the European Antimicrobial Resistance Surveillance System (EARSS) (*8*). We present an authoritative joint analysis of these 2 comprehensive databases. At this highly aggregated level, data are not sensitive enough to unravel the complex interaction between prescribing and resistance. The goal of this study is to give an overview of the situation in the European region and explore whether a relationship between antimicrobial drug use and resistance can be supported by empirical data pooled at national levels.

[1]National representatives of these 2 project groups in 2005 were as follows: Austria: H. Mittermayer, S. Metz, W. Koller (European Antimicrobial Resistance Surveillance System [EARSS]); Belgium: E. Hendrickx (EARSS), H. Goossens; Bulgaria: B. Markova; Croatia: A. Tambic-Andrasevic, Igor Francetic (European Surveillance of Antimicrobial Consumption [ESAC]), S. Kalenic (EARSS); Cyprus: D. Bagatzouni (EARSS); Czech Republic: P. Dvorak (ESAC), P. Urbaskova (EARSS); Denmark: D. Monnet, A. Anker Nielsen (ESAC); Estonia: P. Naaber (EARSS); Finland: P. Huovinen (ESAC), P. Paakkari (ESAC), O. Lyytikainen (EARSS), A. Nissinen (EARSS); France: P. Maugendre (ESAC), D. Guillemot (ESAC), B. Coignard, (EARSS), V. Jarlier (EARSS); Germany: W. Kern (ESAC), H. Schroeder (ESAC), W. Witte (EARSS), K. Heckenbach (EARSS); Greece: H. Giamarellou (ESAC), A. Antoniadou (ESAC), A. Tsakris (EARSS), A. Vatopoulos (EARSS); Hungary: G. Ternak (ESAC), M. Fuzi (EARSS); Iceland: K. Kristinsson; Ireland: E. Smyth (ESAC), R. Cunney (ESAC), D. Igoe (EARSS), O. Murphy (EARSS); Israel: R. Raz (EARSS); Italy: G. Cornaglia, A. Pantosti (EARSS), P. D'Ancona (EARSS); Latvia: S. Berzina (ESAC), A. Balode (EARSS); Lithuania: R. Valenteliene (ESAC), J. Miciulevicience; Luxembourg: R. Hemmer, M. Bruch (ESAC); Malta: M. Borg, P. Zarb (ESAC); the Netherlands: R. Janknegt (ESAC), M. Filius (ESAC), H. de Neeling (EARSS), E. Tiemermsa, J Degener (EARSS); Norway: H. Salvesen Blix (ESAC), A. Hoiby (EARSS), G. Simonsen (EARSS); Poland: W. Hryniewicz, P. Grzesiowski; Portugal: L. Caldeira (ESAC), M. Canica (EARSS); Romania: I. Codita; Slovakia: V. Foltan (ESAC), T. Tesar (ESAC), L. Langsadl (EARSS); Slovenia: M. Cizman (ESAC), M. Mueller-Premru (EARSS), J. Kolman (EARSS); Spain: J. Campos, F. Baquero (EARSS); Sweden: O. Cars (ESAC), G. Skoog (ESAC), B. Liljequist (EARSS), G. Kahlmeter (EARSS); Turkey: S. Unal (ESAC), D. Gür (EARSS); United Kingdom: P. Davey (ESAC), A. Johnson (EARSS), R. Hill (EARSS), H. Hughes (EARSS), M. Coyne (EARSS).

Materials and Methods

Consumption of Antimicrobial Agents

ESAC collects data on antimicrobial drug use in ambulatory care and hospital care in Europe. Currently, 24 countries report data on ambulatory care consumption to ESAC (*9*). Prescribed drugs are grouped by the active substance as the number of defined daily doses (DDD) per 1,000 inhabitants (DID) according to the World Health Organization definition of Anatomical Therapeutic Chemical Classification (ATC) defined daily dose (ATC-DDD version 2005 (*10*). A complete description of the data providers and details of the methods used by ESAC have been published (*7*,*11*,*12*). The performance and methodologic approach of the ESAC system, which aimed to collect comparable and reliable data on antimicrobial drug use, were studied by Vander Stichele et al. (*7*). The collected data were screened for bias caused by errors in assigning medicinal product packages to the ATC; errors in calculations of DDD per package; bias by over-the-counter sales and parallel trade; and bias in ambulatory care/hospital care mix. The study indicated that of the 31 participating countries, 21 delivered ambulatory care data suitable for cross-national comparison (*7*).

For the present study, the total country-specific antimicrobial drug use in ambulatory care and a breakdown into the following major antimicrobial classes were extracted from the ESAC database: penicillins (J01C); other β-lactam antimicrobial agents (cephalosporins, monobactams and carbapenems, J01D); macrolides, lincosamines, and streptogramins (MLS-class, J01F); and fluoroquinolones (J01MA).

Resistance to Antimicrobial Agents

EARSS performs continuous surveillance of antimicrobial drug susceptibility for 7 major bacterial pathogens that cause invasive infections. Data are provided by >900 microbiologic laboratories that serve ≈1,400 hospitals from 32 countries with an overall hospital catchment population estimated to include >100 million inhabitants (*13*). All EARSS participating laboratories perform routine antimicrobial drug susceptibility tests according to standard protocols (*14*) and interpret their susceptibility results according to harmonized national and international guidelines as sensitive, intermediately resistant, and resistant (*15*). More details about the data acquisition and analysis have been published elsewhere (*13*,*16*,*17*). The antimicrobial susceptibility test (AST) results reported by the laboratories are collected by using standardized protocols as described in the EARSS manual (www.rivm.nl/earss). Data that do not meet the requirements of these species-specific protocols are not

accepted. To assess the comparability of results between laboratories participating in EARSS, an external quality assessment exercise is organized every year. A set of 6 strains is provided to each laboratory in collaboration with the UK National External Quality Assurance Scheme. These exercises illustrate that routinely reported results, as collected by EARSS, have sufficient accuracy to provide good estimates of overall resistance prevalences and trends (*18*).

For the present study, AST results of primary blood culture isolates of *Escherichia coli* and *Streptococcus pneumoniae* were extracted from the EARSS database to determine the proportions of penicillin- and erythromycin-nonsusceptible *S. pneumoniae* (PNSP and ENSP, respectively) and proportions of fluoroquinolone-resistant *E. coli* (FQRE) bacteria. Nonsusceptible isolates included both intermediate resistant and resistant isolates. A country-specific resistance score was calculated as the sum of the quartile ranks of resistance against all 3 compound pathogen combinations (PNSP, ENSP, and FQRE). For trend analysis of resistance proportions per country over time, the Cochrane-Armitage trend test was used.

Ecologic Analysis

The strength of association between antimicrobial drug use and resistance was determined by univariate and multiple linear regression analysis. The proportion of resistance (R) in a country was transformed to the natural logarithm of the odds of resistance (ln[R/1–R]), to get a range from –∞ to +∞. The log odds of resistance (as the dependent variable) can then be expressed as a simple linear function of the independent variable (consumption) (*19*,*20*). To give equal weight to small countries with flawless data collection and not give the unequal weight to larger countries with sometimes less-optimal data, the linear regression analysis was not weighed.

To determine the delay between antimicrobial use and resistance, proportions of PNSP, ENSP, and FQRE for 2002–2005 were correlated with the consumption of different antimicrobial drug classes in the same year and the 2 years before. This resulted in 11 different exposure-outcome intervals for each compound–pathogen combination. For further multivariate analysis, the interval with the median correlation coefficient was regarded as representative for the association found in the overall study period.

Only the countries that reported volumes of antimicrobial drug prescriptions in ambulatory care from 2000 through 2004 and susceptibility data for the selected compound–pathogen combinations from 2002 through 2005 were included for linear regression analysis. Countries that provided yearly susceptibility data for <20 isolates were excluded. Data analysis was conducted by using SAS version 9.1 software (SAS Institute Inc., Cary, NC, USA).

Results

Consumption of Antimicrobial Agents

We included in the study 21 European countries, which provided data on the use of antimicrobial agents in ambulatory care to the ESAC database for the period 2000–2004 (including the 15 long-standing European Union (EU) member states). These also included 3 of the 10 nations that joined the EU in May 2004, the Czech Republic, Slovakia, and Slovenia; 2 applicant countries, Bulgaria and Croatia; and 1 European Free Trade Association country, Iceland. Total outpatient antimicrobial drug use differed significantly between countries. Use tends to be low in northern, moderate in central, and high in southern Europe and varied by a factor of 3.4 between Greece (33.4 DID) and the Netherlands (9.7 DID) in 2004 (Figure 1, Table 1).

During the observation period (2000–2004), antimicrobial drug use decreased (≥15%) in Bulgaria, Czech Republic, France, and Germany and increased (≥15%) in Croatia, Denmark, Greece, and Ireland. Penicillins (including broad-spectrum penicillins, ATC category J01C) represented the most widely used antimicrobial class in Europe. This class showed consumption patterns similar to the total outpatient antimicrobial drug use, as did the second most widely used category, which consists mainly of macrolides but also includes lincosamines and streptogramins (MLS class, ATC category J01F). The third most widely used ATC category (J01D, other β-lactams) consists of cephalosporins, monobactams, and carbapenems. Cephalosporins make up the bulk of the antimicrobial agents included in this

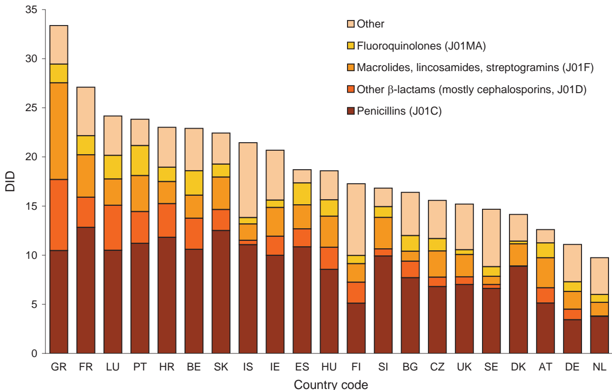

Figure 1. Total antimicrobial drug consumption in ambulatory care in defined daily doses per 1,000 inhabitants per day (DID) by antimicrobial class in 21 European countries in 2004. See Table 1 footnote for country designations.

Table 1. Difference in outpatient antimicrobial drug use DID in 21 European countries, 2004, and changes in use, 2000–2004*†

Substance class (ATC category)	Antimicrobial use, DIDs, 2004			Changes in antimicrobial drug use, 2000–2004	
	Maximum (country)	Minimum (country)	fd	≥15% increase	≥15% decrease
Total use (J01)	33.4 (GR)	9.7 (NL)	3.4	HR, DK, GR, IE	BG, CZ, DE, FR
Penicillins (J01C)	12.8 (FR)	3.4 (DE)	3.8	HU, DK	CZ, FR, DE, SK
Cephalosporins, monobactams, carbapenems (J01D)	7.2 (GR)	0.05 (NL)	>100	SI	BE, BG, CZ, FR, IS NL, ES, SE
Macrolides, lincosamines, streptogramins (J01F)	9.9 (GR)	0.8 (BG)	12.4	BG, HR, GR, IE, NL	BE, FR, DE, LU, ES
Fluoroquinolones (J01MA)	3.04 (PT)	0.28 (DK)	10.9	AT, BG, CZ, DK, FI, DE, HU, IE, LU, UK	SI

*DID, defined daily dose/1,000 inhabitants; ATC, Anatomic Therapeutic Chemical classification; fd, factor difference.
†Country designations: AT, Austria; BE, Belgium; BG, Bulgaria; CZ, Czech Republic; DE, Germany; DK, Denmark; ES, Spain; FI, Finland; FR, France; GR, Greece; HR, Croatia; HU, Hungary; IE, Ireland; LU, Luxembourg; NL, the Netherlands; PT, Portugal; SE, Sweden; SI, Slovenia; SK, Slovakia; UK, United Kingdom.

group. Antimicrobial agents belonging to this category are more commonly used in hospitals; however, in some countries they are also extensively prescribed in ambulatory care. For this reason, use rates in Europe varied >100-fold between countries. The use of this ATC category decreased by ≥15% in 8 countries but increased in Slovenia. Fluoroquinolones hold the fourth position in the European market but showed the most dynamic increase, with growth rates of ≥15% in almost half of all countries (10/21). In terms of overall control of antimicrobial drug consumption, France most consistently reduced its use of 3 of the 4 most frequently prescribed antimicrobial drug classes (Figure 1, Table 1).

Resistance to Antimicrobial Agents

Large differences in the proportions of resistance were reported for the same countries. The highest antimicrobial drug resistance was found in Spain, Hungary, and France and the lowest in Sweden and the Netherlands in 2005 (Figure 2). Resistance proportions in 2005 differed by a factor of 27.7 for PNSP between France (36%) and the Netherlands (1.3%), by 20.5 for ENSP between France (41%) and the Czech Republic (2%), and by 9.7 for fluoroquinolone resistance in *E. coli* between Portugal (29%) and Iceland (3%). From 2001 through 2005, resistance levels remained relatively stable for PNSP but increased for the other 2 compound pathogen combinations (Table 2). Spain and the United Kingdom were the only countries that reported any significant decrease in antimicrobial drug resistance rates. In Spain, penicillin nonsusceptibility fell from 37% to 25% and in the United Kingdom, from 5% to 3.8%. For ENSP a significant increase was observed in Hungary (from 19% to 37%), Finland (from 12% to 20%), and the Netherlands (5% to 11%). The most consistent trend was observed for fluoroquinolone resistance in *E. coli*, which increased in most European countries (Table 2).

Combining Antimicrobial Drug Use with Susceptibility Data

Greece (33.0 DID), France (27.1 DID), Luxembourg (24.2 DID), Portugal (23.8 DID), Croatia (23.0 DID), and Belgium (22.9 DID) were the countries that reported the highest use of antimicrobial agents in ambulatory care. Four of these high-consumer countries—France, Luxemburg, Belgium, and Portugal—were also among the 6 countries with the highest resistance proportions. Croatia occupied an intermediate resistance rank, owing to more modest levels in fluoroquinolone resistance. For Greece, susceptibility data for *S. pneumoniae* were not available, which precluded a meaningful ranking. Although Spain (18.7 DID) and Hungary

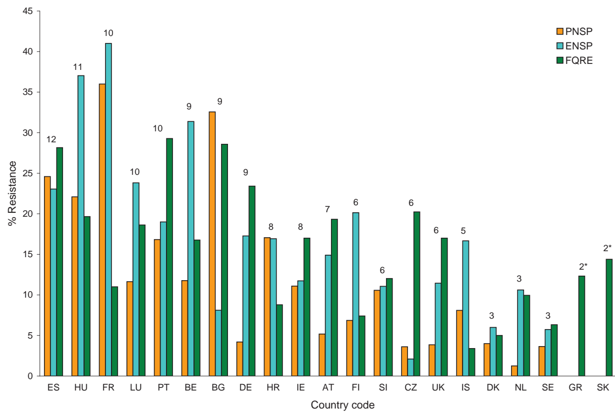

Figure 2. Proportion of penicillin-nonsusceptible *Streptococcus pneumoniae* (PNSP), erythromycin-nonsusceptible *S. pneumoniae* (ENSP), and fluoroquinolone-resistant *Escherichia coli* (FQRE) in 2005, ranked in descending order by country-specific resistance score indicated above bars. *For Greece and Slovakia, data on *S. pneumoniae* resistance were not available. Country (total no. of *S. pneumoniae* isolates reported/ total no. of *E. coli* isolates reported): ES (740/2993); HU (86/468); FR (632/6028); LU (43/188); PT (202/1086); BE (1539/1461); BG (43/196); DE (119/957); HR (129/637); IE (397/1411); AT (290/2049); FI (525/1743); SI (208/657); CZ (194/2233); UK (1373/2359); IS (37/117); DK (1081/1283); NL (802/2140); SE (1017/3035); GR (0/1136); SK (0/132). See Table 1 footnote for country designations.

Table 2. Differences in the proportion of antimicrobial drug resistance in 21 European countries, 2005, and significant trends, 2001–2005

Compound-pathogen†	Antimicrobial drug resistance				
	Europe, %, 2005			Trends, 2001–2005*	
	Maximum (country)	Minimum (country)	fd‡	Increase (p<0.05)	Decrease (p<0.05)
PNSP	36 (FR)	1.3 (NL)	27.7	BG	ES, UK
ENSP	41 (FR)	2 (CZ)	20.5	FI, HU, NL	
FQRE	29 (PT)	3 (IS)	9.7	AT, BE, BG, CZ, DE, ES, FI, HR, HU, LU, NL, PT, SE	

*No trend analysis was performed for Denmark and France, and for Ireland and the United Kingdom for proportion of *Escherichia coli* resistant to fluoroquinolones (FQRE), because data were not available for all years of the study period (2001–2005). See Table 1 footnote for country designations.
†PNSP, proportion of *Streptococcus pneumoniae* not susceptible to penicillin; ENSP, erythromycin-nonsusceptible *S. pneumoniae*.
‡fd, factor difference.

(18.6 DID) were not among the countries with the highest use of antimicrobial agents, both countries did have the highest antimicrobial drug resistance proportions in 2005. The United Kingdom (15.2 DID), Sweden (15 DID), Denmark (14.1 DID), Austria (12.5 DID), Germany (11 DID), and the Netherlands (10 DID) reported the lowest antimicrobial drug use in outpatient settings. Of these, Sweden, the Netherlands, Denmark, and the United Kingdom also were among the 6 countries with the lowest resistance proportions. Germany and Austria reported medium to high rates especially for ENSP (17% and 15%, respectively) and FQRE (23% and 19%, respectively) (Figures 1, 2). Because inspection of the data suggested a relation between antimicrobial drug consumption and resistance, this assumption was formally tested by using simple linear regression.

Because little is known about the delay that can be expected between the change in antimicrobial drug exposure and its effect on antimicrobial resistance at a population level, different intervals were chosen to explore the potential association between use and resistance. Intervals were explored for same-year data, a 1-year delay, and a 2-year delay between exposure and outcome. Thus, the consumption data available for 2000 through 2004 and resistance data for 2002–2005 provided the means to explore the correlation coefficients of 11 exposure-outcome intervals. Only the 17 countries that provided data for all years were included in the linear regression analysis. Table 3 shows the range and median correlation coefficient for all exposure-outcome intervals. Since no statistically significant time dependence was observed, the median correlation coefficient was regarded as representative for the association found for the entire study period (Table 3).

The occurrence of PNSP in European countries correlated with the country-specific use of penicillins, which explained 61% of the observed variance ($p \leq 0.01$) (Figure 3). The second best correlation was provided by the total antimicrobial drug use in ambulatory care, which explained 46% of the observed variance ($p \leq 0.01$). Both associations were robust and remained significant, regardless of the interval between the ascertainment of antimicrobial drug use and the recording of antimicrobial resistance. A notably less consistent association was found when we correlated the use of MLS-class antimicrobial agents or fluoroquinolones with the occurrence of PNSP (Table 3). ENSP occurrence in Europe correlated most compellingly with the country-specific use rate of ATC category J01D (other β-lactams), which explained 48% of the observed variance ($p \leq 0.01$) (Table 3). However, this effect appeared to be confounded by the use of MLS-class antimicrobial agents and fluoroquinolones. By fitting use data for these antimicrobial agents into the model, the effect estimates for the former decreased by 40% (Table 4), indicating that part of the effect attributed to the use of other β-lactam antimicrobial agents appeared to be exerted by MLS-class antimicrobial agents and fluoroquinolones.

Proportions of FQRE in European countries were best explained by the country-specific use data for fluoroquinolones. Fluoroquinolone consumption as reported to the ESAC network explained 36% of the variance observed in EARSS data ($p \leq 0.01$; Figure 4). This effect appeared to be specific and was not associated or confounded by consumption of the other antimicrobial classes.

Discussion

We compared the trends in antimicrobial drug consumption patterns and the antimicrobial drug resistance proportions for 2 major pathogens, *S. pneumoniae* and *E. coli,* in Europe from 2000 through 2005. Antimicrobial drug use in outpatient settings was ascertained by the most comprehensive network for European surveillance of antimicrobial consumption (ESAC), and antimicrobial resistance data were obtained from the European surveillance system (EARSS). The data suggested that in Europe the variation of consumption coincides with the occurrence of resistance at country level. Using simple linear regression analysis, we formally explored whether a relation between country-specific antimicrobial drug use and antimicrobial resistance can be inferred at na-

Table 3. Range and median correlation between the occurrence (logodds) of PNSP, ENSP, and FQRE in 2002–2005 and antimicrobial drug consumption, Europe, 2000–2004*

E consumption	O-resistance phenotype	No. E–O intervals with significant association†	Correlation coefficients (*r*) Median *r* (CI)	Median E–O year	Minimum *r* (CI)	Minimum E–O year	Maximum *r* (CI)	Maximum E–O year
Total use (J01)	PNSP	11	0.68 (0.30–0.87)	2003–2003	0.61 (0.17–0.84)	2001–2003	0.73 (0.39–0.90)	2002–2002
	ENSP	9	0.55 (0.07–0.82)	2001–2003	0.37 (–0.11 to 0.75)	2004–2005	0.71 (0.33–0.89)	2003–2003
Penicillins (J01C)	PNSP	11	0.78 (0.48–0.92)	2003–2004	0.69 (0.28–0.87)	2003–2005	0.82 (0.55–0.93)	2004–2004
	ENSP	3	0.37 (–0.15 to 0.74)	2003–2005	0.26 (–0.29 to 0.66)	2001–2002	0.60 (0.15–0.84)	2003–2003
Cephalosporins, monobactams, carbapenems (J01D)	PNSP	8	0.57 (0.13–0.83)	2002–2003	0.41 (–0.07 to 0.74)	2002–2004	0.64 (0.23–0.86)	2000–2002
	ENSP	11	0.69 (0.30–0.88)	2001–2002	0.50 (0.00–0.79)	2003–2005	0.79 (0.48–0.92)	2004–2004
Macrolides, lincosamides, streptogramins (MLS class J01F)	PNSP	4	0.42 (–0.08 to 0.75)	2004–2004	0.26 (–0.22 to 0.67)	2004–2005	0.53 (0.07–0.81)	2002–2002
	ENSP	9	0.56 (0.08–0.82)	2001–2002	0.35 (–0.19 to 0.71)	2004–2004	0.67 (0.27–0.88)	2003–2004
Fluoroquinolones (JO1MA)	PNSP	9	0.51 (0.04–0.80)	2004–2004	0.36 (–0.10 to 0.74)	2003–2005	0.57 (0.12–0.82)	2002–2002
	ENSP	10	0.62 (0.18–0.85)	2001–2002	0.48 (–0.04 to 0.78)	2004–2005	0.69 (0.29–0.89)	2004–2004
	FQRE‡	9	0.60 (0.17–0.84)	2004–2004	0.44 (–0.05 to 0.76)	2003–2005	0.70 (0.33–0.88)	2001–2002

*PNSP, penicillin-nonsusceptible *Streptococcus pneumoniae;* ENSP, erythromycin-nonsusceptible *S. pneumoniae*; FQRE, fluoroquinolone-resistant *Escherichia coli*; E, exposure; O, outcome; CI, 95% confidence interval; MLS, macrolides, lincosamines, and streptogramins.
†Exposure outcome intervals include all 11 possible time windows, considering the data for consumption (exposure) and resistance (outcome) for the same year as well as for intervals of 1 to 2 y between exposure and outcome. p<0.05 was significant.
‡Significant correlations of fluoroquinolone consumption were found only with FQRE. Other correlations were therefore not shown.

tional aggregation levels and found that the association between antimicrobial drug use and resistance was specific and robust for 2 of the 3 compound pathogen combinations under study, stable over time, but not sensitive enough to explain all of the observed variation.

There was a high degree of consistency between penicillin use and penicillin nonsusceptibility in pneumococci as well as for fluoroquinolone use and an increase in fluoroquinolone resistance in *E. coli.* Simple linear regression showed that these effects were highly specific and robust, as inclusion of the use of other antimicrobial substances did not improve correlation or was not confounding the overall effect estimates (Tables 3, 4; Figures 3, 4). The mechanisms for acquiring resistance against both substances have some features in common. These include successive alterations of chromosomally located genes by either homologous recombination or point mutations, resulting in a stepwise modification of the molecular targets, which first leads to reduced susceptibility and eventually to complete resistance (*21*,*22*). In contrast to many other resistance mechanisms, no mobile genetic elements are involved, and a physical linkage to other resistance determinants is unlikely. It is therefore expected that before phenotypes with stable combined resistance evolve, antimicrobial drug selection will specifically favor homologous resistance.

A nonhomologous effect was observed in the case of ENSP, since the variance in ENSP occurrence was

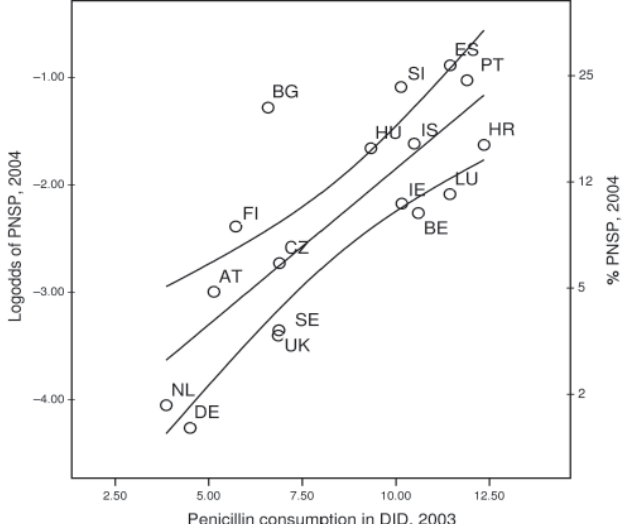

Figure 3. Occurrence of penicillin-nonsusceptible *Streptococcus pneumoniae* (PNSP) plotted against outpatient use of penicillins in 17 European countries including 95% confidence intervals. DID, defined daily doses per 1,000 inhabitants. See Table 1 footnote for country designations.

Table 4. Results of multiple linear regression for the occurrence of PNSP and ENSP*

Models	Exposure	Parameter estimate	p value	R^2
	Outcome variable: logodds PNSP			
Model 1				
Intercept		–4.75		
Gradient	Penicillins	0.29	0.0002	0.61
Model 2				
Intercept		–4.8		
Gradient	Penicillins	0.33	0.002	
	Other β-lactams	–0.05	0.808	
	Fluoroquinolones	–0.11	0.73	0.62
	Outcome variable: logodds ENSP			
Model 1				
Intercept		–2.82		
Gradient	Other β-lactams	0.41	0.003	0.48
Model 2				
Intercept		–3.26		
Gradient	Other β-lactams	0.25	0.14	
	MLS class	0.15	0.39	
	Fluoroquinolones	0.30	0.35	0.56

*PNSP, penicillin-nonsusceptible *Streptococcus pneumoniae*; ENSP, erythromycin-nonsusceptible *S. pneumoniae*; MLS, macrolides, lincosamines, and streptogramins.

best explained by the country-specific use rates of the ATC category of other β-lactams, consisting mainly of cephalosporins. This observation could be either causal, coincidental, or both. In fact, the results of multiple regression models indicate a degree of confounding, as part of the effect attributed to other β-lactams could be explained by MLS-class antimicrobial agents and fluoroquinolones (Table 4). This confounding effect implies that the effect of other β-lactams is mixed with the effect of MLS-class antimicrobial agents and fluoroquinolones used. Data recorded by ESAC suggest that most countries with high use of other β-lactams also have a high consumption of MLS-class antimicrobial agents (r = 0.78, p≤0.01) as well as fluoroquinolones (r = 0.65, p≤0.01). Moreover, countries with the highest levels of other β-lactam use—such as Luxembourg, Croatia, Portugal, Belgium, and France—and high levels of ENSP (23%, 19%, 20%, 31%, and 41%) also reported high levels of combined nonsusceptibility to both erythromycin and penicillin (12%, 9%, 10%, 9%, and 32%). Any increase in selection pressure exerted by β-lactams would also co-select for ENSP under these conditions of combined nonsusceptibility, which could also explain the absence of a direct relationship between use of MLS-class antimicrobial agents and ENSP.

For all compound pathogen combinations that showed significant correlations, the association between the volume of antimicrobial agents used and proportions of resistance was for the most part stable, i.e., independent of the time lag between recording of consumption and the recording of resistance (Table 3). This is not surprising because in the absence of nationwide interventions that would abruptly change the use pattern for an entire country, no major trend changes would be expected, or as other authors have already stated, it is likely that a country with more use or resistance than others in one year, will also have more use or resistance in the next (*19*). Likewise, the steady decline in the consumption in some of the antimicrobial drug classes such as penicillins, as happened in the Czech Republic, France, Germany, and Slovakia, was not reflected by a concomitant decline of penicillin resistance in the pathogens under selective pressure. Mathematical models as well as empirical data suggest that after a reduction in prescribing, resistance will take longer to decline than it took to rise (*23*). In the same way, no decline in resistance against co-trimoxazole was observed in the United Kingdom even 10 years after it abandoned its prescribing, which in this instance was attributed to the co-selection of genetically linked resistance determinants by alternative antimicrobial pressure (*24*). EARSS data show a significant reduction of penicillin resistance in Spain (*25*) and the United Kingdom over the past 5 years (2001–2005), however, no corresponding decline in penicillin use has become apparent that could explain this favorable development (Tables 1, 2). Alternatively, data aggregated at country level by established surveillance networks may not be sensitive enough to identify subtle changes in the complex interaction between antimicrobial drug prescribing and resistance.

EARSS data consist of antimicrobial drug resistance proportions of bacteria that cause invasive bloodstream

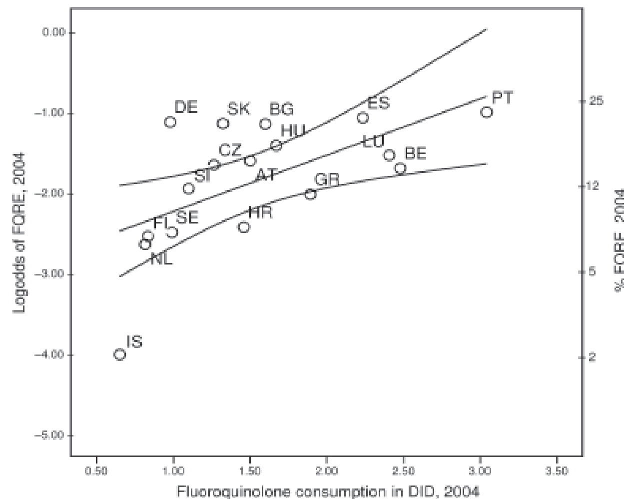

Figure 4. Occurrence of fluoroquinolone-resistant *Escherichia coli* (FQRE) plotted against outpatient use of fluoroquinolone antimicrobial agents in 17 European countries including 95% confidence intervals. DID, defined daily doses/1,000 inhabitants. See Table 1 footnote for country designations.

infections but do not include information from other potentially relevant patient materials. This omission limits the wealth of data but improves the comparability between participating laboratories because it reduces bias introduced by differential case ascertainment. *S. pneumoniae* is the main cause of community-acquired bacteremic pneumonia (*26*), and invasive *E. coli* infections are mainly caused by the translocation of intestinal colonizing strains (*27*). Thus, we believe that resistance among *S. pneumoniae* and *E. coli* blood culture isolates would sufficiently reflect the ecological pressure exerted by the antimicrobial drug use in outpatient settings.

There is little doubt that antimicrobial drug consumption is important in the dissemination of antimicrobial drug resistance. However, additional or alternative factors need to be taken into account (*28*).

We could not control for country-specific differences in hygiene, diagnostic habits, community infection control, and vaccination policies that could provide alternative explanations for some of the observed differences. Moreover, inconsistencies in the sampling population covered by the 2 surveillance systems may introduce inaccuracies that hamper the internal validity of this type of analysis (*29*). In general, data at this high aggregation level are probably not sensitive enough to reflect subtle changes in the complex interaction between antimicrobial drug prescribing and resistance. In this respect, increasing the geographic resolution of data collection by addressing antimicrobial drug use and resistance at the level of health districts would improve the analysis and degree of causal inference that these studies could provide. A higher geographic resolution could also foster interventions by making local extremes of use apparent. However, despite these drawbacks, the data suggest that a specific, robust, and stable association exists between antimicrobial drug use and the occurrence of resistance at country level in the European Union. Our results therefore support interventions that encourage healthcare professionals and healthcare authorities to take firm steps toward promoting prudent use and careful restriction of antimicrobial drug prescription and to monitor the effect of these interventions toward the restoration of the antiinfective activity essential to the success of modern medicine.

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Dr van de Sande-Bruinsma has been part of the EARSS management team since 2001. Her research interest is antimicrobial drug resistance.

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Replacement of Sublineages of Avian Influenza (H5N1) by Reassortments, Sub-Saharan Africa

Ademola A. Owoade,[1] Nancy A. Gerloff,[1] Mariette F. Ducatez,[2] Jolaoso O. Taiwo, Jacques R. Kremer, and Claude P. Muller

Eight new full-length sequences from highly pathogenic avian influenza virus (H5N1) from 4 states in southwest Nigeria were analyzed. All gene sequences were more closely related to the first strains found in Nigeria in 2006 than to any strain found outside the country. Six viruses had evolved by at least 3 reassortment events ($AC_{HA/NS}$, AC_{NS}) from previously identified sublineages A (EMA 2) and C (EMA 1). Our results suggest that highly pathogenic avian influenza virus (H5N1) initially imported into Nigeria in 2006 have been gradually replaced by various reassortments. In all reassortants, nonstructural genes were derived from sublineage C with 2 characteristic amino acids (compared with sublineage A). If the high prevalence of reassortants was typical for West Africa in 2007, the absence of such reassortants anywhere else suggests that reintroductions of influenza A (H5N1) from Africa into Eurasia must be rare.

Highly pathogenic avian influenza (HPAI) virus subtype H5N1 in Africa was first reported from northern Nigeria in February 2006. Phylogenetic analysis of the complete genome showed that these viruses were clearly distinct from the 2 lineages that were found during the same period in southwestern Nigeria (*1*,*2*). The 3 sublineages (referred to as A, B, and C), 2 of which emerged from a common node, had evolved from subtype H5N1 strains that were originally found around Qinghai Lake in 2005. These strains clustered with viruses isolated from 2006 from southern Russia, Europe, and the Middle East (clade 2.2, www.who.int/csr/disease/influenza/tree_large.pdf) but not with the strains prevalent in southeast Asia (*3*). The timeline, the observed influenza A (H5N1) substitution rates in Africa, and the phylogenetic relationship suggested that the sublineages were independently introduced into the country (*1*,*2*). These sublineages were later found throughout Africa with a distinct geographic distribution (*2*,*4*). Sublineage A was also found in Niger and Togo (hemagglutinin [HA] sequence); sublineage B was detected in Egypt and in a human patient in Djibouti (partial HA sequence), and sublineage C was found in Burkina Faso, Sudan, Côte d'Ivoire, Ghana (HA and neuraminidase [NA] sequences) (*5*) and Cameroon (NA sequence) (*6*). Sublineage A strains were also referred to as EMA 2, and both sublineages B and C belong to EMA 1 (*3*). In 2006, one strain with reassorted genes was reported among 35 full-length sequences of the European–Middle Eastern–African lineage (*1*–*4*). We describe new HPAI (H5N1) strains collected in southwestern Nigeria during the second half of 2007, most of which were different reassortants of sublineages A and C.

Materials and Methods

Cloacal swabs were obtained from 8 chicken farms in Lagos (1), Ogun (5), Oyo (1) and Ekiti (1) States from June through November 2007. RNA extraction from cloacal swabs, reverse transcription–PCR amplification, and gene sequencing were conducted as described (*1*). For most viruses, complete sequences were obtained for all gene segments. Kimura distances were calculated on the basis of complete or partial gene sequences by including the maximum sequence length available from all strains included in the comparison. Phylogenetic trees were calculated by using PAUP version 4.0 beta 10 (*7*) with the maximum-like-

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lihood method. The best model was determined by using MODELTEST (*8*). The sequences have been submitted to GenBank with the accession nos. FM160635–FM160642 and FM164800–FM164855.

Results

Reassortants

All genes of A/chicken/NIE/EKI15/2007 and A/chicken/NIE/OYO14/2007 clustered phylogenetically with sublineage A strains (Figures 1, 2). The Kimura distances between the genes of these viruses were 0.4%–1.4%. Among all subtype H5N1 virus sequences published in the Influenza Sequence Database (*5*), NIE/EKI15/2007 and NIE/OYO14/2007 gene sequences were most closely related to those found throughout 2006 and 2007 in Nigeria. Thus, these viruses have most probably evolved from a sublineage A virus initially imported into the country in 2006. This finding is also corroborated by published substitution rates from Africa (*2*).

Five viruses had HA and nonstructural (NS) genes grouping with sublineage C virus genes, whereas the other gene segments were most closely related to sublineage A viruses (e.g., A/chicken/NIE/OG2/2007 and OG5/2007, Figures 1, 2). These viruses evolved by reassortment from sublineages A and C viruses ($AC_{HA/NS}$ reassortment, Figure 2).

Another virus (A/chicken/NIE/LAG6/2007) also showed evidence of reassortment between sublineage A and sublineage C. However, in this virus only the NS gene belonged to sublineage C (Figures 1, 2). The other 7 gene segments of A/chicken/NIE/LAG6/2007 were derived from sublineage A (AC_{NS} reassortant).

Reassortments between Reassortants

Four of the $AC_{HA/NS}$ reassortants (A/chicken/NIE/OG2/2007, A/chicken/NIE/OG4/2007, A/chicken/NIE/OG10/2007, and A/chicken/NIE/OG11/2007), all of which were from Ogun State, had similar sequences in all genes (Kimura distances 0%–0.7 %). The AC_{NS} reassortant A/chicken/NIE/LAG6/2007, obtained from a chicken farm in Lagos State, diverged by 0.9 % in the complete NS gene (derived from C lineage) and by 0.7% to 1.4 % in sublineage A–related gene segments from the latter 4 $AC_{HA/NS}$ reassortants. Some gene segments of the $AC_{HA/NS}$ reassortant A/chicken/NIE/OG5/2007 were most closely related to the other 4 $AC_{HA/NS}$ reassortants, whereas, other gene segments were closer to the AC_{NS} reassortant A/chicken/NIE/LAG6/2007. Matrix protein, HA, NS, NA, and nucleocapsid protein (NP) genes of NIE/OG5/2007 showed a maximal Kimura distance of only ≤0.4% to $AC_{HA/NS}$ reassortant genes but a distance of 0.6%–1.5% to the AC_{NS} reassortant (A/chicken/NIE/LAG6/2007). In contrast, RNA polymerase B protein (PB2), PB1, and PA genes were more

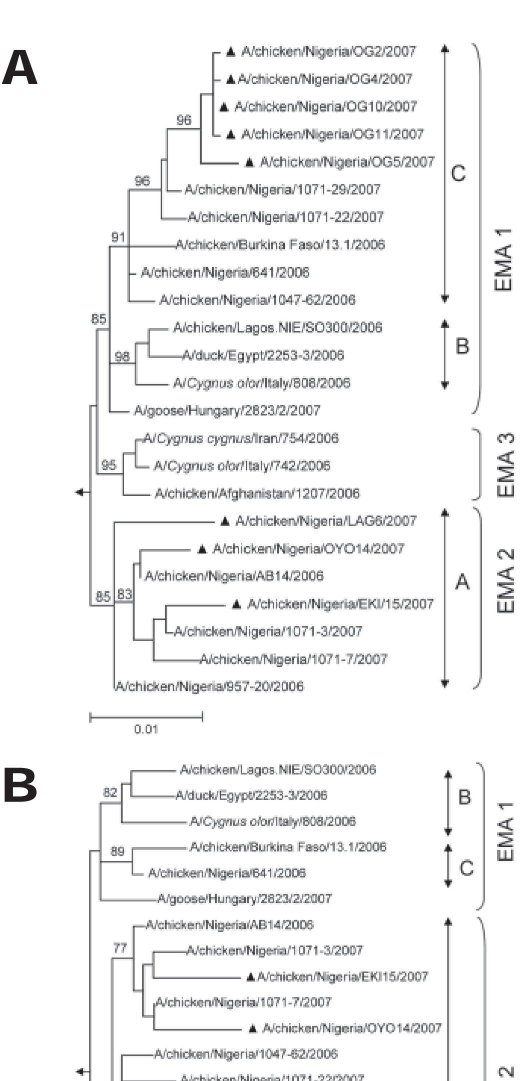

Figure 1. Phylogeny of hemagglutinin (A) and neuraminidase (B) genes from 8 HPAI (H5N1) viruses collected in Nigeria during the second half of 2007 (▲), in comparison with previously identified sublineage A (EMA 2), sublineage B and C (EMA 1), and EMA 3 strains (*1,3*). The tree was calculated by using the maximum likelihood method implemented in PAUP 4.0 (*7*). The substitution model was obtained by using MODELTEST (*8*). Bootstrap values (%) were calculated with the maximum-likelihood method with 1,000 replications and are indicated on key nodes. Scale bars represent ≈1% of nucleotide changes between close relatives. A/duck/Anyang/AVL-1/2001 was used as an outgroup.

closely related to the AC_{NS} reassortant (maximum Kimura distance: <0.6%) than to $AC_{HA/NS}$ reassortants (minimum Kimura distance for the different genes: 0.7%–0.8%). For instance, A/chicken/NIE/OG5/2007 differed by 12 nucleotides in the PA gene from the most closely related $AC_{HA/NS}$ reassortant (A/chicken/NIE/OG2/2007) but by only 1 nucleotide from the AC_{NS} reassortant (A/chicken/NIE/LAG6/2007). On the other hand, A/chicken/NIE/OG5/2007 had 15 nucleotides in the NP gene different from the A/chicken/NIE/LAG6/2007 but only 1 nucleotide difference compared with the closest $AC_{HA/NS}$ reassortant (A/chicken/NIE/OG11/2007) (Figure 2). This finding strongly suggests that A/chicken/NIE/OG5/2007 is the result of an additional reassortment event involving an exchange of genes between the $AC_{HA/NS}$ and AC_{NS} reassorted viruses.

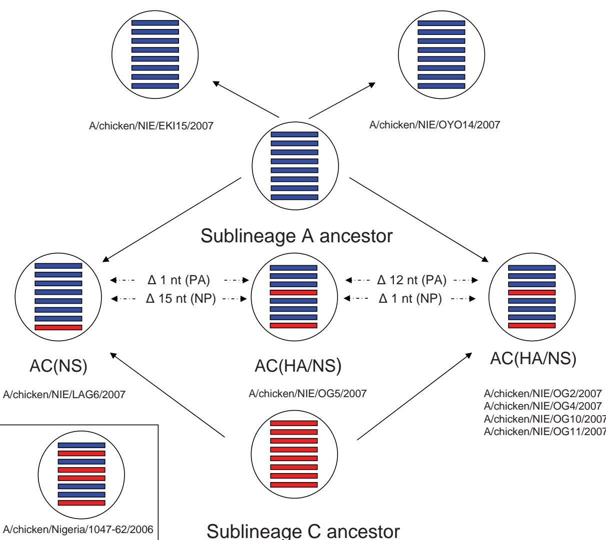

Figure 2. Schematic presentation of sublineage A–derived highly pathogenic avian influenza viruses (H5N1) and reassortants of sublineage A– and sublineage C–derived viruses identified in Nigeria in 2007. The reassortant reported from Salzberg and others in 2007 (*3*) is also shown. Sublineage A–derived gene segments are shown in blue; sublineage C–derived gene segments are shown in red. Gene segments are represented in the following order (from top): PB2, PB1, PA, HA, NP, NA, M, NS.

Mutations

The amino acid sequences of the HA cleavage site (PQGERRRKKRG) of the strains described here are identical to those of all HPAI (H5N1) strains reported from West Africa. All viruses had identical amino acids in all positions of the HA protein that are associated with preferential binding to α2,3-linked sialic acid (*9,10*) as described (*2*).

As for all HPAI (H5N1) strains from Africa, the above viruses had the virulence marker lysine (K) in position 627 of PB2 associated with accelerated viral replication, reduced host defense, higher mortality rate in mice (*11*), and a wider host range of subtype H5N1 strains (*12*). None of the known markers in the matrix 2 gene associated with resistance to amantadine (*13*) and in the NA gene associated with resistance to oseltamivir (H274Y) (*14*) were detected.

Discussion

Gene sequences of all 8 HPAI viruses (H5N1) described here were more closely related to sublineages A or C strains found in Nigeria than to any other published H5N1 virus subtypes. In particular, they were more closely related to the first strains found in Nigeria in the beginning of 2006 than to any strains found outside the country. Thus, the viruses detected in southwestern Nigeria during the second half of 2007 probably evolved from the first viruses brought into the country in early 2006 (*1*), suggesting that HPAI (H5N1) has continuously circulated and is endemic to Nigeria. Sublineage A viruses have continued to circulate in Nigeria, whereas sublineage B was found only once on 1 farm (SO layer farm, Lagos, January 2006), and sublineage C viruses were no longer detected in 2007. Sublineage A viruses have been detected in northeastern Nigeria in February 2007 (*15*) and in 2 states of southwestern Nigeria during the last quarter of 2007 (A/chicken/NIE/EKI15/2007 and A/chicken/NIE/OYO14/2007). Sublineages B and C viruses may have been eliminated in Nigeria by effective countermeasures.

All $AC_{HA/NS}$ described here were obtained from chicken flocks in Ogun State from June through August 2007. These results are similar to those found in the beginning of 2007 in other states of Nigeria (*15*). In addition, we identified an AC_{NS} reassortant in Lagos State (A/chicken/NIE/LAG6/2007) distinct from the latter strain. At least 2 distinct reassortment events were necessary to generate sublineages A and C reassortants $AC_{HA/NS}$ and $AC_{NS,}$ which probably had occurred already in 2006, as suggested by the conspicuous absence of sublineage C in 2007. Although it is obviously more difficult to demonstrate reassortment events between genetically similar viruses, the asymmetry in gene divergence of A/chicken/NIE/OG5/2007 compared with the other $AC_{HA/NS}$ and AC_{NS} reassortants suggests that additional reassortment events have taken place.

In 2006, only 1 reassorted strain was found among 35 European–Middle Eastern–African strains, including 19 viruses reported from Nigeria, belonging to 3 parent sublineages (*1–4*). In the beginning of 2007, 10 of 12 from northern, southern, and central states all belonged to the same $AC_{HA/NS}$ reassortants (*15*), distinct from the $AC_{PB1/HA/NP/NS}$ reassortant detected in 2006 (*3*). Similar reassortants were also found in other regions of sub-Saharan Africa (unpub. data). During the second half of 2007, we found 6 reassortants including 3 distinct reassortants among 8 strains collected from 8 farms located in 4 contiguous Federal States of Nigeria (Figure 2). These results suggest that reas-

sortants have largely replaced the initial sublineages from which they were derived and that reassortments are pervasive. This finding confirms that reassortments between subtype H5N1 viruses occur frequently when different strains cocirculate in the same region (*16*) and is of particular concern if the increasing prevalence is the result of adaptation to the African environment.

Although segments of the replication complex (PB1, PB2, PA, and NP) may reassort individually without affecting viral fitness (*16*), there seems to be a coordinated evolution of the HA and NA genes (*17*). In all but 1 of the Nigerian reassortants, HA and NA genes originated from different sublineages (C and A), suggesting compatibility between phenotypes of both sublineages. All reassortants from Nigeria included sublineage C–derived NS genes, which may suggest a higher fitness of these viruses. Sublineage C–derived NS1 and NS2 sequences from all Nigerian reassortants and 11 unpublished sequences from $AC_{HA/NS}$ reassortants identified in other sub-Saharan regions showed 2 amino acids (NS1 V194 and NS2 R34), which were never identified in sublineage A viruses. It has been shown that modifications in the NS proteins, including amino acids adjacent to V194, may modulate the virulence of HPAI (H5N1) (*18*,*19*). Alternatively, the observation that all reassortants in West Africa have sublineage C–derived NS genes may suggest a better adaptation to the African environment of viruses that came from the cold temperatures of central Asia. Thus, the influence of differences in ecology between Africa and Eurasia on viral selection and dynamics deserves further attention.

Although no reassortments have been reported among clade 2.2 viruses (www.who.int/csr/disease/influenza/tree_large.pdf) in Central Asia, Europe, and the Middle East since their emergence from Qinghai Lake region in 2005, reassortments of these viruses seem to be rampant in sub-Saharan Africa, where they have become the critical determinant of genetic diversity of HPAI (H5N1). Because of low prevalence, mainly in wild birds, clade 2.2 viruses have few opportunities to reassort in Eurasia. In contrast, opportunities to reassort seem to be frequent in sub-Saharan Africa because of great difficulties in setting up a sensitive surveillance system in a complex socioeconomic environment, where backyard farms and large commercial farms with variable biosafety levels coexist, and where culling may threaten the livelihood and survival of the farm.

If the high prevalence of reassortants was typical for West Africa in 2007, the absence of such reassortants anywhere else suggests that reintroductions of subtype H5N1 from Western Africa into Eurasia must be rare. Moreover, all HPAI (H5N1) strains from Nigeria in 2007 were more similar to those found in Nigeria in 2006 than to even the closest relative from Europe in 2007 (Hungary). Although subtype H5N1 has been found in wild birds from Africa, such as vultures (*4*), HPAI (H5N1) has so far not been reported in long-distance migrating birds in West Africa. Thus, the exchange of subtype H5N1 between Eurasia and Africa seems to be a rare event, which in 2006 may have been triggered by unusual bird migration as a result of the central Asian cold spell.

The biological significance of reassortments between genetically similar viruses may be arguable, but the frequency of reassortment events is an important marker of virus endemicity in a region. Moreover, endemicity of HPAI (H5N1) and a high propensity of reassorting in a region where seasonal influenza is unchecked are essential ingredients of the anticipated pandemic.

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The official designation of sublineages A, B, and C can be found in the WHO HPAI (H5N1) clade nomenclature update, to be published soon (www.who.int).

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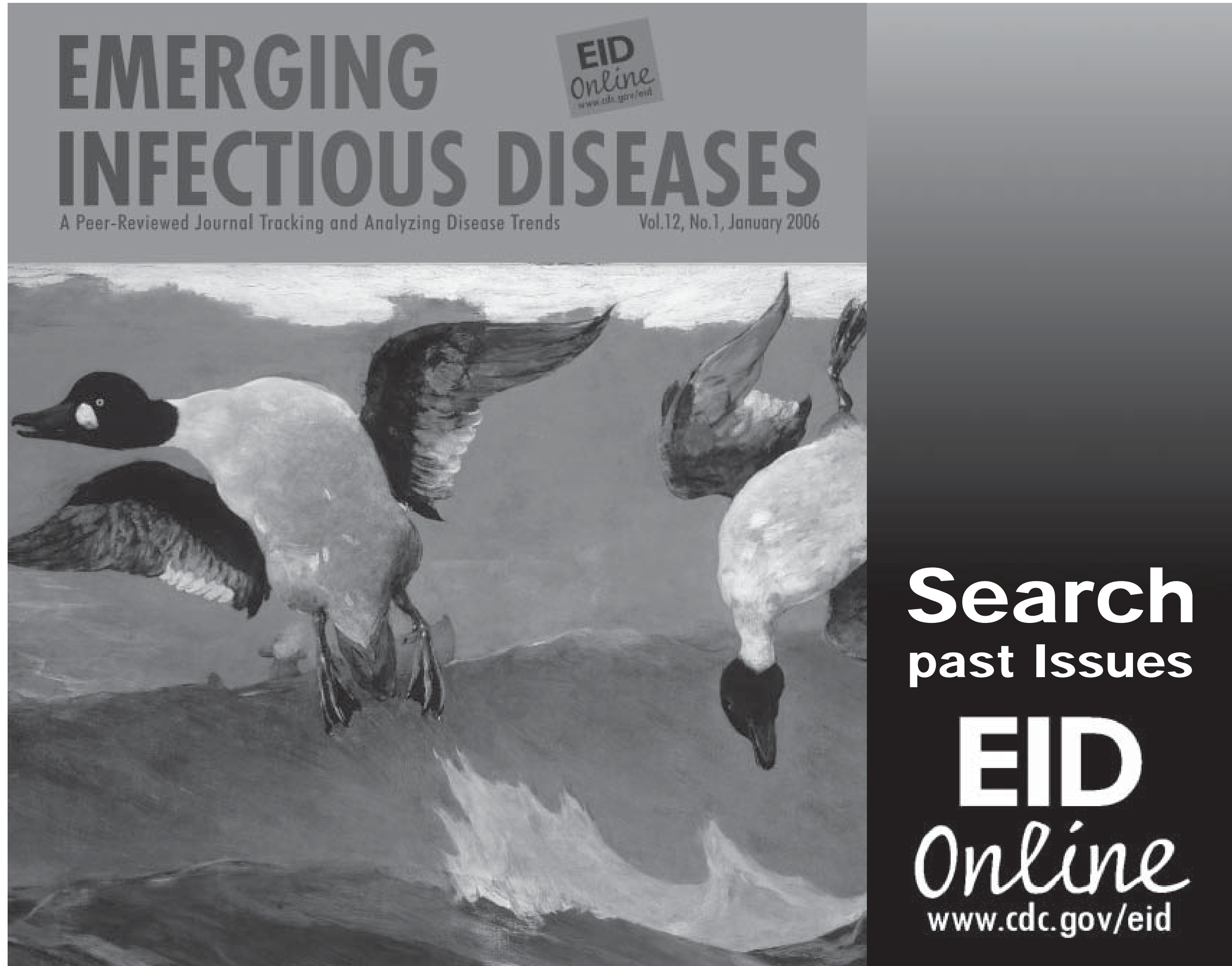

Domestic Pigs and Japanese Encephalitis Virus Infection, Australia

Andrew F. van den Hurk, Scott A. Ritchie, Cheryl A. Johansen, John S. Mackenzie, and Greg A. Smith

To determine whether relocating domestic pigs, the amplifying host of Japanese encephalitis virus (JEV), decreased the risk for JEV transmission to humans in northern Australia, we collected mosquitoes for virus detection. Detection of JEV in mosquitoes after pig relocation indicates that pig relocation did not eliminate JEV risk.

Japanese encephalitis virus (JEV) is a major cause of viral encephalitis in Southeast Asia; >50,000 cases are reported annually (*1*). Ardeid wading birds are the primary maintenance hosts, pigs are the main amplifying hosts, and *Culex* mosquitoes are the primary mosquito vectors. Suppression of JEV disease in humans is generally considered to be best achieved through vaccination of humans or swine, mosquito control, or a combination of these strategies (*2*). An alternative approach of moving domestic pigs away from human habitation has been suggested as a potential method of reducing JEV transmission to humans (*1*,*3*). Although this strategy could be considered a logical way to limit human exposure to infected vectors, the actual effect that removing domestic pigs would have on mosquito infection rates has not been established.

Since the emergence of JEV in northern Australia in 1995, we (the authors) have investigated the ecology of JEV on Badu Island in the Torres Strait, where most human, pig, and animal infections have occurred. Intense transmission on this island has been linked to domestic pigs, which until late 1998 were housed in small backyard pens (Figure 1, panel A), and high populations of *Culex sitiens* subgroup mosquitoes (*4*). In Australia, members of the *Cx. sitiens* subgroup, from which *Cx. annulirostris* is considered to be the most important species, are the primary Australian JEV vectors (*5*). During an outbreak in 1998, the virus was shown to be widespread on Badu Island; isolates were obtained from mosquitoes collected throughout the community (*6*). A vaccination program initiated on the outer Torres Strait islands in 1995, including Badu Island, appears to have limited the number of human clinical cases (*5*).

To further reduce JEV risk for Badu Island residents, after the 1998 outbreak domestic pigs were removed from the Badu Island community to a piggery located ≈2.5 km away (Figure 1, panel B). This relocation led to a significant reduction in the proportion of *Cx. annulirostris* feeding on pigs, and speculation was that this might reduce the number of JEV-infected mosquitoes (*7*). We report on the effects of pig relocation away from human habitation on virus infection rate in *Cx. sitiens* subgroup mosquitoes.

The Study

In response to JEV activity, as evidenced by human clinical cases or the seroconversion of sentinel pigs, adult mosquitoes were collected on Badu Island during 1995, 1998, and 2003 (*6,8,* and this article, respectively). Badu Island is located at 10º07′S and 142º09′E in the central western region of the Torres Strait and is a granite island of ≈101 km^2; its ecology has been described (*6*–*8*).

The mosquitoes were collected with Centers for Disease Control (CDC) miniature light traps (Model 512; John

Figure 1. Pig housing in Badu Island. A) Typical backyard pig pen in community before removal in 1998 and B) Badu Island piggery, where pigs have been housed since late 1998.

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W. Hock Co., Gainesville, FL, USA) baited with either CO_2 alone or in combination with 1-octen-3-ol. Mosquitoes were killed on dry ice and placed in liquid nitrogen dry shippers or on dry ice in insulated containers for transport to Cairns for storage at –70ºC. Mosquitoes were placed on a refrigerated table for species or taxonomic group identification before being placed in pools of ≤200 mosquitoes and sent to Queensland Health Forensic and Scientific Services or the University of Queensland, Brisbane, for JEV detection.

The virus isolation protocols used in 1995 and 1998 have been described (*6*,*8*). In 2003, virus RNA was detected by using a real-time TaqMan reverse transcription–PCR (*9*).

To facilitate the comparison of virus distribution on Badu Island, trap locations were grouped into 3 general areas: within 1.2 km of the piggery, within the area of human habitation (the community), and at a rubbish dump located ≈1.5 km from the community and 4.0 km from the piggery (Figure 2). Dumps are a potential focus of JEV activity because mosquitoes, feral pigs, and wading birds congregate at them (*10*). Maximum-likelihood estimation of mosquito infection rates with 95% confidence intervals were calculated for each of these general areas by using the PooledInfRate statistical software package (*11*).

Because of the difficulty in morphologically separating the members of the *Cx. sitiens* subgroup, data for the 3 members of the group found in Australia—*Cx. annulirostris*, *Cx. palpalis*, and *Cx. sitiens*—were pooled for analysis. However, PCR restriction fragment length polymorphism analysis of a subsample of 135 *Cx. sitiens* subgroup mosquitoes collected in 2003 showed that *Cx. annulirostris* was the dominant member of this group on Badu Island and comprised 94.1% of polymorphic specimens processed; the other 4.4% and 1.5% were identified as *Cx. palpalis* and *Cx. sitiens*, respectively.

A total of 44,328 *Cx. sitiens* subgroup mosquitoes were processed for detection of JEV; 2,871, 24,592, and 16,865 were processed from 1995, 1998, and 2003, respectively (Table). JEV was detected in 66 pools of *Cx. sitiens* subgroup mosquitoes; the highest maximum-likelihood estimation of mosquito infection rate was obtained from mosquitoes collected at the dump in 1998. Despite removal of the domestic pigs, JEV was still detected in 5 pools of *Cx. sitiens* subgroup mosquitoes collected within the community in 2003. However, the point estimates of infection rates were lower than those obtained in 1995 and 1998, when domestic pigs were present within the community, although the slight overlap in 95% confidence intervals indicates that this difference in infection rate was not significant.

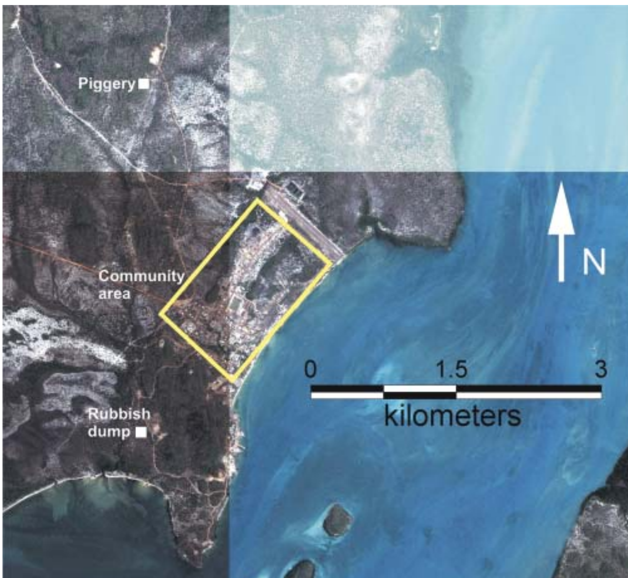

Figure 2. Aerial photograph of Badu Island showing the location of the community, piggery, and rubbish dump.

Conclusions

We demonstrated that although removing domestic pigs from areas of human habitation may reduce contact between amplifying hosts and vectors (*7*), it does not eliminate the presence of JEV-infected mosquitoes. Thus, pig removal does not negate JEV risk for humans. Indeed, evidence for low-level virus transmission to humans is provided by Hanna et al., who found a low level (32%) of natural boosting immunity in Badu residents who had received an inactivated mouse brain–derived JEV vaccine 3 years earlier (*12*).

As has been observed elsewhere in the absence of pigs (*13*), mosquitoes may have become infected by feeding on viremic herons and egrets, populations of which are found on Badu Island (*14*). Analysis of host feeding patterns demonstrated that birds accounted for 23% of blood meals of *Cx. annulirostris* identified from the dump in 2003 (S. Hall-Mendelin and A.F. van den Hurk, unpub. data). Alternately, feral pigs in the community and at the dump are a potential source of virus for mosquitoes, although <1% of mosquito blood meals were from swine at these locations.

Mosquitoes could become infected by feeding on viremic pigs at the piggery and then disperse to other areas on the island. The mean flight distance of *Cx. annulirostris* is 4.4 km; some females traverse up to 12 km (*15*), which is considerably farther than the 2.5 km between the piggery and the community on Badu Island. Solomon recommends that domestic pigs be moved >5 km from human habitation to limit JEV transmission to humans (*3*). Indeed, if infected mosquitoes were flying from the piggery to the community, then the data from our study support this recommendation. However, we suggest that domestic pigs be removed far enough away from human habitation to encompass the flight range of the local *Culex* vectors.

Table. Mosquito infection rates during 3 recognized incursions of Japanese encephalitis virus, Badu Island, northern Australia*

Collection location	Pigs located within community						Pigs relocated outside community		
	1995			1998			2003		
	No.†	No. detected‡	Infection rate (95% CI)	No.†	No. detected‡	Infection rate (95% CI)	No.†	No. detected‡	Infection rate (95% CI)
Community	2,871	8	3.02 (1.43–5.74)	23,467	38	1.69 (1.21–2.29)	7,019	5	0.75 (0.28–1.66)
Piggery	NS	NS	NS	NS	NS	NS	3,316	5	1.61 (0.61–3.56)
Dump	NS	NS	NS	1,125	4	3.68 (1.20–8.85)	6,530	6	0.99 (0.41–2.07)

*Mosquito infection rates determined by maximum-likelihood estimation; 1995, Apr 8–9 and 20–21, 30 trap nights; 1998, Mar 5–6, 25 trap nights; 2003, Mar 13–19, 92 trap nights; CI, confidence interval; NS, mosquitoes not sampled from this location during the year of collection.
†Total no. mosquitoes processed.
‡No. Japanese encephalitis virus–positive pools detected by virus isolation or TaqMan reverse transcription–PCR.

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Influenza Virus (H5N1) in Live Bird Markets and Food Markets, Thailand

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A surveillance program for influenza A viruses (H5N1) was conducted in live bird and food markets in central Thailand during July 2006–August 2007. Twelve subtype H5N1 viruses were isolated. The subtype H5N1 viruses circulating in the markets were genetically related to those that circulated in Thailand during 2004–2005.

In Thailand, from 2004 through 2008, 6 major outbreaks of avian influenza occurred (January–March 2004, July–October 2004, October–December 2005, January–March 2006, November–March 2007, and January 2008). We report on a 14-month avian influenza surveillance program and its finding of influenza virus (H5N1) in live bird and food markets in Thailand.

The Study

From July 2006 through August 2007, an influenza (H5N1) surveillance program was conducted in live bird and food markets in 10 provinces of central Thailand (Figure 1). Cloacal swabs (n = 381) were sampled from live chickens, ducks, pigeons, and house sparrows. Visceral organs and bird meats (n = 549) were collected from carcasses of chickens, ducks, quails, water cocks, water hens, swamp hens, crakes, parakeets, and moor hens at local food markets (Tables 1, 2). An average of 4 markets (range 1–6) were visited each month, and ≈18 samples were collected from each market. All samples were from backyard animals or local meat birds. None were from birds from standard farming systems with high biosecurity.

The viruses were propagated by embryonated egg inoculation (*1*). Allantoic fluids were tested for influenza subtype H5N1 by hemagglutination (HA). Multiplex reverse transcription–PCR (RT-PCR) was performed to amplify H5, neuraminidase (N) 1, and matrix (M) genes from HA-positive samples (*2*).

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Influenza virus (H5N1) was identified in 12 of 930 samples tested. In November 2006, a total of 5 samples with influenza virus (H5N1) were isolated from 1 healthy chicken and 4 visceral organs obtained from 1 live bird market (chicken) and 3 different food markets (moor hen, water cock, and quail). In December 2006, a total of 5 samples with influenza virus (H5N1) were isolated from 5 visceral organs (quail, water cock) from 1 food market. In January 2007, a total of 2 samples with influenza virus (H5N1) were isolated from 2 healthy ducks obtained from 1 live bird market. In the study, 7 isolates were sequenced for whole genome analysis, and the remaining 5 samples were sequenced for H5 and N1 genes. The respective viruses were designated as A/moorhen/Thailand/CU-317/2006 (GenBank accession nos. EU616825–EU616826), A/moorhen/Thailand/CU-318/2006 (EU616827–EU616828), A/watercock/Thailand/CU-319/2006 (EU616829–EU616830), A/quail/Thailand/CU-320/2006 (EU616831–EU616832), A/chicken/Thailand/CU-321/2006 (EU616833–EU616834), A/quail/

Figure 1. A) Poultry at live bird market; B) house sparrows at live bird market; C) chicken meat at food market; and D) moor hen meat at food market.

Table 1. Test results for samples collected during influenza virus (H5N1) surveillance program in live bird and food markets, by collection date, central Thailand

Date sample collected	Location, no. samples		Total no. samples	No. positive results
	Live bird market*	Food market†		
2006				
Jul	7	0	7	–
Aug	6	21	27	–
Oct	8	26	34	–
Nov	20	26	46	5
Dec	18	9	27	5
2007				
Jan	22	35	57	2
Feb	13	36	49	–
Mar	57	66	123	–
Apr	63	74	137	–
May	53	85	138	–
Jun	84	40	124	–
Jul	10	64	74	–
Aug	20	67	87	–
Total	381	549	930	12

*Sample source: swabs.
†Sample source: visceral organs and meats.

Thailand/CU-330/2006 (EU616851–EU616858), A/quail/Thailand/CU-331/2006 (EU616859–EU616866), A/quail/Thailand/CU-332/2006 (EU616867–EU616874), A/quail/Thailand/CU-333/2006 (EU616875–EU616882), A/water-cock/Thailand/CU-334/2006 (EU61683–EU616890), A/duck/Thailand/CU-328/2007 (EU616835–EU616842), and A/duck/Thailand/CU-329/2007 (EU616843–EU616850).

To analyze the isolates, nucleotide sequences were compared with those of influenza subtype H5N1 viruses in Thailand, People's Republic of China, Vietnam, Indonesia, Lao, Myanmar, and Cambodia. The sequences were aligned by using the DNASTAR program (*3*) to elucidate and compare the genetic changes. Phylogenetic analysis was conducted by applying the PAUP program (*4*) with the neighbor-joining algorithm and using branch swapping and bootstrap analysis with 1,000 replicates.

Conclusions

In the course of the 14-month surveillance program, we isolated influenza virus (H5N1) from 12 samples from live birds and from bird meats obtained from the markets. Bird meats were the source of 9 virus-containing samples (5 quail, 2 moor hens, and 2 water cocks), which indicates a risk for influenza virus (H5N1) contamination in bird meats, especially quail. In addition, 3 highly pathogenic avian influenza viruses were isolated from healthy live poultry (1 chicken and 2 ducks). However, the samples that contained influenza virus subtype H5N1 were detected only during the 3-month winter season (November–January). A possible explanation for virus contamination in live bird and food markets may be animal movement from outbreak areas to the markets as well as an attempt to sell infected (dead or dying) birds, especially quail, as bird meat. In addition, most animals or meats in the markets came from backyard farms, where they were in unavoidably close contact with wild birds.

Phylogenetic analysis of the virus HA and NA genes indicated that all 12 subtype H5N1 isolates were part of the Vietnam and Thailand lineage (clade 1). The viruses were closely related to those investigated in Thailand (2004–2005) as well as to other subtype H5N1 isolates in clade 1. In contrast, they differed from influenza subtype H5N1 viruses of the south China and Indonesia lineages (clade 2) (Figure 2). In this study, we did not discern any Thailand isolates closely related to the south China lineage, as previously established in Lao and Cambodia (*5*). Phylogenetic analysis of 6 remaining genes showed them to be also closely related to the Vietnam and Thailand isolates.

Analysis of the deduced amino acid sequences of the HA and NA proteins indicated that the viruses had characteristics of highly pathogenic avian influenza. The HA cleavage site consists of multiple basic amino acids RERRRKKR (in 1 isolate, CU-329, RE<u>K</u>RRKKR). All influenza subtype viruses harbor Glu-222 and Gly-224 at the receptor binding site, indicating preferential binding to the avian receptor SA-α-2, 3-Gal. In addition, the virus sequences contain 7 glycosylation sites as previously identified in most isolates from Thailand (*6*). A glycosylation site adjacent to receptor binding sites may help increase virus infectivity in host cells (*7*). In some isolates, polymorphisms of amino acids related to antigenic properties of the viruses at position V86A, L138Q, and K140N were observed. All 12 subtype H5N1 viruses had a 20-aa deletion in the NA protein, typical for the NA stalk region of recent subtype H5N1 isolates (2003–2007) (*8,9*). None of the subtype H5N1 isolates had any amino acids indicating oseltamivir resistance at the crucial positions 119 (E), 275

Table 2. Test results for samples collected during the influenza virus (H5N1) surveillance program in live bird and food markets, by bird species, central Thailand

Bird species	Location, no. samples		Total no. samples	No. positive results
	Live bird market*	Food market†		
Chicken	204	3	207	1
Duck	59	2	61	2
Quail	–	396	396	5
Pigeon	6	–	6	–
House sparrow	112	6	118	–
Water cock	–	27	27	2
Water hen	–	33	33	–
Swamp hen	–	1	1	–
Crake	–	1	1	–
Moor hen	–	80	80	2
Total	381	549	930	12

*Sample source: swabs.
†Sample source: visceral organs and meats.

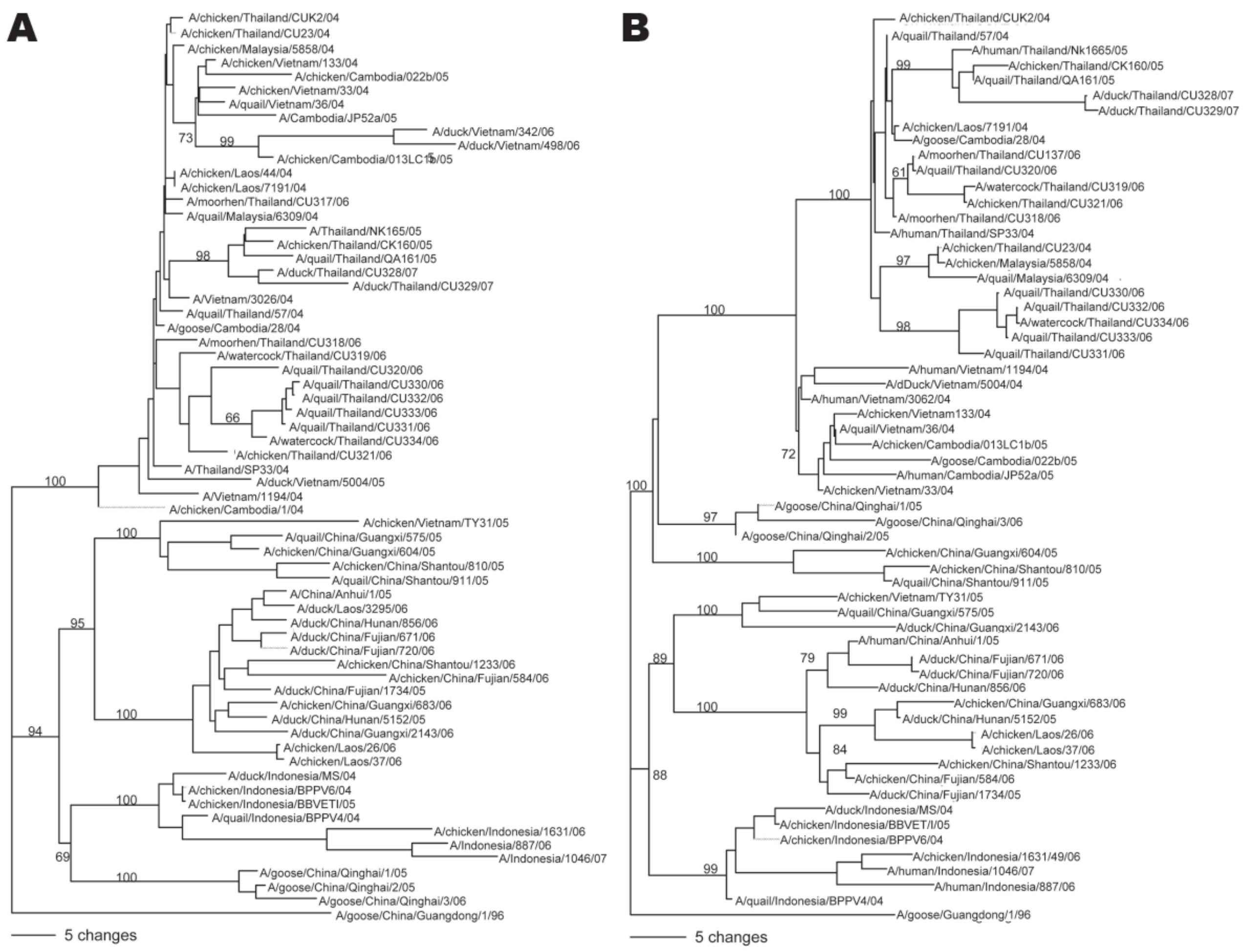

Figure 2. Phylogenetic analysis of the hemagglutinin (A) and neuraminidase genes (B) of influenza virus (H5N1) isolates. Phylogenetic trees were generated by using the PAUP computer program (*4*) and applying the neighbor-joining algorithm with branch swapping and bootstrap analysis with 1,000 replicates. The trees were rooted to A/goose/China/Guangdong/1/96 (H5N1).

(H), 293 (R), and 295 (N) of the NA protein. In summary, the 12 viruses isolated from this study were similar to the viruses from other sources in Thailand, which indicates that the viruses are endemic to Thailand, are circulating in the country, and can be found in any exposed species.

The route of influenza virus (H5N1) introduction into the markets remains to be established. We suspect that this contamination might have occurred as a consequence of animal movement from outbreak areas or from virus-contaminated cages, trucks, and equipment. Unfortunately, the original sources of animals in the markets could not be identified because birds from different sources were housed in 1 or several cages. Fortunately, no human infection was found during 2007–2008 in those provinces where the viruses were isolated.

It has been known that live bird and wet markets play a major role in facilitating emergence or reemergence of influenza and some other respiratory diseases (*10*–*12*). Monitoring of live bird and food markets as an early warning system should be implemented in Asian countries where such markets are still commonplace, and routine surveillance of these markets should be conducted year-round. In addition, raw bird meats should be handled with caution, and consumption of raw bird meats should be avoided. Increased public awareness about the risks for influenza virus (H5N1) in association with live bird and food markets will help prevent and control subtype H5N1 infection in humans.

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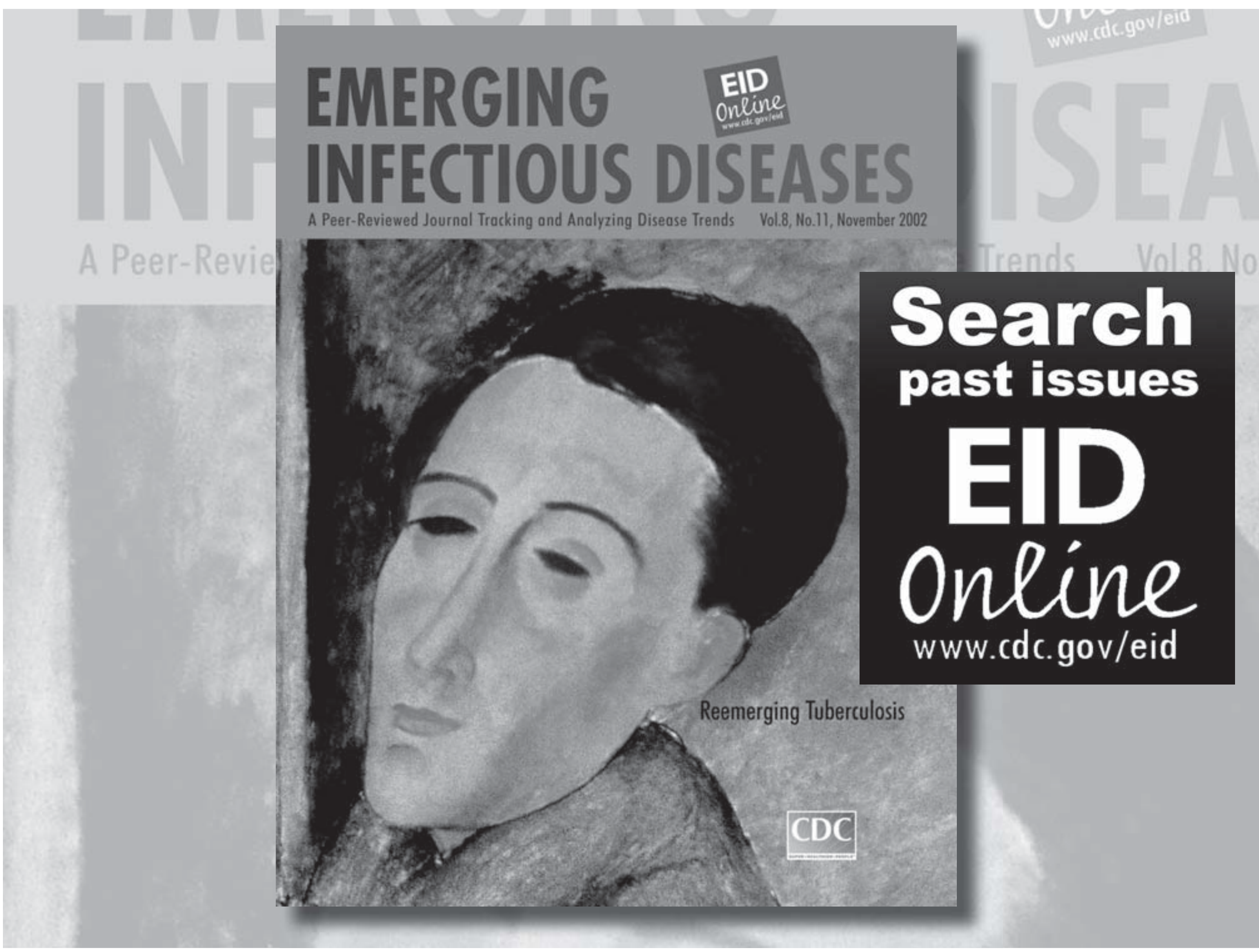

Successful Treatment of Disseminated *Acanthamoeba* sp. Infection with Miltefosine

Alexander C. Aichelburg, Julia Walochnik, Ojan Assadian, Helmut Prosch, Andrea Steuer, Gedeon Perneczky, Govinda S. Visvesvara, Horst Aspöck, and Norbert Vetter

We report on an HIV-negative but immunocompromised patient with disseminated acanthamoebiasis, granulomatous, amoebic encephalitis, and underlying miliary tuberculosis and tuberculous meningitis. The patient responded favorably to treatment with miltefosine, an alkylphosphocholine. The patient remained well with no signs of infection 2 years after treatment cessation.

A 25-year-old man from India, who had been living in Austria for 7 years and had no previous history of major illnesses, was brought by ambulance to the hospital for dyspnea, cough, fever, and weight loss. During neurologic examination, a hearing impairment was suspected. The patient was unable to walk because of severe ataxia. Skin examination showed several necrotic ulcers with purulent discharge and black eschars, measuring 0.5 cm to 3 cm, located on the skull, back, neck, and arms (Figure 1, panels A and B). Miliary tuberculosis (TB) of the lungs, liver, spleen, and kidneys was suspected on the basis of chest radiography and computed tomography (CT) of chest and abdomen. Ziehl-Nielsen (ZN) staining for acid-fast bacilli in sputum, bronchial secretions, and lavage obtained through bronchoscopy was negative. PCR for *Mycobacterium tuberculosis* in bronchial secretions and serum was positive. Culture on Loewenstein agar resulted in growth of nonresistant *M. tuberculosis* after 31 days. Blood cultures were negative for aerobic/anaerobic bacteria, mycobacteria, and fungi. Results of serologic tests were negative for *Aspergillus, Candida, Cryptococcus, Histoplasma, Blastomyces,* and *Coccidioides* spp. Severe immunosuppression with a CD4+ lymphocyte count of 182 cells/µL made HIV infection probable, but HIV testing results were negative. Cranial CT showed multiple small enhancing lesions in cerebral cortex and underlying white matter, pons, midbrain, and around most of the cisterns. On magnetic resonance imaging (MRI), the lesions appeared as high T2 signal areas that enhanced heterogeneously or in a ringlike manner. These findings were compatible with the diagnosis of meningoencephalitis with intracerebellar abscess formation.

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Cerebrospinal fluid (CSF) obtained through lumbar puncture was negative for *Toxoplasma gondii, Encephalitozoon cuniculi,* and *Enterocytozoon bieneusi* by PCR and for *Trypanosoma gambiense* by indirect hemagglutination assay. Staining and antigen testing (enzyme immunoassay) for *Cryptococcus neoformans* was negative, as was *Treponema pallidum* antibody testing. No viruses (herpes simplex 1 and 2, varicella zoster, enterovirus) could be detected by PCR. Cultures were negative for aerobic/anaerobic bacteria and fungi. ZN staining detected acid-fast bacilli that were confirmed to be nonresistant *M. tuberculosis* after culture for 38 days. PCR for *M. tuberculosis* was positive. An *Acanthamoeba*–specific PCR (*1*) and DNA sequencing of the PCR product showed *Acanthamoeba* genotype T2 (corresponding to group III). High immunoglobulin (Ig) G (2,000) and IgM (1,000) titers against *Acanthamoeba* spp. could be demonstrated serologically. The organism could not be grown in culture (*2*).

Two skin-biopsy specimens were obtained; they showed necrotizing granulomatous inflammation affecting the entire dermal thickness and subcutis. Stains and culture were negative for *Mycobacterium* spp., fungi, and *Acanthamoeba* spp. but the specimens tested positive for acanthamoebae by PCR. In addition to a standard tuberculostatic 5-drug regimen including intravenous streptomycin, empiric anti-amoebic treatment based on data retrieved from the few case reports of successful treatment of systemic *Acanthamoeba* infections (*3*–*5*) was initiated. The regimen included a combination of parenteral trimethoprim/sulfamethoxazole (later changed to oral sulfadiazine) and parenteral fluconazole. CSF samples drawn 2 and 8 weeks after initiation of therapy tested negative for mycobacteria by ZN staining, PCR, and culture but remained positive for *Acanthamoeba* sp. by PCR.

Transbronchial lung biopsy specimens from a second bronchoscopy performed 1 month after admission tested negative for mycobacteria by ZN staining, PCR, and culture but positive for *Acanthamoeba* sp. by PCR. Acanthamoebae could not be cultivated from bronchial secretions or biopsy sample; immunostaining that used a polyclonal rabbit anti–*A. castellanii* (genotype T4) serum was negative.

Within 12 weeks after initiation of tuberculostatic therapy, complete clinical and radiologic resolution of miliary

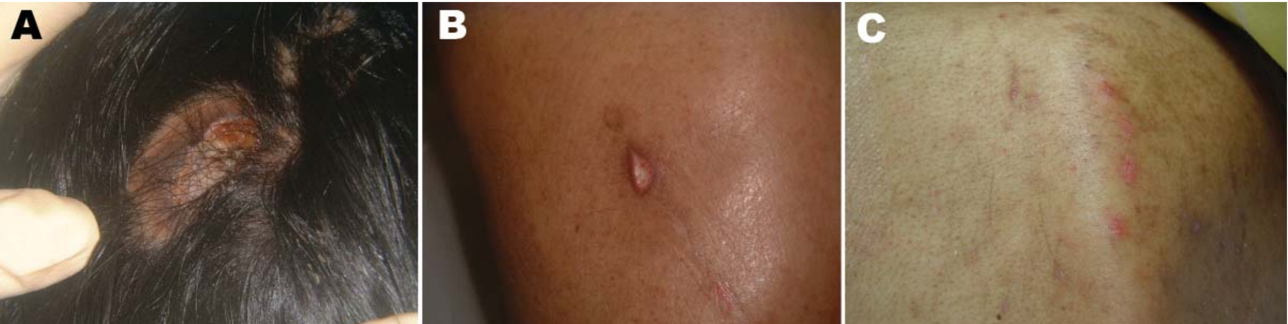

Figure 1. Ulcer with purulent discharge located on the skull (A) and on the back at first examination (B) and 17 days after topical treatment with miltefosine was initiated (C).

TB of lungs, liver, spleen, and kidneys could be achieved, and the CD4+ lymphocyte count increased to 421 cells/µL. Nevertheless, the neurologic status of the patient deteriorated, even after liposomal amphotericin B and flucytosine had been added to the regimen. Consecutive cranial CT and cranial MRI scans demonstrated progression of the lesions, with the biggest lesion (1.8 cm in diameter) located in the right cerebellopontine angle and cerebellum (Figure 2, panels A and B).

As skin lesions were also gaining size, treatment with miltefosine, a phosphocholine analog that has proven successful in treating visceral leishmaniasis (*6*) and is highly effective against acanthamoebae in vitro (*7,8*), was initiated topically as a solution, 60 mg/mL, 1 drop applied directly to each skin lesion 2 times a day. After dramatic improvements of the skin lesions were observed within only 3 weeks (Figure 1, panel C), our patient began peroral miltefosine 100 mg/day (2.5 mg/kg); all other drugs except the tuberculostatic 5-drug regimen were stopped.

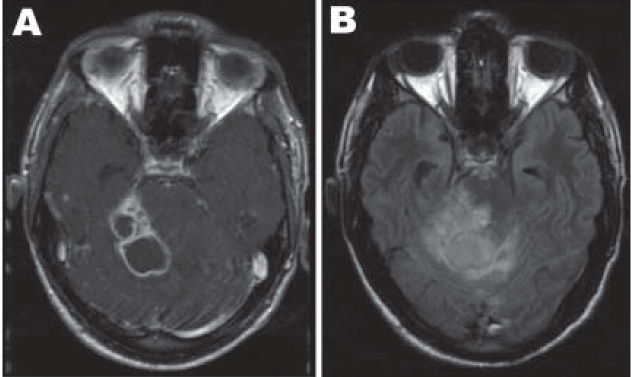

Figure 2. Axial brain magnetic resonance imaging at the level of the cerebellum performed 6 weeks after initial consultation. A) Contrast-enhanced T1 weighted image showing several ring-enhancing lesions in the right cerebellar hemisphere and the right cerebellar peduncle. B) The corresponding fluid attenuation inversion recovery image illustrates the extensive perifocal edema exerting a severe mass effect through compression and displacement of the fourth ventricle with consecutive enlargement of the lateral ventricle.

On MRI scan, performed 8 weeks after admission, the brain abscess located in the right cerebellopontine angle had reached a size of 4 cm, leading to a rise of intracranial pressure that could not be controlled by intravenous administration of mannitol and dexamethasone. Nine weeks after admission, an Ommaya Reservoir was implanted, and up to 100 mL of CSF was drained daily to control intracranial pressure. Amikacin, the only other drug that seemed to have some efficacy against *Acanthamoeba* spp. in vitro (*2*), was given intrathecally and intravenously (20 mg/2 mL intrathecally + 1,000 mg/d intravenously) in place of the streptomycin originally included in the 5-drug regimen, under continuous monitoring of peak and trough levels in CSF and blood. A surgical excision of the abscess was performed 3 weeks later. The histologic specimen of this lesion was again positive for *Acanthamoeba* sp. by PCR but negative by immunostaining. Neither acanthamoebae nor mycobacteria could be grown in culture, despite positive ZN staining of the specimen (Table).

Under ongoing therapy with miltefosine, amikacin, and 4 more tuberculostatic drugs, the patient improved. The remaining cerebral lesions regressed in size. Healing of the dermal lesions was achieved within 6 weeks; topical miltefosine treatment was stopped after 8 weeks. Intrathecal amikacin and oral miltefosine therapy was halted 6 and 12 weeks, respectively, after initiation.

A 2-drug tuberculostatic therapy was maintained for 1 year after the patient had been discharged from hospital. Two more lumbar punctures were performed 23 and 29 weeks after the patient was initially evaluated. For the first time neither mycobacteria nor acanthamoebae could be detected. CT scan and MR imaging of the brain showed no major pathology. Serologic titers, which had gradually declined after initiation of miltefosine therapy, reached normal levels. The patient was transferred to a specialized neurologic institution for rehabilitation. Ataxia and hearing impairment did not improve. During the next 24 months, the patient was regularly seen in our outpatient clinic. No

Table. Diagnosis of *Acanthamoeba* sp. and mycobacterial infection*

Organism, patient specimen	Microscopy	PCR	Culture	Immunostaining	Serology
Acanthamoeba sp.					
Cerebrospinal fluid	Negative	Positive	Negative	NA	NA
Brain biopsy	Negative	Positive	Negative	Negative	NA
Bronchial secretions	Negative	Positive	Negative	NA	NA
Lung biopsy	Negative	Positive	Negative	Negative	NA
Skin biopsy	Negative	Positive	Negative	NA	NA
Blood	NA	NA	NA	NA	Positive
Mycobacterium tuberculosis					
Cerebrospinal fluid	Positive	Positive	Positive	NA	NA
Brain operative specimen	Positive	Positive	Negative	NA	NA
Sputum	Negative	Positive	NA	NA	NA
Bronchial secretions	Negative	Positive	Positive	NA	NA
Lung biopsy	Negative	Negative	Negative	NA	NA
Blood	NA	Positive	Negative	NA	NA

*NA, not available.

signs of infection could be found, and *Acanthamoeba* immunoreactivity remained below cutoff.

Disseminated acanthamoeba infection is a rare disease characterized by widespread granulomatous infiltration of the skin and extracerebral organs; it usually occurs in immunocompromised patients. Most reported cases have progressed to granulomatous amoebic encephalitis (GAE). The incidence of GAE is low in spite of the ubiquity of these amoebae. Although <200 cases of GAE have been described worldwide, it is still of substantial medical relevance because it is usually fatal due to diagnostic difficulties (*9*,*10*) and lack of effective treatment.

In our patient, co-infection with *M. tuberculosis* with severe immunosuppression may have contributed to his susceptibility to acanthamoeba infection but not to the disease progression and clinical deterioration seen even after TB could be controlled. Problems in culturing acanthamoebae from clinical specimens have been reported frequently, and isolation of amoebae from CSF is generally uncommon (*11*). In our case no reactivity to immunofluorescence was seen, either because the biopsy missed the area of active infection or because of the low sensitivity of polyclonal antibodies available. Nevertheless, early diagnosis of acanthamoeba infection in our patient was achieved by molecular methods that proved to be more sensitive than microscopy and culture.

GAE and cutaneous acanthamoeba infections have been empirically treated with a wide array of antimicrobial agents. The outcome has been mostly failure (*3*,*11*,*12*), except for a few cases that occurred in immunocompetent patients (*10*,*13–15*). Few case studies reported successful treatment of patients with a solitary brain lesion or initiation of therapy before the infection entered the brain (*5*,*10*). The first successful treatment of AIDS-related GAE was reported in 2000 (*4*).

The condition of our patient deteriorated under empirical treatment with antimicrobial agents previously used to treat acanthamoeba infection. When anti-amoebic therapy was changed to peroral and topical miltefosine, the skin lesions healed and the brain lesions regressed. After the remaining brain lesion had been surgically excised, the patient could be discharged from the hospital. Two years after treatment ended, the patient is partly rehabilitated with no signs of amoebic or mycobacterial infection.

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Dr Aichelburg is a respiratory and infectious diseases physician at the Otto Wagner Hospital in Vienna. He specializes in HIV medicine. His other research interests include tuberculosis, malaria, and opportunistic infections.

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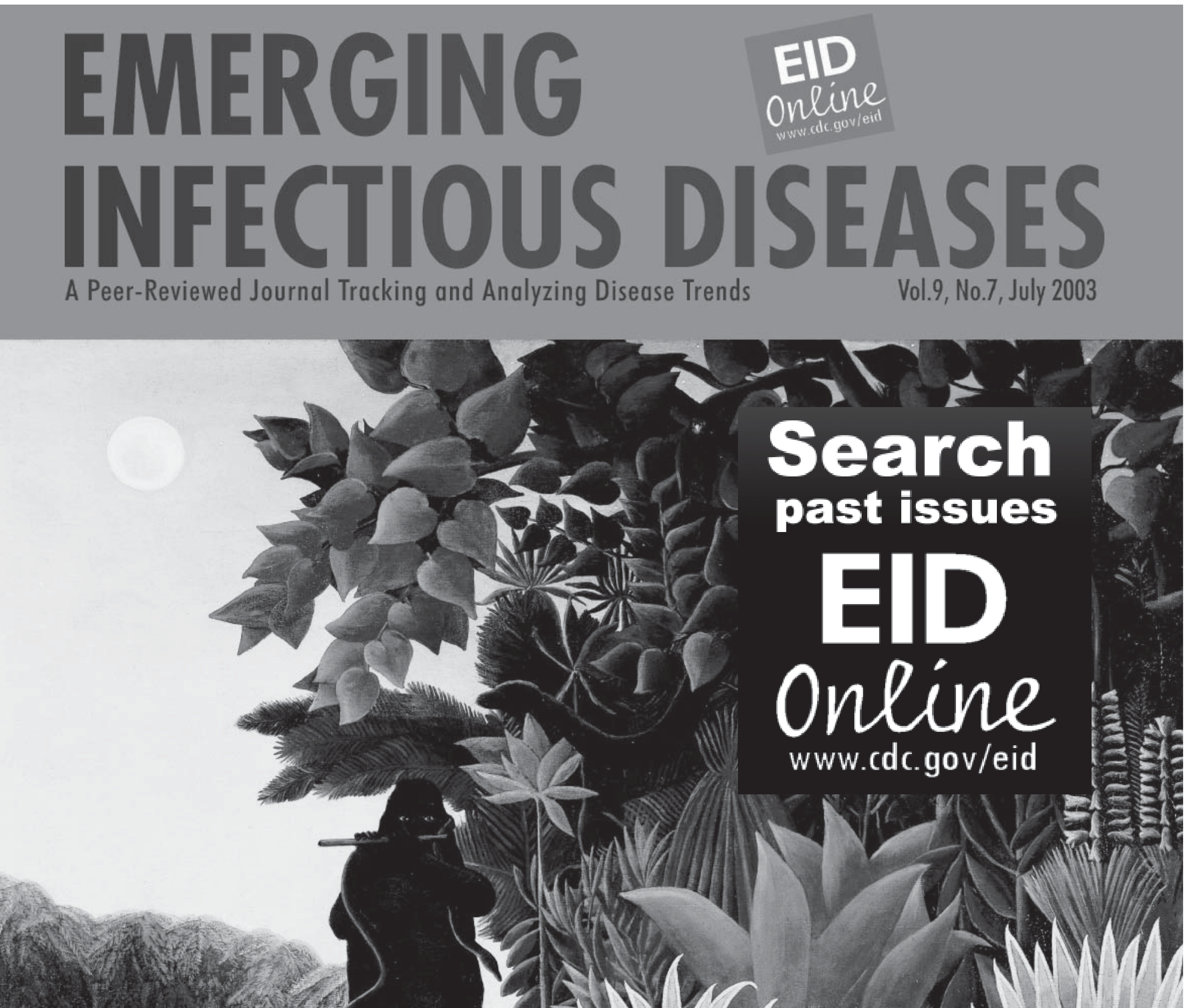

Delinquent Mortgages, Neglected Swimming Pools, and West Nile Virus, California

William K. Reisen, Richard M. Takahashi, Brian D. Carroll, and Rob Quiring

Adjustable rate mortgages and the downturn in the California housing market caused a 300% increase in notices of delinquency in Bakersfield, Kern County. This led to large numbers of neglected swimming pools, which were associated with a 276% increase in the number of human West Nile virus cases during the summer of 2007.

Although West Nile virus (WNV) (family *Flaviviridae*, genus *Flavivirus*) has remained epidemic in California since its arrival in 2003 (*1*), the cascade of events enabling local outbreaks remains poorly understood. WNV is amplified enzootically among several passeriform bird species within concurrent rural and urban cycles and is tangentially transmitted to humans by several *Culex* mosquito species (*2*). *Culex tarsalis* is the primary rural vector whose abundance relies on the availability of surface water created by precipitation and agricultural irrigation, whereas members of the *Cx. pipiens* complex are urban vectors whose abundance is dependent on underground drainage systems, wastewater, or anthropogenic peridomestic sources (*3*). Surveillance data useful in tracking WNV risk include temperature anomalies, mosquito abundance and infection rate trends, sentinel chicken seroconversions, dead bird reports and necropsy results, and the numbers of reported equine and human cases. Each of these factors are assigned a risk score, averaged, and ranked in terms of overall risk from 1 (normal season) to 5 (epidemic conditions) (*4*).

The Study

An outbreak with 140 laboratory-confirmed human cases of WNV was centered in the Bakersfield area of Kern County, California, during 2007 (incidence = 17.5/100,000 population). This case cluster formed the WNV epicenter for California during 2007, was the largest mosquito-borne encephalitis virus outbreak in Kern County since the 1952 epidemic of western equine encephalitis virus (*5*) and represented a 205%–280% increase in the numbers of confirmed WNV cases observed since 2004 (*6*). The 2007 outbreak was unanticipated on the basis of surveillance data. Winter and spring weather was exceptionally dry (40% of expected rainfall) and hot (mean March–June temperatures ranged from 0.5°C–2.0°C above 30-year normal values). Rural *Cx. tarsalis* populations remained below 5-year averages (–32% to –76% of average during weeks 19–29) because of decreased rainfall, snow pack, and water allotments to agriculture. The Kern River, which flows through Bakersfield, remained mostly dry during spring and summer; key bird species decreased in abundance because of the drought (overall catch of free-ranging birds in 2007 was 31% of catch at the same traps during 2006) and the previous negative effect of WNV infection; and surviving birds in key species had high herd immunity to WNV (house finch WNV seroprevalence = 22%, n = 182; western scrub jay = 44%, n = 27) acquired during previous seasons.

Despite these findings, the infection incidence in *Cx. pipiens quinquefasciatus* increased rapidly to 18.5 females/1,000 mosquitoes in June 2007 at traps within Bakersfield, a month earlier than observed in previous summers (online Appendix Figure 1, available from www.cdc.gov/EID/content/14/11/1747-appF1.htm). With reduced competition from house finches and predation on nestlings by western scrub jays, house sparrow populations increased dramatically. This expanding population was dominated by hatching year birds, had limited protective immunity (4.1%, n = 311), and comprised 23% of 124 WNV-positive dead birds reported by the public. Early season high risk of WNV infection in birds was followed closely by human cases, and this and several other case clusters of high incidence stimulated an emergency appropriation of $6.2 million by the Governor's Office of the State of California to enhance surveillance and mosquito control.

Careful examination of service requests for mosquito control made to the Kern Mosquito and Vector Control District (KMVCD) and an aerial survey of Bakersfield showed an extensive number of green or neglected pools, most of which were producing mosquitoes. The likely reasons for neglected pools are the adjustable rate mortgage and associated housing crises in Kern County and throughout California, which have led to increased house sales, notices of delinquency of payment, declarations of bankruptcy and home abandonment. Kern County was especially affected (Figure 1), with a 300% increase in notice of delinquency in the spring quarter of 2007 compared with that of 2006. Associated with home abandonment was the expanding number of neglected swimming pools, Jacuzzis (hot tubs), and ornamental ponds. As chemicals deteriorated, invasive algal blooms created green swimming pools that were

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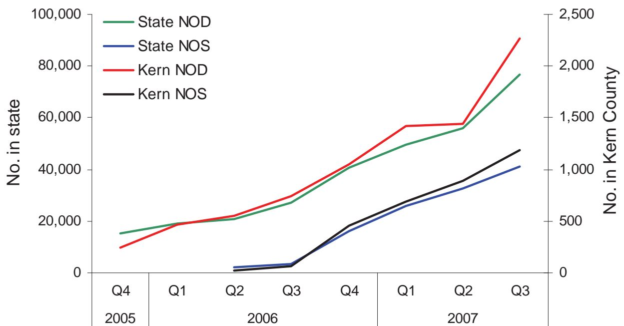

Figure 1. Notice of delinquency (NOD) and notice of sale (NOS) for homes in Kern County and California by quarter (Q) per year, 2005–2007.

exploited rapidly by urban mosquitoes, thereby establishing a myriad of larval habitats within suburban neighborhoods that were difficult to locate from the ground. These pools frequently were located within new housing tracts and not confined to old neighborhoods. An aerial photograph of a representative Bakersfield neighborhood shows the extent of the problem, with 17% of the visible 42 pools and Jacuzzis appearing green and likely producing mosquitoes (Figure 2). The extent of this problem also was indicated by the marked increase in the number of pools that required treatment by the KMVCD (online Appendix Figure 2, available from www.cdc.gov/EID/content/14/11/1747-appF2.htm). The increase in August 2007 followed an aerial survey of Bakersfield that enabled identification of previously unknown problem pools.

By law, all swimming pools or properties with pools have to be surrounded by 2-m high fencing and gates that must be locked when the homeowner is not present. These locked fences provided a formidable obstacle for mosquito control personnel to overcome for surveillance and treatment. Public awareness of this problem has been enhanced by education programs and media information, and local residents have begun to notify the KMVCD and other local agencies about neglected pool problems. Alarmingly, during 2008, many of these unmaintained pools previously positive for *Cx. p. quinquefasciatus* were now occupied by *Cx. tarsalis*, a more competent vector of WNV than *Cx. p. quinquefasciatus* (*7,8*). Collections of immature mosquitoes from 31 neglected pools taken during February–August 2008 produced 8,978 emerging adults, of which 59% were *Cx. tarsalis* and 41% were *Cx. p. quinquefasciatus*. Ongoing surveillance continues to monitor the extent of this problem in Kern County and throughout California and its affect on the ongoing WNV epidemic.

Figure 2. Aerial photograph of a representative Bakersfield, California, neighborhood taken during August 2007. Red arrows indicate neglected or green swimming pools. Letters (F, G, H, J) are photographic reference points.

Conclusions

Anthropogenic landscape change historically has facilitated outbreaks of pathogens amplified by peridomestic vectors such as *Cx. pipiens* complex mosquitoes and associated commensals such as house sparrows. The recent widespread downturn in the housing market and increase in adjustable rate mortgages have combined to force a dramatic increase in home foreclosures and abandoned homes and produced urban landscapes dotted with an expanded number of new mosquito habitats. These new larval habitats may have contributed to the unexpected early season increase in WNV cases in Bakersfield during 2007 and subsequently have enabled invasion of urban areas by the highly competent rural vector *Cx. tarsalis*. These factors can increase the spectrum of competent avian hosts, the efficiency of enzootic amplification, and the risk for urban epidemics.

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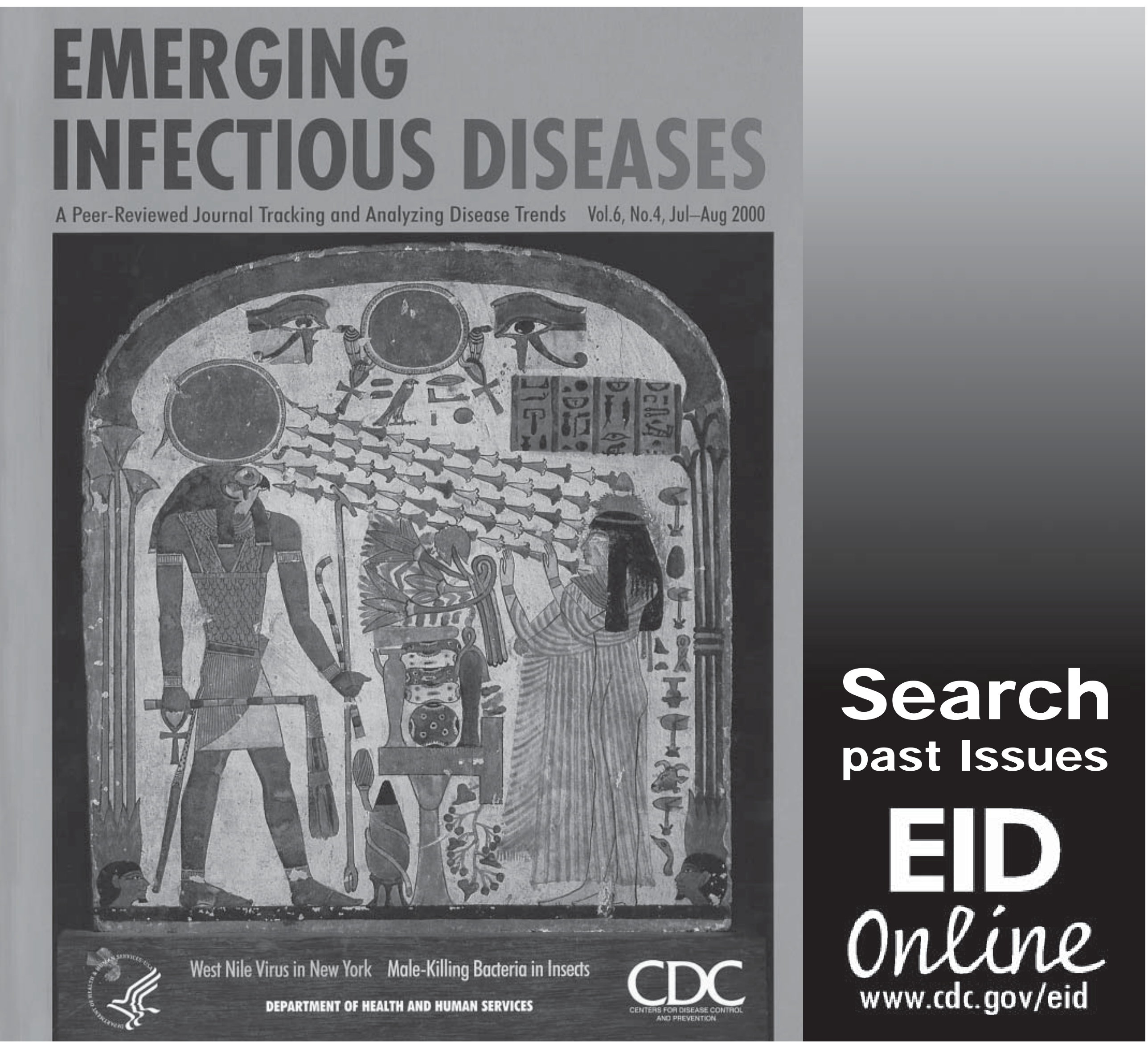

Use of Malaria Rapid Diagnostic Test to Identify *Plasmodium knowlesi* Infection

Thomas F. McCutchan, Robert C. Piper, and Michael T. Makler

Reports of human infection with *Plasmodium knowlesi*, a monkey malaria, suggest that it and other nonhuman malaria species may be an emerging health problem. We report the use of a rapid test to supplement microscopic analysis in distinguishing the 5 malaria species that infect humans.

Recent reports of *Plasmodium knowlesi* infections in humans in Sarawak and Sabah in Borneo and in the Pahang Peninsula of Malaysia have focused attention on the potential of monkey malarias to be a human health issue (*1,2*). As much as 70% of malaria infections in regional hospitals in Borneo are the result of *P. knowlesi* infection; similar infections have been found in Thailand, the Philippines, and Singapore (*3–5*). To date, only patients in hospitals are being screened for the disease. To better understand the epidemiology of this apparent outbreak of *P. knowlesi* in humans, one needs a method to rapidly screen both monkeys and humans in areas of high disease prevalence, regardless of their present health status. Thus, a rapid test that could detect and distinguish among the primate malarias would not only benefit individual patients but would also provide an important epidemiologic tool to monitor the overall risk and prevalence of malaria.

We have known for nearly 8 decades that, under laboratory conditions, several monkey malarias are capable of infecting humans and that *P. knowlesi* can be transmitted to humans by mosquito bite (*6,7*). Work in Malaysia by a team from the National Institutes of Health nearly 50 years ago reported that transmission to humans was not occurring to any prevalent extent. Currently, we see major foci of the disease, which can be life-threatening. Although the current overall incidence of *P. knowlesi* infection in humans is low, an exacerbating problem is that it can be consistently misdiagnosed by microscopy as the more benign human malaria, *P. malariae* (*1,2,8,9*). The rapid replication rate of *P. knowlesi* and the resulting high level of parasitemia warrant immediate and aggressive treatment, whereas *P. malariae* does not. Although the use of PCR has been essential to defining the problem, a more rapid diagnosis would be an important tool for prompt medical treatment. Furthermore, incorporating the capability to detect *P. knowlesi* into existing rapid tests already capable of detecting the other 4 *Plasmodium* species that infect humans (*P. falciparum, P. vivax, P. ovale*, and *P. malariae*) would be beneficial.

P. knowlesi is transmitted by members of the *Anopheles leucosphyrus* group of mosquitoes that resides in the upper canopy of the forests in large areas of Southeast Asia; these *Anopheles* mosquitoes have infrequent contact with humans (*10*). With increasing encroachment into the forest areas to provide farmland, however, humans are likely to increase their exposure to this vector. The potential for *P. knowlesi* infection as well as other monkey malarias to expand into the human population is real. While the *P. knowlesi* parasite is carried by zoophilic mosquitoes, some monkey malarias such as *P. cynomolgi* and *P. inui* are transmitted by the same mosquito vectors that carry human malaria and therefore represent an even wider threat.

One important test developed for detecting human malarias is an antigen-capture test based on monoclonal antibodies (MAbs) to plasmodium lactate dehydrogenase (pLDH). The 4 human malarial LDH isoforms have been cloned, and >20 MAbs have been raised that differentially recognize epitopes among the isoforms (*11,12*). The specificity of a subset of these antibodies is shown in Figure 1. Of the 4 human *Plasmodium* spp., antibodies such as 17E4 and 7G9 specifically bind only to *P. falciparum* LDH, whereas antibodies such as 11D9 and 13H11 bind only to *P. vivax* LDH.

Using this panel of antibodies, we show that we can distinguish *P. knowlesi* from *P. malariae*. *P. knowlesi* binds to both the "falciparum-specific" (17E4/7G9) and the "vivax-specific" (11D9/13H11) antibodies (Figure 1, panels A and B). Furthermore, *P. knowlesi* does not react with 10D12 (an antibody specific for *P. ovale*), 7E7 (an antibody that reacts strongly with *P. malaria*e and weakly with *P. falciparum*), or 9C1 (an antibody that reacts exclusively with *P. ovale* and *P. malariae*). Detecting *P. knowlesi* in monkeys, which often are co-infected with several other malaria parasites, is also important and can be achieved with the same panel of antibodies. We have tested the reactivity of the *P. falciparum*–specific antibody (17E4/7G9) with the other monkey malarias known to be indigenous to Malaysia (*P. cynomolgi*, *P. inui,* and *P. fieldi*) and found that none react (Figure 1, panel C). This then serves as a basis for distinguishing *P. knowlesi* from the other prevalent forms of monkey malaria.

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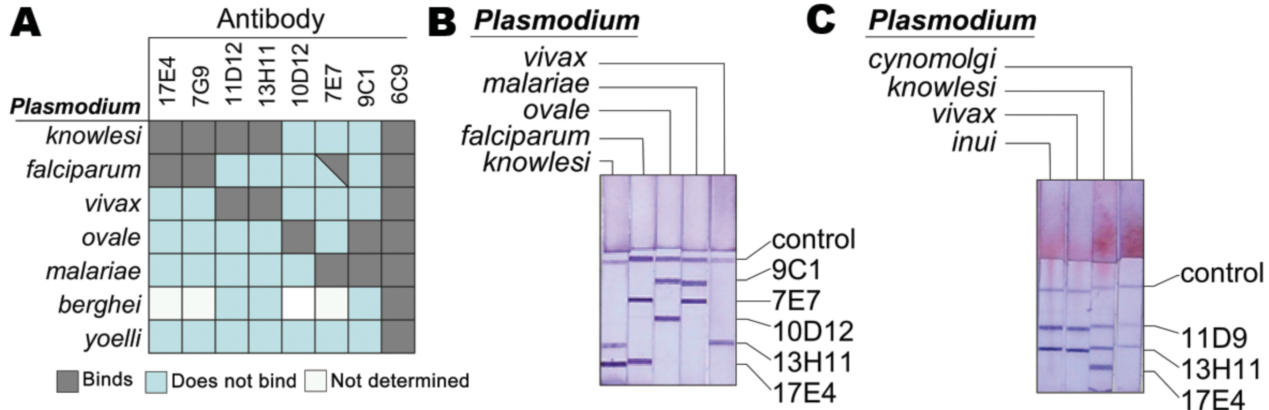

Figure 1. Binding specificity of different anti–*Plasmodium* lactate dehydrogenase (pLDH) antibodies. A) Shown are the reactivities of the indicated monoclonal antibodies (MAbs) to the LDH from 7 *Plasmodium* spp. Reactivity was determined by using an immunocapture assay as previously described (*9*). B) Example of an immunodipstick assay that detects *P. knowlesi*. An immunochromatographic strip assay containing the indicated antibodies was allowed to wick lysed blood infected with *P. vivax, P. falciparum*, *P. knowlesi, P. ovale*, or *P. malariae.* Blood was wicked in the presence of colloidal gold conjugated to antibody 6C9, which binds all pLDH isoforms. *P. vivax* LDH is immobilized only by 11D9 and 13H11, and *P. falciparum* LDH was only immobilized by 17E4. *P. knowlesi* LDH was immobilized by 11D9 and 13H11 antibodies and also by 17E4. C) An immunochromatographic strip assay containing the indicated antibodies was allowed to wick lysed blood infected with *P. vivax, P. cynomolgi, P. inui,* and *P. knowlesi.* Blood was wicked in the presence of colloidal gold conjugated to antibody 6C9, which binds all pLDH isoforms. Both *P. cynomolgi* and *P. inui* show the same epitope profile as *P. vivax*.

The unexpected pattern of antibody recognition on which we based our tests led us to examine the molecular basis of recognition (Figure 2). As expected, *P. knowlesi* LDH is highly similar to the known pLDH isoforms. We found that only a few residue differences could account for the epitope differences detected by the 17E4/7G9 and 11D9/13H11 antibodies. We first generated a 3-dimensional model of *P. knowlesi* LDH and then mapped surface-exposed residues that were uniquely shared by *P. falciparum* or *P. vivax* isoforms. The protein structure was calculated

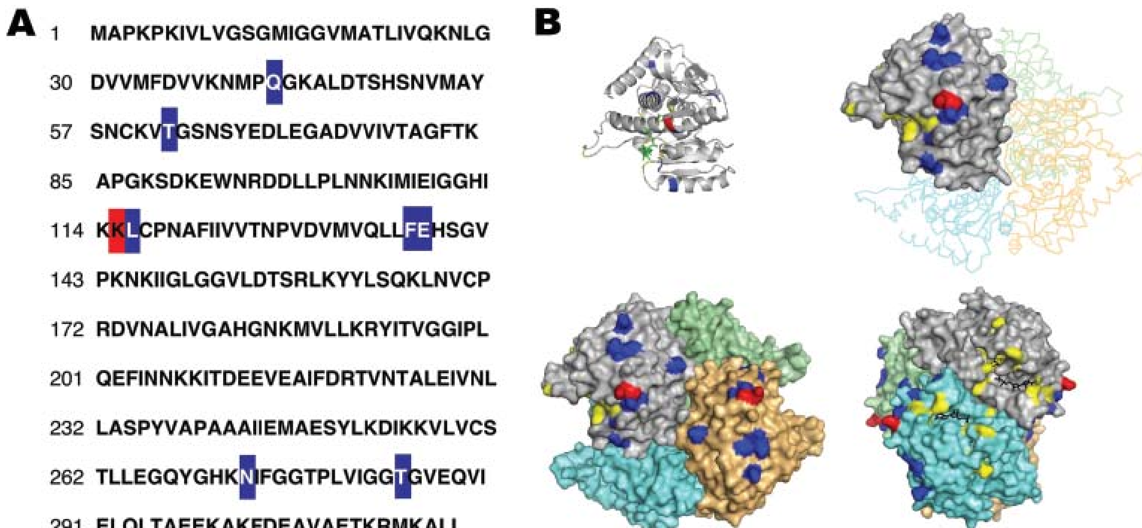

Figure 2. Modeling of the analysis of *Plasmodium knowlesi* lactate dehydrogenase (LDH). A) Sequence of LDH from *P. knowlesi* deduced from genomic DNA fragments sequenced by the Sanger malaria genome project (www.sanger.ac.uk/Projects/P_knowlesi). LDH isoforms from *P. vivax, P. malariae, P. ovale, P. berghei, P. yoelli*, and *P. falciparum* were compared with that of *P. knowlesi.* Residues unique to *P. knowlesi* and *P. vivax* are shown in blue; residues unique to *P. knowlesi* and *P. falciparum* are shown in red. B) Model of *P. knowlesi* LDH and specific epitopes. A model for *P. knowlesi* LDH was calculated by using WURST protein threading server (www.zbh.uni-hamburg.de/wurst/index.php) and the *P. falciparum* and *P. vivax* crystal structures (PDB: 2A94 and 3 2AA3). Shown is the monomer, as well as the assembled tetramer, aligned to the backbone of the *P. vivax* tetramer using pymol. The nicotinamide adenine dinucleotide cofactor analog 3-acetyl pyridine adenine dinucleotide is shown in black. Residues important for substrate binding and catalysis are shown in yellow. *P. knowlesi* residues shared only with *P. vivax* are shown in blue and indicate where the 11D9/13H11 epitopes could be. *P. knowlesi* residues shared only with *P. falciparum* are shown in red and indicate a critical determinant of the 17E4/7G9 epitopes.

by using the structures of *P. falciparum* and *P. vivax* LDH (PDB: 2A94 and 2AA3) and the WURST threading server (*13*). Here, only a few patches of residues were found to describe the *P. vivax*–specific epitope, and only 1 residue (K115) was found to describe the *P. falciparum*–specific epitope (Figure 2, panel B). Thus, the existing MAbs perform well at distinguishing pLDH isoforms despite only a small number of different surface-exposed residues.

These data show that pLDH antibodies that detect *P. falciparum* and *P. vivax* can also be used to detect and distinguish *P. knowlesi.* The 1 major caveat is that a *P. knowlesi* infection cannot be distinguished from a mixed infection with both *P. vivax and P. falciparum* in the blood. Mixed infections of this description, however, are infrequent, as these species do not proliferate concurrently when both are present in the blood (*14,15*). Furthermore, any confusion would be resolved by microscopic examination of blood that, while inadequate to distinguish *P. knowlesi* and *P. malariae*, would serve to distinguish *P. knowlesi* from mixed infections.

Obviously, an antibody specific for *P. knowlesi* would be optimal if the threat of *P. knowlesi* increases. Although development of a specific antibody would be a considerable investment, our epitope analysis discussed here indicates that only small sequence differences in pLDH isoforms are required to generate antibody panels capable of uniquely distinguishing animal pLDH isoforms.

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Dr McCutchan is head of the Regulation of Growth and Development Section of the Laboratory of Malaria and Vector Research at NIAID. His work on malaria, which spans over 25 years, has resulted in the publication of >100 manuscripts on such subjects as vaccine development, parasite diagnosis, and antimalaria drug development.

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Phylogenetics and Pathogenesis of Early Avian Influenza Viruses (H5N1), Nigeria

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Three highly pathogenic avian influenza subtype H5N1 and 4 Newcastle disease viruses were isolated from sick or dead chickens in southwestern Nigeria. Sequencing and phylogenetic analysis placed them within H5N1 subclade 2.2.2. Intravenous and intranasal pathogenicity tests produced systemic disease with vascular endothelial cell tropism in chickens.

The first official report of avian influenza virus (H5N1) in Africa was made in January 2006 (*1*). Before then, surveillance was ongoing at the poultry clinic of the University of Ibadan Veterinary Teaching Hospital to identify causes of death in chickens in Nigeria.

The Study

Nasopharyngeal and cloacal swab samples were collected from sick and dead birds found on farms near Ibadan, Nigeria, and injected into 10-day-old embryonating chicken eggs. Retrospective analysis of isolates obtained in or near January 2006 identified influenza virus (H5N1) in 3 samples. In addition, 4 Newcastle disease virus isolates were obtained, which highlights the cocirculation of Newcastle disease virus and influenza viruses (H5N1) in Nigerian poultry and emphasizes the need for specific virologic testing to distinguish the clinically similar poultry diseases caused by these 2 pathogens.

The 3 avian influenza (H5) isolates were designated influenza A/chicken/Nigeria/228-5/2005, A/chicken/Nigeria/228-6/2006, and A/chicken/Nigeria/228-10/2006. Nucleotide sequences of the coding regions of all 8 segments of the 3 viruses demonstrated that all isolates possessed a multiple basic amino acid at the hemagglutinin (HA) cleavage site with the sequence PQGERRRKKR. Sequences at this site were identical to those of highly pathogenic avian influenza (HPAI) subtype H5N1 viruses from Europe, Russia, Asia, and from recent isolates from the Lagos state of Nigeria (*2*); it lacks a single basic residue when compared with HA from strains from southeastern People's Republic of China, Vietnam, Cambodia (PQRERRRKKRG), and Thailand (PQREKRRKKRG) (*3*). Other notable features of the sequences were the absence of the H274Y genetic change associated with high-level resistance to oseltamivir in influenza neuraminidase 1 (N1) viruses (*4*). Similarly, known amantadine resistance–linked mutations were absent. The nonstructural (NS) 1 open reading frame encodes a 5-aa deletion at positions 80–84, as has been observed since 2001 in subtype H5N1 isolates from poultry. One of the isolates (A/chicken/Nigeria/228-10/2006) also has a C-terminal amino-acid extension, which is predicted to affect the function of the PDZ-ligand domain otherwise present at the C terminus of the NS1 protein (*5*,*6*). This sequence change did not detectably affect the ability of NS1 to block interferon induction when expressed transiently in 293T cells (data not shown). The polymerase basic 2 protein (PB2) of these viruses possesses a lysine residue at position 627, an amino acid previously implicated in mammalian adaptation of subtype H5N1 viruses (*7*–*9*).

Phylogenetic analysis based on the HA sequence and on complete genome sequences of HPAI (H5N1) strains grouped the 3 new isolates from Nigeria with other isolates from Europe, the Middle East, and Lagos state of Nigeria. According to recent classification by the H5N1 Evolution Working Group, the viruses belong to clade 2.2.2 (previously referred to as the European-Middle Eastern-African clade 1) (*10*). The online Technical Appendix, available from www.cdc.gov/EID/content/14/11/1753-Techapp.pdf, shows the phylogenetic trees generated on the basis of the sequences of HA, NA, nucleocapsid protein (NP), and NS segments. On the basis of phylogenetic analyses of all 8 segments, no evidence of reassortment was observed among the newly sequenced HPAI isolates from Nigeria. Although prior reports (*2*,*3*,*11*) have suggested 3 independent introductions of HPAI viruses into Nigeria, our analysis of viruses in this study and in GenBank identified only 2 clades (clades 2.2.2 and 2.2.3) among the Nigeria isolates, suggesting 2 unique introductions into Nigeria.

The virulence of the HPAI virus isolated in poultry was assessed by intravenous injection of a 1:10 dilution of allantoic fluid into groups of 4-week-old SPF White Leghorn chickens, 8 per group, as previously described (*12*). According to results of this assay, all 3 isolates were highly pathogenic (Table); mean times to death were 1.0, 1.3, and

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Table. Virus isolation titers from chickens inoculated with highly pathogenic influenza A/chicken/Nigeria/228-5/2005 (H5N1) and controls*

Sample	Virus-positive swabs/swabs tested, no. (mean titer† as $\log_{10}$ EID_{50}/mL)		Virus-positive tissues/tissues tested, no. (mean titer† as $\log_{10}$ EID_{50}/g)				
	Oropharyngeal	Tracheal	Heart	Kidney	Lung	Muscle	Brain
Sham	0/2	0/2	0/2	0/2	0/2	0/2	0/2
Experimental							
1 dpi	2/2 (2.4)	1/2 (1.7)	1/2 (2.9)	1/2 (4.5)	1/2 (4.9)	1/2(3.9)	1/2 (3.5)
2 dpi	2/2 (6.1)	2/2 (6.8)	2/2 (8.7)	2/2 (7.9)	2/2 (7.6)	2/2 (6.8)	2/2 (5.6)

*Virus isolated from tissues and swabs sample on 1 and 2 days postinoculation (dpi). EID_{50}, mean embryo infectious doses.
†Limit of detection $\leq 10^{1.9}$ EID_{50}/g of tissue or $\leq 10^{0.9}$ EID_{50}/mL sample.

1.4 days, similar to times previously reported for other European-Asian lineage HPAI (H5N1) viruses (*13*).

To determine infectivity and pathogenicity, using a simulated natural respiratory route of exposure, we intranasally inoculated ten 4-week-old White Leghorn chickens with strain A/chicken/Nigeria/228-5/2005 (10^6 mean embryo infectious doses). The caged birds were inspected daily for clinical signs and death. Two chickens that were not inoculated and were maintained as negative controls were euthanized (100 mg sodium pentobarbital/kg body weight) at 0 days postinoculation (dpi). At 1 dpi, 2 of the injected birds were euthanized. On 2 dpi, postmortem examinations were performed on 2 birds that had died, and samples were collected for virus isolation and for histopathologic and immunohistochemical examination of a variety of tissues. For immunohistochemical examination, a monoclonal antibody against influenza A nucleoprotein was used as previously described (*14*). Virus isolation and titration were conducted with brain, heart, lung, kidney, breast muscle, oropharyngeal, and cloacal samples; embryonating chicken eggs were used (*12*).

After intranasal inoculation with A/chicken/Nigeria/228-5/2005, all birds died or were euthanized for severe illness within 3 dpi (mean time to death 2.125 days). At 1 dpi, both intranasally inoculated chickens sampled were infected, as evidenced by low virus titers in oropharyngeal swabs; however, only 1 chicken had systemic infection with moderate titers of virus in tissues (Table). Histologically, both chickens lacked lesions, but the spleen of 1 chicken had a few avian influenza virus–positive histiocytes. The intranasally inoculated chickens that died and were necropsied on 2 dpi had exhibited mild to moderate listlessness, a hunched posture, and partial closure of eyelids a few hours before death. Gross lesions included scattered petechia in epicardial fat within the coronary groove (Figure, panel A), a few petechia in mucosa of the ven-

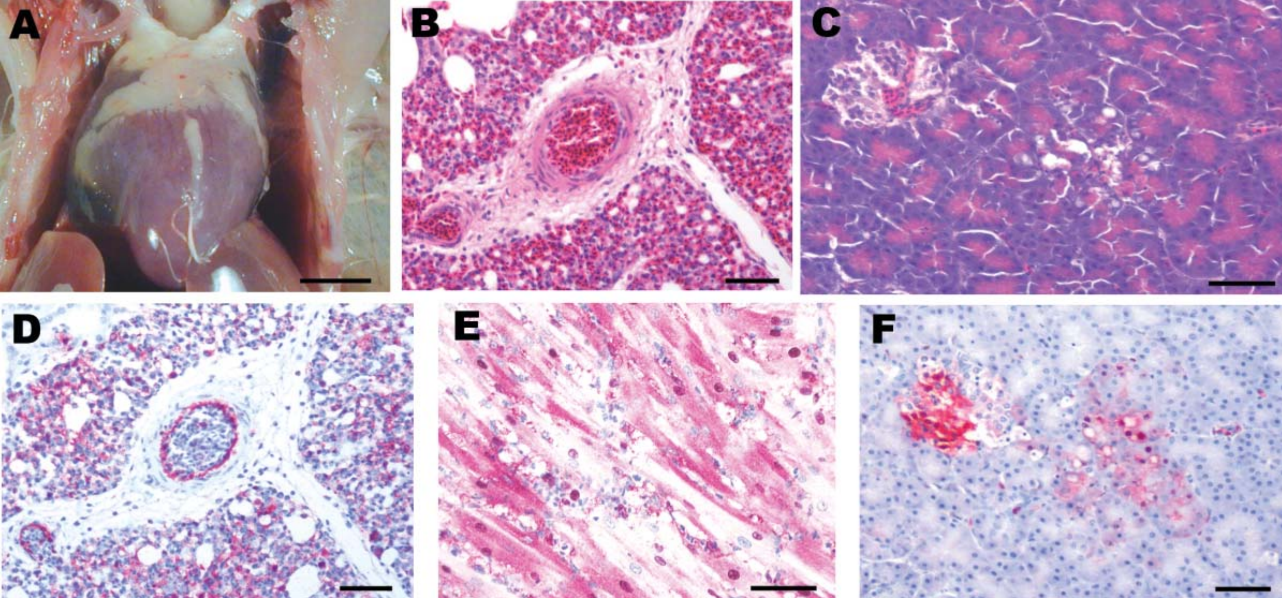

Figure. Experimental studies in chickens inoculated intranasally with highly pathogenic influenza A/chicken/Nigeria/228-5/2005 (H5N1) and sampled 2 days postinoculation. A) Photograph (scale bar = 1 cm) of a gross lesion, showing petechia in epicardial fat in the coronary groove. B,C) Photomicrographs (scale bars = 50 μm) of tissue sections stained with hematoxylin and eosin, showing severe histiocytic and heterophilic interstitial pneumonia with moderately interlobular edema (panel B) and pancreatic acinar epithelium (panel C). D–F) Photomicrographs (scale bars = 50 μm) of tissue sections stained immunohistochemically to demonstrate avian influenza virus nucleoprotein, showing avian influenza virus antigen in pulmonary and blood vessel endothelial cells and histiocytes (panel D), nuclei and cytoplasm of cardiac myocytes (panel E), and pancreatic acinar epithelium and islet cells (panel F).

triculi (gizzards), slight increase in pericardial fluid, and swollen kidneys. Swabs from respiratory and alimentary tracts and the tissues had high titers of the virus (Table). Histologically, tissues with lesions coincided with sites of virus replication and indicated severe systemic infection. The most severe lesions were severe interstitial pneumonia with edema (Figure, panel B), moderate to severe myocyte degeneration and necrosis in the heart, moderate nonsuppurative encephalitis, moderate necrotizing rhinitis, moderate lymphohistiocytic depletion and apoptosis in the spleen, and mild to moderate degeneration and necrosis of pancreatic acinar epithelium (Figure, panel C). Scattered lesions of necrosis and inflammation were seen in liver, cloacal bursa, thymus, proventriculus, ventriculus, and pancreatic islets. Influenza A virus was localized to necrotic cells, which most frequently included blood vessel endothelium throughout the body (Figure, panel D), cardiac myocytes (Figure, panel E), pulmonary histiocytes and heterophils (Figure, panel D), neurons and glial cells in the brain, splenic histiocytes and cellular debris, and renal tubular epithelium. Virus was less frequently identified in Kupffer cells and hepatocytes; histiocytes in lamina propria of alimentary tract, thymus, and cloacal bursa; pancreatic acinar and islet epithelium (Figure, panel F); proventricular epithelium; and bone marrow myeloid cells.

Conclusions

The 3 early HPAI (H5N1) isolates from Nigeria, which belonged to clade 2.2.2, produced in chickens a systemic infection characterized by virus replication and associated necrotic and inflammatory lesions in critical internal organs such as the heart, brain, and lungs. A prominent vascular tropism of the virus was evidenced by widespread replication in blood vessel endothelium throughout the body and is typical of other HPAI viruses (H5N1) of the Asian lineage.

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Growth and Geographic Variation in Hospitalizations with Resistant Infections, United States, 2000–2005

Marya D. Zilberberg, Andrew F. Shorr, and Marin H. Kollef

From 2000 through 2005, hospitalizations with resistant infections (methicillin-resistant *Staphylococcus aureus*, *Clostridium difficile*–associated disease, vancomycin-resistant enterococcus, *Pseudomonas aeruginosa,* and *Candida* infection) nearly doubled, from 499,702 to 947,393. Regional variations noted in the aggregate and by individual infection may help clarify modifiable risk factors driving these infections.

Over the past decade we have witnessed a rise in the antimicrobial drug–resistance epidemic in the United States and worldwide. Not only are resistant organisms such as methicillin-resistant *Staphylococcus aureus* (MRSA) and *Clostridium difficile* encountered with increasing frequency (*1*,*2*), but as their susceptibility to antimicrobial agents is waning, their overall virulence is on the rise (*3*,*4*). Additionally, though traditionally thought of as a nosocomial pathogen, MRSA, for example, is now a well-recognized community-acquired infection (*5*).

With the rapid growth of resistance, and the added associated illnesses and deaths (*6*,*7*), these infections exert a considerable strain on the US healthcare system, specifically on hospitals. Although important to understand for individual infections, the aggregate volume of resistance is an important factor in illustrating the problem as a whole and in helping identify the potential resources needed to deal with the epidemic. To understand the full extent of resistant infections in US hospitals, we examined their longitudinal trends from 2000 through 2005, focusing further on regional patterns of resistance during this time frame.

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The Study

We identified all hospitalizations carrying a diagnosis of MRSA, *Clostridium difficile*–associated disease (CDAD), vancomycin-resistant enterococcus (VRE), *Pseudomonas aeruginosa,* and *Candida* infections for 2000–2005 from the National Inpatient Sample data. These data are available on the Healthcare Costs and Utilization Project net [HCUPnet] website, administered by the Agency for Healthcare Research and Quality (*8*). We used the corresponding diagnosis codes from the International Classification of Diseases, 9th revision, Clinical Modification (online Appendix Table, available from www.cdc.gov/EID/content/14/11/1756-appT.htm). Because few reports of vancomycin-resistant *Staphylococcus aureus* exist (*9*), we assumed that most cases with the code V09.8 represented VRE infections. We limited hospitalizations in which *Candida* organisms had been identified to deep-seated infections, including candidiasis of the lung, disseminated candidiasis, candidal endocarditis, meningitis, esophagitis, and enteritis. The numbers of discharges per year for infections associated with each organism and in aggregate were stratified by census region. We obtained regional estimates of all US hospitalizations in the corresponding years from the HCUPNet (*8*), and censal and intercensal data on the US population for 2000–2005 from the US Census Bureau. We calculated region-specific hospitalization incidence rates associated with the resistant pathogens. Because large numbers would predispose the study to type I error, we did not perform formal significance testing; rather, we focused on clinical and policy-relevant trends.

The overall volume of resistant infections increased by 89.6% from year 2000 through 2005 (Table). As a proportion of the total volume growth, the increases across regions were comparable. The southern region had the highest raw volume of resistant infections for the study period (2000, 37.3%; 2005, 39.1%). The West had the smallest contribution in 2000 (19.0%) and 2005 (19.5%). However, the Northeast had the highest relative incidence per 1,000 hospitalizations with 14.00 in year 2000; its incidence of 19.98 in 2005, however, was lower than that in the South, 20.76/1,000 (Table). Regional disparities in the population-based incidence of hospitalizations with resistant organisms also occurred (Table). Thus, the incidence in the Northeast was not only the highest for 5 of the 6 years examined, but compared to that seen in the lowest-incidence region, the West, was higher by as much as 41.9% in 2003. This gap shrank in 2004 and 2005 to 29.9% and 27.7%, respectively.

When the incidences of individual component infections were examined, several patterns emerged. While the Northeast led other regions in the incidence of CDAD hospitalizations over the entire period examined (Figure, panel A), the South exhibited the highest population incidence

Table. Volume, incidence of, and hospitalizations for infections with resistant organisms in the United States, by census region, 2000–2005

Hospitalizations and incidence	2000	2001	2002	2003	2004	2005
Annual no. hospitalizations						
All US	499,702	559,728	639,468	699,140	783,601	947,393
Northeast	102,913	119,799	132,607	151,306	152,881	188,306
Midwest	115,623	122,122	144,647	161,166	179,547	204,351
South	186,320	212,450	245,933	256,420	305,822	370,348
West	94,846	105,357	116,281	130,247	145,353	184,390
Incidence/1,000 hospitalizations						
All US	13.72	15.05	16.92	18.29	20.27	24.19
Northeast	14.00	16.17	17.74	20.01	19.98	24.29
Midwest	13.72	14.10	16.47	18.27	20.14	22.65
South	13.59	15.04	17.09	17.62	20.76	24.79
West	13.69	15.07	16.25	17.88	19.74	24.76
Incidence/100,000 population						
All US	177.08	196.32	222.12	240.71	267.27	320.18
Northeast	191.76	222.22	244.98	278.51	280.73	345.49
Midwest	179.27	188.40	222.21	246.63	273.59	310.31
South	185.27	208.55	238.33	245.54	288.87	344.88
West	149.45	163.31	177.59	196.33	216.17	270.56

of MRSA and *Pseudomonas* hospitalizations. Although temporal patterns of regional population incidence varied somewhat for hospitalizations in which VRE and *Candida* spp. infections were diagnosed, by year 2005 the Northeast emerged as the region with the highest incidence of VRE, while the South had the highest incidence of *Candida* spp. hospitalizations. The lowest incidence of VRE hospitalizations was consistently seen in the southern region in each of the studied years. The incidence of hospitalizations with pseudomonal infections remained relatively stable regionally over time (Figure, panels B, C, D).

Conclusions

We have demonstrated a substantial rise in the absolute number, incidence, and geographic variations across the United States in hospitalizations in which infections have been caused by pathogens exhibiting antimicrobial resistance. The Northeast consistently outpaced the other regions in the aggregate volume of resistant infections in 5 of the 6 years examined. For individual infections, a region's having a relatively high incidence of 1 organism does not guarantee it will have a high incidence of another organism, as illustrated by the reversal of the regional incidence patterns for MRSA and CDAD, for example. Most troubling, however, is the general finding of a ubiquitous, substantial, and continuing increase in the incidence of hospitalizations with resistant infections.

A notable pattern in our study is that the regions with the higher incidence of CDAD (Northeast and Midwest) also exhibited higher incidence of VRE in at least half of the study period, consistent with the observation that infection with CDAD can facilitate transmission of VRE (*10*,*11*). The South had the highest incidence of MRSA and lowest incidence of VRE. Since both pathogens share similar risk factors, why this pattern should be present is biologically unclear (*12*,*13*), although a recent report noted a similar

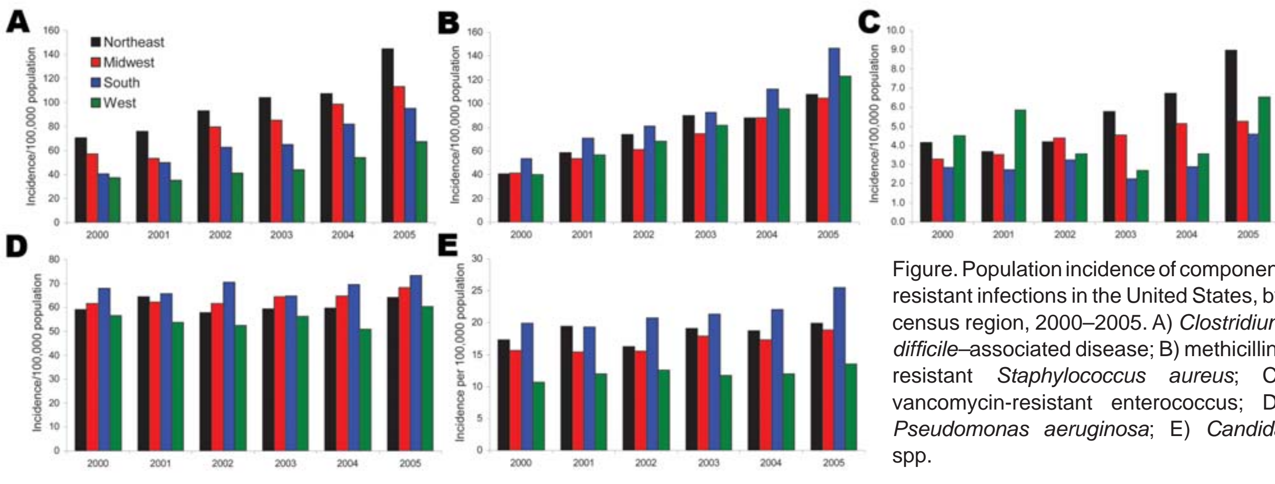

Figure. Population incidence of component resistant infections in the United States, by census region, 2000–2005. A) *Clostridium difficile*–associated disease; B) methicillin-resistant *Staphylococcus aureus*; C) vancomycin-resistant enterococcus; D) *Pseudomonas aeruginosa*; E) *Candida* spp.

pattern of concomitant increases in MRSA and decreases in VRE incidence between 1999 through 2005 (*14*). This potential inverse relationship should be investigated further. Lastly, we noted that, although substantially discrepant regionally, the incidence of hospitalizations with *P. aeruginosa* infections, consistent with others' observations, has remained relatively stable over the 6-year period (*15*). We cannot illuminate the reasons for the patterns of infection incidences we have uncovered. Further studies should encompass much more granular geographic data to confirm our findings and raise hypotheses to explain them.

The most important limitation of our study is that case ascertainment was performed by using administrative coding, rather than clinical and microbiologic data, and we were unable to verify diagnostic accuracy either across time or geographic areas; therefore, the observed increases may be partially due to increased awareness of resistance. However, administrative coding has been used to track the epidemiology of both MRSA and CDAD (*1*,*2*). Furthermore, temporal trends in case volume are similar to trends reported from clinical studies. At least a proportion of the case-patients we identified likely had overlapping infections with multiple organisms. Nevertheless, the aggregate number of infections that we have described has implications for hospital resource use because persons with multiple infections likely require more care than those with a single pathogen. Finally, we were unable to differentiate between community-acquired and nosocomial infections.

In summary, we have demonstrated a notable increase in the incidence of hospitalizations with resistant organisms in the United States. Regional variations in the incidence may yield clues for future research efforts to ascertain what modifiable risk factors drive decreases in the incidence of these deadly infections. The nearly 1 million annual hospitalizations in 2005 with resistant infections and their relentless upward trajectory in the United States are undesirable and unsustainable. Aggressive and coordinated efforts to reduce inappropriate use of antibimicrobial agents in humans and livestock and to encourage development of novel therapeutics are urgently needed to stem this public health hazard in the United States and throughout the world.

Dr Zilberberg is a health services researcher at the University of Massachusetts, Amherst, and the president of Evi*Med* Research Group, LLC. Her research interests include reducing complications and optimizing the efficiency of healthcare delivery in the hospital setting.

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Pyemotes ventricosus Dermatitis, Southeastern France

Pascal Del Giudice, Véronique Blanc-Amrane, Philippe Bahadoran, Eric Caumes, Pierre Marty, Mariléna Lazar, Christian Boissy, François Desruelles, Arezki Izri, Jean-Paul Ortonne, Evelyne Counillon, Olivier Chosidow, and Pascal Delaunay

We investigated 42 patients who had unusual pruritic dermatitis associated with a specific clinical sign (comet sign) in 23 houses in southeastern France from May through September 2007. *Pyemotes ventricosus,* a parasite of the furniture beetle *Anobium punctatum,* was the cause of this condition.

In 2006, we described an outbreak of unusual dermatitis in southeastern France (*1*). Patients affected had highly erythematous pruritic macules typical of arthropod bites, sometimes associated with a linear erythematous macular tract that we called the comet sign (Figure 1). The cause of this outbreak remained unknown. In May 2007, during an entomologic ecoenvironmental investigation conducted inside the homes of some of these patients, we found wooden furniture, which harbored live furniture beetles (*Anobium punctatum*) (Figure 2, panel A), and small amounts of wood dust on the floor. Because *A. punctatum* does not bite humans or cause contact dermatitis (*2*), it was not considered as the direct causative agent. However, stereomicroscope examination of the wood dust identified the mite *Pyemotes ventricosus* (Figure 2, panel B). Because *Pyemotes* spp. can cause dermatitis (*3*), they were considered as the hypothetical agent causing the eruption. We then conducted an observational and entomologic study of the new cases.

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The Study

From May through September 2007, all patients referred to the dermatology department at Fréjus Hospital (Fréjus, France) with suspected arthropod-bite dermatitis were examined; patients were also checked for the comet sign. Patients were asked about their outdoor activities (walking in the countryside, gardening), homes, and whether they had wooden furniture potentially infested with furniture beetles. Skin eruptions were photographed. Detailed clinical and histologic descriptions of the lesions have been reported (*1*).

Whenever possible, ecoenvironments (surrounding terrain and home interior) of patients with typical macules associated with the comet sign were investigated. Household pets were examined by veterinarians. When wormholes were found in wooden furniture, *A. punctatum* was noted as present in the immediate environment. Mites were collected and examined under a stereomicroscope (magnification ×80). Removal of furniture was recommended if it was infested with *A. punctatum*.

From May through October 2007, 42 patients with typical eruption formed 23 clusters, comprising 1–5 patients each living in the same home. Nineteen patients lived ≤50 km from Fréjus and 23 were vacationing in this city. Among the 23 homes in the cluster, 14 were entomologically and ecoenvironmentally investigated. *A. punctatum* (Figure 2, panel A) was found in all 14 homes and *P. ventricosus* was found in 12 (83%). Females (Figure 2, panel B), some gravid, and rare males were seen. Entomologic investigations outside homes found no evidence of any other insect pests. Veterinarians who examined the 2 pets living in these homes excluded dog ectoparasites. Dermatitis persisted or recurred for weeks in all patients until infested furniture was removed or patients left their homes. Oral prednisone (0.5 mg/kg) rapidly (within 48 hours) attenuated the pruritus.

One of us (P.D.) volunteered to place *P. ventricosus*–infested wood dust on 1 cm^2 of abdominal skin under an occlusive bandage for 4 hours. Two negative controls consisted of placing uncontaminated wood dust under a bandage and the bandage alone. Twenty-four hours later, an erythematous macular pruritic lesion was observed that lasted for 7 days, but no comet sign developed. No cutaneous reaction was observed under the 2 negative-control bandages.

Natural dermatitis also occurred in 2 investigators during collection of ecoenvironmental samples. Typical lesions with the comet sign (Figure 1, panels E and F) developed in these investigators after they touched wood dust and infested furniture (1 of the investigators had only entered a room with infested furniture but had not touched it). Skin scrapings of the investigators did not contain parasites. A skin-biopsy specimen from 1 investigator showed a sub-

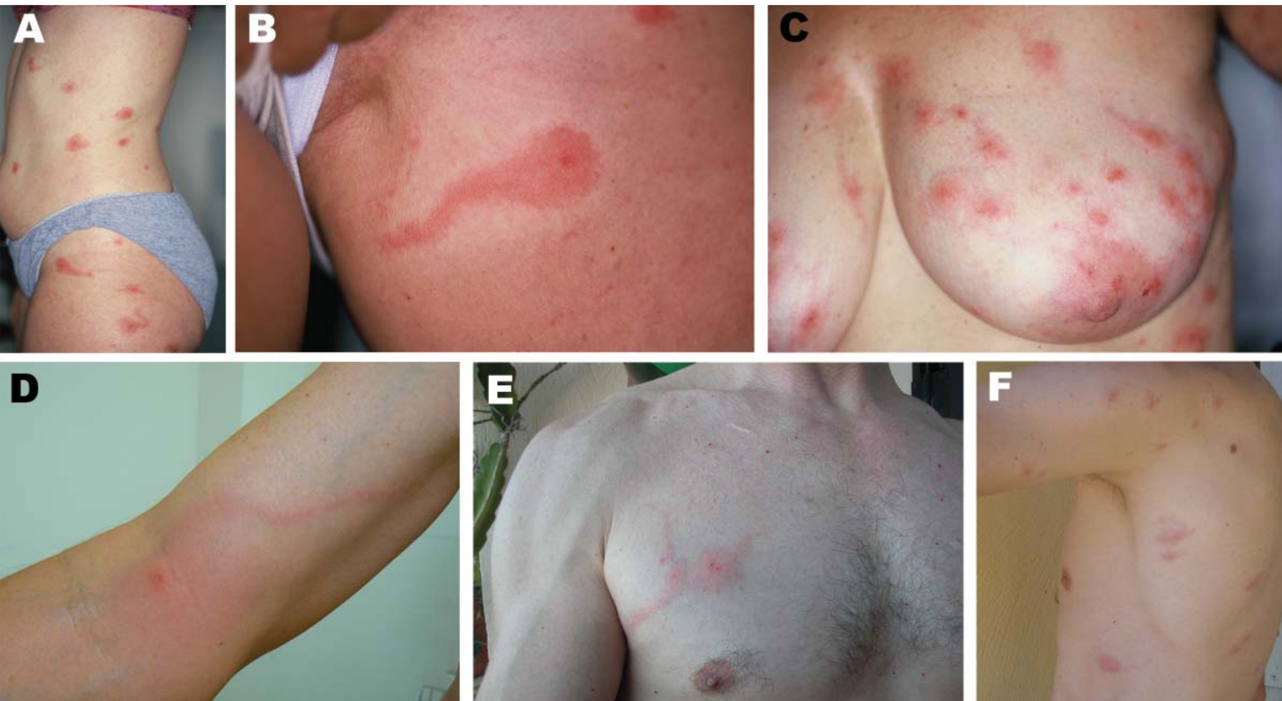

Figure 1. A–F) Photographs of 6 persons with skin lesions of *Pyemotes ventricosus* dermatitis. Note the central microvesicles, ulcerations or crusts, and some lesions with the comet sign. D) Lymphangitis-like dermatitis. E, F) Lesions resulting from natural infection of 2 of the investigators.

corneal ulceration but no parasite. Skin lesions were examined dermoscopically (magnification ×40) and showed a microulceration or vesicle in the center of the macule. In contrast, in vivo confocal laser scanning microscopy (CLSM) of a microvesicle from 1 investigator showed an ovoid foreign body with morphologic features suggestive of *P*. *ventricosus* (Figure 2, panels C and D).

Conclusions

In 2006, we described an outbreak of an unusual dermatitis associated with a specific clinical sign that we called the comet sign (*1*). In 2007, similar cases occurred and we demonstrated that the mite *P*. *ventricosus* was the causative agent of the condition. *P*. *ventricosus* (Newport, 1850) (phylum Arthropoda, class Arachnida, order Acarina, suborder Prostigmata, family Pyemotidae) is an ectoparasite of arthropod larvae. This mite has been reported to be associated with *A*. *punctatum* (De Geer, 1774), the common furniture beetle (*2*).

This mite was associated with 2 investigators' macular lesions that were acquired during experimental or natural infection. Natural infestation gave rise to macules frequently associated with the comet sign. We visualized, by using in vivo CLSM, a microvesicle, an ovoid foreign body with morphologic features suggestive of the mite. An entomologic ecoenvironmental investigation found *P*. *ventricosus* and its host (*A*. *punctatum*) in 83% of the patients' homes investigated. Although *Pyemotes* spp.–related dermatitis outbreaks have been described, the outbreak we describe showed an emergent pattern with documented intradomiciliary infestation and the comet sign.

Although *Pyemotes* spp. have been known since the beginning of the 20th century to cause dermatitis (*5*), recent reports are scarce. In all recorded outbreaks, ectoparasites of insect larvae feeding on plants were responsible for dermatitis in workers exposed to agricultural products (*3*–*7*). Recent reports of dermatitis caused by *Pyemotes* spp. are even rarer (14 clinical reports since 1961), which make this dermatitis an almost forgotten disease (*8*–*13*).

The outbreak was associated with home interior infestations of *P*. *ventricosus* associated with *A*. *punctatum.* Fine and Scott described the first cases of dermatitis caused by *P*. *ventricosus* parasitizing *A*. *punctatum* (*8*). In Great Britain, Hickin found that up to 60% of woodworm larvae in damp locations were parasitized with *Pyemotes* spp., which were considered responsible for some skin irritations in woodsmen (*2*). More recently, Rodriguez-Casado et al. (*11*) described a *P*. *dermatitis* outbreak associated with *A*. *punctatum*–infested wood desks in a school.

Our cases were concentrated from May through September. The fact that we observed most cases in July and August might reflect the mite's life cycle, which is activated when temperatures reach 80°F (26°C) (*3*). Moreover, many patients (23/42 in 2007) were on summer vacation,

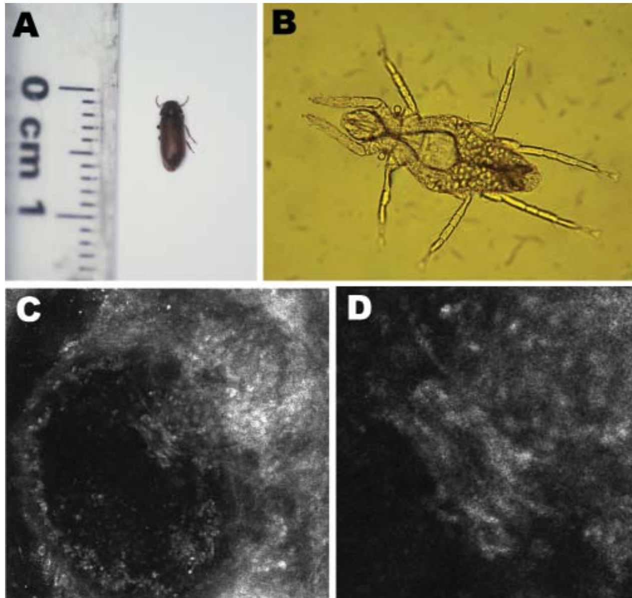

Figure 2. Organisms involved in transmission of *Pyemotes ventricosus* dermatitis. A) Common furniture beetle (*Anobium punctatum*) (5 × 2 mm). B) Nongravid female *P. ventricosus* mite (210 × 40 μm). C) Confocal laser scanning microscopy (CLSM) image (CLSM Vivascope 1500 microscope; Lucid Inc., Rochester, NY, USA) of a *P. ventricosus* mite (210 × 40 μm) in its microvesicle. D) Higher magnification of the microvesicle in panel C (light area in the center) (magnification ×4).

and most of them were living in homes that had been closed for months (i.e., not cleaned regularly), making it likely that the interior mite concentration would be high.

Pyemotes spp. dermatitis has been described as a pruritic erythematous rash with maculopapules with a central microvesicle (*7*). We initially reported that the typical pruritic erythematous macules were sometimes associated with a linear tract (comet sign) (*1*). Several hours after exposure, a linear erythematous macular tract arose from patients' lesions. Whether this sign is specific to *Pyemotes* spp. or *P*. *ventricosus* remains unknown. The epidermis along the linear tract was clinically and histologically intact (*1*), thereby making contact dermatitis or epidermal migration of *P*. *ventricosus*, as in human scabies, unlikely. In vivo CLSM detected features suggestive of the mite inside the cutaneous microvesicle. A comet sign might represent onset of specific lymphangitis, as suggested for 1 patient (Figure 1, panel D). Two recent similar cases in southern France, which were considered specific atypical lymphangitis (*14*,*15*), might have been *P*. *ventricosus* dermatitis.

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Change in Japanese Encephalitis Virus Distribution, Thailand

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Japanese encephalitis virus (JEV) genotypes in Thailand were studied in pigs and mosquitoes collected near houses of confirmed human JEV cases in 2003–2005. Twelve JEV strains isolated belonged to genotype I, which shows a switch from genotype III incidence that started during the 1980s.

The origin of Japanese encephalitis virus (JEV) was recognized before 1935, and JEV was isolated in Japan in 1935. The virus has since spread from India to Indonesia and within the past 3 decades has reached previously unaffected parts of Asia and northern Australia (*1,2*). JEV is one of the most widespread causes of viral encephalitis worldwide; an estimated 3 billion persons are at risk for infection, and 10,000 to 15,000 die annually (*3*). Although most human infections are asymptomatic (1/1,000), 1/300 infections causes symptomatic infections, and 1/4 patients seeking treatment have symptoms of brain inflammation, which can lead to permanent neurologic sequelae and a 1/4 death rate (*4*).

JEV is a flavivirus transmitted by *Culex* mosquitoes to birds and pigs; humans are dead-end incidental hosts. On the basis of nucleotide sequencing of capsid/premembrane protein (C/PrM) and envelope (E) genes, 5 virus genotypes have been identified, including genotypes I to III (GI, GII, GIII). These have been found distributed all over southern Asia; a GIV strain was isolated from eastern Indonesia, and an isolate originating in Malaysia may represent a fifth genotype (*5*).

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Table 1. Location of Japanese encephalitis virus study sites, Thailand

Site no.	Province	Latitude N	Longitude E
1	Phuket	43º36′90″	88º67′10″
2	Chiang Mai	50º10′10″	21º15′757″
3	Ratchaburi	58º41′09″	15º19′015″
4	Nakhon Pathom	59º38′49″	15º46′044″
5	Khon Kaen	18º63′72″	18º28′276″
6	Chumphon	09º58′09″	99º02′87″
7	Samut Songkham	13º26′24″	100º00′00″

Three vaccines, derived from JEV GIII strains, are currently in use. Since the 1960s JEV immunization campaigns have dramatically reduced the effects of the disease in southern and Southeast Asia (*6*). In Thailand, JEV immunization began as a part of childhood vaccination program in the northern provinces in 1990; this program rapidly expanded to 36 provinces that had reported a persistent incidence of encephalitis (*7*).

The Study

To study the JEV genotype distribution in Thailand and to eventually detect changes in Japanese encephalitis epidemiologic patterns, we conducted a 3-year survey (2003–2005) of JEV incidence in 7 provinces representative of the 4 regions of Thailand (north, Chiang Mai Province; northeast, Khon Khen Province; central plain, Nakhon Pathom, Ratchaburi, and Samut Songkram Provinces; south, Phuket and Chumphon Provinces). Pig farms and rice fields within a 2-km radius around houses of confirmed human cases of

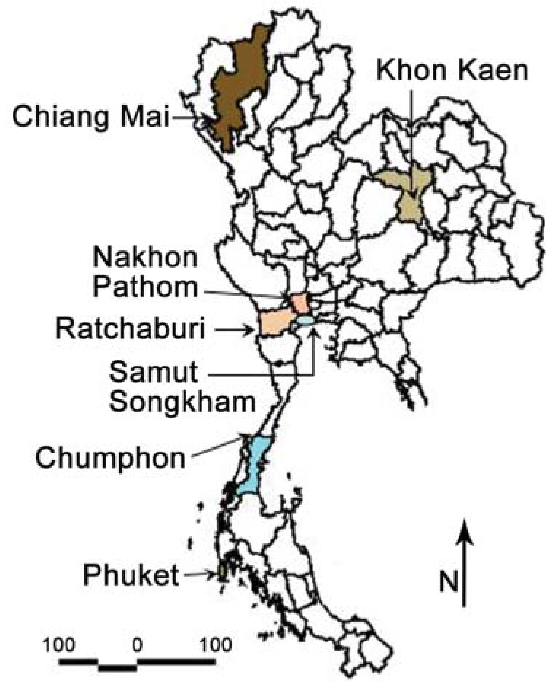

Figure 1. Provinces of Thailand showing study sites in Phuket, Chiang Mai, Ratchaburi, Nakhon Pathom, Khon Kaen, Chumphon, and Samut Songkham.

Japanese encephalitis were targeted for sample collection. Ten healthy sentinel piglets (10 weeks of age) were surveyed in each province, and blood samples were collected weekly for 14 weeks. Adult mosquitoes were collected on a monthly basis according to the targeted pig farm and availability of breeding sites for vectors (Table 1; Figure 1) by using both the CDC gravid trap (Model 1712) and the CDC light trap (P. Reiter, Centers for Disease Control and Prevention, Fort Collins, CO, USA).

Fifty microliters each from pig serum specimens and from filtered suspension of crushed mosquitoes were used for virus isolation on C6/36 cells. We tested JEV propagation by immunofluorescent assay. RNA extraction was done from a supernatant of JEV-positive cell culture, after first passage, according to manufacturer's protocol as well as RNA reverse transcription–PCR (RT-PCR) (GIBCO-BRL, Gaithersburg, MD, USA). RT-PCR was performed on 4 μL of cDNA template by using 2.5 units of Ampli-Taq Gold DNA Polymerase (PerkinElmer, Foster City, CA, USA). Overlapping JEV E gene fragments were amplified with 2 sets of primers: Ea forward primer (5′-ATA GTA GCT ATG TGT GCA AAC AAG G 5-3′), Ea reverse primer (5′-GAA TTC RGT YGT GCC YTT CAG AGC-3′); and Eb forward primer (5′-AGC TCA GTR AAG TTR ACA TCA GG-3′), Eb reverse primer (5′-GAA TTC AAT GGC ACA KCC WGT GTC-3′), respectively (*8*). The 1,216 nucleotides generated partial sequences of the JEV E gene that were compiled by using Sequence-Alignment Editor software version 2.0a11 (A. Rambaut, Department of Zoology, University of Oxford, Oxford, UK); pairwise genetic distances were calculated with MEGA software version 2.0 (*9*) (Table 2; Figure 2).

Twelve JEV strains were isolated, 3 from mosquitoes in 2003 and 10 from pigs in 2004 and 2005. The new JEV sequences were analyzed with a group of 22 previously published JEV strain sequences, including 6 from Thailand

Table 2. Strains of Japanese encephalitis virus used for phylogenetic analysis*

Strain	Year	Location	Source	Genotype	GenBank accession no.
FU	1995	Australia	Human	II	AF217620
P3	1949	China	Mosquito†	III	AY243844
Beijing-1	1949	China	Human brain	III	L48961
JKT7003	1981	Indonesia	Mosquito†	IV	U70408
JKT5441	1981	Indonesia	Mosquito†	II	U70406
Nakayama	1935	Japan	Human brain	III	AF112297
JaOArS7485	1985	Japan	Unavailable	III	AB028259
JaNAr0102	2002	Japan	Pig blood	I	AY377577
K94P05	1994	Korea	Mosquito†	I	U34929
WTP	1970	Malaysia	Mosquito†	II	U70421
DH20	1985	Nepal	Human brain	III	U03690
PhAn1242	1984	Philippines	Pig	III	U70417
HK8256	1982	Taiwan	Mosquito†	III	U70396
Chiang Mai	1964	Chiang Mai, N Thailand†	Human	III	U70393
P19Br	1982	Chiang Mai, N Thailand	Human brain	I	U70416
KPPO34-35CT	1982	Khon Khen, NE Thailand†	Mosquito†	III	U03693
B1065	1983	South Thailand	Pig	II	U70388
B2239	1984	Chiang Mai, N Thailand	Pig blood	I	U70391
ThCMAr4492	1992	Chiang Mai, N Thailand	Mosquito†	I	D45362
JE_CM_1196	2005	Chiang Mai, N Thailand	Pig	I	DQ238602
JE_KK_80	2004	Khon Khen, NE Thailand	Pig	I	DQ111784
JE_KK_82	2004	Khon Khen, NE Thailand	Pig	I	DQ111785
JE_KK_83	2004	Khon Khen, NE Thailand	Pig	I	DQ111787
JE_KK_87	2004	Khon Khen, NE Thailand	Pig	I	DQ111788
JE_KK_577	2005	Khon Khen, NE Thailand	Pig	I	DQ238601
JE_KK_580	2005	Khon Khen, NE Thailand	Pig	I	DQ238600
JE_KK_1116	2005	Khon Khen, NE Thailand	Pig	I	DQ343290
JE_RT_36	2003	Ratchaburi, Central plain Thailand	Mosquito‡	I	DQ087975
JE_CP_49	2004	Chumphon, S Thailand	Pig	I	DQ087974
JE_CP_67	2004	Chumphon, S Thailand	Pig	I	DQ087972
JE_PK52	2004	Phuket, S Thailand	Mosquito§	I	DQ084229
VN118	1979	Vietnam	Mosquito†	III	U70420
02VN22	2002	Vietnam	Pig blood	I	AY376465
Murray Valley E.1-51	1951	Australia	Human		AF161266

*N, northern; NE, northeastern; S, southern.
†Unidentified species.
‡*Culex tritaeniorhynchus.*
§*Cx. quinquefasciatus.*

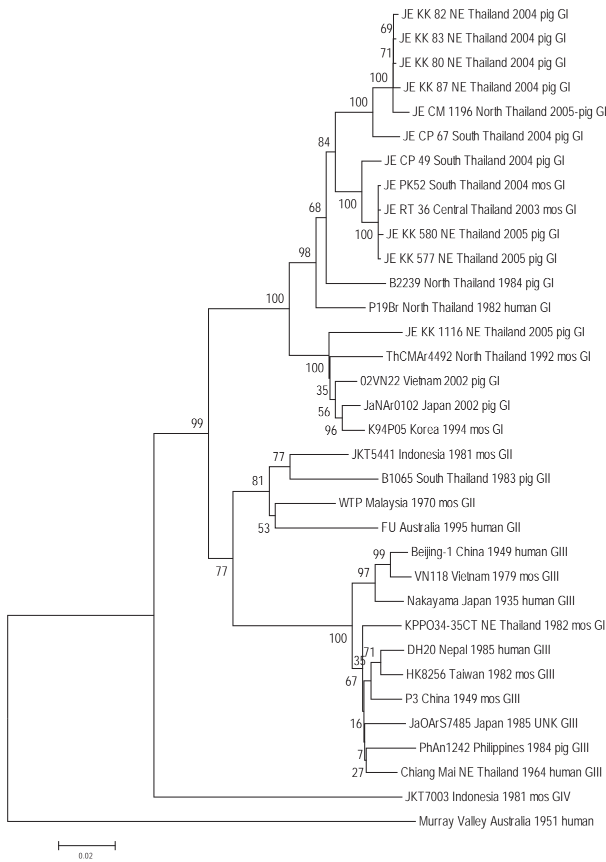

Figure 2. Sequence phylogeny based on E (envelope) gene nucleotide sequence of Japanese encephalitis virus isolates from pigs and mosquito hosts in Thailand during 2003–2005, with reference to other Southeast Asian isolates. Phylogenetic analysis was performed by using nucleotide alignments, the Kimura 2-parameter algorithm (for the calculation of pairwise distances), and the neighbor-joining method (for tree reconstruction), as implemented in MEGA software (*9*). The tree was rooted within the Japanese encephalitis serogoup by using Murray Valley virus (GenBank accession nos. E1–51). The robustness of branching patterns was tested by 1,000 bootstrap pseudoreplications. Each strain is abbreviated, followed by the country of origin (and the region of origin in Thailand, e.g., NE = northeast) and year of isolation. Bootstrap values are indicated above the major branch; 33 taxa comprised the ingroup, and all taxa were rooted with Murray Valley virus. A unique gap was treated as a "fifth base." The character state optimization was chosen as accelerated transformation. Consistence index 0.572; retention index 0.7528. Scale bar indicates no. nucleotide substitutions per site.

and 16 from other Asian countries. A phylogenetic tree was generated, and all 12 JEV new isolates fit into the same GI cluster, as did 3 other Thai strains previously isolated in 1982, 1984, and 1992 (Figure 2). Eleven of the newly identified isolates formed a subcluster (Figure 2, GIa) with 2 other strains previously isolated from Chiang Mai and Khon Ken in 1984 and 1982, respectively (B2239NThailand, P19Br NThailand); the remaining new isolate (JE KK 1116NEThailand2005) was associated with another subcluster (Figure 2, GIb), including strains isolated in 1992 from Chiang Mai (ThCMAr4492) and 3 others isolated from Vietnam, Japan, and Korea (O2VN22; JaNAr0102; and K94P05). Both subclusters were supported by 1,000 bootstrap replications and were consistent with the taxa distance (data not shown) showing introductions of GI in 1982 (within the GIa subcluster followed by a recent dispersion all over the country), and in 1992 for the GIb subcluster followed by local transmission.

GI strains appeared to cluster phylogenetically but not geographically, which suggests virus strains were transported over noncontiguous domains at variable geographic distances. Major environmental changes have occurred since the early 1950s with the increase in local and international transportation systems. Some researchers (*10*) consider the increase of the virus incidence in the human population to be associated with increased commercial activity. However, because of the low level of viremia in humans, traditionally considered dead-end hosts for JEV, it is more likely that the virus was spread within the country and to neighboring countries by migratory birds, infected domestic pigs, or infected mosquitoes (or their eggs) (*11,12*).

Although GIII strains were historically reported to circulate mostly in northern Thailand in the early 1980s, GI and GIII were found co-circulating from the north to the south; thereafter, only the GI strain was isolated in Thailand (*13*). The same genotype shift of GIII to GI, dating back to the early 1990s, was reported by several other Asian countries, including Japan and Korea in 1991 and Vietnam in 2001 (*14*); a steady emergence and dispersion of GI was also noticed in China in 1979, in Taiwan in the 1980s (*13*), and in Australia in 2000 (*2*). Altogether, such unique endemic expansion of GI occurred over a 25-year period in several countries of Southeast Asia, replacing the GIII genotype, which was present all over the region since the beginning of the virus genotype identification (prospectively and retrospectively).

Conclusions

In Thailand, the epidemiologic pattern of Japanese encephalitis first showed a visible decline in incidence with the development of immunization programs, but this decline also corresponded to the late 1980s when the practice of raising pigs in the backyard evolved into industrialized pig farming and the high rate of piglet seroconversion showed an intense virus circulation. The dramatic increase of industrial pig farming and trading must have played a major role in the dispersion of JEV genotypes within past decades in Asia. Concurrently with pig farming, the culicid main vectors have changed (*14*) and such factors as their ecology, trophic preferences, host competence, and virus fitness could play a role in an evolving rural environment.

Moreover, further studies are needed to clarify the expansion of JEV GI strains, including the efficiency of a human and pig GIII-derived vaccine and the role of potential cross-immunity between another circulating flavivirus (*13*).

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Role of Human Polyomaviruses in Respiratory Tract Disease in Young Children

Rachel L. Wattier, Marietta Vázquez, Carla Weibel, Eugene D. Shapiro, David Ferguson, Marie L. Landry, and Jeffrey S. Kahn

KI virus was detected in respiratory secretions of 8/367 (2.2%) symptomatic and 0/96 asymptomatic children (p = 0.215). WU virus was detected in 26/367 (7.1%) of symptomatic children and 6/96 (6.3%) asymptomatic children (p = 1.00). These human polyomaviruses may not independently cause respiratory tract disease in young children.

In 2007, 2 new human polyomaviruses, KI virus (KIV) and WU virus (WUV), were identified by molecular screening of respiratory secretions from children <2 years of age with symptomatic respiratory tract disease (*1,2*). Both viruses have since been detected in asymptomatic children and in those concurrently infected with other respiratory viruses, suggesting that KIV and WUV may not cause respiratory tract disease (*3–5*). To further understand the epidemiology of these viruses in young children and to clarify their association with symptomatic respiratory tract infections in this age group, we screened respiratory specimens from both asymptomatic and symptomatic children for the presence of KIV and WUV.

The Study

Respiratory specimens from 2 groups of children (all <2 years of age) were collected in 2004 and screened for KIV and WUV. The first group comprised symptomatic children whose respiratory specimens were submitted to the Clinical Virology Laboratory, Yale–New Haven Hospital, New Haven, Connecticut. These respiratory specimens tested negative for respiratory syncytial virus (RSV), parainfluenza viruses (types 1–3), influenza viruses A and B, and adenovirus by direct fluorescence antibody assay. The second group comprised asymptomatic children at the hospital-affiliated pediatric clinic for well-child care. Nucleic acids were extracted from each specimen by using QIAamp nucleic acid purification kits (QIAGEN, Valencia, CA, USA). Samples were screened by nested PCR for both KIV and WUV (for WUV, the first primers were those used by Gaynor et. al., and the nested primers were 5′-GCGCATCAAGAGGCACAGCTACTATTTC-3′ and 5′-GCGCCTAGCCTGTGAACTCCATC-3′). The G/C clamp for each primer is underlined. (*1,2*). Positive and negative controls were included in each set of PCRs. All PCR products were sequenced. Any child who had multiple specimens with positive results was included once in the total number of children whose specimens tested positive for a given virus.

Specimens from symptomatic children who tested positive for KIV or WUV were also screened for human bocavirus (HBoV); human metapneumovirus (hMPV); human coronaviruses (HCoV) 229E, NL63, and HKU1; and human picornaviruses (including rhinoviruses [HRV]) by using previously described methods (*6–12*). To screen for human parainfluenzavirus type 4 and HCoV OC43, RNA extraction and reverse transcription were performed as previously described (*7*). The primers used to amplify hPIV4 were 5′-GCGAGAGGATCCAGCTGGTGGC-3′ and 5′-GCGCCCTAATCTTTCCTGTTGATGG-3′. The primers for HCoV-OC43 were 5′-GCATAAGCCCC GCCAGAAGAGGAG-3′ and 5′-GCGCTGACGCTGTG GTTTTGGACT-3′.

We tested 423 direct fluorescent antibody–negative respiratory specimens, from 367 children, for KIV and WUV. The results of screening are summarized in the Table. Of the 367 symptomatic children, there were 8 (2.2%; 95% confidence interval [CI] 1.0%–4.3%) whose specimens tested positive for KIV and 26 (7.1%; 95% CI 4.7%–10.2%) whose specimens tested positive for WUV. One child had 2 specimens that tested positive for WUV. None (0%; 95% CI 0%–4.0%) of the 96 specimens from asymptomatic children tested positive for KIV. Specimens from six (6.3%; 95% CI 2.3%–13.1%) of the 96 asymptomatic children tested positive for WUV. The odds ratio for the proportions of symptomatic and asymptomatic children positive for WUV was 1.14 (95% CI 0.46–2.86; p = 1.0, Fisher exact test). The odds ratio for the proportions of symptomatic and asymptomatic children positive for KIV is undefined (none of the specimens from children in the asymptomatic group tested positive). The difference was not statistically significant (p = 0.215). The distribution of number of samples screened per month was similar for the asymptomatic and symptomatic groups.

The monthly distribution of children positive for KIV or WUV is shown in Figure 1. The age distribution of KIV- and WUV-positive children is shown in Figure 2. The youngest KIV-positive child was 4 months of age. The youngest WUV-positive child was 12 days of age.

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Table. Detection of KIV and WUV in children <2 years of age with and without respiratory tract disease*

Patient group	No. KIV-positive children/total no. tested (%)	No. WUV-positive children/total no. tested (%)
Respiratory tract disease	8/367 (2.2)†	26/367 (7.1)‡
Asymptomatic	0/96 (0)	6/96 (6.3)

*KIV, KI virus; WUV, WU virus.
†p = 0.215, compared with asymptomatic children.
‡p = 1.0, compared with asymptomatic children.

Viral co-infection was detected in 2 (25%) of the 8 KIV-positive specimens; 1 specimen tested positive for both HBoV and hMPV, and the other tested positive for HBoV. Of the 6 KIV-positive children whose specimens tested negative for other respiratory viruses, all had evidence of respiratory tract disease or fever. Five (63%) of the KIV-positive children had underlying illnesses.

Viral co-infection was detected in 13 (50%) of the 26 WUV-positive symptomatic children; 7 (26%) were positive for hMPV, 4 (15%) for HRV, 2 (7%) for HCoV-NL63, and 2 (7%) for HBoV. Two WUV-positive specimens tested positive for both hMPV and HBoV. Of the 13 WUV-positive children whose specimens tested negative for the other respiratory viruses, 12 had records available for review. Nine (69%) had evidence of respiratory tract disease, including fever. Two of these children had potential noninfectious explanations for their symptoms. One female child had end-stage pulmonary hypertension, and it was unclear whether her respiratory distress was due to worsening pulmonary hypertension or an infectious process. Respiratory distress developed in a male child after suspected aspiration; his symptoms resolved within 6 hours.

Three of the WUV-positive children had been hospitalized since birth; specimens from 1 of these children tested positive for WUV on 2 occasions. The interval between the first and second specimen was 98 days, and during that time 3 other specimens from this child tested negative for WUV. The child was hospitalized throughout this period and received mechanical ventilation for chronic lung disease. This child and 2 others were hospitalized in the same respiratory care unit at the time of WUV detection. Another child whose specimens tested positive for WUV had recently been discharged from that respiratory care unit.

Of 25 WUV-positive children whose records were available for review, 11 (44%) had underlying illnesses. Of the 6 asymptomatic children whose specimens tested positive for WUV, 1 had a history of prematurity and the other 5 had no underlying illnesses.

Conclusions

We detected KIV and WUV in respiratory samples obtained from children in Connecticut in 2004. The rates of detection for the symptomatic children in our study are similar to those observed in prior studies (*1,3,4*). As in studies by others, we detected WUV in asymptomatic and symptomatic children (*3–5,13*). This finding provides further evidence that asymptomatic infection of WUV may occur. KIV was not detected in any of the asymptomatic children we tested. However, it is possible that subclinical infection with KIV occurs and that we failed to detect any cases because of the low prevalence of KIV in our study population. Our study had only 15% power to detect a difference between 2.2% and 0% for KIV, whereas it had adequate power (90%) to detect a difference between 7.1% and 0% for WUV.

Co-infection with other respiratory viruses was a common finding in both KIV- and WUV-positive children. Because specimens from symptomatic children were tested for common viruses by antigen testing rather than PCR, which is presumably more sensitive, the co-infection rate could, in fact, be greater. The high rates of co-infection observed in our study and the studies of others support the notion that KIV and WUV may not cause respiratory tract disease. However, KIV or WUV was the only virus detected for a small number of children with evidence of respiratory tract disease, so it is still possible that these viruses contribute to respiratory tract disease in susceptible children.

Detection of WUV in 3 patients hospitalized since birth suggests the potential for nosocomial, congenital, or perinatal infection. One of these children had 2 respiratory

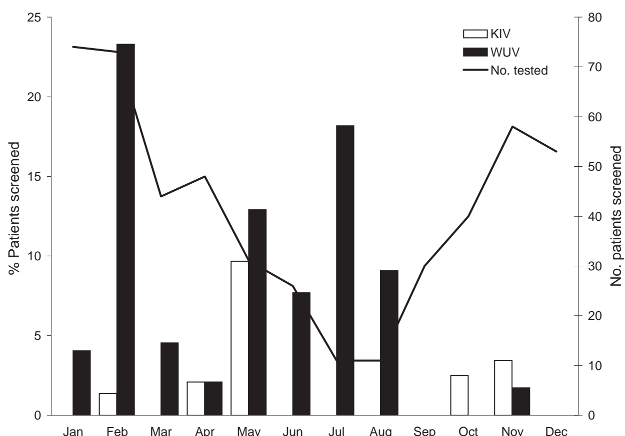

Figure 1. Monthly distribution of children positive for KI virus (KIV) and WU virus (WUV). The WUV-positive children include both asymptomatic and symptomatic children whose specimens tested positive for WUV. One child who tested positive for WUV in February and March is represented in both months. The superimposed line graph represents the number of children tested in each month.

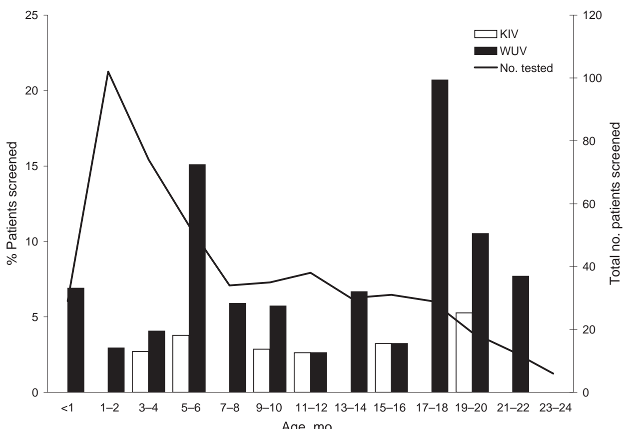

Figure 2. Age distribution of children positive for KI virus (KIV) and WU virus (WUV). The WUV-positive children include both asymptomatic and symptomatic children. One child whose specimens tested positive at age 6 months and again at age 9 months is represented in both age groups. The superimposed line graph represents the number of children tested in each age group.

specimens positive for WUV. Le et al. recently reported detection of WUV in multiple samples obtained from 2 immunocompromised patients (*13*). This finding may represent persistent or latent infection.

In conclusion, we have established that 2 recently discovered polyomaviruses, KIV and WUV, are circulating in Connecticut. Because of the high rates of viral co-infection and detection of WUV in both asymptomatic and symptomatic children, these viruses may not be respiratory pathogens.

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Ms Wattier is a Doris Duke Clinical Research Fellow and a candidate for the MD/Master of Health Science degree at Yale University School of Medicine. She plans to pursue postgraduate training in pediatrics.

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Identification of Potential Environmentally Adapted *Campylobacter jejuni* Strain, United Kingdom

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In a study of *Campylobacter* infection in northwestern England, 2003–2006, *C. jejuni* multilocus sequence type (ST)–45 was associated with early summer onset and was the most prevalent *C. jejuni* type in surface waters. ST-45 is likely more adapted to survival outside a host, making it a key driver of transmission between livestock, environmental, and human settings.

Human campylobacteriosis shows a marked seasonality with a peak during the early summer months in many countries (*1*). The driving factors for this seasonality are not understood. Studies have shown a coincident seasonality of infection in chicken, livestock, and humans, and the possibility of a common environmental trigger has been suggested (*2*). In a recent study of the influence of climate on seasonality in England and Wales, incidence of campylobacteriosis was correlated with air temperature (with higher temperature indicating more cases at key points of the year) (*3*). This finding may relate to animal husbandry practices, especially animal housing (*4*).

Studies have attempted to identify environmental reservoirs of infection in water sources; *Campylobacter* organisms have been successfully cultured from surface water (*5*), and campylobacteriosis has been linked with exposure to untreated water (*6*). We were interested in identifying the factors driving the early summer increase of cases in the United Kingdom and in investigating the role of environmental reservoirs. Preliminary data identified multilocus sequence type (ST)–45 complex as a strain with possible transmission from environmental sources (*7*), and we have analyzed this complex in more detail.

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The Study

The study population was defined as all human cases of laboratory-confirmed *Campylobacter* infection with onset from April 2003 through March 2006, reported by residents in 4 local authorities in northwestern England, as previously described (*7*). All case-patients were asked detailed questions about their illnesses and possible exposures.

Water samples were collected at least each fortnight from October 2003 through December 2005 as 2-L grab samples from sampling points on 2 rivers associated with the study area (River Mersey and River Wyre). Water samples were transported to the Food and Environmental Microbiology Laboratory, Royal Preston Hospital. *Campylobacter* species were isolated by the addition of 10 mL of the water sample to 90 mL of warmed *Campylobacter* enrichment broth (product CM0983, Oxoid Ltd, Basingstoke, UK) and incubated at 37°C for 24 hours, followed by incubation at 42°C for 24 hours. The enrichment broths were subcultured onto *Campylobacter* blood-free selective agar (charcoal cefoperazone deoxycholate agar product CM0739, Oxoid Ltd) at 37°C for 48 hours microaerobically, by using a microaerobic gas generating kit (product CN0025, Oxoid, Ltd). *Campylobacter* colonies were identified by morphologic features and confirmed by microaerobic and aerobic growth on blood agar. The colonies were then placed in Amies transport and sent to the laboratory Health Protection Agency regional laboratory in Manchester, UK, for DNA extraction and characterization. *C. jejuni* isolates were identified to species and typed by multilocus sequence typing as previously described (*7*).

Case–case methodology was used to compare exposures between sequence types of *Campylobacter* (*8*). Statistical analysis was performed by using STATA version 9.2 (StataCorp, College Station, TX, USA). Two-way tabulations and Fisher exact test were used to estimate the direction and size of association of individual variables with sequence types. Logistic regression was performed to estimate the significance of these findings and to examine them in a multivariate model. Data were collected from 2 distinct locations, and, thus, area of residence was controlled for in analysis as a stratified variable. Likelihood ratio tests were used to assess the significance of including or excluding variables from multivariate analysis.

Among the typed isolates of *C. jejuni* (n = 1,104), ST-45 (n = 49) was the third most prevalent sequence type reported in the study (after ST-257 and ST-21) and highly restricted to cases reported between late April (week 17) and early August (week 32) (Figure 1). When week of diagnosis was categorized into 2 seasons (early summer [weeks 17–28] and all other weeks), logistic regression of season by sequence type confirmed that, when compared with all other typed cases of *C. jejuni*, cases of ST-45 were more

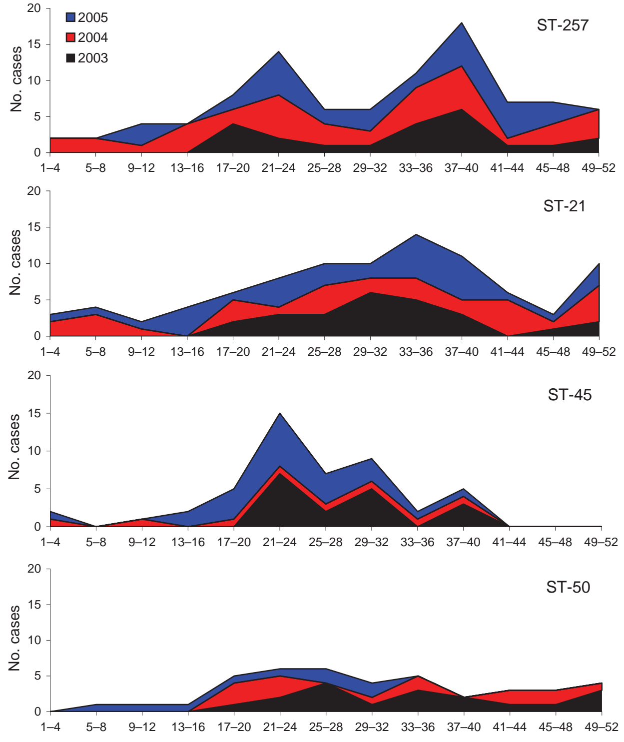

Figure 1. Stacked area charts showing the number of human case reports of the 4 most prevalent sequence types (STs) during the study, by 4-week intervals.

likely to be reported in early summer than during the rest of the year (odds ratio 2.79, confidence interval 1.56–4.99, p = 0.001; 1,008 observations), and this relationship was not seen with other prevalent sequence types.

Seventy-four (37%) of 198 river water samples were positive for *C. jejuni*, and among these, 28 sequence types (Table 1) were identified, 11 of which were also reported among human cases in the study population. The most prevalent sequence type identified was ST-45, and the seasonality of isolation appears to closely correlate with the seasonality of human disease onset caused by this type (Figure 2).

Univariate logistic regression analysis identified that, in addition to illness during early summer, the statistically strongest associations (p<0.1) with ST-45 were being <5 years of age and living in the more rural part of the study area. These 3 factors remained significantly and independently more associated with ST-45 infection than with other sequence types when combined in a multivariate model (tested by using likelihood ratio tests) (Table 2). When this model was used, subsequent estimation of the association of food and exposure variables showed that consumption of chicken at least once was less associated and consumption of home-delivered milk and going fishing were more associated with ST-45 than with other types. This finding was sustained in a multivariate model (also including the demographic variables previously mentioned) (Table 2).

Table 1. Isolates of *Campylobacter jejuni* recovered from river water samples in 2004 and 2005, showing clonal complex and ST*†

Clonal complex/ST	Water isolates	Human cases	Prevalence among human cases, %
ST-45			
45	21	Y	4.83
2405	2		–
2406	1		–
230	1		–
714	1		–
2219	1		–
137	1	Y	1.08
ST-21			
21	2	Y	9.37
53	1	Y	3.55
UA	1		–
ST-48			
48	3	Y	3.85
475	1	Y	1.48
ST-42			
42	2	Y	1.38
1751	1		–
ST-1332			
2404	1		–
696	1		–
ST-508			
2187	2		–
ST-179			
220	1		–
ST-460			
606	1	Y	0.10
ST-403			
415	1		–
ST-658			
UA	1		–
ST-677			
677	1	Y	0.69
ST-257			
257	1	Y	9.76
ST-1388			
177	1		–
ST-61			
61	1	Y	1.38
UA			
UA	4		–
448	2		–
2408	1		–
789	1		–
947	1		–
2407	1		–
Total	61		

*ST, sequence type; UA, unassigned or newly identified sequence type at time of publication; –, blank values.
†Those STs also found in human cases of *C. jejuni* among the study population are indicated with a Y and their prevalence indicated as a percentage of all typed cases of *C. jejuni* in the study population.

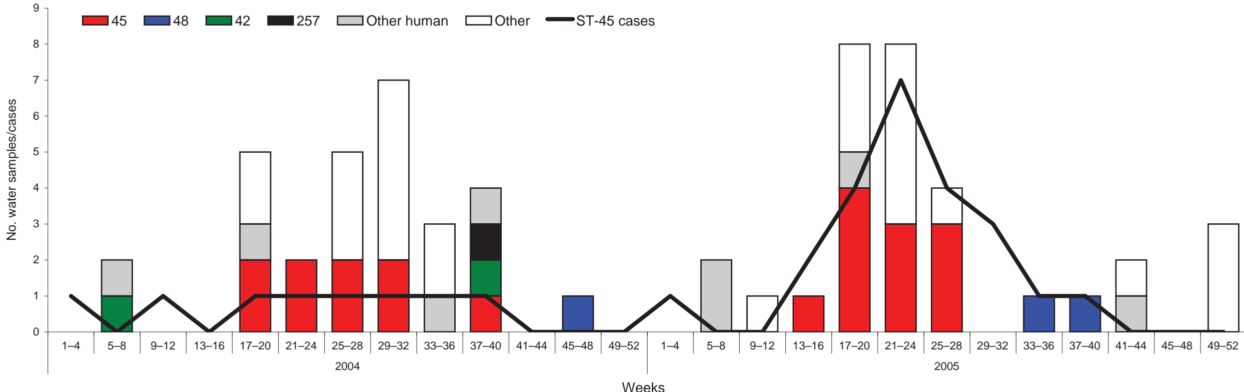

Figure 2. Numbers and sequence types (STs) of isolates of *Campylobacter jejuni* from surface water samples compared with numbers of human cases of ST-45 (line) for 2004 and 2005, by 4-week interval. Only the 4 most prevalent human types also identified in water samples are distinguished (ST-257, ST-45, ST-21, and ST-48). The "Other human" category includes all other *C. jejuni* sequence types found both in human cases in the study and in water samples. The "Other" category includes other *C. jejuni* sequence types found in water samples but not in human case-patients in the study.

However, information on exposure variables was not available for all 1,008 cases of *C. jejuni*, which resulted in a loss of statistical power.

Conclusions

We have shown that a single prevalent human strain of *C. jejuni*, ST-45, is strongly associated with the early summer seasonal peak of campylobacteriosis incidence described previously in northwestern England (*7*). This strain is also frequently isolated from recreational surface waters adjacent to the populations studied, from which other prevalent strains have rarely been isolated. The concordance between period of ST-45 isolation in water and reported incidence in humans is striking and suggests a relationship between the presence of this strain in the environment and human infection. When compared with case-patients infected by other sequence types of *C. jejuni*, persons infected with ST-45 are more likely to live in rural areas, to be <5 years of age, to have gone fishing before illness, or to have consumed home-delivered milk, and were much less likely to have eaten any chicken in the 2 weeks before illness.

The coincident seasonal presence of ST-45 in both surface water and in case-patients may arise simply through seasonal excretion of ST-45, resulting from well-characterized seasonal infection in humans (*1*,*3*,*7*) or livestock. The river systems sampled were both urban and rural in character, and contamination through human sewage discharge or animal feces from adjoining pastures is possible. However, other common human and animal *Campylobacter* sequence types were largely absent from the water sampled.

Although ingestion of untreated surface waters has previously been shown to be a risk factor for campylobacteriosis in a UK-wide case-control study (*9*), these new data are not sufficiently robust to demonstrate a causal link for ST-45 infection. Although data were collected on proxy exposures to water, reported exposures were very low. Fur-

Table 2. Two-step multivariate logistic regression analysis of epidemiologic variables associated with cases of ST-45, in comparison with all other sequence types of *Campylobacter jejuni* *

Variable	Univariate			Multivariate		
	OR	CI	p value	OR	CI	p value
Initial model (obs = 1,008)†						
Onset during early summer	2.79	1.56–4.99	0.001	2.72	1.52–4.89	0.001
Age <5 y	3.48	1.54–7.86	0.003	3.32	1.45–7.61	0.005
Rural residence	1.96	1.10–3.51	0.023	2.03	1.13–3.66	0.018
Final model (controlled for above)‡				Final multivariate (obs = 580)		
Going fishing in the 2 weeks before illness (obs = 737)	3.29	0.69–15.80	0.137	3.95	0.71–22.0	0.118
Consumption of home-delivered milk (obs = 633)	1.99	0.85–4.64	0.113	2.45	0.97–6.17	0.058
Consumption of chicken at least once (obs = 645)	0.23	0.08–0.66	0.006	0.21	0.07–0.63	0.006

*obs, number of observations for each analysis (i.e., no. cases in the study with available information); OR, odds ratio, CI, confidence interval.
†Initial analysis of basic demographics (obs = 1,008) showed being ill in the early summer (weeks 17–28) and being <5 y of age were independently associated with cases of infection with ST-45, when controlled for the stratified variable of residential area.
‡Further analysis of exposures (controlled for the initial model) showed going fishing, consuming chicken, and consuming home-delivered milk to be independently associated with cases of infection with ST-45, and these associations remained in the final multivariate model, including all variables listed in the table. For the full regression model, only 580 of the possible 1,008 cases had information on all the variables of interest.

thermore, systematic water sampling would be required to confirm the apparent seasonal positivity of ST-45 in these data. The potential role of pets, and in particular dogs, in bridging the gap between exposure to surface water (for example, while being exercised) and domestic exposure settings remains to be investigated. Although cases of infection caused by ST-45 were no more associated with owning dogs than were cases of other sequence types (data not shown), the exposures of those dogs were not recorded. In a recent study, cases of ST-45 clonal complex were more associated with contact with pet dogs and cats than were other clonal complexes identified (*10*); pet-mediated transmission of ST-45 might be supported in this study by the observed association of ST-45 with young children.

Despite the evidence presented of a potential environmental transmission route for ST-45, it is also a type well recognized to colonize poultry (*10–12*), and the incidence among humans may be a result of consumption of seasonally contaminated poultry (*2*). However, the absence of strong early summer seasonality among other recognized chicken-adapted sequence types suggests that this is not the case (data not shown). Also, the evidence from our study is strong that consumption of chicken (a common human exposure) is less associated with human ST-45 infection than with other types.

An explanation for these observations may be that ST-45 represents a strain of *Campylobacter* that is comparatively well adapted to survival outside an animal host, as has been hypothesized for some strains of *C. coli* (*13*). Other studies have reported that ST-45 is more widely distributed in terms of host and ecologic niche, including water, than other common sequence types (*12*,*14*). Evidence also has indicated that ST-45 is more resilient to physical stress than other sequence types (*15*). This would certainly support the hypothesis that ST-45 was more available to infect humans through transmission routes other than food, either through direct exposure to water or the countryside through outdoor activities or by indirect exposure through pets. The hypothesis is further supported by the association of human ST-45 with more rural area of residence in these data. The availability of ST-45 to humans due to its hypothesized adaptive survival outside animal hosts would apply even more so to poultry, because of their increased environmental exposure, and 1 study has demonstrated that contamination of a new flock with ST-45 arose from an isolate in a puddle outside a chicken house (*12*). Thus, through an as-yet-uncharacterized adaptation, ST-45 may be a strain of *C. jejuni* that is able to bridge the various recognized environmental, livestock, and human transmission settings for disease, making it a key target for intervention in reducing *Campylobacter* prevalence. It may also be a key driver for the early summer rise in human incidence, both through nonfoodborne exposure and contamination of food animals.

Acknowledgments

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Dr Sopwith is an epidemiologist leading research and development at the regional unit of the Health Protection Agency, based in Liverpool. His current interests include the molecular epidemiology of campylobacteriosis in northwestern England, the epidemiology of cryptosporidiosis, the prevalence of rotavirus in children, and the zoonotic transmission of *Corynebacterium ulcerans*.

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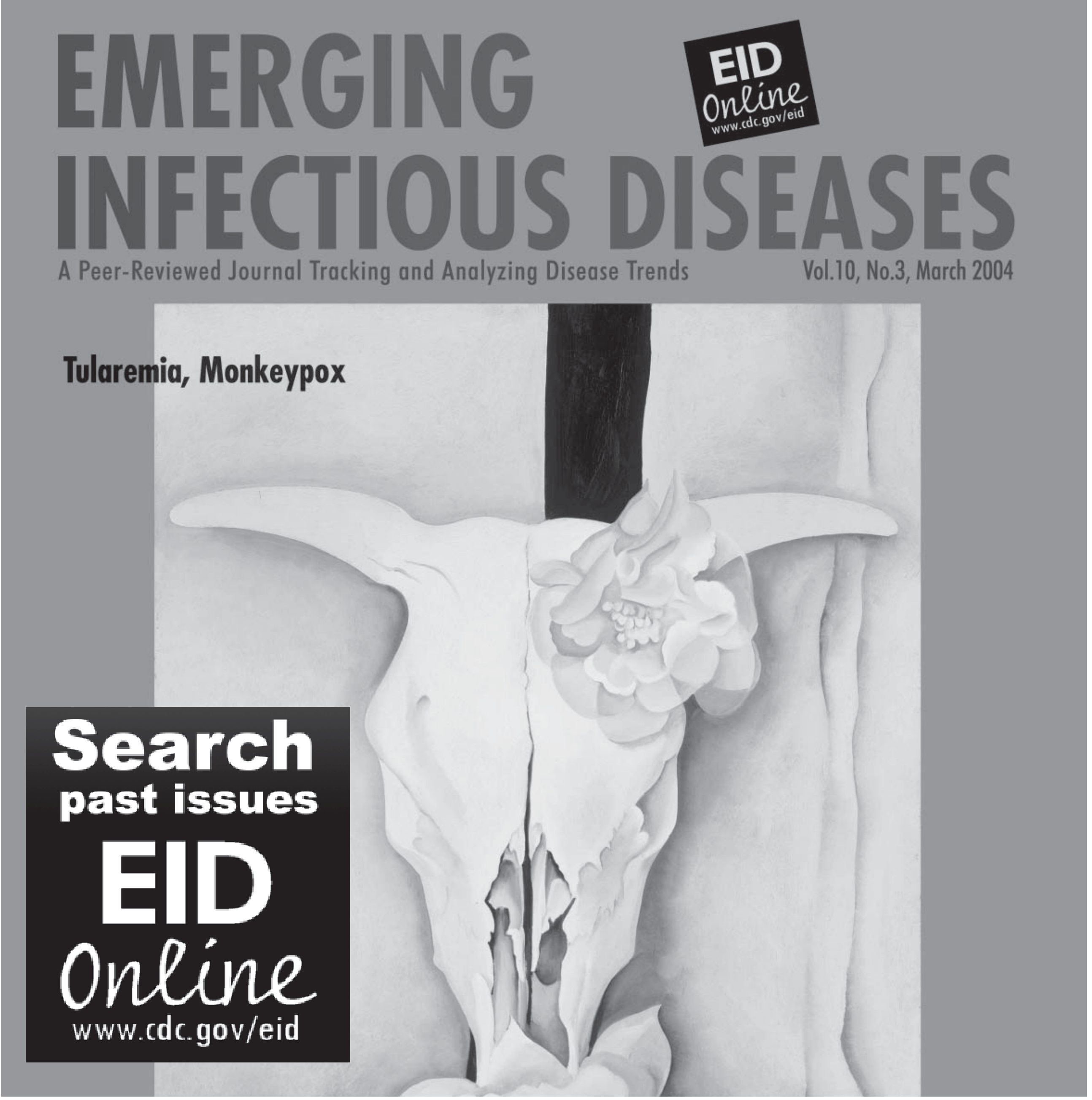

Porcine Respiratory and Reproductive Syndrome Virus Variants, Vietnam and China, 2007

Youjun Feng, Tiezhu Zhao, Tung Nguyen, Ken Inui, Ying Ma, Thi Hoa Nguyen, Van Cam Nguyen, Di Liu, Quang Anh Bui, Long Thanh To, Chuanbin Wang, Kegong Tian, and George F. Gao

We characterized isolates from porcine respiratory and reproductive syndrome virus epidemics in Vietnam and China in 2007. These isolates showed ≈99% identity at the genomic level. Genetic analysis indicated that they share a discontinuous deletion of 30 aa in nonstructural protein 2, which indicates that identical variants emerged in Vietnam and China.

Porcine respiratory and reproductive syndrome (PRRS) is one of the most economically influential infectious diseases in the industry of swine cultivation. The etiologic agent is PRRS virus (PRRSV), a member of the family *Arteriviridae* in the order Nidovirales (*1,2*). Genomic analysis of PRRSVs has shown that the virus genome varies from 15 kb to 15.5 kb and comprises at least 8 open reading frames (ORFs) that encode nearly 20 mature proteins (*3*). PRRSVs with different geographic origins can be classified into 2 major genotypes, the European type (type I, EU-type) (*4*) and North American type (type II, NA-type) (*5*). Nonstructural protein 2 (Nsp2) (*6*) and glycoprotein 5 (encoded by ORF5) (*7*), are regarded as 2 regions of high heterogeneity that are involved in the pathogenicity of PRRSV strains.

In general, PRRSV has caused either respiratory failure in neonates or abortions in sows during sporadic PRRS outbreaks worldwide (*2*). The unprecedented large-scale PRRS outbreaks in 2006 swept over nearly half of the People's Republic of China and involved >2,000,000 pigs, which posed great concern to the global swine industry and to public health (*8*). Subsequent genomic analysis showed that all the PRRSVs isolated from this outbreak (*8*) share a unique discontinuous deletion of 30 aa in Nsp2. In previous studies, only 2 virulent NA-type PRRSV strains (P129 and MN184) had been suggested to carry deletions in the Nsp2 protein (different from our 30-aa deletion), a highly variable region that contributes to the virulence and PRRSV genotyping (*9*,*10*).

A suspected PRRS outbreak was observed in Vietnam in 2007, and further spread of the disease has been found in China in 2007. To elucidate the characteristics of these outbreaks and the PRRSVs isolated, we conducted a detailed investigation.

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The Study

In March 2007, the suspected PRRS-like disease was initially found in Hai Duong Province, the northern province of Vietnam. The disease later spread to nearly the entire country and affected at least 65,000 pigs. Meanwhile, the recurrence of PRRSV infections in China was officially announced in May 2007 by the Chinese Ministry of Agriculture.

The affected pigs exhibit the clinical features of high fever, depression, and shivering. Viscera (e.g., brain, kidney, lung, heart, liver, and spleen) were sampled from those dead pigs from different provinces in Vietnam and China. Pathologic examination showed severe lesions viscera, for example, blood spots in the kidney and hemorrhages in the lung (Figure 1, panels A, B), findings similar to those observed in the 2006 PRRS outbreaks in China (*8*,*11*). The

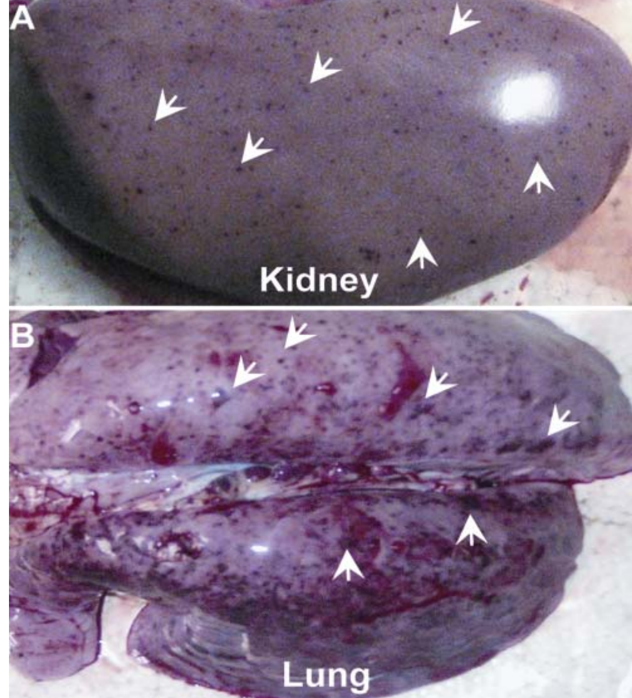

Figure 1. Pathologically dissected specimens from pigs infected by porcine respiratory and reproductive syndrome viruses. Blood spots in kidneys (A) and lung hemorrhages (B) are indicated by white arrows.

inocula (from each representative specimen) were propagated in Marc-145 cells (QIAGEN, Hilden, Germany) for the viral genomic RNA isolation. Based on our previous observation of the 2006 PRRS epidemics in China, we designed 2 pairs of specific primers (*Nsp2*-F: 5′-AAA GAC CAG ATG GAG GAG GA-3′ and *Nsp2*-R: 5′-GAG CTG AGT ATT TTG GGC GTG-3′; *orf5*-F: 5′-ATG TTG GGG AAG TGC TTG ACC-3′ and *orf5*-R: 5′-CTA GAG ACG ACC CCA TTG TTC CGC-3′). They correspond to the DNA fragment covering a putative discontinuous deletion of 30 aa in Nsp2 (666 bp), and ORF5 in full length, respectively. PCR-based detection showed that all the samples collected so far, are positive for PRRSV, but negative for African swine fever, classical swine fever, and foot-and-mouth disease viruses.

To test the virulence of Vietnam PRRSV isolates, 6 specific pathogen–free piglets were challenged with a representative strain (termed 07QN, which had been isolated from a dead pig in Quang Nam Province, Vietnam). The piglets were monitored for clinical signs every 6 hours. All procedures were conducted in a facility qualified with Biosafety Level 3 and approved by Vietnamese Committee for Approval of Drugs and Cosmetics ethics committee. As we expected, the piglets reproduced the serious symptoms similar to those observed in our field investigation, which indicated that the viral agent is highly pathogenic.

To better understand the genetic relationship of Vietnamese PRRSV isolates to the 2006 and 2007 Chinese isolates, 5 strains of PRRSVs (1 isolate from Vietnam, 07QN, and 4 newly isolated isolates from China, 07HEBTJ, 07BJ, 07HEN, and 07NM) were subjected to whole genome sequencing as described (*8*). The viral genomes were found to be >15,300 bp (GenBank accession numbers are available from the authors). Bioinformatics analysis further revealed that the 2007 Vietnamese isolate (07QN) and 2007 viruses (e.g., 07BJ) have 99% identity to 2006 PRRS isolates in China at the level of nucleic acid sequences. Whole genome–based phylogenetic relationship also showed that all these viral isolates are grouped into the same subclade in the type II genotype (Figure 2). All of the 2007 strains, together with those Chinese viruses collected in 2006, were found to be similar to 3 Chinese strains reported previously (HB-1, HB-2, and CH-1a) (*3*,*12*) and 1 NA-type virulent strain, P129 (*8,9*).

In addition, >30 sequences of *orf5*, a highly variant gene, were sequenced, and ORF5-based genotyping also supported the classification of these 2007 isolates (from Vietnam and China) into a subgroup of type II, while far from other subclades with VR-2332, a prototype of type II (not shown). Moreover, the multiple alignments of Nsp2 demonstrated that the 2007 PRRSV isolates from Vietnam (51 total), together with 2007 Chinese strains (6 total) are nearly identical and share a discontinuous deletion of 30 aa at the position of 482 aa and 534–562 aa, which is consistent with deletions in the strains from the 2006 epidemic in China (*8*).

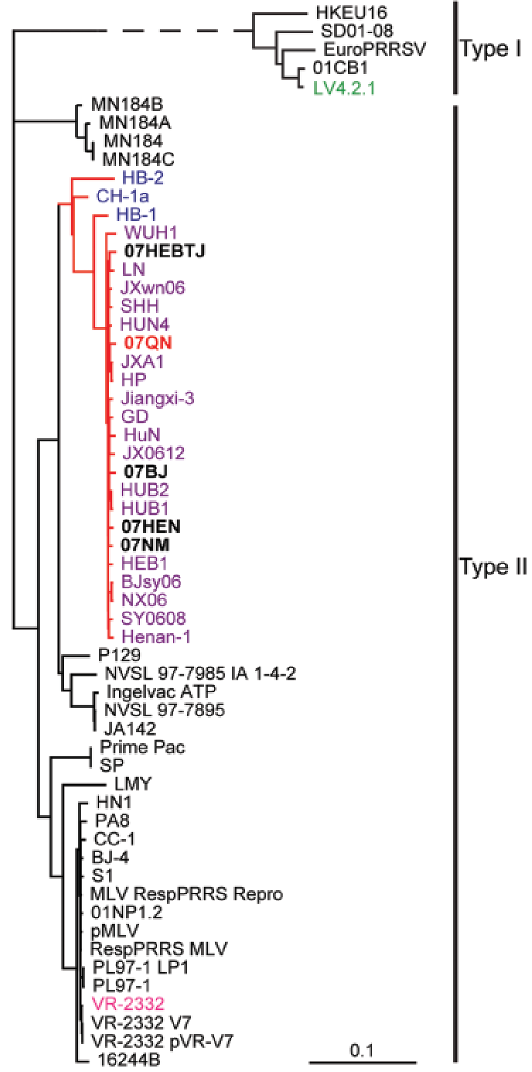

Figure 2. Whole genome–based phylogenetic relationship of porcine respiratory and reproductive syndrome viruses (PRRSVs) LV4.2.1, the prototype of type I (European PRRSV) (shown in green), and VR2332, the standard strain of type II (North American PRRSV) (shown in pink). Three Chinese isolates reported before 2006 (HB-1, HB-2, and CH-1a) are shown in blue. Chinese 2006 PRRSV isolates are shown in purple. Chinese 2007 isolates are shown in black **boldface**, and a representative of 2007 Vietnamese strains (07QN) is shown in red **boldface**. The 2007 Vietnamese and Chinese PRRSV isolates are classified into the same subclade of type II, as are all the 2006 Chinese PRRSV strains.

Conclusions

PRRS has become a serious challenge to the global pig industry, causing serious economic losses (*2*). In particu-

lar, the unparalleled 2006 PRRS outbreaks engulfed China, and adult pigs were not exempt, which indicates that these variants may have evolved to exhibit new characteristics of pathogenicity (*8*). These outbreaks caused extensive concern worldwide (*13*). Even though the deletion of 30 aa in Nsp2 has been proposed as a potential virulence factor (*8*), the molecular mechanisms underlying its high virulence are yet to be elucidated.

Origin of these lethal variants in Vietnam is still obscure, although Kamakawa et al. (*14*) suggested that PRRSV may have been present in Vietnam before 1999. Convergent, but separate, evolution of PRRSVs in Vietnam and China may explain in part the emergence of the nearly identical PRRSV variants in these 2 neighboring countries, although this hypothesis does not rule out the possibility that 2006 Chinese PRRSV variants were transmitted into Vietnam and then circulated rapidly. Additionally, intraprovincial and interprovincial transportation of live pigs and the similar climate in Vietnam and China may have contributed to these outbreaks (*15*).

In summary, our findings provided robust evidence that nearly identical variants of NA-type PRRSVs are the causative pathogens that triggered PRRS epidemics in Vietnam and China in 2007. This finding highlights the importance of prevention and control of this highly transmissible infectious agent.

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Possible New Hepatitis B Virus Genotype, Southeast Asia

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We conducted a phylogenetic analysis of 19 hepatitis B virus strains from Laos that belonged to 2 subgenotypes of a new genotype I. This emerging new genotype likely developed outside Southeast Asia and is now found in mixed infections and in recombinations with local strains in a geographically confined region.

As a result of mutations and recombinations, hepatitis B virus (HBV) has evolved into 8 known genotypes (A–H), with a putative new genotype I recently found in Asia (*1*,*2*). Some genotypes have been associated with distinct clinical patterns, and their detection and identification are important for virus and disease surveillance.

In Laos, 8.7% of the population are chronically infected with HBV, and perinatal transmission is the most common route of infection. Here, we present the phylogenetic analysis of 19 related strains found in voluntary blood donors from Laos that cluster with the new genotype I.

The Study

Phylogenetic analysis of sequences obtained from hepatitis B surface antigen–positive first-time blood donors from donation centers in Vientiane City and central provinces of Laos showed that 163 (42.2%) strains were of genotype B, and 204 (55.4%) were of genotype C. Subgenotypes included B2 (18), B3 (1), B4 (128), and B5 (16), as well as C2 (190), C3 (1), and C5 (13) (Figure 1, panel A). Nineteen strains, including 15 complete sequences, did not group with any of the known genotypes A–H. These sequences formed 2 clusters, which were emerging from a common node (bootstrap value of 100%; Figure 1, panel A). One of the clusters grouped with a recently reported single strain from Vietnam, for which we had previously defined a new genotype I (*2*). The 2 new groups from Laos will be referred to here as subgenotypes I1 and I2. Notably, all I1 strains were of serotype *adw*, whereas all I2 subgenotypes were of serotype *ayw*. With 1 exception, all genotype I strains were found in donors living in Vientiane City. Strains recovered from Hanoi, Vietnam, 8 years ago and reported as aberrant strains (*3*) also group with subgenotype I1.

Detailed analysis of full genome sequences showed that genotype C strains as a group were most closely related to genotype I (average Kimura distance of 7.89%, Table 1). The closest subgenotype was C3 with a 7.0% average Kimura distance (data not shown). The bootstrap value of the separating node was 92% (Figure 1, panel A), which is well above the bootstrap value of the G/DE node. On the S gene level, genotype I was most closely related to genotype G with a distance of 4.23% and a bootstrap value of 96% at the separating node (data not shown). Within the 2 subgenotypes I1 and I2, an average diversity over the complete genome of 1.19% and 0.94% was calculated; this difference increased to 2.33% when all strains were considered as a single group. The maximal genetic distance between 2 full-length genotype I strains was 4.3%. All cluster-

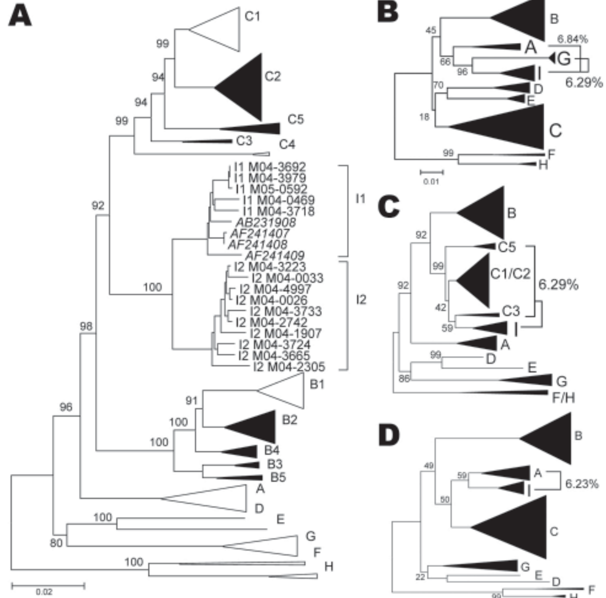

Figure 1. A) Phylogenetic comparison of all complete genotype I genomes (n = 15) obtained and compared to sequences of all known genotypes and subgenotypes. Non–genotype I genotypes identified in Lao People's Democratic Republic in the present study are shown as full triangles. Numbers indicate bootstrap values of important nodes. B–D) Phylogenetic comparison of positions 400–1400 (left), 1400–3000 (middle), and 3000–400 (right), of all genotype I strains with all known genotypes and subgenotypes. Percentages indicate average genetic distances between genotype I and G, C, or A, respectively. Scale bars indicate number of substitutions per site.

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Table 1. Average Kimura distances (in %) within (**boldface**) and between reference complete genome sequences of genotypes A to H and the putative new genotype I and subgenotypes I1 and I2

Genotype	A	B	C	D	E	F	G	H	I
A	***4.26***								
B	10.27	**4.14**							
C	9.95	10.17	**4.79**						
D	10.89	11.84	11.6	**2.95**					
E	10.88	12.33	12	8.2	**1.2**				
F	16.16	16.3	15.98	16.07	15.96	**7.18**			
G	12.48	14.28	14.2	12.46	11.75	17.56	**0.33**		
H	16.58	16.79	16.31	15.96	16.87	9.46	17.37	**2.29**	
I	8.43	9.7	7.89	10.95	11.23	15.74	12.13	16.21	**2.33**
I1	8.37	9.69	7.85	11.03	11.21	15.5	12.1	12.1	
I2	8.49	9.72	7.93	10.89	11.26	15.94	12.16	12.16	

ings were verified by maximum likelihood tree construction (data not shown). Thus, in accordance with published criteria (*4*), these values warrant the definition of a new genotype I with 2 subgenotypes I1 and I2.

Most current genotypes of HBV seem to be the result of 1 or several recombination events (*5*). In particular, this is evident for the B/C recombinant, which has spread in mainland Asia (*6*) and has been defined as genotype Ba. Also genotypes B and C show some similarity with each other (Figure 2). Bootscan analysis (*7*) of all genotype I strains, including M04–3665, amplified by complete genome PCR, showed similarities with genotype C (nt 1400–3000), A (nt 3000–400), and G (nt 400–1400) by using a window size of 800 nt (Figure 2). Smaller bootscan windows tended to blur the relatedness. Phylogenetic reconstruction (Figure 1, panels B–D) and BLAST searches (http://blast.ncbi.nlm.nih.gov) (Table 2) of the above fragments of genotype I sequences confirmed the results of the bootscan analysis, which suggests that this genotype may also have evolved from a series of recombination events in a distant past.

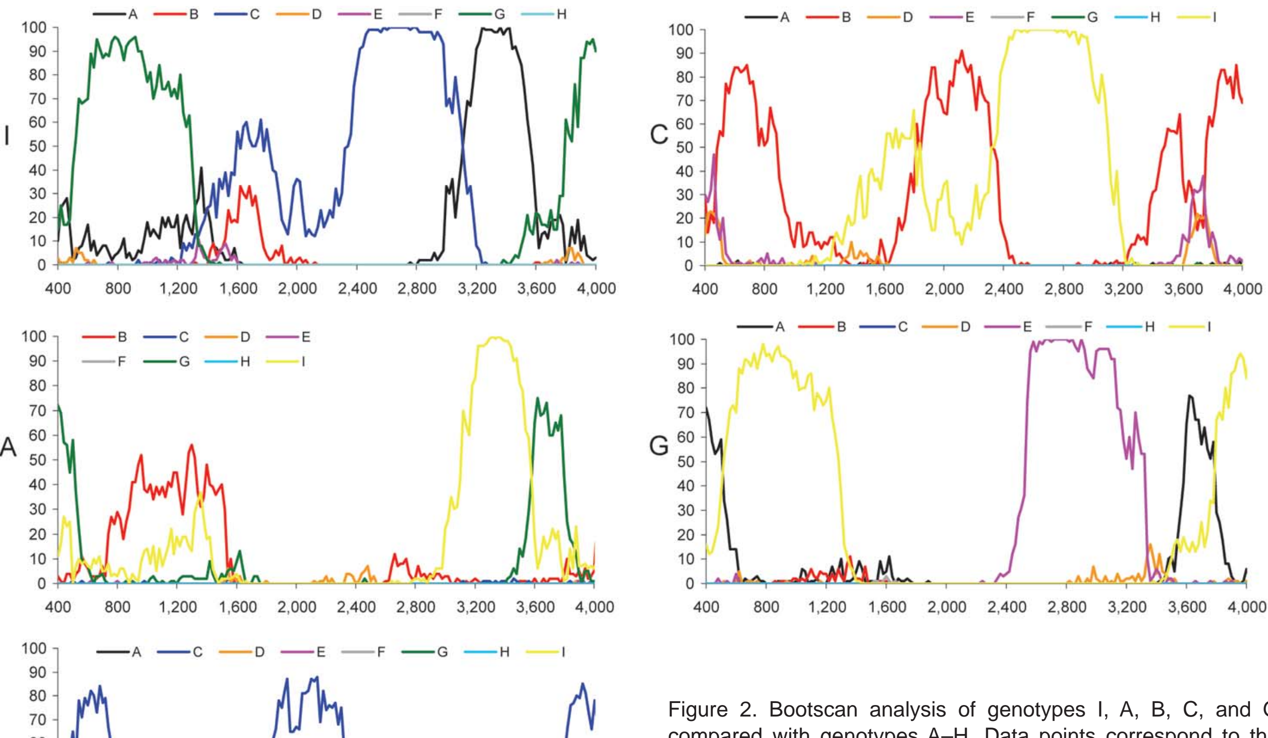

Figure 2. Bootscan analysis of genotypes I, A, B, C, and G compared with genotypes A–H. Data points correspond to the center of sequence windows of 800 bp. For the analysis of the first 400 nt, the beginning of the genome was duplicated at the end of the sequence: nt 3200–4000 represent positions 0–800.

Table 2. Characteristics of strains most closely related to different recombinant fragments of genotype I (M04-0469): genotypes and genetic distances

Characteristic	nt 400–1400	d	nt 1400–3000	d	nt 3000–400	d
Most similar genotype	G	6.29*	C	6.29	A	6.23
2nd most similar genotype	A	6.84	B	9.14	C	8.61
Most similar subgenotype	NA		C3	5.42	A4	5.23
Most similar strain	G, AF160501	5.51	C3, X75656	4.1	A3, AM184126	4.5
2nd most similar strain	G, AB056513	5.62	C1,AB222715	4.3	A4, AY934764	4.7

*Distances (in %) to genotypes of subgenotypes are average distances, that is, the A-like sequence was in average most similar to subgenotype A4 but the minimal distance observed was to an A3 strain. nt, nucleotide; d, distance; NA, not applicable.

The genotype C–like fragment was most closely related to subgenotype C3 (Table 2), thus far found only in the Pacific, except for a single incomplete strain in Laos (from this study). C1, found in Japan, Korea, the People's Republic of China, and Uzbekistan (*8,9*), is the second most closely related subgenotype. The genotype A–like fragment was most similar to subgenotypes A3 and A4, recently found by us and confirmed by others in sub-Saharan Africa (*10–12*). The apparent relatedness with the defective genotype G is even more surprising, since it has only been found sporadically in the United States, Japan, Germany, and France (*13–15*). Thus, none of the contributing genotypes or subgenotypes have ever been identified in Southeast Asia, which evokes questions about the origin of genotype I in this region.

To further exclude artifacts of mixed infections, various genome regions of 15 genotype I strains were cloned. The 92 clones analyzed clustered with the same group as the uncloned parent sequences. In just 2 cases, for which only the preS/S gene was available for analysis (M05–0659 and M04–2769), a mixed infection with several genotypes was found. The first (M05–0659) contained an I1 and I1/B4 recombinant sequence (B4 on the last 300 nt), while the second (M04–2769) contained 5 different species: 1 B5/C2 recombinant (C2 on the last 400 nt), 1 C5/C2 recombinant (C2 on the last 400 nt), 1 genotype C2 sequence, 4 genotype C5 sequences, and 1 C/I2 recombinant (C subgenotype unclear; I2 on nt 200–600).

Four strains with no signs of mixed infections in the sequence electropherograms clustered inconclusively in the phylogenetic analysis of partial sequences (data not shown). These strains were cloned and identified by bootscan analysis as recombinants between several genotypes and/or subgenotypes. In 3 recombinants, only subgenotypes I1 and I2 were involved (M04–3739, M04–2531, and M04–0309). The 2 latter recombinants had identical bootscan patterns (I1 between nt 1400–1900), while in the first recombinant, the I2 sequence was shifted downstream by 200 nt (data not shown). Strain C04–0790 showed a similar pattern to M04–3739, but the I1 sequence was replaced by a C2 sequence. Bootscan analysis of a previously reported strain (GenBank/EMBL/DDBJ no. AB231909) showed a B4/I1 recombinant with the I1 sequence identified between positions 600 and 1864. The most similar strains for each of the distinct regions of these recombinants are virtually all circulating in Laos (this study). In fact, the C2 sequence of the C04–0790 recombinant strain was identical to the C04–1257 strain found in a different district of Vientiane. This strain could have been a potential donor strain.

Conclusions

In a preliminary report, we discussed the need to assign a new genotype (I) to strains that we had found in Laos (*1*). More recently, Tran et al. defined a new genotype I on the basis of a single, similar strain from Vietnam (*2*). Here we analyzed a larger number of new sequences which formally comply with the definition of a new genotype (I) and 2 subgenotypes of it. A complex recombination pattern suggests that genotype I was formed by recombinations outside of Southeast Asia before spreading within Laos and Vietnam, and giving birth to a new genotype with subgenotypes, which later recombined with regional strains. Identification and analysis of genotype I strains provide further evidence of the importance of recombination in the evolution and genesis of new HBV genotypes, a complexity not fully acknowledged by the current genotype classification. Nucleotide sequences from this study have been submitted to international public databases under accession nos. FJ023546–48, FJ023553–60, FJ023566–68, FJ023572–73, FJ023577–630, FJ023642, FJ023659–83, FJ023700–07, FJ023854, FJ023878–85, FJ023936, FJ023968, FJ023994 and FJ358584–98.

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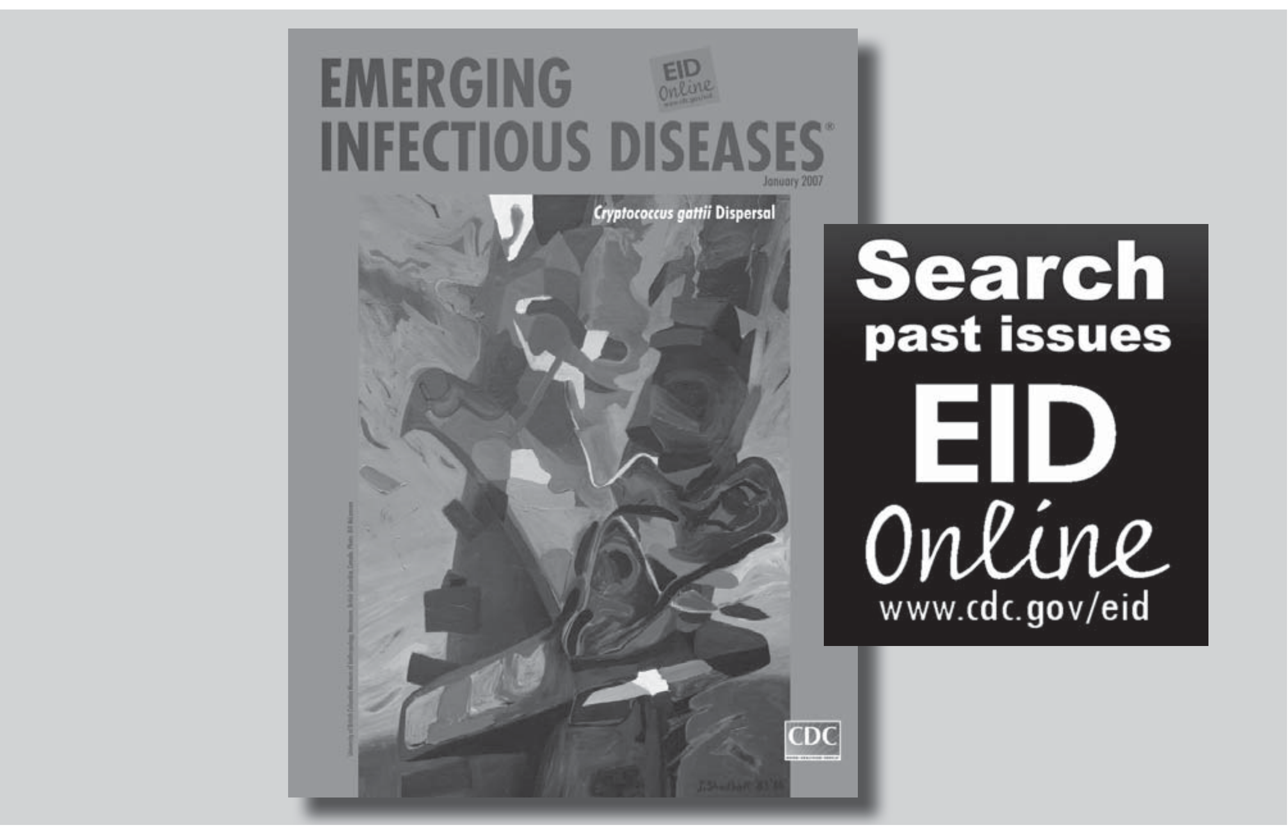

Tourism and Specific Risk Areas for *Cryptococcus gattii*, Vancouver Island, Canada

Catharine Chambers, Laura MacDougall, Min Li, and Eleni Galanis

We compared travel histories of case-patients with *Cryptococcus gattii* infection during 1999–2006 to travel destinations of the general public on Vancouver Island, British Columbia, Canada. Findings validated and refined estimates of risk on the basis of place of residence and showed no spatial progression of risk areas on this island over time.

Cryptococcus gattii is a fungus that infects the lungs and central nervous system of mostly immunocompetent humans and animals (*1*). In 1999, *C. gattii* emerged on the east coast of Vancouver Island (VI), British Columbia (BC), Canada (*2*), and is now considered endemic in the environment (*3*,*4*), affecting human (*5*) and animal populations (*6*). Travel histories of patients have been used to monitor fungal spread (*5*) and to estimate the incubation period of this disease (*7,8*).

Intra-island travel on VI is common, and fungal exposure may not occur near residences of case-patients. Incidence rates calculated by using patient residence have suggested areas along the east coast of the island that may pose increased risk for infection (Figure) (*9*). Environmental sampling has provided evidence of the fungus over a large part of eastern VI. However, this sampling was not performed randomly and may not accurately identify areas of highest risk (*3*,*4*).

The Study

This study compared travel histories of *C. gattii*–infected case-patients with travel patterns of the general public to validate and refine these risk areas on VI. We also examined spatial progression of these areas over time to assess whether *C. gattii* spread from a single focal point since its emergence in 1999.

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C. gattii–infected case-patients were defined as BC residents with culture-confirmed *C. gattii* infection or HIV-negative residents of BC with *Cryptococcus* sp. infection diagnosed by antigen detection or histopathologic analysis. Analysis included all cases diagnosed from January 1999 through December 2006 in which the patient had documented travel history on VI. Case-patients were interviewed by using a standard questionnaire and asked about travel to any city outside their city of residence in the 12 months before symptom onset or diagnosis (*8*).

Tourism BC (www.hellobc.com/en-CA/default.htm) provided aggregated monthly visitor volume to 14 visitor centers in major tourist destinations (Figure) on VI during 2000–2006. Visitors were counted if they spoke with visitor center counselors. Only visitors classified as BC residents were included in these analyses; additional personal attributes of visitors were not collected (C. Jenkins, pers. comm.). Seasonal visitor centers that had only partial data available for certain months were excluded.

Proportion of visits to each visitor center city was defined as number of visits to a visitor center city divided by total number of visits to all visitor center cities. For case-patients, the proportion was similarly defined. In both instances, visits to multiple cities by the same person were counted multiple times. Differences between proportion of case-patient visits and Tourism BC visits were evaluated by Fisher exact test and StatXact software (Cytel Inc., Cambridge, MA, USA). Analysis was conducted for all years combined and in 2 four-year increments (1999–2002 and 2003–2006) to assess potential spread of *C. gattii* on VI over time. Because Tourism BC visitor data were unavailable for 1999, case data for 1999–2002 were compared with aggregated Tourism BC visitor data from 2000 through 2002. Analysis was also conducted for a subset of

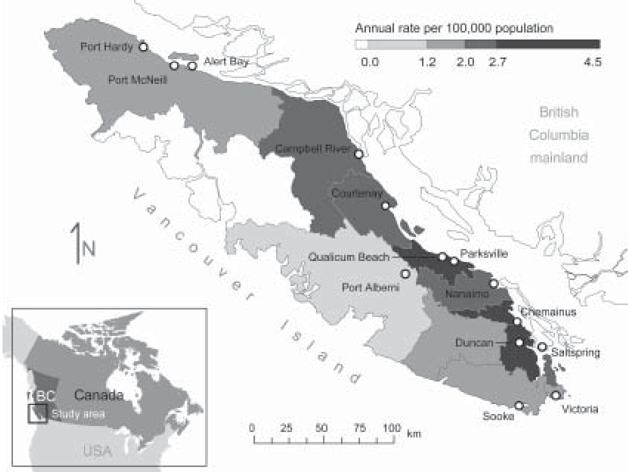

Figure. Annual rate of infection with *Cryptococcus gattii* by local administrative area, 1999–2006 (*9*), and distribution of visitor center cities on Vancouver Island, British Columbia (BC), Canada. Only visitor centers that were included in the analysis are shown.

case-patients who resided on the mainland because they represented travel exposures uncontaminated by potential exposure in place of residence. The α value for significance was adjusted to account for testing multiple visitor center cities (p = 0.05/14 visitor center cities = 0.0036). Maps were created by using ArcMap version 8.2 (Environmental Systems Research Institute Inc., Redlands, CA, USA).

Travel history data were available for 104 (60.1%) case-patients. Eighty-two (78.8%) had traveled to ≥1 visitor center city. Of these, 62 (75.6%) resided on VI and 20 (24.4%) lived on the BC mainland. A significantly greater proportion of visits to Parksville (18.7% vs 7.2%; p<0.0001) and Nanaimo (21.4% vs 7.4%; p<0.0001) were reported for patients than for Tourism BC visitors (Table). Similar results were obtained when analysis was restricted to earlier (1999–2002) and later (2003–2006) periods (Table).

When analysis was restricted to data concerning mainland residents (patients with travel-associated exposure but no residential exposure to the fungus), a greater proportion of mainland case-patients visited Courtenay (19.4% vs 7.6%; p = 0.017), Parksville (30.6% vs 8.3%; p = 0.0001), Nanaimo (11.1% vs 6.9%; p = 0.313), and Qualicum Beach (8.3% vs 4.7%; p = 0.239) than did Tourism BC visitors during 1999–2006; however, only Parksville reached statistical significance. Because of the small number of patients who resided on the mainland (n = 20), we could not further restrict this subset analysis to earlier (1999–2002) and later (2003–2006) periods.

Residents of VI may be exposed in their place of residence, in addition to their travel destination. However, we could not accurately weight patient exposure in the home environment to exposure at the travel destination. Minor differences in results obtained for all case-patients compared with only mainland patients may be caused by this limitation or by differences in travel preferences between these groups.

Although travel history data were unavailable for 39.9% of the case-patients, they were not significantly different in terms of mean age (p = 0.303, by F test) or sex (p = 0.574, by χ^2 test). A higher proportion of included patients resided in central VI. However, travel patterns of central VI residents did not differ from travel patterns of other VI residents (data not shown). Our analysis assumes that travel patterns of Tourism BC visitors represent those of the general BC public. However, characteristics and activities of persons who use Tourism BC visitor centers may differ from those of persons who do not. Therefore,

Table. Proportion of cases of *Cryptococcus gattii* infection compared with proportion of BC residents who visited Tourism BC visitor centers, by location, Vancouver Island, British Columbia, Canada, 1999–2006*

	1999–2002			2003–2006			All years		
Location	No. (%) cases	No. (%) visits	% Difference	No. (%) cases	No. (%) visits	% Difference	No. (%) cases	No. (%) visits	% Difference
Nanaimo	26 (20.3)	20,160 (6.5)	13.8†	14 (23.7)	35,169 (8.0)	15.7†	40 (21.4)	55,329 (7.4)	14.0†
Parksville	25 (19.5)	24,095 (7.8)	11.8†	10 (16.9)	30,070 (6.9)	10.1	35 (18.7)	54,165 (7.2)	11.5†
Duncan	12 (9.4)	20,484 (6.6)	2.8	4 (6.8)	25,973 (5.9)	0.9	16 (8.6)	46,457 (6.2)	2.3
Victoria	24 (18.8)	58,092 (18.8)	0	16 (27.1)	94,452 (21.6)	5.6	40 (21.4)	152,544 (20.4)	1.0
Qualicum Beach	8 (6.3)	14,197 (4.6)	1.7	3 (5.1)	26,429 (6.0)	–0.9	11 (5.9)	40,626 (5.4)	0.4
Port McNeill	1 (0.8)	5,985 (1.9)	–1.2	0	6,378 (1.5)	–1.5	1 (0.5)	12,363 (1.7)	–1.1
Courtenay	8 (6.3)	35,051 (11.3)	–5.1	4 (6.8)	30,859 (7.0)	–0.3	12 (6.4)	65,910 (8.8)	–2.4
Saltspring Island	4 (3.1)	19,093 (6.2)	–3.0	1 (1.7)	20,744 (4.7)	–3.0	5 (2.7)	39,837 (5.3)	–2.7
Chemainus	3 (2.3)	13,374 (4.3)	–2.0	1 (1.7)	23,273 (5.3)	–3.6	4 (2.1)	36,647 (4.9)	–2.8
Port Alberni	4 (3.1)	23,466 (7.6)	–4.5	4 (6.8)	30,760 (7.0)	–0.2	8 (4.3)	54,226 (7.3)	–3.0
Sooke	3 (2.3)	15,450 (5.0)	–2.6	0	19,485 (4.4)	–4.4	3 (1.6)	34,935 (4.7)	–3.1
Alert Bay	0	7,891 (2.5)	–2.5	0	18,107 (4.1)	–4.1	0	25,998 (3.5)	–3.5†
Campbell River	8 (6.3)	29,219 (9.4)	–3.2	2 (3.4)	49,830 (11.4)	–8.0	10 (5.3)	79,049 (10.6)	–5.2
Port Hardy	2 (1.6)	23,106 (7.5)	–5.9	0	26,616 (6.1)	–6.1	2 (1.1)	49,722 (6.6)	–5.6†
All centers	128	309,663	–	59	438,145	–	187	747,808	–

*Visitor centers that were only opened seasonally were not included in the analysis. BC, British Columbia.
†Significant differences after adjustment for multiple comparisons according to Fisher exact test (p≤0.0036).

caution is necessary when generalizing results to the entire BC population. Our interpretation is limited by its inability to account for duration of time spent in each visitor center city and specific activities of persons while there, factors that may contribute to exposure risk.

Conclusions

Our findings suggest that the opportunity for *C. gattii* exposure in the areas studied has existed since the beginning of its emergence and that minimal spatial progression of risk areas has occurred over time. Areas of higher risk near Parksville and Nanaimo are consistent with distribution of environmental samples, which shows a high number of *C. gattii*–positive samples in these areas (*3*). Results are also consistent with annual incidence rates for *C. gattii* infection based on place of residence, which are highest along the central eastern coast (Figure) (*9*).

When compared with areas on the basis of place of residence, more refined geographic risk areas associated with our analysis may result from potential reporting bias that produced reported percentage differences that are larger than expected. BC residents may be more likely to visit or travel through Nanaimo, a commercial center on VI and transportation gateway to the rest of the island (*10*), than shown in Tourism BC data. Case-patients may be more likely to report traveling to Parksville, a popular tourist destination, because it was often mentioned in media reports of the initial *C. gattii* outbreak. Alternatively, results may indicate a true increase in travel-associated risk in areas near Parksville and Nanaimo. Some case-patients who resided in areas with high incidence rates may have acquired their infections by travel to these 2 areas. Although Parksville and Nanaimo may represent areas of higher risk, environmental sampling suggests fungal colonization in southern and central eastern VI, and travelers can be exposed to *C. gattii* in these regions (*3*).

To determine travel-related risk for malaria (*11*) and gastrointestinal illness (*12–14*), travel patterns of case-patients have been compared with those of the general public. Use of visitor center information and tourism surveys is a cost-effective solution to derive comparison data during a retrospective investigation. This approach shows promise in assessing risk for environmental pathogens where location of exposure is unclear.

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Metagenomic Diagnosis of Bacterial Infections

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To test the ability of high-throughput DNA sequencing to detect bacterial pathogens, we used it on DNA from a patient's feces during and after diarrheal illness. Sequences showing best matches for *Campylobacter jejuni* were detected only in the illness sample. Various bacteria may be detectable with this metagenomic approach.

Infectious diseases are caused by various pathogens, including as-yet unidentified microorganisms. Because procedures for detecting and identifying pathogens vary according to the target microorganism, clinical examinations require a variety of media, reagents, and culture methods. In addition, conventional examination protocols usually require much labor, time, and skill, thus forming an obstacle to a prompt diagnosis.

Newly developed, "next-generation" DNA sequencers can determine >100 megabases of DNA sequences per run (*1*). These new technologies eliminate the bacterial cloning step used in traditional Sanger sequencing; instead, they amplify single isolated DNA molecules and analyze them with massively parallel processing. To develop a new system to promptly detect and identify various infectious pathogens, we tapped into the potential of these novel sequencers. We directly detected the causative pathogenic microbe in a clinical human sample (diarrheic feces) by means of unbiased high-throughput DNA sequencing.

The Study

A 34-year-old man had become ill after eating dinner out with his family. After 3 days, severe diarrhea, stomach ache, and shivering developed in the only 3 persons (the patient plus 2 family members) who had eaten undercooked chicken that night. Four days after onset of clinical signs, feces were collected from the patient and stored in a freezer at –80°C. At a clinical laboratory in Osaka, Japan, conventional culture methods were used to examine the sample for possible bacterial enteropathogens (*2*), and specific reverse transcriptase–PCR was used to test for norovirus (*3*); however, no candidate pathogens were detected.

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We therefore analyzed this fecal sample for possible pathogens by means of high-throughput DNA sequencing. DNA was extracted from the diarrhea sample (hereafter referred to as the illness DNA sample) with a QIAamp DNA Stool Mini Kit (QIAGEN, Valencia, CA, USA). After the man had completely recovered 3 months later, another fecal sample was collected (hereafter referred to as the recovery DNA sample) and maintained at –80°C until DNA extraction. Both DNA samples were subjected to unbiased high-throughput DNA sequencing with a GS20 sequencer (454 Life Sciences, Branford, CT, USA) (*4*).

Sequencing produced 96,941 effective sequences for the illness DNA sample and 106,327 for the recovery sample. The average length of the sequences was 102.1 bp. The DNA sequences obtained were searched with the BLASTN program for the National Center for Biotechnology Information nucleotide sequence database (http://blast.ncbi.nlm.nih.gov). The BLASTN output was then analyzed by using a classification system consisting of the Center's taxonomy database and its searching system. This system, devised with the aid of Perl language (www.perl.com) and the MySQL database (www.mysql.com), facilitates the identification of scientific names and statistical analysis. The Figure shows the organisms from which the sequences in the database were derived that showed the best matches for the sequences queried (expect [E]-value $<10^{-5}$). For both DNA samples, ≈20% of the total sequences showed the best matches for the currently reported bacterial DNA sequences. The Table shows the frequency distributions of species from which close matches for the sequences were derived (E-value $<10^{-40}$). The most frequently detected bacterial species in both samples belonged to the phylum Bacteroidetes, the normal flora of the human intestine. No major differences were found in the frequency of the species between the illness and recovery DNA samples.

A striking difference between the 2 samples, however, was that 156 sequences of the illness DNA sample showed best matches for the sequences derived from *Campylobacter jejuni*, but no sequences of the recovery DNA sample showed any such significant matches. The *C. jejuni* sequences from the illness DNA sample included many housekeeping genes, such as the genes for the ribosomal RNAs and DNA polymerases (online Appendix Table, available from www.cdc.gov/EID/content/14/11/1784-appT.htm); thus, they strongly suggested the presence of *C. jejuni* in the illness fecal sample.

Because *C. jejuni* is a bacterium that causes acute gastroenteritis and is normally not present in the intestines of

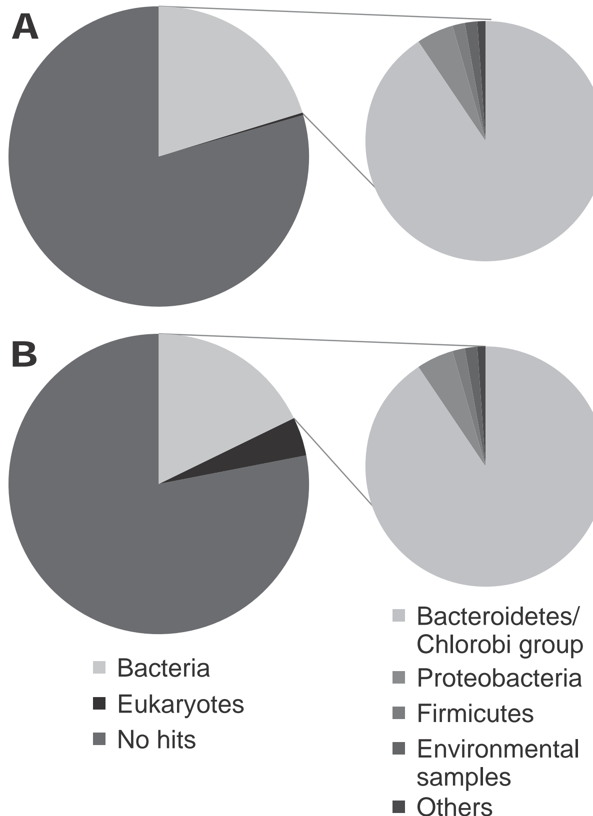

Figure. Comparison of the organisms from which the best matches for the sequences were derived from a BLASTN (http://blast.ncbi.nlm.nih.gov) search with an expect-value cutoff of 10^{-5}. A) DNA from nondiarrheic fecal sample collected 3 months after patient had recovered. B) DNA from diarrheic fecal sample collected while patient was ill.

healthy persons (*5*,*6*), these results prompted us to reexamine the illness fecal sample for *C. jejuni*. For the illness sample but not the recovery DNA sample, *Campylobacter*-specific PCR (*7*) produced a typical banding pattern that is unique to *C. jejuni* (data not shown). The recovery rate of *Campylobacter* spp. from patient specimens substantially decreases when the specimens are frozen before isolation (*8*). To obtain higher recovery of *Campylobacter* spp. and thus validate the presence of *C. jejuni* in the illness sample, we performed cultures with enrichment and selective media again on the frozen illness fecal sample (*5*). *C. jejuni*–like bacteria with corkscrew motility grew on selective agar plates. Biochemical identification using the API Campy kit (API-bioMérieux, Marcy L'Etoile, France) demonstrated that the organism was *C. jejuni,* thus proving its presence in the illness fecal sample.

Conclusions

We directly detected a bacterial pathogen in a patient sample by using high-throughput DNA sequencing. This finding implies that basically any kind of bacterial pathogen may be detectable with a common procedure. The method is directly applicable not only to fecal samples but also to other types of clinical samples; it could detect and identify bacterial pathogens that are usually difficult to ascertain with conventional examination procedures. Because this novel approach can be expected to have major potential for detection of pathogens in various infectious diseases, it warrants further investigation.

The approach reported here also enabled us to directly analyze the ratio of pathogenic to commensal bacteria in the human intestine. Assessment of the relative population of intestinal bacteria would enable us to investigate the dynamics of bacterial pathogens in human intestines, in relation to associated intestinal microbial flora, during infectious disease processes.

Many causative agents of emerging infectious diseases are of animal origin, and many are previously identified microbes (*9*,*10*). Because a vast amount of genome information about various microorganisms is continually being accumulated in databases, the approach we used will become increasingly useful. Recent metagenomic studies have identified unknown virus pathogens (*11–13*). Using the present approach to analyze various clinical cases, especially of outbreaks of infectious diseases with as-yet unidentified causative agents, may lead to the discovery of novel bacteria that are currently not known to be pathogenic to humans.

The current cost for high-throughput sequencing may limit the use of this method to specialized purposes, such as the hunt for novel pathogens for research or detection of bioterrorism (*14*). However, because the progress of DNA sequencing technology has been rapid (*1*), the cost, time, and labor for sequencing have been greatly reduced, and this trend will likely continue for the foreseeable future

Table. Frequency distributions of species in fecal samples taken from patient during illness and after recovery, as determined by BLASTN*

Organism	No. (%)	
	Illness†	Recovery‡
Bacteroides vulgatus	5,944 (50.5)	4,743 (56.5)
Homo sapiens	2,955 (25.1)	84 (1.0)
Parabacteroides distasonis	818 (6.9)	1,283 (15.3)
B. thetaiotaomicron	767 (6.5)	1,046 (12.5)
B. fragilis	759 (6.4)	842 (10.0)
Uncultured bacterium	195 (1.7)	227 (2.7)
Campylobacter jejuni	156 (1.3)	0
B. ovatus	48 (0.4)	63 (0.8)
Uncultured *Bacteroides* spp.	20 (0.2)	19 (0.2)
B. uniformis	14 (0.1)	8 (0.1)

*BLASTN available from http://blast.ncbi.nlm.nih.gov. Expect-value cutoff 10^{-40}.
†Diarrheic fecal sample collected while patient was ill. Total sequences 96,941; total (100%) BLAST matches 11,777.
‡Nondiarrheic fecal sample collected 3 mo after patient had recovered. Total sequences 106,327; total (100%) BLAST matches 8,397.

(*15*). Therefore, high-throughput DNA sequencing may soon be adopted as the main method for examining microorganisms in major clinical laboratories. The data presented here represent an example of this major innovation in the field of clinical examination for causative agents of infectious diseases.

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This study was approved by the ethical review committees of the Research Institute for Microbial Diseases, Osaka University, and RIKEN. The sequencing data reported here are available in the Short Read Archive database at the National Center for Biotechnology Information under accession no. SRA001127.

Dr Nakamura is a researcher in the Section of Bioinformatics, Thailand-Japan Research Collaboration Center on Emerging and Reemerging Infections, Research Institute for Microbial Diseases, Osaka University. His research interests have included crystallographic analysis for biomacromolecules, which he currently applies to his work in bioinformatics.

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Prevalence and Pathogenicity of WU and KI Polyomaviruses in Children, the Netherlands

Marieke M. van der Zalm, John W.A. Rossen, Bart E. van Ewijk, Berry Wilbrink, Petra C.H.M. van Esch, Tom F.W. Wolfs, and Cornelis K. van der Ent

A longitudinal study in 2004 and 2005 detected polyomaviruses WU and KI in 44% and 17% of children with and without respiratory symptoms, respectively, in the Netherlands. In some children both viruses were detected for long periods. In several symptomatic children no other respiratory pathogen was detected.

High-throughput sequencing techniques have revealed 2 new polyomaviruses called the WU virus (WUPyV) (*1*) and KI virus (KIPyV) (*2*). WUPyV and KIPyV have been reported in respiratory samples of uncontrolled studies of small groups of hospitalized patients (*1–5*). However, the clinical relevance of these viruses in humans is unclear because data are lacking on these viruses in otherwise healthy persons outside a hospital setting (*6–8*). Whether these newly identified viruses also occur in healthy children and whether they should be seen as causative agents for clinical respiratory disease are not known. This study determined the prevalence of WUPyV and KIPyV in young children in the Netherlands with and without clinical respiratory symptoms.

The Study

During a 6-month period (November 2004–April 2005), we performed a systematic survey on WUPyV and KIPyV and closely monitored respiratory symptoms in a prospective longitudinal cohort of 18 young children (<1–7 years of age, mean age 3.4 years from throughout the Netherlands). The study coordinator contacted parents twice a week (by telephone or email) to ask about the presence of any signs or symptoms of respiratory tract illness. Respiratory signs and symptoms were defined as coryza (rhinorrhea or nasal congestion), sore throat, ear ache with or without ear discharge, cough, sputum production or dyspnea, in the presence or absence of fever (>38°C). Every 2 weeks, samples for virus detection were collected regardless of any respiratory symptoms. A sample was defined as asymptomatic if the child had no respiratory symptoms 1 full week before and 1 full week after sampling. A sample was defined as symptomatic if the child had any respiratory symptoms 1 week before and 1 week after sampling. Parents collected the samples for virus detection by rubbing 1 nostril and posterior oropharynx of their child with separate cotton-tipped swabs. Feasibility of virus sampling by the parents has been described earlier (*9*). The study was approved by the local medical ethics committee, and the parents gave written informed consent.

In total 230 samples of symptomatic and asymptomatic samples were collected and tested for WUPyV and KIPyV by real-time PCR using an ABI 7500 System (Applied Biosystems, Foster City, CA, USA) (*10*) and for other respiratory pathogens by PCR (*11*) (Figure 1). In 119 samples (52%), the following nonpolyomavirus respiratory pathogens were detected: rhinovirus (32%); enterovirus (3%); respiratory syncytial viruses A and B (2%); coronaviruses OC43, 229E, and NL63 (17%); influenza viruses A and B (1%); human metapneumovirus (1%); adenovirus (<1%); *Mycoplasma pneumoniae* (3%); and *Chlamydophila pneumoniae* (5%).

WUPyV was found in 21 (9%) of 230 samples from 8 (44%) of 18 children (Figure 1). In 5 episodes WUPyV was the only pathogen detected and might therefore have been responsible for the observed respiratory symptoms. KIPyV was found in 6 (3%) of 230 samples in 3 (17%) of 18 children. In 2 symptomatic samples positive for KIPyV, no other pathogens were detected.

To track possible reinfections and/or prolonged infections of WUPyV and KIPyV during the observation period, we constructed Figure 2. For pediatric patients 1, 2, 3, and 6, WUPyV was found in >1 positive sample. In addition, prolonged presence of WUPyV (in ≥2 successive samples, equal to ≥2 weeks) was detected in 3 children (1, 2, and 6). The youngest child of the group (patient 1) had 2 periods of a prolonged infection with WUPyV (1 period of 3 successive positive samples and 1 period with 4 positive samples) as well as 3 successive KIPyV-positive samples. In the other children, KIPyV positivity was limited to 1 period.

Most infections with WUPyV and KIPyV were seen in the youngest children; 95% of the WUPyV infections and 83% of the KIPyV infections were seen in children ≤4 years of age (patients 1–10). For the children with a WUPyV infection, the median age was 2.6 years (interquartile range

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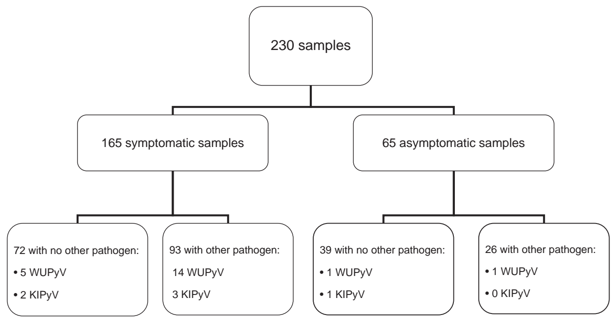

Figure 1. Flow chart of the respiratory samples taken in the study. Samples were collected during November 2004–April 2005, throughout the Netherlands. Samples were taken during symptomatic and asymptomatic episodes. Results show WU polyomavirus (WUPyV)and KI polyomavirus (KIPyV) detections in samples simultaneously negative for other respiratory pathogens and in samples in which ≥1 other respiratory pathogen(s) were detected.

0.7–3.9); for a KIPyV infection the median age was 3.0 years (interquartile range 0.4–4.0) (Figure 2)

Conclusions

This unique prospective longitudinal cohort study shows a high occurrence of WUPyV and KIPyV in children. WUPyV and KIPyV were repeatedly observed as the only detectable pathogen in children with respiratory symptoms, which may suggest that both viruses have pathogenic potential. In addition, younger age was associated with a higher occurrence of WUPyV and KIPyV infections.

Our overall percentages of WUPyV and KIPyV are somewhat higher than percentages reported in most other studies (*1–8*). This finding may partially be explained by the fact that samples were collected during the respiratory disease season. In addition, our study had a longitudinal design, and, therefore, our prevalences cannot be compared with those of other studies. In our study WUPyV was the third most prevalent pathogen (9%) after rhinoviruses (32%) and coronaviruses (17%). KIPyV was the fifth most prevalent pathogen (3%), comparable with enteroviruses.

The pathogenic role of WUPyV and KIPyV in respiratory disease is a subject of dispute. Some researchers suggest an association between WUPyV and KIPyV and respiratory symptoms (*1*,*3*,*5*), whereas others question the association between these viruses and disease (*7*,*8*). Here we report that WUPyV and KIPyV were the only viruses found in several samples, most of them originating from symptomatic episodes. Although these findings suggest a pathogenic role for both viruses, more extensive data are needed to establish their definite role in respiratory disease. In addition, we stress that we did not look for parainfluenza virus, human bocavirus, and coronavirus HKU1 in our study. We cannot exclude the fact that these viruses might be present in the samples in which only KIPyV or WUPyV was detected.

Because latent infections with subsequent asymptomatic reactivation are described as a feature of the polyomaviruses BK and JC (*12*), we were interested in the longitudinal course of WUPyV and KIPyV infections. Because most previous studies have a cross-sectional design, reinfections and persistence of WUPyV and KIPyV are usually missed. One study reported that WUPyV was detected in sequential samples of 2 immunocompromised patients for 6–8 weeks; 1 child was 16 months and the other 4 years of age (*5*). In the present study, positive episodes of WUPyV were interrupted by intervals during which no virus was detected in some children; however, the low viral load in these samples (high cycle threshold values) may have been below the detection limit in these negative intervals. In addition, we cannot exclude the possibility that poor collection techniques of samples with low viral loads resulted in failure to detect WUPyV.

We observed periods of successive positive samples for both WUPyV and KIPyV. These results might indicate that both viruses are able to persist in the respiratory tract. However, these positive samples could also represent new infections of WUPyV and KIPyV. Genetic analysis is needed to investigate whether this observed persistence is actual persistence or whether it represents new infections

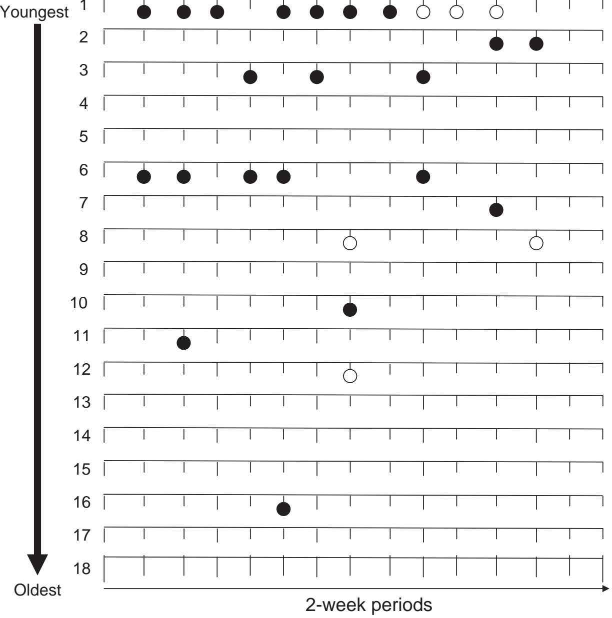

Figure 2. Timelines of WU polyomavirus (WUPyV) and KI polyomavirus (KIPyV) in 2-week samples, taken regardless of symptoms. Samples were collected during November 2004–April 2005, throughout the Netherlands. Each line represents a child in order of increasing age (patients 1–18, aged <1–7 years); the time between 2 vertical lines accounts for ≈2 weeks. The solid symbols are WUPyV infections; the open symbols are KIPyV infections.

with different WUPyV and KIPyV subtypes. We conclude that WUPyV and KIPyV are frequently present in young children. Additional studies are needed to confirm the suggestion from this study that both viruses may be associated with respiratory disease.

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etymologia

Chimera

[ki-mir′ə]

From the Greek *Khimaira*, Latin *Chimaera*; she-goat. In Greek mythology: a composite creature with the body and head of a lion, a goat's head rising from its back, and a serpent's tail. In science: an individual organism whose body contains cell populations derived from different zygotes, of the same or different species. Each population of cells keeps its own character, and the resulting animal is a mixture of tissues. Chimera also refers to a substance created from proteins or genes of 2 species, as by genetic engineering. Chimerism is rare in humans; ≈40 cases have been reported.

Source: Dorland's illustrated medical dictionary, 31st edition. Philadelphia: Saunders; 2007; http://www.merriam-webster.com; http://www.medicinenet.com/script/main/hp.asp

New Foci of Buruli Ulcer, Angola and Democratic Republic of Congo

Kapay Kibadi, Mbutu Panda, Jean-Jacques Muyembe Tamfum, Alexandra G. Fraga, Adhemar Longatto Filho, Gladys Anyo, Jorge Pedrosa, Yoshinori Nakazawa, Patrick Suykerbuyk, Wayne M. Meyers, and Françoise Portaels

We report 3 patients with laboratory-confirmed Buruli ulcer in Kafufu/Luremo, Angola, and Kasongo-Lunda, Democratic Republic of Congo. These villages are near the Kwango/Cuango River, which flows through both countries. Further investigation of artisanal alluvial mining as a risk factor for Buruli ulcer is recommended.

Buruli ulcer (BU), which is caused by the bacterium *Mycobacterium ulcerans*, is an indolent necrotizing disease of skin, subcutaneous tissue, and bone. BU is the third most common mycobacterial disease of humans, after tuberculosis and leprosy (*1*,*2*). Africa is the most affected continent, particularly in its tropical, central, and western regions (*1*).

BU was first reported in the Democratic Republic of Congo (DRC) in 1950 (*1*). The disease has been reported in 5 of 11 provinces in DRC (Lower Congo, Bandundu, Maniema, Katanga, and South-Kivu) (*3*).

BU was first reported in Angola in Caxito, Bengo Province, in 1998 (Figure) (*4*). Reports of BU in newly arrived Angolan refugees at Kimpese (Lower Congo) since the 1960s (*5*) suggest that Angola has long been an area endemic for BU. However, no cases have been reported along the Kwango/Cuango River in DRC or Angola. This river, known as the Kwango River in DRC and the Cuango River in Angola, is the boundary between Angola and DRC from Luremo to Kasongo-Lunda (Bandundu Province) (Figure).

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This study describes 3 laboratory-confirmed cases of BU. These cases were most likely acquired near the Kwango/Cuango River.

The Study

We studied 3 patients suspected of having BU who were admitted to the Dr Lelo Medical Center in Kinshasa (patient 1) and the Mother Teresa Buruli Ulcer Treatment Center in Kinshasa (patients 2 and 3). The study was reviewed and approved by the ethics committee of the Institute of Tropical Medicine, Antwerp, and the Public Health School of the Kinshasa University, Kinshasa, Ministère de l'Enseignement. The 3 patients provided verbal consent to participate in the study. Patients 1 and 2 were men 30 and 28 years of age, respectively, and patient 3 was a girl 13 years of age.

Characteristics of the patients are shown in the Table. Laboratory tests were performed on surgically excised tissues and exudates according to World Health Organization (WHO) recommendations (*6*). Patients were treated with rifampin and streptomycin for 12 weeks according to WHO recommendations (*7*). Four weeks after the beginning of treatment, surgical debridement was performed, followed by split-skin grafting. The patients were followed up at the 2 treatment centers and were considered cured when the lesions had completely healed.

All patients were residents of Kinshasa (DRC) and had no contacts with areas endemic for BU before traveling to areas along the Kwango/Cuango River where the BU skin lesions first appeared. However, all patients had frequent contact through alluvial diamond mining (patients 1 and 2) or domestic activities (patient 3) along the Kwango/Cuan-

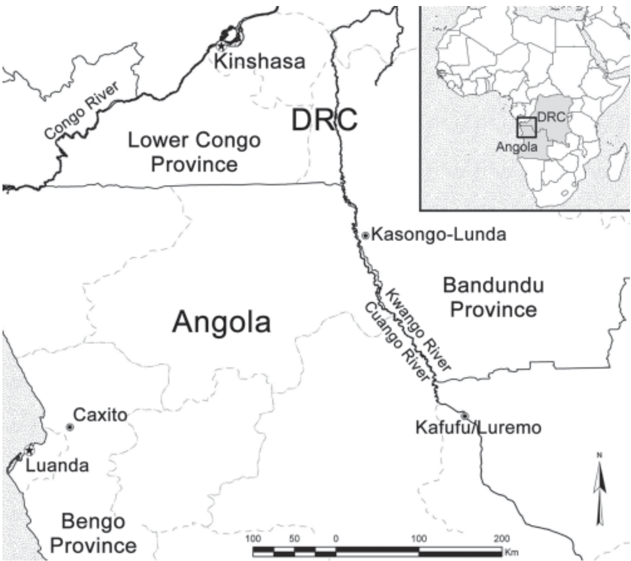

Figure. Locations in Democratic Republic of Congo (DRC) (Kasongo-Lunda) and Angola (Kafufu/Luremo) where 3 patients with Buruli ulcer were detected.

Table. Characteristics of 3 Buruli ulcer patients infected along the Kwango/Cuango River, DRC and Angola*

Characteristic	Patient 1	Patient 2	Patient 3
Age, y, sex	30, M	28, M	13, F
Origin	Kinshasa, DRC	Kinshasa, DRC	Kinshasa, DRC
Location where infected	Kafufu/Luremo, Angola	Kafufu/Luremo, Angola	Kasongo-Lunda, DRC
Patient delay,† y	2	2.5	2.5
Date of first symptoms	2002 Oct	2003 Jan	2003 Jan
Date care was sought	2004 Sep	2005 Jun	2005 Jul
Lesion			
Type	Ulcer	Ulcer	Ulcer
Size, cm^2	320	150	896
Site	Right thigh	Right arm	Left leg
Test results			
Ziehl-Neelsen staining	+	+	+
Culture	–	–	–
IS*2404* PCR	+	+	+
Histopathologic changes	ND	ND	Extensive areas of necrosis with clumps of AFB
Duration of hospitalization, mo	3	6	7
Follow-up period with no relapse, mo	42	30	28
Outcome	Cured	Cured	Cured

*DRC, Democratic Republic of Congo; +, positive; –, negative; IS, insertion sequence; ND, not done; AFB, acid-fast bacilli.
†Time between appearance of first signs or symptoms and care being sought at a medical center.

go River in DRC (Kasongo-Lunda) or in Angola (Kafufu/Luremo) (Figure).

Patients reported that their lesions had started 2–2.5 years earlier as nodules that later ulcerated. These patients were first treated locally near the Kwango/Cuango River by traditional healers and with 2% Dakin fluid (sodium hypochlorite solution) to cleanse the wounds. These measures were unsuccessful, and the patients returned to Kinshasa for treatment at the 2 medical centers, where they were admitted in September 2004 (patient 1), June 2005 (patient 2), and July 2005 (patient 3).

On admission, all 3 patients had large ulcers (150–896 cm^2). Patient 1 had an ulcer on the right thigh, patient 2 on the right arm, and patient 3 on the left leg (Table). BU was confirmed by Ziehl-Neelsen staining for acid-fast bacilli and a positive IS*2404* PCR result (Table). Cultures remained negative after incubation for 12 months at 32°C. For patient 3, BU was also confirmed by histopathologic analysis performed before treatment. A specimen showed a predominantly neutrophilic inflammatory infiltrate near extensive areas of necrosis associated with calcification and clumps of extracellular acid-fast bacilli.

The 3 patients were considered cured after 3, 6, and 7 months, respectively, of hospitalization. No relapses were observed after follow-up periods of 42, 30, and 28 months, respectively.

Conclusions

BU patients in our study had advanced disease with ulcers >10 cm in diameter. They were cured by treatment with antimicrobial drugs and surgery in accordance with WHO recommendations for treatment of BU (*6*,*7*). The patients were most likely infected during alluvial mining and use of water from the Kwango/Cuango River for domestic activities. Artisanal diamond mining in alluvial deposits along river banks consists of informal digging with basic equipment (often with unprotected hands and feet). Miners often work for long periods extracting diamonds from alluvial deposits along river banks. In Angola, mining areas are located in swamps that border the Kwango/Cuango River. Water sources used for domestic purposes along this slow-flowing river are unprotected, and proper hygienic procedures are lacking.

Epidemiologic studies have established a close association of BU and wetlands, especially those with slow-flowing or stagnant water (ponds, backwaters, and swamps) (*8*–*10*). In Uganda and in Benin, use of unprotected sources of water for domestic purposes increased the risk for contracting BU (*10*–*12*). Environmental factors, including poor hygienic conditions, along the Kwango/Cuango River make this region an area of high risk for contracting BU (*10*,*13*).

Studies have linked increased incidence of BU to human-made modifications such as expanded agricultural activities, deforestation, or construction of dams (*14*). Activities of both large mining enterprises and individual miners are responsible for environmental changes that may play a role in increased incidence of BU. Diamond-mining pits may become pools of stagnant waters that are a dangerous source of waterborne diseases.

In the 1950s in the Belgian Congo (now DRC), several cases of BU were reported in alluvial gold miners working in the mining camp of Kakerifu between the Nzoro and Kibali Rivers (*1*). Currently, in the gold-mining area

of Amansie West District in Ghana, many BU infections occur among workers in contiguous alluvial mining operations (*15*). Diamond mines along the Cuango River in Angola may have influenced the emergence of BU cases along this river basin. However, whether the Cuango River floodplain is a region endemic for BU or if this region became endemic after diamond-mining activities is not known.

The frequency of BU in Angola is not documented partly because of political changes after the country's independence in 1975. Surveys are urgently needed to determine the endemicity of BU in Angola. Our findings emphasize the need for further investigation of diamond, gold, and any other gemstone mining as a risk factor for contracting BU, particularly in West and Central Africa, where mining is common. All areas along the Kwango/Cuango River in DRC and Angola should be investigated for foci of BU. The association of artisanal alluvial mining with BU draws attention to a disease that further diminishes the quality of life of persons who are already living under the precarious circumstances experienced by those who mine diamonds.

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Novel Human Rhinoviruses and Exacerbation of Asthma in Children[1]

Nino Khetsuriani, Xiaoyan Lu, W. Gerald Teague, Neely Kazerouni,[2] Larry J. Anderson, and Dean D. Erdman

To determine links between human rhinoviruses (HRV) and asthma, we used data from a case–control study, March 2003–February 2004, among children with asthma. Molecular characterization identified several likely new HRVs and showed that association with asthma exacerbations was largely driven by HRV-A and a phylogenetically distinct clade of 8 strains, genogroup C.

Human rhinovirus (HRV) infection triggers asthma exacerbation (*1*), but there are no data on links between specific HRVs and asthma. Molecular sequence–based methods enabled recent identification of several novel HRVs (*2–9*) and have made it practical to look for genogroup and genotype-specific correlations with disease. In a previous study, we found a significantly higher prevalence of HRVs in children with asthma exacerbations than in children with well-controlled asthma (*10*). In this study, we used molecular characterization methods to examine HRVs from these patients with asthma.

The Study

The case–control study was conducted in metropolitan Atlanta, Georgia, USA, during March 2003–February 2004, among children with asthma who were ≥2 years of age (*10*). Case-patients were defined as patients with asthma exacerbation; controls were defined as patients with stable asthma. Information on symptoms of acute viral respiratory illness was also collected. The definitions, epidemiologic and laboratory methods, and clinical description of patients are available from Table 1 and the previously published report (*10*).

HRVs were detected in nasopharyngeal swab specimens by seminested reverse transcription–PCR (RT-PCR) targeting the 5′-noncoding region (NCR) (*10*). For further genetic characterization, HRV-positive samples were extracted from a previously unopened aliquot and amplified by using a nested RT-PCR that targeted the virus capsid protein 1 (VP1) gene at positions 2432–2781, based on HRV 1B (GenBank accession no. D00239) for species A and positions 2531–2799, based on HRV 14 (GenBank accession no. NC_001490) for species B. We used Sequencher 3.1.1 software (Gene Codes, Ann Arbor, MI, USA) for sequence assembly and editing. Nucleotide and predicted amino acid sequences were aligned with previously published HRV VP1 sequences (GenBank accession nos. AY355180–AY3552831, EF186077, EF077279, EF077280, EF582385–EF582387) by using ClustalW as implemented in BioEdit (version 7.0.5) (www.mbio.ncsu.edu/BioEdit/bioedit.html).

Phylogenetic trees were constructed by using the neighbor-joining algorithm implemented in PAUP* version 4.0.d10 (*11*). Partial VP1 sequences for the novel HRV strains were submitted to GenBank (accession nos. EU312093–EU312101).

As reported previously (*10*), HRVs were detected by a 5′-noncoding region seminested RT-PCR in 53 (37%) of 142 children with asthma, including 39 (60%) of 65 case-patients and 14 (18%) of 77 controls. Of these, the HRVs from 29 (55%) (24 [62%] of the 39 HRV-positive case-patients and 5 [36%] of the 14 HRV-positive controls) were subsequently genotyped. VP1 sequences from the remaining 24 HRV-positive specimens could not be obtained because of low amplicon yield (Table 2). Specimens from patients with symptoms of acute viral respiratory infection (Table 1) were more likely than those from patients without viral symptoms to yield sufficient VP1 amplicon for genotyping (percent genotyped 85% and 36%, respectively; odds ratio [OR] 9.1; 95% confidence interval [CI] 2.1–50.0; $p<0.05$).

Of the 29 HRVs successfully genotyped, species A accounted for 18 (62%) strains, species B accounted for 3 (10%), whereas 8 (28%) strains formed a phylogenetically distinct clade, which we provisionally named "genogroup C" (Table 2, Figure). Of the 18 HRV-A strains, 17 showed close genetic relatedness (80.7%–93.8% nucleotide and 89.6%–98.8% predicted amino acid sequence identity) to HRV prototype strains. One HRV-A strain (GA23584) was highly divergent from the closest prototype, HRV80 (73.2% nucleotide and 73.0% amino acid sequence identity), which suggests that it could represent a distinct previously undescribed HRV. The 3 HRV-B strains were closely related to prototype strains (84.0%–88.6% nucleotide and 89.7%–93.4% predicted amino acid sequence identity).

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Table 1. Criteria and definitions used in the study of children with asthma, March 2003–February 2004 (*10*)

Category	Criteria
Current persistent asthma:	
In children 2–5 y of age	All of the following: 1. Physician diagnosis of asthma 2. ≥2 previous episodes of cough, wheeze, and/or respiratory distress 3. Current treatment with asthma medications 4. Parent or sibling with current or past diagnosis of asthma or allergy, and/or current or past evidence of atopy (defined by seasonal rhinitis, eczema, or food hypersensitivity)
In children 6–17 y of age	All of the following: 1. Physician diagnosis of asthma 2. Symptoms of asthma in the past 12 mo 3. Current treatment with asthma medications
Case (asthma exacerbation)	Current persistent asthma, hospital admission or clinic visit for asthma exacerbation, and all of the following: 1. Signs and symptoms of airflow obstruction (i.e., cough, wheeze, shortness of breath, chest tightness) within past 48 h 2. Increased asthma symptoms resulting in hospital admission or clinic visit 3. Repeated use of short-acting β-agonists within past 48 h 4. Increased dose or addition of a new asthma controller therapy within past wk
Control (well-controlled asthma)	Current persistent asthma, routine clinic visit for asthma, and all of the following: 1. No systemic steroid therapy in past 4 wk 2. No increase in dose and no new controller medications in past wk 3. No change in the frequency of use of short-acting rescue medications in past wk 4. No increase in asthma symptom frequency in past wk
Acute respiratory viral illness	≥2 of the following: fever, stuffy/runny nose, headache, muscle aches, and pain or redness of eye(s) at the time of clinic visit or hospital admission

The partial VP1 sequences of genogroup C strains were phylogenetically distinct from HRV species A and B and showed a substantial intragroup diversity (Figure). VP1 sequence identity of these viruses with the closest match

Table 2. Human rhinoviruses identified in 53 pediatric patients with asthma, March 2003–February 2004, Atlanta, Georgia, USA*

HRVs	Receptor-binding group	No. among all HRV+ patients, n = 53	No. among HRV+ case-patients, n = 39		No. among HRV+ controls, n = 14
			Virus symptoms, n = 20	No virus symptoms, n = 19	
Total no. genotyped†		29	17	7	5
Species A		18	12	3	3
HRV12	Major	1	1	0	0
HRV30	Minor	2	2	0	0
HRV36	Major	1	0	1	0
HRV39	Major	1	0	0	1
HRV43	Major	1	1	0	0
HRV44	Minor	2	1	0	1
HRV46	Major	1	1	0	0
HRV49	Minor	2	1	1	0
HRV53	Major	1	0	1	0
HRV54	Major	1	1	0	0
HRV61	Major	1	1	0	0
HRV65	Major	1	1	0	0
HRV66	Major	1	0	0	1
HRV68	Major	1	1	0	0
GA23584‡	Unknown	1	1	0	0
Species B		3	1	0	2
HRV48	Major	1	0	0	1
HRV99	Major	2	1	0	1
Genogroup C§	Unknown	8	4	4	0
Not genotyped	Unknown	24	3	12	9

*HRV, human rhinovirus; case-patients, asthma patients with exacerbations; controls, asthma patients without exacerbation.
†HRV genotype based on partial virus capsid protein (VP1) gene sequence. Serotype designation based on ≥90% VP1 amino acid sequence identity with respective prototype strains.
‡Strain GA23584 showed 73.0% amino acid sequence identity with HRV80.
§Genogroup C HRVs form a clade phylogenetically distict from species A and B HRVs.

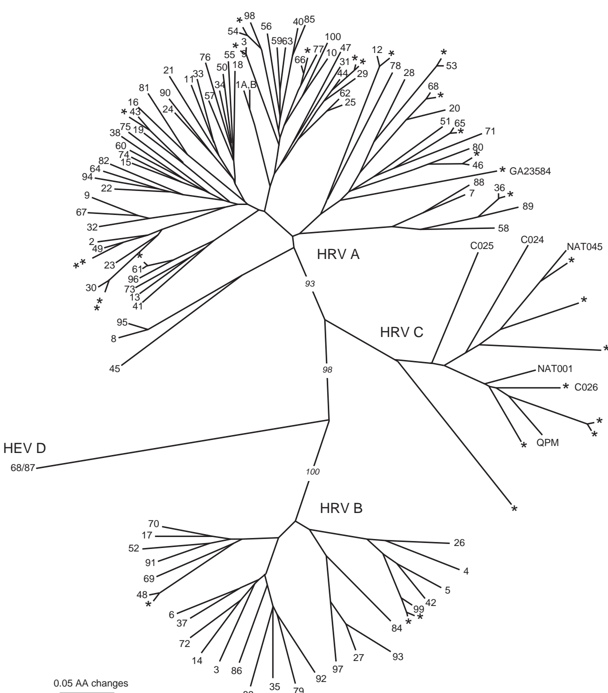

Figure. Phylogenetic tree of partial virus capsid protein 1 (VP1) amino acid sequences of human rhinoviruses (HRVs) identified in 29 HRV-positive pediatric asthma patients, March 2003–February 2004, Atlanta, Georgia, USA (designated *), previously published sequences of strains QPM (GenBank accession no. EF186077), C024-C026 (accession nos. EF582385–EF582387), and NAT001 and NAT045 (accession nos. EF077279–EF077280). HRV prototype strains designated 1A, 1B, 2-100. Human enterovirus (HEV) 68/HRV87 (designated 68/87) is included as outgroup. Tree construction and bootstrap values determined with PAUP* (*11*).

within the same genogroup ranged from 68.4% to 74.6% for nucleotide and from 68.5% to 85.5% for amino acid sequences. These novel viruses were related to other recently described HRVs: HRV–QPM detected in specimens from Australia (*4*), C024–C026 detected in specimens from Hong Kong (*6*), and NAT001 and NAT045 detected in specimens from California (*8*) (Figure). Their identity scores compared with HRV–QPM were 66.0%–82.7% for nucleotide and 65.2%–86.9% for amino acid sequences. One of the strains (GA23592) was almost identical in partial VP1 sequence to C026 (Figure). The degree of genetic diversity among the genogroup C viruses far exceeded that between HRVs defined as distinct serotypes by classical serologic methods, which suggests that at least 7 of 8 of these viruses are antigenically distinct from each other rather than minor variants of the same serotype. The genogroup C HRV identity scores were substantially lower when compared with their closest matches from species A and B: 48.2%–51.1% for nucleotide and 38.5%–49.8% for amino acid sequences, and 35.9%–42.8% for nucleotide and 29.3%–35.8% for amino acid sequences, respectively.

Conclusions

In our study, the association of asthma exacerbations with HRV infection appeared to be largely driven by the novel genogroup C, which was found exclusively in case-patients, and species A. The association was statistically significant for species A (detected in 15 [23%] of 65 case-patients vs. 3 [4%] of 77 controls; OR 7.4; 95% CI 1.9–43.1; p<0.001) and for genogroup C (detected in 8 [12%] case-patients vs. 0 controls; OR undefined; p<0.010) but not for infrequently identified species B (detected in 1 [2%] case-patient vs. 2 [3%] controls, p>0.05) or for HRVs that could not be genotyped (15 [23%] cases vs. 9 [12%] controls; p>0.05). The distribution of HRVs between case-patients and controls still differed when the analysis was limited to the HRV-positive group (p = 0.05) or to genotyped HRVs only (p<0.05). The results of the only other study that reported novel HRVs in asthma patients (2 of which, NAT001 and NAT045, were related to genogroup C viruses in our study) are difficult to interpret because that study of adults with "cold" symptoms showed an unexpected lack of association of HRVs with asthma exacerbation (*8*).

Patients infected with genogroup C HRVs had lower forced expiratory volumes during the first second (FEV1) than did those infected with other HRVs (median 58.5% vs. 93%; p = 0.01), but the distribution of demographic and other clinical variables did not differ significantly between the 2 groups. Lower FEV1 with genogroup C infection than with other HRVs suggests a potentially greater severity of asthma exacerbation in patients infected with these HRVs. When one considers the great variation among HRV serotypes in levels of sensitivity to candidate antiviral compounds (*12*,*13*), genogroup-related differences in associated disease patterns have implications for clinical management of HRV infections in asthma patients and for development of antiviral drugs against HRVs. Preliminary data suggest that HRV-QPM and related HRV-C strains from Hong Kong share certain VP1 sequence characteristics with HRVs that are resistant to a candidate antipicornavirus drug, pleconaril (*4*,*6*,*13*). These data raise the possibility that these novel HRVs might also be resistant to this compound.

The HRV-positive specimens from which VP1 gene sequences could not be obtained derived predominantly from patients without symptoms of acute respiratory viral illness. The absence of symptoms in HRV-infected persons likely reflects subclinical, asymptomatic infection, which is common for HRVs (*14*), or HRV persistence after a recently resolved infection (*15*), both of which are likely associated with lower viral loads (as opposed to acute symptomatic infections), thus leading to lower detection rates in a VP1 assay that uses highly degenerate primers.

In conclusion, we found a striking genetic diversity of HRVs among children with asthma and confirmed the ex-

istence and wide geographic distribution (USA, Australia, Hong Kong) of HRVs distinct from both previously recognized HRV species, A and B. Our finding supports the role of the novel HRVs as human pathogens. Additional studies are needed to further explore clinical and public health implications of these findings.

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Methicillin-Resistant *Staphylococcus aureus* in a Beauty Salon, the Netherlands

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An outbreak of community-associated USA300 methicillin-resistant *Staphylococcus aureus* occurred in a beautician and 2 of her customers. Eight other persons, who were either infected (n = 5) or colonized (n = 3), were linked to this outbreak, including a family member, a household contact, and partners of customers.

The reported number of community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) infections is increasing rapidly. CA-MRSA is increasingly isolated from patients who lack traditional risk factors for colonization or infection. CA-MRSA often contains the virulence factor Panton-Valentine leukocidin (PVL), which causes skin and soft tissue infections.

The CA-MRSA USA300 strain is known to cause outbreaks among population groups (*1*), such as native Americans, prison inmates, military personnel, men who have sex with men, and competitive sports participants, and accounts for 97% of MRSA isolates obtained in emergency departments across the United States from patients with soft tissue infections (*2*). CA-MRSA is associated with invasive infections, including necrotizing fasciitis (*3*), sepsis (*4*), and pneumonia (*5*). The USA300 strain, which is also found in Europe (*6*), was first isolated in the Netherlands in 2002.

Overall prevalence of MRSA in the Netherlands is low (2%) (*7*). In 2006, 3.8% (n = 76) of all MRSA isolates (1 per patient) sent to the National Institute for Public Health were identified as the USA300 strain. We report an outbreak of the USA300 strain related to a beauty salon in the Netherlands, in a beautician, a family member, a household contact, and customers and their partners.

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The Study

In September 2005, a medical microbiologist from the regional medical microbiology laboratory reported to the municipal health department a recurring MRSA infection in a beautician. From December 2004 onwards, the woman had recurrent infections on the legs, buttocks, and groin resulting in incision and drainage of lesions. When an abscess developed in the genital area in July 2005, MRSA was cultured from a wound swab. In December 2005, the beautician was declared MRSA-free after antimicrobial treatment. Swabs were taken 3 times in 1-week intervals from nose, throat, perineum, and wound and used for enrichment culture of MRSA. In March 2006, the woman was tested again for MRSA colonization; test results showed that she had been reinfected or that therapy had failed. The beautician also had eczema. Because of the "hands on" nature of her work, she was advised to temporarily stop providing services to customers.

The municipal health department conducted a risk assessment of the woman's household contacts and the beauty salon. The Netherlands does not require that MRSA infections be reported. Therefore, the municipal health department depends upon the consent and full cooperation of index patients and contacts for further investigation of outbreaks. Consequently, in this instance, household contacts for screening were identified but had not presented themselves for screening. Contacts who had complaints sought treatment at the emergency department, where the observant infection control practitioner (ICP) and microbiologists related them to the MRSA outbreak. Nurses obtained specimens by swabbing each patient's nose, throat, and wounds. A case was defined as a patient who had a culture-confirmed MRSA infection during the outbreak period July 2005–December 2006 and a direct epidemiologic link to the index patient.

In April 2006, a salon customer was hospitalized with an abscess of the breast caused by MRSA; in July 2006, another customer who had had boils since February 2006 was found to be MRSA positive. Both customers had been given wax treatments by the beautician during the period in which she had an infected hair follicle in her armpit. Swabs taken from this site showed that the beautician was infected with the same MRSA strain as before. Concern arose about the risk for infection to customers through instruments, materials (wax), or contact with other employees. The index patient and the other 6 employees of the salon regularly provided services to each another.

A nurse and ICP of the municipal health department visited the salon in June 2006 to check on hygiene proto-

cols and to advise on preventive measures to reduce risk for further transmission. All working procedures and protocols were investigated, and the salon was advised to clean and disinfect instruments and procedure rooms. More specifically, the ICP observed a total waxing procedure performed by the staff. Ten swabs were taken from used wax, wax implements, and the treatment room. All 6 employees were screened and informed about MRSA and the current situation. Arrangements were also made to test 22 regular customers who had received wax treatments by the index patient in the previous 2 months. In the following weeks, these customers were screened at the municipal health office and informed about MRSA. Of the 22 regular customers, 21 completed a questionnaire and 19 were actually screened for MRSA by culturing samples from nares and throats.

All employees and the 19 selected regular customers were negative for MRSA colonization. All environmental swabs were also negative for MRSA. It was noted that the 70% alcohol used to disinfect the skin after waxing was diluted with water because customers had complained about the stinging effect of the alcohol on treated skin. Furthermore, it became apparent that after performing waxing treatments the beautician would touch the waxed skin of customers with ungloved hands to check for remaining hairs. She did not wash her hands after removing the gloves.

During the outbreak investigation, more background information became available from those who were MRSA colonized or infected and who could be indirectly linked to the beautician or her customers. During the week that the first infected customer was identified (in April 2006), another customer was hospitalized with an abscess in the groin. Unfortunately, no culture was taken from this patient. The partner of the second infected customer was also infected with MRSA that was related to an abscess on his leg. By the end of 2006, a MRSA-positive couple was identified as a contact of the second infected customer. In August 2006 another couple was reported to be MRSA positive; both had abscesses on the thighs. Because no further epidemiologic data could be obtained, whether the couple's infection was linked to the beauty salon is not clear.

A total of 45 persons who had been in direct or indirect contact with the beautician were screened for MRSA: 3 family members, 3 roommates, 11 other persons (including secondary contacts), 6 beauty salon employees, and 22 customers (including regular customers). Fifteen persons had skin infections and 10 of them were colonized with MRSA (beautician, family member, roommate, ex-partner of the roommate, customers, and partners of customers). Although skin infections never developed in the beautician's family members, tests did show MRSA colonization in one of them. The beautician's boyfriend, a native of the United States, had already lived for >2 years in the Netherlands. Although he had skin lesions, no *S. aureus* was found. The girlfriend of a sport mate who regularly exercised with the partner of a customer was colonized with MRSA at the end of 2006. She had immigrated recently from the United States to the Netherlands, but her first screening test results were negative. The mean age of the patients was 29 years (range 21–40 years).

Eleven people were found to be MRSA positive. Of these 11, 3 persons with a direct link to the beauty salon (the beautician and 2 customers) (Table), 6 with an indirect link (family member, roommate, ex-partner of roommate, partner of a customer, sport mate of partner of a customer and his partner), and a couple from whom no epidemiologic data could be obtained were infected with the same MRSA strain as the beautician. To characterize the MRSA isolates, the following typing methods were used: pulsed-field gel electrophoresis (PFGE), staphylococcal protein A (*spa*) typing, multilocus sequence typing, staphylococcal cassette chromosome *mec* (SCC*mec*) typing, PCR for PVL genes (*LukS-LukF*), and a recently described assay for the USA300 strain-specific arginine catabolic mobile element (ACME)–encoded *arcA* gene (*8*).

All MRSA isolates were identical and identified as the well-known CA-MRSA USA300 strain. All strains were PFGE type 218 (according to the Dutch PFGE classification system = USA300), *spa* type t024, sequence type (ST)8 (1 isolate was characterized as ST879, a singleton of ST8), SCC*mec* IVa, and positive for PVL and ACME. All MRSA isolates had identical susceptibility patterns: resistant to oxacillin (and thus to all β-lactam antimicrobial drugs) and erythromycin, and susceptible to rifampicin, ciprofloxacin, gentamicin, clindamycin, vancomycin, teicoplanin, tetracycline, cotrimoxazole, mupirocin, and fusidic acid.

Conclusions

Outbreaks of CA-MRSA strains have been reported with increased frequency. Several reports involved out-

Table. Characteristics of MRSA outbreak related to a beauty salon, the Netherlands*

Case no.	Case-patient	Age, y	Gender	Type of infection	Site of infection	Date of first positive MRSA culture	MRSA sample test results
1	Beautician, index	21	F	Abscess	Leg, buttock, groin	2005 Jul	W+
2	Customer no. 1†	36	F	Abscess	Breast	2006 Mar	W+
3	Customer no. 2	29	F	Boils, abscess	Genitals	2006 Jul	N+, T+, P+, W+

*MRSA, methicillin-resistant *Staphylococcus aureus*; W, wound; N, nose; T, throat; P, perineum; +, positive.
†Hospitalized.

breaks among competitive sports participants, military personnel, men who have sex with men, prisoners, native Americans, and drug users (*1,9,10*). Skin treatments in a beauty salon likely led to MRSA transmission as a result of contact with an infected beautician.

Clearly, our study and others show that CA-MRSA is an emerging problem in the community setting. In the Netherlands, patients are generally only tested after recurrent infections. Unless outbreaks occur in a defined group, MRSA remains undetected in the general population because reporting is not mandatory. Although the prevalence of MRSA in the Netherlands is low, local microbiologic laboratories should report outbreaks, when detected, to the local municipal health department for further investigation. More research is necessary to better understand the risk factors involved in these outbreaks.

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Unusual *Cryptosporidium* Genotypes in Human Cases of Diarrhea

Guy Robinson, Kristin Elwin, and Rachel M. Chalmers

Several *Cryptosporidium* spp. are known to infect humans, but most cases of illness are caused by *C. hominis* or *C. parvum*. During long-term genotyping in the United Kingdom, we identified 3 unusual *Cryptosporidium* genotypes (skunk, horse, and rabbit) in human patients with diarrhea.

Cryptosporidium spp. are frequently a cause of diarrheal disease in immunocompetent as well as immunocompromised humans. Over the past decade molecular methods have enabled the characterization and identification of species and genotypes within the genus. The taxonomy is under continual review, but so far 20 valid species and numerous genotypes have been described. Many are named after the original host from which the isolate was recovered and are often referred to as "host-adapted" (*1,2*). Most human infections are caused by *C. hominis* or *C. parvum* but *C. meleagridis*, *C. felis*, *C. canis*, *C. suis*, *C. muris*, *C. andersoni*, *C. hominis* monkey genotype, cervine genotype, and the chipmunk genotype I have also been detected (*1–6*). The immune status of the host is not necessarily linked to infection with other species/genotypes (*1,7*). We describe 3 unusual *Cryptosporidium* genotypes detected in human patients with diarrhea.

The Study

Since 2000, the UK Cryptosporidium Reference Unit has maintained a national collection of *Cryptosporidium* oocysts (*8*). Over 16,000 *Cryptosporidium*-positive human fecal samples have been submitted by primary diagnostic laboratories and characterized by the Reference Unit to identify the infecting species. In addition to the expected *C. hominis*, *C. parvum,* and small number of *C. meleagridis*, *C. felis*, *C. canis,* and cervine genotype isolates, 3 other genotypes (skunk, horse, and rabbit) were identified in separate samples from individual patients after the onset of diarrhea in 2000 (sample W971), 2003 (sample W6863), and 2007 (sample W16103). A routinely collected minimum dataset was submitted with each sample, and further exposure data were collected for each patient from the local Consultant in Communicable Disease Control.

To prepare isolates for molecular characterization, oocysts were concentrated by saturated salt flotation, disrupted by boiling for 1 hour and the DNA purified by using a QIAamp DNA Mini Kit (QIAGEN, Valencia, CA, USA) as previously described (*9*). All 3 isolates were characterized by PCR–restriction fragment length polymorphism (RFLP) or bidirectional sequencing (GeneService Ltd., Cambridge, UK) at the small subunit (SSU) rRNA (≈800-bp product) (*10*), *Cryptosporidium* oocyst wall protein (COWP) (≈550-bp product) (*11*) and heat shock protein (HSP) 70 (≈450-bp or ≈325-bp products) (*12*) genes. Sequences were compared with GenBank submissions by using the BLAST algorithm (www.ncbi.nlm.nih.gov/Education/BLASTinfo/BLAST_algorithm.html).

To confirm identification, phylogenetic analysis was conducted in TREECON (www.bioinformatics.psb.ugent.be/software/details/3) with other known *Cryptosporidium* spp. and genotypes by using alignments generated in ClustalX version 2.0 (ftp://ftp.ebi.ac.uk/pub/software/clustalw2) and manually edited in BioEdit version 7.0.9 (www.mbio.ncsu.edu/BioEdit/bioedit.html). All sequences generated in this study have been submitted to GenBank under accession nos. EU437411–EU437418.

At the SSU rRNA and HSP70 genes, sequence analysis confirmed that W971, W6863, and W16103 were skunk, horse, and rabbit genotypes, respectively (Table). Isolate W971 was homologous with genotype W13 found in storm water, which, in turn, is the skunk genotype (*6*). Initially, the BLAST search for isolate W6863 erroneously indicated *C. parvum* as the most probable identity at the SSU rRNA gene, but this was due to the short length (484 bp) of the only horse genotype sequence available (AY273770) for comparison. Thus, *C. parvum* isolates that spanned our whole query sequence (787 bp) were calculated to have greater identities by BLAST. However, a detailed comparison between AY273770 and W6863 showed only 2-bp differences (including 1 insertion in our sequence) compared with 7-bp differences between W6863 and *C. parvum*. W6863 was confirmed as a variant of the horse genotype by HSP70 gene sequence analysis and SSU rRNA gene phylogenetic analysis (Figure).

PCR-RFLP analysis of the SSU rRNA and COWP genes differentiated the skunk and horse genotypes from the most common human pathogens. However, identifying the rabbit genotype by PCR-RFLP at these loci was more problematic because of this genotype's close relationship with *C. hominis*. The sequence and restriction pattern are identical at the COWP gene and, with only 4-bp substitutions (2 occurring in *SspI* cut-sites), the pattern is similar at

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Table. Descriptive epidemiology and identification of 3 unusual *Cryptosporidium* genotypes in patients, United Kingdom*

Sample ID	Patient details and exposures 2 weeks before illness	Identification at 3 genes (similarity to GenBank isolates)		
		SSU rRNA	HSP70	COWP
W971	25-year-old woman, swam regularly in a pool, holiday in UK forest park	W13 (790/790-bp homology to AY737559)	Skunk genotype (279/279-bp homology to AY120917)	Sequence data unavailable
W6863	30-year-old woman, immunocompetent, foreign travel, swam in a pool	Horse genotype (483/485 bp, 99.6%, similarity to AY273770)	Horse genotype (389/389-bp homology to AY273774)	*C. parvum* (503/506 bp, 99.4%, similarity to DQ388390)
W16103	48-year-old woman, immunocompetent, foreign travel (Spain), contact with animals (birds)	Rabbit genotype (784/784-bp homology to AF120901)	Rabbit genotype (279/279-bp homology to AY273775)	*C. hominis* (506/506 bp to DQ388389)

*ID, identification; SSU, small subunit; HSP, heat shock protein; COWP, *Cryptosporidium* oocyst wall protein.

the SSU rRNA gene. Increasing the resolution by running the agarose gel at an appropriate concentration and for as long as possible is important for the separation of the *C. hominis* diagnostic band (449 bp) from the rabbit genotype (472 bp).

Conclusions

Information on possible risk factors was collected for the 2 weeks before the onset of illness, but we cannot be sure how these 3 persons became infected with the unusual genotypes. The skunk genotype was found in a 25-year-old woman from a rural area of southwest England, who reported no foreign travel and no contact with animals. She worked and swam regularly at an adult daycare center and had spent a week during the incubation period with clients at a holiday forest park in her region. There was no information to suggest that she was immunocompromised. The horse genotype was found in a 30-year-old immunocompetent woman also from a rural area of southwest England, who reported swimming and foreign travel (destination unknown) but no contact with animals during the incubation period. The rabbit genotype was found in a 48-year-old immunocompetent woman from a rural area of northwest England, who reported foreign travel to southern Spain and contact with wild birds (feeding ducks and geese) but no contact with other animals.

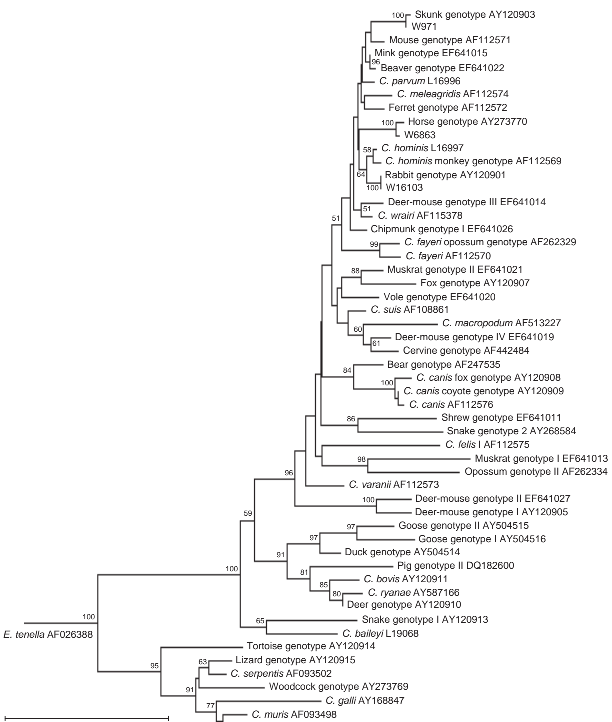

Figure. Phylogenetic relationships between 3 unusual *Cryptosporidium* genotypes and known *Cryptosporidium* species/genotypes as inferred by a neighbor-joining analysis of the small subunit rRNA gene. Evolutionary distances were calculated by the Kimura 2-parameter model with *Eimeria tenella* as an outgroup. Bootstrapping values >50% from 1,000 pseudoreplicates are shown at branches.

Previously, these 3 genotypes were known to cause infections only in wild or zoo animals (*13,14*). Wild animals are known to be an important source of *Cryptosporidium* oocysts in environmental samples and we have detected the rabbit genotype in surface waters and septic tank samples (unpub. data), but the source is unknown. Since many isolates have yet to be found in humans and although little is actually known about them, they are assumed to be insignificant to public health (*6,15*). The importance of unusual genotypes in humans who seek treatment for diarrheal disease warrants further investigation.

Acknowledgments

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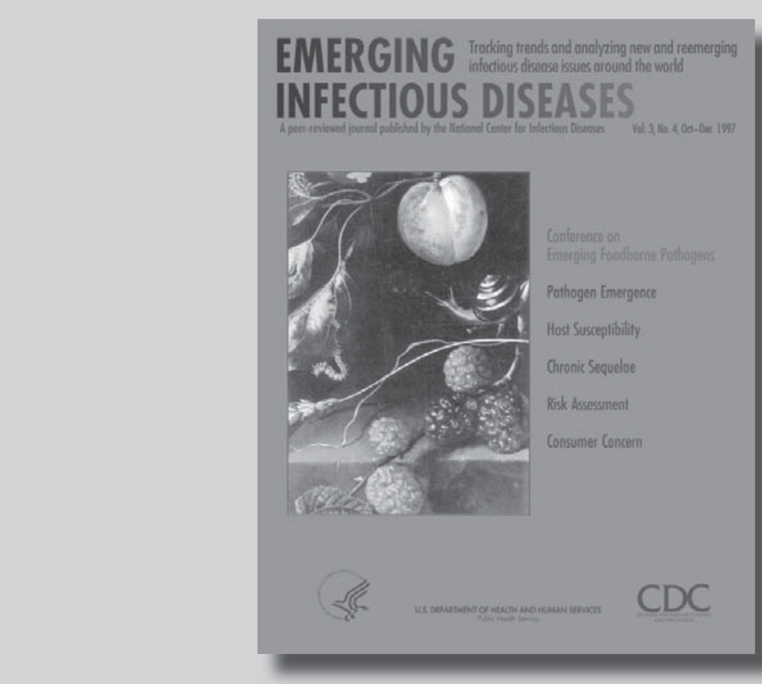

Shiga Toxin–producing *Escherichia coli* Serogroups in Food and Patients, Germany

Dirk Werber, Lothar Beutin, Rohtraud Pichner, Klaus Stark, and Angelika Fruth

We compared 61 Shiga toxin–producing *Escherichia coli* (STEC) serogroups from 448 food isolates with 71 STEC serogroups from 1,447 isolates from patients in Germany. Two thirds (41/61), representing 72% of food isolates, were also found in patients. Serogroups typically isolated from patients with hemolytic uremic syndrome were rarely found in food.

Shiga toxin–producing *Escherichia coli* (STEC) of serogroups other than O157 (non-O157 STEC) account for 80% of STEC gastroenteritis reports in Germany's national surveillance database (*1*). Some of the non-O157 serogroups unequivocally cause disease comparable in severity to that caused by STEC O157, such as the hemolytic uremic syndrome (HUS) (*2*). Numerous, but not all, STEC serogroups have been linked with human disease.

Food is an important transmission vehicle for human STEC infection, especially in outbreaks (*3*), and many different STEC serogroups are isolated from food (*4*). Yet the public health relevance of many of these STEC serogroups, which includes their ability to cause human disease and the frequency with which this may occur, has not been investigated.

In Germany, identification of STEC in patients' stool and in food is based on detection of Shiga toxin or of a Shiga toxin gene and subsequent isolation of STEC strains (*4*,*5*). This allows, in principle, ascertainment of all STEC strains, independent of their serogroup. To assess the public health relevance of STEC isolated from food, we compared those strains with those isolated from patients.

The Study

Information on STEC isolates from food came from 2 sources. The first source was the Federal Institute for Risk Assessment, which received isolates from German governmental food inspection laboratories for strain characterization from 2005 through 2007 (food source 1). These STEC isolates originated from routine food samples taken by food safety authorities across Germany, according to a nationwide sampling scheme that focused during the sampling period mainly on red meat, ground raw meat, and stabilized meat products. The second source was the Max-Rubner Institute in Germany, which had conducted a series of investigations in conveniently selected meat-processing companies in Germany from 1996 through 2004 (food source 2). Information on STEC isolates from patients came from a laboratory-based sentinel in existence from 1999 through 2004, coordinated by Germany's National Reference Center. The sentinel has been described elsewhere (*6*). In brief, private laboratories across Germany agreed to screen stool specimens of gastroenteritis patients for the presence of Shiga toxin 1 and Shiga toxin 2 with an enzyme immunoassay if predefined criteria were met (e.g., patients with diarrhea were <5 years of age, bloody diarrhea was mentioned on the laboratory request form). Positive samples were sent to the National Reference Center, where STEC strains were isolated and subtyped by various methods (including serotyping).

We calculated frequencies and proportions of STEC serogroups separately for food and patient isolates. Serogroups were compared for matches in both groups. Because the clinical outcome associated with human STEC infection was not systematically recorded, we additionally compared serogroups of food isolates with a compilation (available on the Internet) of literature reports of STEC serotypes and their association with human illness (*7*). We acknowledged an association with human illness if a symptom at least as severe as diarrhea was specified for a serogroup. The proportion of serogroups in patient and food isolates was compared by using the Wilcoxon signed rank test. Within selected serogroups, we examined serovars (classified by O and H antigen, e.g., O157:H7) to assess comparability between food and patient isolates because the serovar is a better proxy for genomic background of the strains than is the serogroup.

Serogroup information for STEC isolated from food was available for 448 strains (including nontypeable strains [Ont] and self-agglutinating isolates [Orough]), 357 from food source 1 and 91 from food source 2 (Table 1). The most common of the 61 serogroups identified in food isolates were O8 (9%), O91 (6%), and O113 (5%) (Table 2). Commonalities, but also differences, were observed between the food sources. For example, the proportion of serogroups O8 and O91 was high in both food sources, whereas O113 strains were isolated only from food source 1. Notably, STEC isolates from game represented 24% (85/357) of

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Table 1. Foods from which STEC was isolated in Germany, 1996–2004*

Source	Origin	No. ruminant samples†	Total no. samples
Federal Institute for Risk Assessment‡	Ground beef§	112	112
	Game¶	57	85
	Raw milk	55	55
	Beef and beef products	43	43
	Raw spreadable sausages	20	20
	Pork and pork products	0	19
	Cheese	15	15
	Other#	2	7
Max-Rubner Institute**	Raw spreadable sausages	54	54
	Raw sausage meat	34	34
	Other††	2	3
Total		395	448

*STEC, Shiga toxin–producing *Escherichia coli*.
†Samples from food containing ruminant meat or of ruminant origin.
‡Isolates from governmental food inspection laboratories (2005–2007).
§Of these, 12 contained also ground meat from sheep.
¶Deer (n = 56), wild boar (n = 18), hare (n = 10), game unspecified (n = 1).
#Sheep meat (n = 2), duck, salad, flan, minced horse meat, river water (1 each).
**Isolates obtained during a series of investigations in selected meat-processing companies (1996–2004).
††Meat juice (n = 2), horse meat (n = 1).

strains isolated in governmental food inspection laboratories (Table 1). Game also had the highest STEC prevalence among the different food categories routinely sampled by Germany's food safety authorities from 2005 through 2006 (13%, 95% confidence interval 9%–17%) (*8*,*9*). Serogroup information for STEC isolates from patients was available for 1,447 of 1,478 (including Ont and Orough). Overall, 71 different STEC serogroups were isolated, and O157 (18%) was the most frequently serotyped O-group, followed by O103 and O26 (14% each; Table 2). No secular trends were observed during the study period, but proportions of single serogroups varied across years, particularly for STEC O103 isolates (range 6%–24%).

Of the 61 food serogroups, 41 (67%) were also identified in patients (Figure). These serogroups comprised 72% (242/339) of food isolates with a known serogroup. Similarly, 78% (19/25) of serogroups isolated from game, accounting for 70% (44/63) of isolates, occurred also in patients. The Internet search showed a published association with human illness for at least 41 (67%) of all food serogroups; the phrase "at least" is used because 5 serogroups (O174, O176-O179) were officially acknowledged as genuine O-groups after May 2003 (*10*), which according to the website is the date of its last update (*7*). Moreover, some serogroups exclusively found in food in this study (and not listed on the website) have been described as sporadic patient isolates from Germany (*11*) and elsewhere (*12*).

Table 2. Serogroups of STEC most frequently isolated from patients (1999–2004) and food (1996–2007), Germany*

Frequency ranking	Serogroups isolated from patients (%)	Serogroups isolated from food† (%)
1	O157 (18)	O8 (9)
2	O103 (14)	O91 (6)
3	O26 (14)	O113 (5)
4	O91 (10)	O22 (4)
5	O145 (4)	O115 (4)
Total percentage‡	60	28

*STEC, Shiga toxin–producing *Escherichia coli*.
†Food categories from which strains were isolated: O8: raw spreadable sausage (n = 14), pork and products (n = 9), ground beef (n = 7), raw sausage meat (n = 5), raw milk (n = 4), game (n = 2), other (n = 1). O91: raw spreadable sausage (n = 14), ground beef (n = 5), raw milk (n = 3), game (n = 2), raw sausage meat (n = 2), other (n = 2). O113: ground beef (n = 10), cheese (n = 3), game (n = 3), pork and products (n = 2), beef and products thereof (n = 1), raw milk (n = 1), raw spreadable sausage (n = 1). O22: ground beef (n = 8), raw spreadable sausage (n = 6), beef and beef products (n = 1), game (n = 1), ground beef (n = 1), raw milk (n = 1), raw sausage meat (n = 1). O115: raw sausage meat (n = 12), raw spreadable sausage (n = 3).
‡Percentage of all isolates for which serogroup information was available (1,447 isolates from patients and 448 isolates from food).

Overall, a significant inverse correlation was found between the ranking of the serogroup proportion in patients and in food (p<0.01). This finding is illustrated by the following: of the 41 serogroups found in food and in patients, 33 accounted each for <1% of the patient isolates. In total, they represented only 9% of patient isolates but 45% of food isolates. Conversely, the 3 most frequently identified serogroups in patients, O157, O103, and O26, represented 46% of the patient isolates but only 3% of food isolates. These 3 serogroups account for 85% of STEC isolated in pediatric HUS patients in Germany (*2*). Notably, the virulent serogroup O157 was found in 5 (1%) food isolates. This result is compatible with results of studies conducted in other countries that identified only few, if any, O157 strains among STEC strains isolated from ruminant meat, particularly beef (*13*,*14*).

At least 1 serogroup, O91, was frequently isolated from both food and patients. In food, O91 strains were the second (6%), and in patients the fourth (10%), most commonly identified O-group (and the most commonly identified O-group in adults; data not shown). On the serovar level, a comparable distribution was observed between

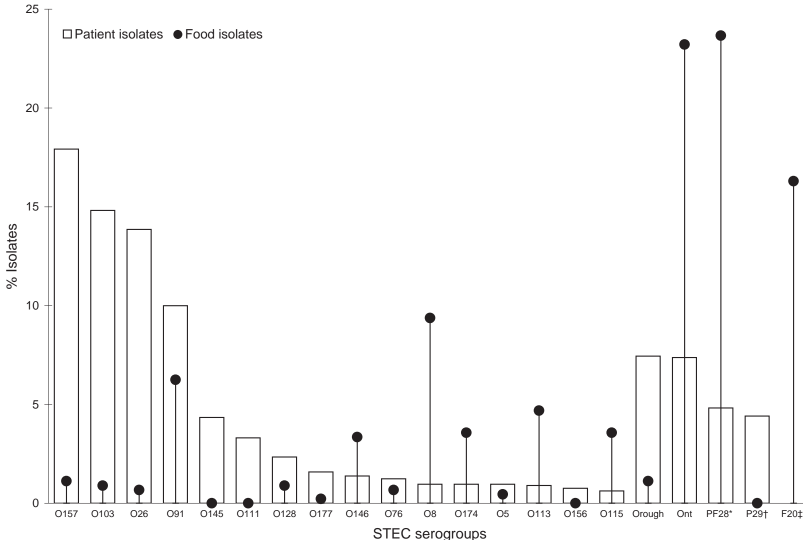

Figure. Proportion of Shiga toxin–producing *Escherichia coli* (STEC) serogroups identified in patients during a laboratory-based sentinel surveillance program (1999–2004) compared with STEC from food (1996–2004), Germany. *Comprises 28 serogroups that each accounted for <0.5% of patient isolates and were also identified in food isolates (O2, O4, O6, O15, O22, O23, O30, O38, O40, O55, O74, O84, O87, O88, O101, O102, O104, O110, O112, O119, O120, O121, O136, O148, O163, O171, O178, O179). †Comprises 29 serogroups that each accounted for <0.5% of patient isolates but were not identified in food isolates (O1, O7, O9, O12, O14, O17, O18, O25, O51, O60, O69, O70, O71, O75, O77, O78, O80, O86, O90, O93, O98, O106, O117, O118, O150, O154, O165, O167, O181). ‡Comprises 20 serogroups identified in food isolates only (O11, O21, O27, O36, O46, O56 O59, O62, O79, O82, O100, O109, O116, O126, O130, O141, O153, O166, O172, O176).

isolates from patients and food: in both groups, the same 3 serovars (O91:H–, O91:H14, O91:H21) could be distinguished; O91:H– was the most common STEC serovar in patient isolates (64%, 94/145) as well as in food isolates (47%, 19/41). A different situation was observed for STEC O113, the third (5%) most frequently serotyped O-group in food. Among patient isolates, STEC O113 was not that frequently isolated: the serogroup ranked 14th and comprised 1% of patient isolates. Furthermore, a greater heterogeneity between patient and food isolates was found in O113 strains. Although the same 3 serovars were identified in both groups (O113:H–, O113:H4, O113:H21), O113:H4 was the predominant STEC serovar in isolates from patients (70%, 9/13), whereas O113:H21 (81%, 17/21) was the most common in food isolates. The latter serovar is frequently isolated from nonpediatric HUS patients (*15*).

Conclusions

Two thirds (41/61) of serogroups from food were also isolated from patients, comprising 72% of food isolates with a known serogroup. These serogroups included, albeit uncommonly, those typically identified in pediatric and nonpediatric HUS patients. An association with human illness has been published for more than two thirds of food serogroups. These findings suggest that many STEC strains isolated from food in Germany are pathogenic for humans. Notwithstanding, the most frequent STEC serogroups in patients, except O91, were only rarely found in food.

The incongruent serogroup distributions of STEC isolates from food and from patients likely reflect the nonprobabilistic sampling schemes and differing sampling periods that underlie these populations. In addition, differences in pathogenicity among serogroups, a different serovar distribution at the serogroup level, and the fact that foodborne transmission is only 1 transmission route (*5*) should also contribute to the observed differences. Game might be a relevant, and as yet underappreciated, source for human STEC infection in Germany. Epidemiologic studies are needed to assess the risk associated with consumption of or contact with game.

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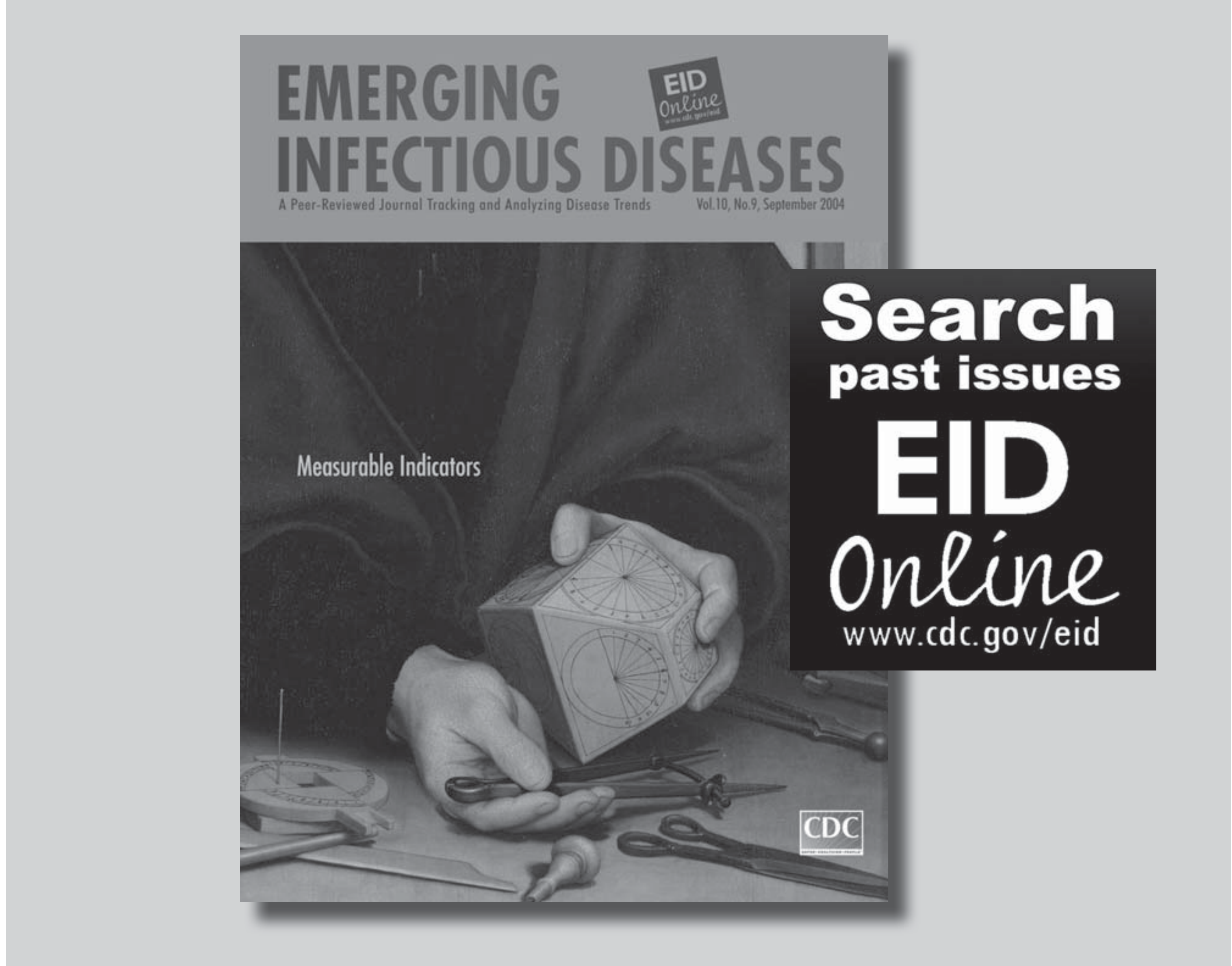

Paralysis Case and Contact Spread of Recombinant Vaccine–derived Poliovirus, Spain

To the Editor: The World Health Organization Polio Eradication Initiative has reduced to 4 the number of countries with endemic transmission of wild polioviruses and has reported the widespread circulation of polioviruses that have evolved from attenuated vaccine (oral poliovirus vaccine [OPV]), so-called vaccine-derived polioviruses (VDPVs) (*1*,*2*). VDPVs can reportedly replicate in immunocompromised persons, in whom they produce paralysis, and can spread to contacts and produce paralytic polio in immunocompetent but incompletely immunized persons; these viruses can even cause some polio outbreaks in areas with a low level of vaccination coverage (*2*). When VDPVs are detected, a careful follow-up of VDPV cases and contacts is essential if spread is to be avoided. Because point mutations or recombination events have been associated with reversion to neurovirulence transmission and thus a greater probability of outbreaks (*2*), meticulous molecular studies of the detected strains are recommended.

Spain progressively adopted vaccination with OPV for children in 2004. As a consequence, the incidence of vaccine-like poliovirus detection in recently vaccinated children, which was relatively common up to that time (51 isolates in 2003) (*3*), began to decrease (15 isolates in 2004 and none in 2005 except the case described here). In July 2005, a 14-month-old boy from Morocco with residual paralysis and major histocompatibility class II immunodeficiency was reported through the Spanish Acute Flaccid Paralysis Surveillance System. The patient had received 2 OPV doses at birth and at 6 months of age in Morocco; 8 months later, meningoencephalitis developed. The case was immediately considered suspicious and was therefore monitored at least monthly until the boy died. Sampling was conducted, coinciding with his visits to the hospital to receive therapy with immunoglobulin (γ globulin 0.5 g/kg). His contacts were studied, environmental surveillance was conducted, and molecular analysis of all detected viruses was performed. Laboratory methods for virus detection and characterization, including 10 new reverse-transcription–PCRs designed to cover the entire genome, are detailed in the Table.

Serotype 2 VDPVs were detected in all 10 stool samples of the patient with residual paralysis for 6 months, until he died, and in 3 of the 7 family contacts analyzed (father and 2 brothers, 11 and 13 years of age, none with confirmed previous vaccination). One of the contacts, considered immunocompetent, shed virus for 216 days (5 fecal samples in which 5 complete genomes were obtained and 1 additional fecal sample in which virus capsid protein 1 [VP1] could be amplified); a stool sample collected on day 284 was negative. Technical problems delayed sewage sampling. When sewage from the area in which the patient and positive contacts lived was sampled on February 8, 2006, no polioviruses were detected; however, an echovirus 30 was detected. Poliovirus viral load fluctuated (10^6–10^9 copies/mL in the paralysis-affected person), decreasing after each immunoglobulin therapy dose (Figure 1 in online Technical Appendix, available from www.cdc.gov/EID/content/14/11/1807-Techapp.pdf). The corresponding level was <10^5 in the contacts. The highest value of viral load was recorded in the patient's final sample, taken before he died. Homology of the VP1 gene with respect to the original vaccine PV2 fluctuated from 97.8% to 98.6% in the case samples but remained constant (98.4%) in the contact samples (Figure 1 in online Technical Appendix). All studied polioviruses featured the following nucleotide substitutions in the 5′ untranslated region: G309A, T344C, T355C, T398C, A481G, T500C, and T743C (Figure 2 in online Technical Appendix). Furthermore, the final sample from the patient had A476C, G505T, T588A, and A738C. Several nucleotide substitutions detected in VP1–4 were common to all samples (Figure 2 in online Technical Appendix); 5 resulted in amino acid changes, including T2909C (VP1 I143T) and G3277A (VP1 V266I). All samples contained 2 noncontiguous recombination fragments Sabin 2/Sabin 1 in the nonstructural genes, including the entire 3C gene and the 3′ half of the 3D-pol (Figure 2 in online Technical Appendix) as in other reports (*7*–*10*). Both fragments, when compared with C species enterovirus, were closely related to Sabin 1 (99.6% and 97.9%, respectively). Specific nucleotide and amino acid comparisons among the isolates are detailed in Figure 3 in the online Technical Appendix.

According to the proposed classification (*2*), all the detected viruses were iVDPVs (isolated from immunocompromised patients) that spread only to close contacts because they were not detected in local sewage. If we assume that the greater the amount of viral excretion in feces that occurs, the higher number of replicating polioviruses (as well as the potential for greater genetic diversity), the patient had a more active infection (that responded to the therapy) than did the contacts. Fluctuation in homology to the parental OPV strain might be due not only to the calculation method (calculation was made on the basis of the majority-base call at each chromatogram position, and case sequences presented many mixed nucleotide positions) but also to immunotherapy. Treatment appeared to have decreased virus replication, probably by its action mainly on species with greater fitness and higher replication rates (those that were more similar to the original

Table. Laboratory methods used for study of vaccine-derived poliovirus case, Spain, 2005*

Procedure	Test	Method†	Sample
Sample preparation	Concentration of sewage for detection of enterovirus in the environment	Concentration with negative charge filters (Millipore, Billerica, MA, USA; 0.45 μm) of 20 L of local sewage	E
	RNA purification from samples (before molecular analysis)	MagAttract Virus Mini-Biorobot (QIAGEN GmbH, Hilden, Germany) from 200 μL of stool sample dissolved in water	S
Classic virology techniques	Cellular culture (Biosafety Level 3) for growing PV	LB20 (transgenic mouse), RD (human rhabdomyosarcoma), HEF (human fibroblast), A-549 (ATCC-CCL185)	S, E
	Immunofluorescence of infected cells	Lim-Beyesh-Melnick A-H and RIVM A-G pools	I
	EV neutralization assay	Antibodies (Chemicon, Temecula, CA, USA)	I
Molecular techniques	Molecular EV detection	RT-nested PCR 5′ UTR (*4*)	S, I, E
	Molecular EV quantification	MutaReal EV real-time PCR kit (Immunodiagnostik AG, Bensheim, Germany)	S
	Molecular EV typing	RT-nested PCR in major VP1 region (*5*)	S, I, E
	PV intratypic characterization	Specific vaccine PV RT-PCR (*6*)	S, I, E
	PV genome sequencing fragment 1	1s: TAAAACAGCTCTGGGGTTGTA (2–22) 1as: CACCACCCAAGAAGCGGCC (1023–1041) 1ns: GCTCTGGGGTTGTACCCACTCC (9–30) 1nas:TAACTCTGGGCAATTCAACGA(1001–1021)	S, I
	PV genome sequencing fragment 2†	2s: CATGCTAAACTCCCCAAAC (945–963) 2as: AGGTGCGCAACATGATGG (1882–1910)	S, I
	PV genome sequencing fragment 3†	3s: CAGACAATTACCAGTCTCC (1814–1832) 3as: ATTACTAAAAATGCATTGGTTCCC (2518–2541)	S, I
	PV genome sequencing VP1 fragment†	VP1s: ACAACACACATTAGTCAAGAGGCTA (2449–2473) VP1as: GGATTTGGACACCAAAACAAAGC (3385–3407)	S, I
	PV genome sequencing fragment 4†	4s:GTGCCCACGACCTCCA (3288–3303) 4as: CTTGGGTGCGACATCTCA (4042–4059)	S, I
	PV genome sequencing fragment 5†	5s: TAATCAAAATTATCTCATCACTTGTG (3962–3987) 5as: CATGAGCGAGTACTCCAGA (4872–4889)	S, I
	PV genome sequencing fragment 6†	6s: CTGGCCAGGAGATTCG (4834–4949) 6as: AAATGATGGAGTTTTGATCGT(5725–5747)	S, I
	PV genome sequencing fragment 7†	7s: AGGCAGGAACTAATCTTGAAA (5630–5650) 7as: CTAAGTATGTAGGCAACAAGAT (6164–6185)	S, I
	PV genome sequencing fragment 8†	8s: CAAAAATGATCCCAGGCTCA (6117–6136) 8as: AAACCTACAAGGGCATAGATT (6917–6937)	S, I
	PV genome sequencing fragment 9†	9s: CAGGCACATCAATTTTTAACTC (6857–6878) 9as: GGTAAATTTTTCTTTAATTCGGGG(7416–7439)	S, I
	Additional PV sequencing primers	447as: CCGGCCCCTGAATGCGGC (447–464) 4666s: CCAGACGGAGCAGACATG (4666–4683)	S, I

*E, local sewage; S, stools; I, isolates; EV, enterovirus; PV, poliovirus; UTR, untranslated region; VP1, virus capsid protein.
†Sense (s) and antisense (as) primers: 5′ → 3' sequence (position according to X00595). n, nested. All reverse transcription–PCR (RT-PCR) systems had the same conditions: 5 μL of clinical samples (case) or isolates (contacts) were added to the reaction mixture (final volume 50 μL): AMV/Tfl 1X reaction buffer, 2 mmol/L $MgSO_4$, 200 μM each dNTP, 1 μM each primer, 5 U of AMV RT, and 5 U of Tfl DNA polymerase (Access RT-PCR System, Promega, Madison, WI, USA). First RT step of 45 min at 48°C, 2 min at 94°C, 45 cycles of denaturation (94°C, 2 min), annealing (53°C, 1 min), and elongation (68°C,1 min 30 s).

Sabin strain). As a consequence, treatment might produce a bottleneck that unmasked more divergent species. Both the case and contact strains had intertypic Sabin 1/Sabin 2 recombination in nonstructural genes and also shared most of the nucleotide and amino acid substitutions. However, pathologic changes occurred only in the patient whose immunologic mechanisms were affected and whose viral load was consequently much higher. A recent report (*1*) suggests that VDPVs can emerge in any country that uses OPV with insufficient vaccine coverage. In a polio-free IPV-user country, poliomyelitis can arise and spread to contacts who are not properly vaccinated. In the case we present here, the high level of vaccination coverage in Spain and the rapid control of close contacts achieved through the surveillance and control programs prevented virus spread. In the global pre-eradication phase, countries are recommended to change vaccination from OPV to IPV. However, IPV-adopting countries commonly share borders with OPV-adopting countries and residents may travel back and forth; thus, although the probability of VDPV circulation decreases, it does not reduce to zero. Therefore, active surveillance, rapid classification of isolates, and molecular characterization of the virus are essential.

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Widespread Oseltamivir Resistance in Influenza A Viruses (H1N1), South Africa

To the Editor: Oseltamivir is the most widely used antiviral drug for influenza; it is a potent inhibitor of influenza virus neuraminidase (NA) protein (*1*). Until recently, oseltamivir resistance occurred in <1% of circulating viruses globally. An increased number of influenza A viruses (H1N1) with resistance to oseltamivir was first reported to the World Health Organization (WHO) by Norway in late January 2008. The viruses carried a specific histidine-to-tyrosine mutation at position 274 (H274Y; H275Y in N1 numbering system) in the NA protein that confers high-level resistance to oseltamivir (*2*). Further surveillance by the European Surveillance Network for Vigilance against Viral Resistance and the WHO Global Influenza Surveillance Network (GISN) showed that 16% of community isolates (0%–67% by country) of influenza A viruses (H1N1) circulating in the 2007–08 season in several other countries were also oseltamivir resistant (*3*). The predominant influenza subtype circulating in South Africa this winter season is H1N1. To determine whether oseltamivir-resistant viruses have spread to South Africa, we examined influenza A (H1N1) isolated during the 2008 winter season for resistance to this antiviral compound.

Specimens were obtained mainly from the National Institute of Communicable Diseases (NICD) active sentinel surveillance program in all 9 provinces. Throat or nasopharyngeal swabs were taken from patients within 48–72 hours of onset of symptoms and sent to NICD laboratories for virus isolation as described (*4*).

Of the H1N1 subtype viruses isolated in May and June, 23 were sent to the WHO Collaborating Centers for Reference and Research on Influenza in London and Melbourne for resistance testing (*5*,*6*). Forty-five of the viruses, which included viruses isolated in July, were tested at NICD by using a modified amplification refractory mutation system PCR (ARMS-PCR) (*7*). This method can simultaneously detect wild-type or mutant virus with the 274 mutation in a single PCR. Partial sequencing of the NA and hemagglutinin (HA) genes was performed to confirm the NA H274Y resistance mutation and to determine genetic drift in HA from the A/Brisbane/59/2007 virus recommended for the Northern Hemisphere 2007–08 vaccine.

At the time of resistance testing, 92 H1N1 subtype viruses had been isolated. The 23 virus isolates sent to the WHO Collaborating Centers were

highly resistant to oseltamivir by the NA inhibition enzyme assay, with 50% inhibitory concentration values of 554 nM to 1,485 nM (A. Hay, I. Barr, pers. comm.). All 45 isolates tested locally were positive by ARMS-PCR for oseltamivir resistance at position 274. The H274Y mutation was confirmed by sequence analysis of the N1 genes. The N1 sequences were closely related to those isolated in Europe and elsewhere in the 2007–08 winter season. However, the presence of 1 or 2 aa mutations in viruses from South Africa (M23L and N73K in the stalk region) compared with resistant European isolates indicated that some genetic drift of N1 from the older strains had occurred. Although most 2008 isolates were closely related to the A/Brisbane/59/2007 strain, several of the isolates from South Africa had mutations in an additional 2 or 3 aa residues at positions 183, 185, and 189, which mapped close to the receptor binding site of HA. (GenBank accession nos. for nucleotide sequences obtained in this study are EU914901–EU914916.)

Before the 2007–08 Northern Hemisphere winter, surveillance by GISN laboratories showed that oseltamivir-resistant H1N1 subtype viruses were extremely rare. Low numbers of drug-resistant viruses carrying the H274Y mutation usually followed oseltamivir treatment and showed reduced fitness with poor transmission (*8*). Consequently, fitter nonresistant viruses appear to have predominated. In contrast, no evidence indicated that persons from whom resistant viruses were isolated during the European 2007–08 winter season had either been treated or been in close contact with another person who had been treated with oseltamivir (*2*,*8*).

We report oseltamivir-resistant H1N1 subtype viruses in Africa and the Southern Hemisphere. It appears that resistant viruses have spread from the Northern Hemisphere and have undergone extensive transmission within the population. These viruses may soon appear in other countries in the Southern Hemisphere. Ongoing monitoring is needed to understand the further evolution of oseltamivir resistance.

Clinical symptoms of all patients in this study suggest an illness similar to that generally associated with seasonal influenza A virus (H1N1); no complications were reported. This finding is not unexpected because all isolates tested in the study were from outpatients. The policy in South Africa for use of oseltamivir for treatment of severe influenza remains unchanged because 2008 H3N2 subtype viruses are still drug sensitive.

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Human Parvovirus 4 in Kidney Transplant Patients, France

To the Editor: Human parvovirus 4 (PARV4) is a recently identified virus, distantly related to already known members of the family *Parvoviridae* that affect humans and animals. Initially, PARV4 was characterized in the blood of a North American patient who had acute viral syndrome; a sequence-independent amplification approach was used (*1*). Molecular analysis of the viral prototype genome (5,268 nt) identified 2 large, nonoverlapping open reading frames (ORFs) and showed limited homology with human parvovirus B19 (<30% aa similarity). Subsequent phylogenetic analyses have shown that at least 2 genotypes are identifiable, differing by ≈8% at the nucleotide level (*2*).

The first prevalence studies, performed mainly in North America and the United Kingdom, reported finding the virus in plasma samples from febrile patients who had symptoms resembling those of acute HIV infection (6%), from cadavers of hepatitis C RNA–positive intravenous drug users (30%), and in plasma donations from healthy blood donors (5% pooled, 2% individual) (*3–5*). PARV4 was also identified in clotting factor VIII concentrate and in plasma pools negative for parvovirus B19 DNA (*5*,*6*). This new virus appears to not be restricted solely to blood samples; it has been already identified in bone marrow, in various autopsy tissue samples from patients with AIDS, and in liver tissues of persons with liver dysfunctions (*7–9*). Typical amounts of PARV4 DNA identified in the various samples tested ranged from <500 to >10^6 copies/mL.

We investigated PARV4 DNA in plasma samples collected from cohorts of 378 inpatients and 192 healthy blood donors from southeastern France during 2007: 164 kidney transplant patients (55 women, 109 men; mean age 51 ±14 years; mean duration of transplantation 37 ±30 months), 214 hemodialysis patients (88 women, 126 men; mean age 65 ±15 years; mean duration of dialysis 30 ±28 months), and 192 voluntary blood donors (86 women, 106 men; mean age 40 ±21 years).

Blood samples were collected in vacuum tubes (Vacutainer SST; Becton Dickinson, Meylan, France) and then centrifuged. Plasma aliquots were stored at –80°C before DNA extraction. Nucleic acids were extracted from 1-mL volumes of plasma by using a nucleic acid extraction machine (MagNA Pure LC, Roche Diagnostics, Meylan, France) and eluted into 50-µL volumes. Samples were screened for PARV4 DNA by real-time PCR (StepOne Plus, Applied Biosystems, Courtaboeuf, France) by using a consensus TaqMan PCR system composed of conserved primers and a fluorogenic hydrolysis probe located on the ORF2 of the viral genome (*5*). Amplification reactions were performed by using 5 µL of extracted nucleic acids with the TaqMan Fast Universal PCR kit (Applied Biosystems) in a final volume of 20 µL. The amplification conditions were 95°C for 20 s, followed by 50 cycles of 95°C for 1 s and 60°C for 20 s. Using dilutions of a synthetic template corresponding to the target sequence (103 nt), we estimated the sensitivity of the TaqMan assay to be 10 copies of PARV4 DNA.

Positive results were obtained from 5 blood samples, all from kidney transplant patients (5/164; 3.05%). Real-time PCR products were cloned, sequenced, and compared with sequences of PARV4 already deposited in databases. They exhibited 100% nucleotide identity in this ORF2 region with the PARV4 prototype isolate (GenBank accession no. AY622943). The titer of PARV4 DNA in the positive samples was low and did not exceed 500 copies/mL plasma. The 5 patients (1 woman and 4 men, mean age 48 ±18 years, mean duration of transplantation 25 ±21 months, no heterologous blood transfusions) did not show evidence of specific biological or clinical dysfunctions.

Whether these 5 patients were infected by kidney graft was impossible to determine because kidney transplant tissue samples were unavailable for analysis. However, 2 blood samples were available for retrospective analysis for 1 patient (male, 66 years), who was PARV4 positive at 4 months after transplant. These samples, collected 1 and 2 months before transplant, were negative for PARV4 DNA, which suggests possible transmission of the virus by the transplanted organ or reactivation of a latent infection resulting from immunosuppressive treatments.

PARV4 DNA was not detected in any persons in the 2 other cohorts: hemodialysis patients and voluntary blood donors. Investigations of larger cohorts and/or analyses of plasma pools, using optimized molecular approaches, are required for a better understanding of the diffusion of PARV4 in France.

In summary, we found PARV4 in the blood of transplant patients and determined that for 1 of these patients, PAR4 was present only after the transplant procedure. The natural history and clinical features of this new parvovirus remain largely unknown. Further investigations to elucidate the mode of transmission and the potential effect of PARV4 infection in this category of patients are urgently needed.

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Establishment of *Biomphalaria tenagophila* Snails in Europe

To the Editor: Schistosomiasis, known since ancient times, is caused by blood flukes (Trematoda: Schistosomidae). It is a major communicable disease with public health and socioeconomic effects in the developing world (*1*). Among parasitic diseases, schistosomiasis ranks second only to malaria with regard to the number of persons infected and at risk. The life cycle of schistosomes is complex, requiring specific freshwater snails as intermediate hosts for larvae development and multiplication. Among *Schistosoma* species that affect humans, *Schistosoma mansoni* is the most likely to invade new areas mainly because of the adaptability and invasiveness of its intermediate host, *Biomphalaria* snails. Natural populations of these snails are usually found in tropical standing water or freshwater in South America and Africa, but they also reach 30° latitude in subtropical areas (*1*,*2*). Many species of these red-blooded planorbid snails (Gastropoda: Basommatophora) are able to survive a long time when removed from their freshwater habitat (*1*). Of the 34 *Biomphalaria* species, 4 (*B. glabrata, B. pfeifferi, B. straminea*, and *B. tenagophila*) have recently expanded their native ranges (*3*). They have been introduced to areas where other *Biomphalaria* species are endemic (e.g., Congo and Egypt) or to subtropical zones that have no frost period (Texas, Louisiana, Florida, Hong Kong) (*3*,*4*). None of the known invasions, whether peripheral range expansion or long distance dispersal, reached the temperate zone. Spreading of the blood-fluke snails to schistosome-free areas may enable the parasite to colonize new habitats concurrently, expanding the potential area of clinical schistosomiasis.

We collected these snails in spring 2005, autumn 2006, and autumn 2007, near Răbăgani, Romania, Eastern Europe (46°45′1.3″N, 22°12′44.8″E) in a hypothermal spring. Water temperature was 25°C in the spring and 16°C–25°C, gradually decreasing, along the brook course. In and beside an abandoned concrete pool next to the spring, we collected 100 shells and 34 living specimens that macroscopically resembled *Biomphalaria* spp. snails. All 16 dissected animals proved to be fully developed adults, according to the maturity of their genital organs (Figure). Using available identification keys (*5*), we tentatively identified these snails as *B. tenagophila.* Voucher specimens have been deposited in the Hungarian Natural History Museum (accession nos. HNHM96857 and HNHM95433).

DNA was extracted from the foot muscles of 3 specimens by using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). For amplification of the partial mitochondrial 16S ribosomal RNA gene, we used a PCR with primers 16Sar and 16Sbr (*6*). Nucleotide sequences were determined in both directions. PCR products of ≈430 bp were detected from all 3 samples. Automatic cycle sequencing of the randomly selected amplicon (GenBank accession no. EU069412) showed 99.74% similarity to *B. tenagophila* (AF449615, Brazil).

Our morphologic, anatomic, and molecular data unambiguously prove the occurrence of *B. tenagophila* snails in Romania. *B. tenagophila* snails had been found earlier (in 2004) at this location but had presumably been misidentified as dwarf specimens of a common European species, *Planorbarius corneus* (*7*). Consequently, *B. tenagophila* snails have been not only introduced, but also established in Răbăgani, representing the furthest self-sustaining population of this species from the equator.

B. tenagophila is a new species for the European fauna. It could represent a founder population of unknown origin for further spread into Europe, which might easily be accomplished by migrating birds or more likely by plants used in aquariums (*3*). Although no trematode larvae were detected in the observed specimens, clinical schistosomiasis can be imported by immigrants or tourists into Europe, as has been reported in Romania and neighboring Hungary (*8*,*9*). If eggs were released in feces of humans infected with the blood flukes, they could hatch in the environment and the larvae could develop to an infective stage in these snails. The observed local cultural and social factors involving natural water (washing clothes, bathing) in Răbăgani where *B. tenagophila* have been found may also increase the chance of human infection.

We believe that *B. tenagophila* in Europe, together with the global climate change and a possible encounter of these snails with schistosomes, could pose a public health risk. Measures must be taken to prevent the spread of this species into European freshwater. Chemical control is not possible in Răbăgani because it is an area where other rare and endangered snail species are protected (*7*). Therefore, the manual collection and removal of all the *B. tenagophila* specimens in the area seems to be the only possibility for eradication, which might remain in effect for years. To avoid similar establishments, we suggest regular malacologic and parasitologic surveillance of at least the thermal and hypothermal water bodies for these tropical invaders around European settlements.

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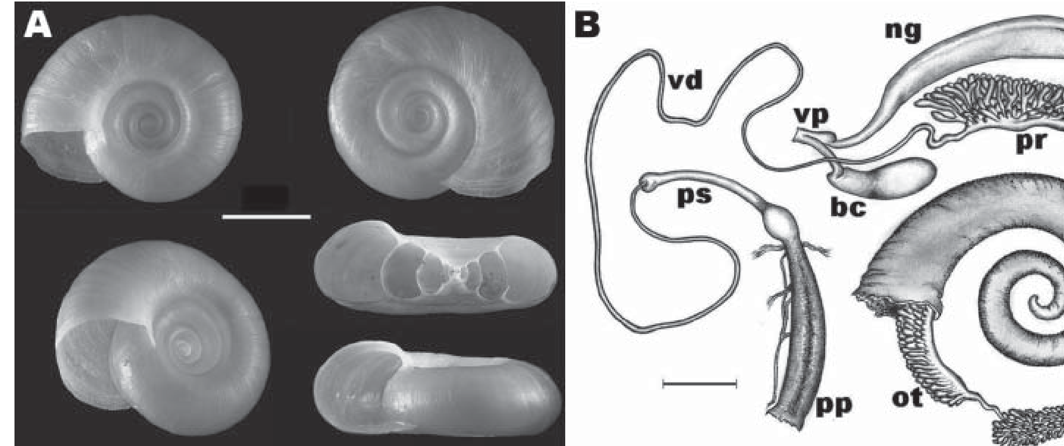

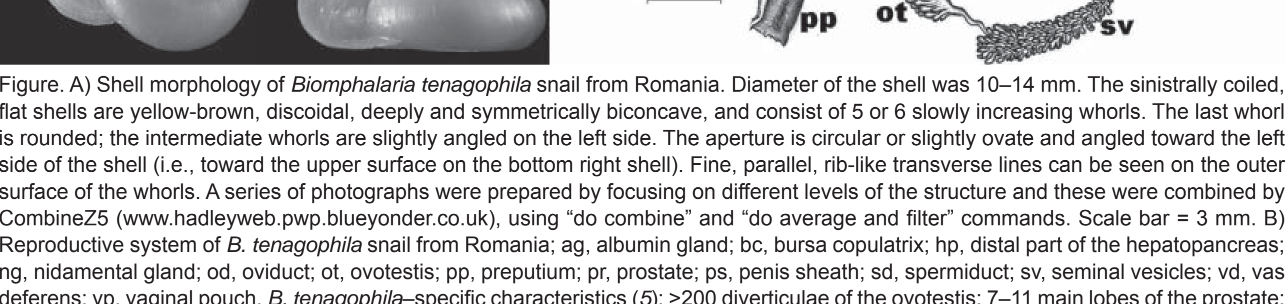

Figure. A) Shell morphology of *Biomphalaria tenagophila* snail from Romania. Diameter of the shell was 10–14 mm. The sinistrally coiled, flat shells are yellow-brown, discoidal, deeply and symmetrically biconcave, and consist of 5 or 6 slowly increasing whorls. The last whorl is rounded; the intermediate whorls are slightly angled on the left side. The aperture is circular or slightly ovate and angled toward the left side of the shell (i.e., toward the upper surface on the bottom right shell). Fine, parallel, rib-like transverse lines can be seen on the outer surface of the whorls. A series of photographs were prepared by focusing on different levels of the structure and these were combined by CombineZ5 (www.hadleyweb.pwp.blueyonder.co.uk), using "do combine" and "do average and filter" commands. Scale bar = 3 mm. B) Reproductive system of *B. tenagophila* snail from Romania; ag, albumin gland; bc, bursa copulatrix; hp, distal part of the hepatopancreas; ng, nidamental gland; od, oviduct; ot, ovotestis; pp, preputium; pr, prostate; ps, penis sheath; sd, spermiduct; sv, seminal vesicles; vd, vas deferens; vp, vaginal pouch. *B. tenagophila*–specific characteristics (*5*): >200 diverticulae of the ovotestis; 7–11 main lobes of the prostate; and presence of vaginal pouch. Scale bar = 1 mm.

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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.

Rickettsia aeschlimannii Infection, Algeria

To the Editor: Only 2 cases of *Rickettsia aeschlimannii* infection have been reported. We report 2 additional cases documented in Algeria by immunofluorescence (IF) assays and confirmed by Western blot (WB) assays and cross-adsorption studies.

Tick-borne rickettsioses are now recognized as emerging or reemerging human infections worldwide. These zoonoses, caused by intracellular bacteria within spotted fever group (SFG) *Rickettsia* spp., share characteristic clinical features including fever, rash, and sometimes inoculation eschar at the bite site (*1*). In North Africa, cases of rickettsioses are rarely documented (*2*). In Algeria, only Mediterranean spotted fever caused by *R. conorii* has been described (*3*).

From 2000 through 2006 in Algeria, all patients with suspected rickettsioses seen at the infectious diseases units of Constantine and Batna hospitals were included in a prospective study; clinical and epidemiologic data and acute-and convalescent-phase serum samples obtained 2–4 weeks later were collected. Serum samples were sent to Marseille, France, where they were analyzed by an IF assay, using 9 SFG rickettsial antigens (*R. conorii conorii*, *R. conorii israelensis*, *R. africae*, *R. sibirica mongolitimonae*, *R. aeschlimannii*, *R. massiliae*, *R. helvetica*, *R. slovaca*, and *R. felis*) and a typhus group antigen (*R. typhi*) (*3*). The IF assay result was considered positive 1) if immunoglobulin (Ig) G titers were ≥128 and/or IgM titers were ≥64 for *R. conorii* and 2) if IgG titers were ≥64 and/or IgM titers were ≥32 for other rickettsial antigens (*3*). When cross-reactions between several antigens were noted, rickettsial antigen was considered to represent the infectious agent if titers of IgG and/or IgM antibody against this antigen were at least 2-fold higher than titers of IgG and/or IgM antibody against other rickettsial antigens (*3,4*). When the difference in titers among several antigens was lower than 2-fold, WB assays and cross-adsorption studies were performed (*4,5*). A total of 135 patients were included in the study. We describe 2 cases of *R. aeschlimannii* infection. Cases caused by other SFG rickettsiae will be reported elsewhere.

An 80-year-old man who reported contact with dogs parasitized by ticks had a 7-day history of high fever, headache, myalgia, and vomiting. On physical examination, a generalized maculopapular rash, 2 eschars (right shoulder and knee), and bilateral hemorrhagic signs on the retina were noticed. Elevated levels of liver enzymes (aspartate aminotransferase 187 U/L, alanine aminotransferase 108 U/L), hyponatremia (sodium 120 mmol/L), and hypokalemia (potassium 2.9 mmol/L) were found. IF assay showed raised levels of IgG/IgM against *R. aeschlimannii* (512/64) and *R. conorii* (128/0).

The second patient, a 36-year-old man, reported a 15-day history of fever with headache and failure of amoxicillin and cotrimoxazole treatments. Oral aphtous, a maculopapular rash, and purpuric lesions on the arms were noticed. IF assay showed raised levels of IgG/IgM at the same titer (2,048/32) against *R. conorii*, *R. aeschlimannii*, and *R. massiliae.* WB assays and cross-adsorption studies confirmed that antibodies were directed against *R. aeschlimannii* (Figure). Both patients recovered after doxycycline treatment (*1*).

R. aeschlimannii was first characterized as a new SFG rickettsia after its isolation from *Hyalomma marginatum marginatum* ticks in Morocco in 1997 (*6*). Thereafter, *R. aeschlimannii* has been detected in this tick species in southern Europe and North Africa (*7*), as well as in *H. m. rufipes* in sub-Saharan Africa (*1*). Preliminary data have suggested that these *Hyalomma*

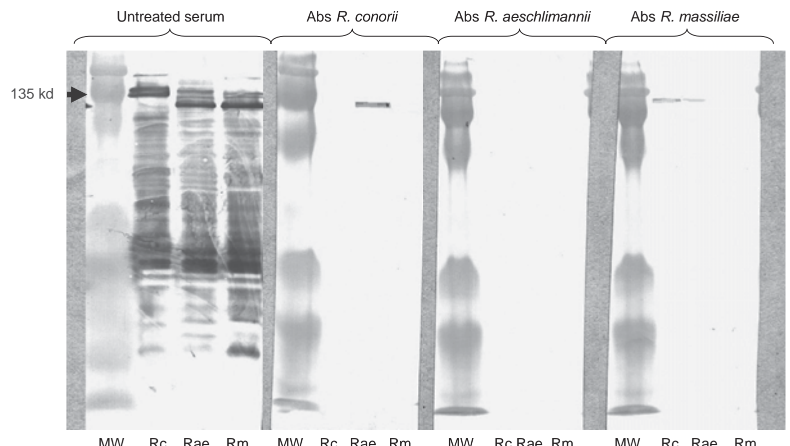

Figure. Western blot assay (WB) and cross-adsorption studies in serum of a patient with rickettsiosis in Algeria. Immunofluorescent assay showed raised levels of immunoglobulin (Ig) G/M at the same titer (2,048/32) against *Rickettsia conorii*, *R. aeschlimannii*, and *R. massiliae*. Lanes Rc, Rae, and Rm: WB assay using *R. conorii*, *R. aeschlimannii*, and *R. massiliae* antigens, respectively. MW, molecular weights are indicated on the left. Untreated serum, late serum samples tested by WB. When adsorption is performed with *R. aeschlimannii* antigens, homologous and heterologous antibodies disappear, but when it is performed with *R. conorii* antigens and *R. massiliae,* homologous antibodies disappear but heterologous antibodies persist. This result indicates that antibodies are specifically directed against *R. aeschlimannii*. Abs, absorbed.

organisms may be not only vectors but also reservoirs of *R. aeschlimannii* and as a consequence, the geographic distribution of *R. aeschlimannii* would be at least that of these ticks throughout southern Europe and Africa (*8*).

Although WB assays and cross-adsorption studies are time-consuming and only available in specialized reference laboratories, new data can be obtained for a better understanding of rickettsioses. We have added the description of 2 more cases of infection with *R. aeschlimannii*. Only 2 cases of human infection caused by this rickettsia had been previously reported, including infection in a patient returning to France from Morocco, and another in a patient in South Africa (*9,10*).

Clinicians should be aware that several tick-borne rickettsial pathogens are present in Algeria. Specific clinical features may be directly influenced by the *Rickettsia* spp. involved, the rickettsial infection rate of the vector, and tick behavior. *H. marginatum* ticks readily bite humans, and persons may receive multiple simultaneous tick bites. Furthermore, the high infectious rate of these ticks by *R. aeschlimannii* has been reported (*1*). Therefore, the probability of being bitten by several infected *H. marginatum* ticks is high and can lead to several eschars in patients, a characteristic of few tick-borne rickettsioses. Finally, although doxycycline is the reference treatment for rickettsioses, rifampin has been used (*1*). However, although *R. conorii* is susceptible in vitro to this drug, *R. aeschlimannii* is resistant. Because patients suspected of having rickettsiosis must receive prompt presumptive treatment, the presence of *R. aeschlimannii* in Morocco reinforces the need to use doxycycline as a first-line drug.

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Severe Malaria and Artesunate Treatment, Norway

To the Editor: Approximately 8,000 cases of imported falciparum malaria are reported in Europe each year (*1*). In a study from Belgium of 1,743 persons with fever acquired in the Tropics, only falciparum malaria resulted in deaths (*2*).

Until recently, the standard treatment of severe malaria was intravenous quinine (*3*). Frequent adverse effects, however, and reports of limited clinical efficacy in some falciparum malaraia–endemic areas preclude its usefulness (*4*). In contrast, artesunate, a water-soluble artemisinin derivate extracted from the plant *Artemesia annua* (quinghao), is considered safe and highly efficacious (*4,5*). Artesunate has the advantage of rapidly killing malaria parasites only a few hours after invading the erythrocyte, and it also reduces cytoadherance (*4*). Resistance to artesunate at the Cambodia–Thailand border has been reported, but until now artesunate resistance has not been considered a problem in most malaria–endemic regions (*5,6*). On the basis of 6 randomized controlled trials comparing artesunate and quinine, a recent Cochrane review recommended artesunate as the first-line treatment in adults with severe malaria in such areas (*7*). Similar recommendations were issued by the World Health Organization (WHO) in 2006 (*8*). Also, the European surveillance network, TropNetEurope, and the Advisory Committee on Malaria Prevention in UK Travelers advocate artesunate as the first-line treatment for severe falciparum malaria in travelers (*9,10*). However, the manufacturers of intravenous (IV) artesunate have not achieved Good Manufacturing Practice certification; currently, the drug is not widely used outside Asia.

In March 2008, an inquiry for patients treated with IV artesunate for severe falciparum malaria was mailed to all major departments of infectious diseases in Norway. All departments responded, and 9 patients treated from February 2006 to May 2008 were identified at 3 centers: 7 at Haukeland University Hospital in Bergen, 1 at Akershus University Hospital in Nordbyhagen, and 1 at Ullevål University Hospital in Oslo. Clinical and laboratory features were retrospectively obtained from the medical records. Artesunate was produced by Guilin Pharmaceutical, Guangxi, China, and provided from IDIS Pharmaceutical, Weybridge, United Kingdom.

With the exception of 1 patient who had become infected while in Myanmar, all patients acquired falciparum malaria in West Africa (Table). Four patients were Norwegian tourists or businessmen; 4 patients were visiting friends and relatives and had lived in Norway for 2, 15, 20, and 40 years, respectively. One patient was a pregnant (third trimester) immigrant. None of the patients had used antimalarial chemoprophylaxis. The patients' symptoms fulfilled up to 5 of the WHO criteria for severe malaria: 1 patient had cerebral malaria, 5 impaired consciousness, 5 jaundice, 2 shock, 2 renal failure, 2 hemoglobinuria,1 hematemesis, and 8 hyperparasitemia (Table). The initial treatment consisted of IV artesunate plus doxycycline (n = 7), IV artesunate monotherapy (n = 1), or IV artesunate plus clindamycin (n = 1). The dosing of artesunate was 2.4 mg/kg at 0 h, 12 h, and 24 h and then daily thereafter. Patient 6 received a 1,200-mg loading dose of quinine before transfer to one of the study hospitals (Table). None of the patients needed exchange transfusions. No adverse effects were attributed to artesunate, and the pregnant patient delivered a healthy child at term. The parasitemia level fell below 1% in all patients within 1–2 days. Treatment was changed to oral antimalarial drugs (artemether–lumefantrine, mefloquine, proguanil–atovaquone, or quinine) within 2.1 days (mean); all patients recovered uneventfully and were discharged from the hospital within 4.2 days (mean) (Table). No episodes of recrudescence were documented posttreatment at 4 weeks follow-up; 7 patients had a negative malaria slide and 2 patients were not examined for parasites but had no clinical recrudescense at follow-up.

Our findings support those of several randomized controlled trials performed in Asia and indicate that therapy with IV artesunate is safe, induces rapid parasite clearing, and usually results in swift clinical cure. Blood exchange transfusion, a labor-intensive and potentially hazardous procedure, was initially considered for 2 of our patients but was deemed unnecessary because of the rapid improvement after artesunate treatment. Artemisinins have short half-lives, and there is an increased risk for recrudescence if used alone. We gave concurrent IV doxycycline or clindamycin to all but 1 of our patients; all patients were treated with an oral drug after IV artesunate, and recrudescence was not noted.

A major obstacle for the use of IV artesunate is its poor availability outside Asia and the fact that its use is not approved in many countries. However, in the United States, artesunate for infusion may now be obtained as an investigational drug from the Centers for Disease Control and Prevention (www.cdc.gov/malaria/features/artesunate_now_available.htm), and in the European Union, artesunate recently received the Orphan Medicinal Drug Designation from the European Medicines Agency (www.emea.europa.eu/pdfs/human/comp/opinion/48693207en.pdf) and may be obtained from IDIS Pharma (www.idispharma.com).

If falciparum malaria is acquired at the Cambodia–Thailand border region, artesunate resistance should be considered; except for this region, where mefloquine resistance also is a problem, artesunate is considered to be an efficacious drug with limited reports of resistance. In conclusion, the current case series suggests that IV

Table. Epidemiologic, clinical, and laboratory data from 9 patients with severe falciparum malaria treated with intravenous artesunate, Norway, 2006–2008*

Patient no. (gender/ age, y)	Reason for travel	Country of disease acquisition	WHO severe malaria criteria	Days from symptom onset to therapy	Initial treatment	Parasitemia level, %					Length of hospital stay, d
						Day 0	Day 1	Day 2	Day 3	Day 4	
1 (M/37)	Tourism	Ghana	Impaired consciousness, bilirubin† 53, hyperparasitemia	10	AS + D	4	<1	0‡			4
2 (M/45)	VFR	Mali	Hyperparasitemia	4	AS + D	5	<1‡	<1	NA	0	4
3 (M/25)	VFR	Ghana	Impaired consciousness, hematemesis, hemoglobinuria, lactate 3.2,§ hyperparasitemia	5	AS + D	15	7	<1	0‡		3
4 (M/41)	Tourism	Ghana	Coma, shock, hemoglobinuria, bilirubin 241, hyperparasitemia	5	AS + D	20	3	<1	NA	0‡	5
5 (F/32)	Immigration	Nigeria	Impaired consciousness, bilirubin 50, hyperparasitemia	3	AS + C (patient pregnant)	7	0	NA‡			3
6 (M/46)	Business	Nigeria	Impaired consciousness, creatinine† 309, bilirubin 58, hyperparasitemia	6	Quinine 1,200 mg loading dose, then AS + D	30	5	0.5‡	0		7
7 (M/35)	Tourism	Myanmar	Impaired consciousness, hyperparasitemia	10	AS	4	<1	0‡	0		5
8 (F/38)	VFR	Liberia	Shock	7	AS + D	1	<1‡	0	0		3
9 (M/55)	VFR	Guinea	Creatinine 315, bilirubin 118, hyperparasitemia	4	AS + D	6	<1	0‡			4

*WHO, World Health Organization; AS, artesunate; D, doxycycline; VFR, visiting friends and relatives; NA, not available; C, clindamycin.
†µmol/L (bilirubin reference range 5–25; creatinine reference range 60–105).
‡Day when intravenous artesunate was discontinued.
§mmol/L (reference range 0.5–2.2).

artesunate is an efficacious and safe treatment option in travelers returning from West Africa with severe falciparum malaria.

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Bacteremia Caused by *Mycobacterium wolinskyi*

To the Editor: *Mycobacterium wolinskyi* is a rapidly growing mycobacterium that belongs to the *M. smegmatis* group, which includes *M. smegmatis* sensu stricto and 2 species described in 1999 (*M. goodii* and *M. wolinskyi*) (*1*). Only 9 cases of infection caused by *M. wolinskyi* have been reported (*1–3*), and these included 3 cases of bone infection and 1 case of infection of a hip prosthesis. All patients had a history of surgery after traumatic injury and all specimens were isolated from the surgical wound. In our study, we used molecular diagnostic tools and report a case of bacteremia caused by *M. wolinskyi*.

In November 2006, we diagnosed non-Hodgkin lymphoma in a 22-year-old woman. A venous port was implanted, and 4 courses of rituximab (anti-CD20 monoclonal antibody) plus additional chemotherapy (cyclophosphamide, epirubicin, vicristine and prednisolone) were administered from December 2006 through May 2007. No unfavorable sequelae occurred after chemotherapy, and the tumor showed a complete response. In August 2007, we admitted the patient to our hospital because of a spiking high fever (up to 40°C), chills, and pain in the left knee. On physical examination, the patient had a tender, warm, erythematous, and swollen left knee. These symptoms progressed to other joints, including the left hip and ankle.

Laboratory data showed a normal leukocyte count (3.4×10^9 cells/L). The patient's C-reactive protein level increased from 1.13 mg/dL (on the day of admission) to 24.95 mg/dL (7 days after admission). We drew 2 sets of blood samples from a peripheral vein for culture and incubated these cultures (BACTEC 9240 Continuous Monitoring Blood Culture System; Becton Dickinson, Sparks, MD, USA) using BACTEC Aerobic Plus and Anaerobic Plus medium (Becton Dickinson). Within 3 days, the cultures tested positive for acid-fast bacilli.

The isolate was identified by 16S rRNA gene amplification of an 880-bp region (corresponding to positions 27–907), as previously described (*4,5*). For amplification, we used broad-range primers 16S-27f (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 16S-907r (5′-CCG TCA ATT CMT TTR AGT TT-3′). For sequencing 16S rDNA, we used either the primer 16S-27f or 16S-519r (5′-GWA TTA CCG CGG CKG CTG-3′). We performed both forward and reverse (5′ and 3′) sequencing. For accurate analysis of the data, a 492-bp variable region (corresponding to positions 27– 519) was carefully analyzed after it was compared with sequences of *Mycobacterium* spp. in the BLAST database (www.ncbi.nlm.nih.gov), as described (*6*). The results showed 99% similarity between our isolate and *M. wolinskyi*.

A few days later, we obtained synovial fluid by needle biopsy and cultured samples in BACTEC Aerobic Plus and Anaerobic Plus medium (Becton Dickinson) and on trypticase soy agar. Within 3 days, these cultures were also positive for *M. wolinskyi*. Arthroscopically assisted arthrocentesis and debridement showed a turbid joint and the debrided tissue showed inflammatory processes within the synovial tissue and the presence of ac-

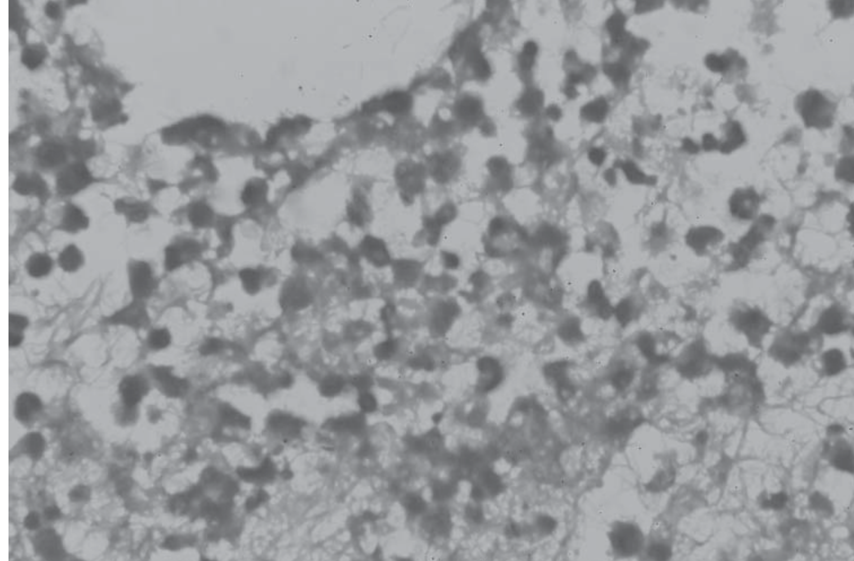

Figure. Histologic image of debrided tissue of the patient, showing inflammatory processes within the synovial tissue and the presence of an acid-fast bacillus (magnification ×400, acid-fast stain).

id-fast bacilli (Figure). We grew cultures of acid-fast bacilli on trypticase soy agar after 2 to 4 days. The colonies were nonchromogenic, smooth to mucoid, and off-white to cream on Middlebrook 7H10 and trypticase soy agar.

We tested the in vitro antimicrobial susceptibility using the broth dilution method (*7*). The isolate susceptible to amikacin, cefoxitin, imipenem, doxycycline, and ciprofloxacin and resistant to sulfamethoxazole, clarithromycin, and tobramycin. We initiated treatment of the patient with moxifloxacin, minocycline, and amikacin 1 day after the athroscopy and the patient's fever subsided within 72 hours. We continued amikacin therapy for 1 month and administered moxifloxacin and minocycline for 6 months.

This patient is unique because she had a case of bacteremia caused by *M. wolinskyi,* and she had no history of major traumatic injury. The bacterium might have been introduced during implantation of the venous port or during minor trauma that went unnoticed. The chemotherapeutic regimen administered to our patient may have played a role in the infection. Immunosuppression by treatment with rituximab (an anti-CD20 monoclonal antibody) and a steroid during chemotherapy may have worsened the patient's B-cell function and thereby weakened her immunity. Surgical debridement followed by antimicrobial therapy for at least 6 months is the suggested treatment for *M. wolinskyi* infection, and we followed this regimen. Because of the frequency of relapse and resistance, we used combination therapy with multiple antimicrobial agents.

This case suggests that immunocompromised patients may be vulnerable to infection by rapidly growing mycobacterium such as *M. wolinskyi*. In such cases, we suggest antimicrobial drug treatment, based on in vitro susceptibility. More data on antimicrobial drug susceptibility should be collected for treatment of this type of infection.

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Incubation Period for Human Cases of Avian Influenza A (H5N1) Infection, China

To the Editor: Since 1997, more than 400 human cases of highly pathogenic influenza A virus (H5N1) infection have been reported worldwide, including 30 from mainland China. Ascertainment of the incubation period for influenza virus (H5N1) is important to define exposure periods for surveillance of patients with suspected influenza virus (H5N1) infection. Limited data on the incubation period suggest that illness onset occurs $\leq$7 days after the last exposure to sick or dead poultry (*1–4*). For clusters in which limited human-to-human virus transmission likely occurred, the incubation period appeared to be 3–5 days (*5–7*) but was estimated to be 8–9 days in 1 cluster (*5*). In China,

exposure to sick or dead poultry in rural areas and visiting a live poultry market in urban areas were identified as sources of influenza A virus (H5N1) exposures (*8*), but the incubation period after such exposures has not been well described.

We conducted a retrospective descriptive study of 24 of 30 influenza virus (H5N1) cases in China to estimate and compare incubation periods for different exposure settings, including case-patients exposed only to sick or dead poultry versus those exposed only to a wet poultry market, where small animals and poultry may be purchased live or slaughtered (see www.searo.who.int/en/Section23/Section1001/Section1110_11528.htm). Exposures may be direct (e.g., touching poultry) or indirect (e.g., no physical contact, but in close proximity to poultry, poultry products, or poultry feces). We excluded 6 cases, including 2 with unavailable epidemiologic data, 1 without an identified exposure source, 2 in a cluster with limited person-to-person transmission (*6*), and 1 in which the patient was exposed to both a wet poultry market and to sick or dead poultry. Epidemiologic data were collected through patients and family interviews and a review of case-patients' medical records.

The incubation period was defined as the time from exposure to symptom onset. The maximum time from first exposure to illness onset was limited to 14 days for biological plausibility. For case-patients with exposures on multiple days, we calculated each case-patient's median incubation period and then calculated the overall median and range of the distribution of these median incubation periods. Similarly, the minimum and maximum incubation periods for case-patients with exposures on multiple days was estimated by using the last or first known exposure day, respectively. The overall incubation period among these case-patients was estimated by determining the median of the distribution of case-patients' median incubation periods. Incubation periods were compared by using the Wilcoxon rank-sum test. All statistical tests were 2-sided with a significance level of $\alpha = 0.05$.

Of the 24 case-patients, 16 (67%) had exposure to sick or dead poultry only (median age = 25 years [range 6–44]; 25% male; 100% lived in rural areas). Eight (33%) had visited a wet poultry market only (median age = 30 years [range 16–41]; 63% male; 88% [7/8] lived in urban areas) (Table). For case-patients with ≥2 exposure days (n = 18), and for case-patients with a single exposure day (n = 6), the overall median incubation period was longer for those who had visited a wet poultry market than for those who were exposed to sick or dead poultry, but the difference was not significant. When data for single and multiple exposure days were combined, the overall median incubation period for case-patients exposed to a wet poultry market (n = 8) was significantly longer than for case-patients (n = 16) exposed to sick or dead poultry (7 days [range 3.5–9] vs. 4.3 days [range 2–9]; p = 0.045).

Our findings are subject to limitations. Proxies for deceased case-patients may not have known all of the case-patient's exposures. Surviving case-patients may not have recalled or identified all exposures that occurred, including environmental exposures. It was impossible to ascertain when infection occurred for case-patients with multiple days of exposures. Our limited data did not permit the use of other methods such as survival analysis to better define incubation periods. We did not quantify exposure duration and could not determine whether repeated exposures (dose-response) or a threshold of exposure to influenza A virus (H5N1) exists to initiate infection of the respiratory tract. Laboratory testing was not performed to confirm that the exposure sources contained influenza virus (H5N1) or to quantify exposures.

Despite exposures of many persons in China to sick or dead poultry or to wet poultry markets, human influenza A (H5N1) disease remains very rare. Our findings suggest that the incubation period may be longer after exposure to a wet poultry market than after exposure to sick or dead poultry, and, therefore, a longer incubation period than the 7 days that is used widely (*4,9*) could be considered for

Table. Estimated incubation period of 24 human cases of infection with avian influenza A virus (H5N1), China*

Exposure data	Case-patients with exposure to sick/dead poultry only	Case-patients with exposure to wet poultry market only	p value	All case-patients
No. case-patients with exposures on multiple days	12	6		18
Overall median incubation period, d (range)	4.5 (2–9.5)	6.3 (3.5–7)	0.276	5 (2–9.5)
Median of minimum incubation period, d (range)	1 (0–5)	0 (0–2)	0.315	0.5 (0–5)
Median of maximum incubation period, d (range)	7.5 (4–14)	11.5 (7–14)	0.108	8.5 (4–14)
No. case-patients with single known exposure	4	2		6
Overall median incubation period, d (range)	3.5 (2–6)	8.5 (8–9)	0.064	5 (2–9)
All case-patients	16	8		24
Overall median incubation period, d (range)	4.3 (2–9)	7 (3.5–9)	**0.045**	5 (2–9.5)
Overall median of minimum incubation period, d (range)	1.5 (0–6)	1 (0–9)	0.752	1.5 (0–9)
Overall median of maximum incubation period, d (range)	6 (2–14)	9 (7–14)	**0.031**	7.5 (2–14)

*__Boldface__ represents significant results (Wilcoxon rank-sum test).

surveillance purposes. However, because of the small number of influenza virus (H5N1) case-patients, our study was too underpowered to draw any firm conclusions; results should be interpreted cautiously. In a study of cases in Vietnam, 5 case-patients did not have any identified exposure ≤7 days of illness onset (*10*). In China, the exposure period for surveillance of suspected cases now includes exposure to a wet poultry market ≤14 days before illness onset. Although data on person-to-person virus transmission are limited, close contacts of patients infected with influenza virus (H5N1) in China are monitored daily for 10 days after the last known exposure. Further studies are needed to quantify the incubation period after exposure to sick or dead infected poultry, a wet poultry market, or to an influenza A virus (H5N1) case-patient and to investigate the basis for any differences.

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Mycobacterium haemophilum Infection after Alemtuzumab Treatment

To the Editor: The immunosuppressive agent alemtuzumab is a DNA-derived, humanized monoclonal antibody directed against the panlymphocyte, cell-surface antigen CD52 (*1*). The drug is approved for the treatment of refractory B-cell chronic lymphocytic leukemia (*2*) and also has been used after stem cell (*3*) and organ transplantations (*4*). Alemtuzumab causes profound and prolonged lymphocyte depletion, which results in a variety of complications involving infections (*5*). However, mycobacteria have rarely been reported to cause infection after alemtuzumab treatment. We describe infections with *Mycobacterium haemophilum*, a fastidious nontuberculous mycobacterium, in 2 patients who experienced cutaneous lesions while they received alemtuzumab.

Patient 1

A 65-year-old man with refractory chronic lymphocytic leukemia had been receiving treatment with alemtuzumab for 3 months. During a 5-week period beginning 15 weeks after the alemtuzumab therapy started, 20–30 tender nodular-ulcerative lesions developed on the patient's extremities. Most of the lesions were distributed along a saphenous vein site (Figure). Immediately before receiv-

ing alemtuzumab, he had been given rituximab for 3 months. A punch biopsy of the cutaneous lesion showed lymphogranulomatous inflammation in the dermis. Acid-fast stains of the skin punch biopsy specimen, as well as aspirated material from the lesions, demonstrated acid-fast bacilli. Cultures on Middlebrook 7H11 agar (Becton Dickinson and Company, Sparks, MD, USA) containing X-factor strips incubated at 30°C showed growth of the acid-fast bacilli after 13 days. The isolate was subsequently identified as *M. haemophilum* by using conventional biochemical profiles and assessment of morphologic features, including an optimal growth temperature of 30°C and a hemin requirement. The patient was treated with 4 drugs (rifampin, doxycycline, clarithromycin, ciprofloxacin), and he rapidly improved. Susceptibility testing, using broth MIC determinations described in Clinical and Laboratory Standards Institute publication M-24A (www.clsi.org/source/orders/free/m24-aa.pdf), indicated that the isolate was sensitive to clarithromycin, ciprofloxacin, clofazimine, and linezolid; intermediately sensitive to rifampin; but resistant to rifabutin, doxycycline, ethambutol, streptomycin, and amikacin. The antimicrobial drugs the patient was receiving were changed to only rifampin, clarithromycin, and ciprofloxacin. He completed a 6-month course of treatment without recurrence of the lesions.

Patient 2

A 17-year-old woman with severe systemic lupus erythematosus and secondary myelodysplastic syndrome received an unrelated T-cell depleted bone marrow transplant. Her conditioning regimen included melphalan, thiotepa, fludarabine, and 2 doses of alemtuzumab. She initially did well posttransplant and was discharged from the hospital Approximately 3 months later, 40–50 tender erythematous papular lesions developed on her extremities. A skin biopsy specimen showed mycobacterial panniculitis. Cultures from skin, blood, and bone marrow grew *M. haemophilum* after 18–19 days' incubation. She was successfully treated with rifampin, clarithromycin, and gatifloxacin; however, she died several months later from unrelated complications.

M. haemophilum was first described in 1978 when it was isolated from cutaneous lesions of a woman from Israel with Hodgkin disease (*6*). *M. haemophilum* most often causes joint, cutaneous, and pulmonary infections in immunocompromised patients (*7*) and lymphadenitis in immunocompetent children (*8*). *M. haemophilum* is a fastidious organism that requires media supplemented with ferric ions in the form of hemin, hemoglobin, or ferric ammonium citrate, and incubation at 30°C–32-°C for several weeks. On the basis of our experience at Memorial Sloan-Kettering Cancer Center (23 cases of *M. haemophilum* infection observed from 1990 through 2000) (*9*), the following specimens are routinely set up for culture: blood smear specimens that are positive for acid-fast bacilli, synovial or joint fluids, skin biopsy specimens, cutaneous lesions, ulcers, abscesses, lymph nodes, and lung biopsy specimens. Culture media include Middlebrook 7H11 agar plates with a hemin-containing paper strip (X-factor) placed on the agar surface that are then incubated at 30°C for 6 weeks. Growth of the organism is usually detected within 2 to 3 weeks, and the isolates are usually susceptible in vitro to the quinolones, macrolides, and rifamycins and resistant to several drugs for tuberculosis, including ethambutol, isoniazid, and pyrazinamide (*9*).

Alemtuzumab has been associated with the development of infections caused by a variety of microorganisms. However, mycobacteria have infrequently been the reported cause. In a review of 547 organ transplant recipients who received alemtuzumab treatment, miliary tuberculosis developed in 1 recipient of a kidney trans-

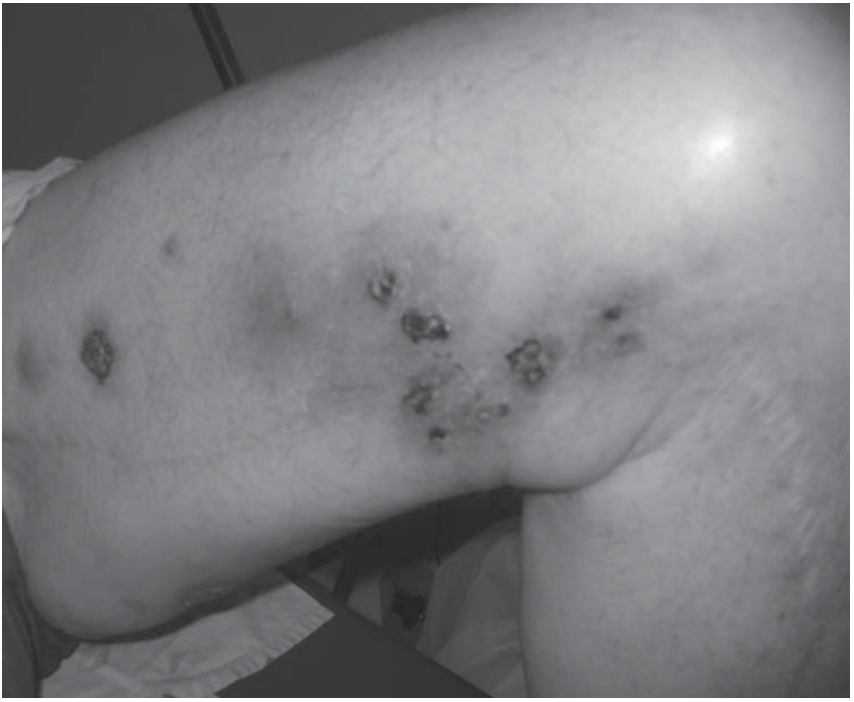

Figure. Nodular-ulcerative skin lesions on the left thigh caused by *Mycobacterium haemophilum* infection in a patient with chronic lymphocytic leukemia (patient 1) whose condition had been treated with alemtuzumab.

plant, and pulmonary infection with *M. kansasii* developed in 2 recipients of lung transplants (*5*). There is also a case report of systemic *M. bovis* infection developing in a patient with relapsing B chronic lymphocytic leukemia after administration of alemtuzumab (*10*).

Although we believe that alemtuzumab is responsible for the severe immunosuppression that predisposed these patients to *M. haemophilum* infection, other explanations are plausible. For example, patient 1 had received rituximab and cyclophosamide for 6 months. These drugs, in addition to his underlying disease of chromic lymphocytic leukemia, may have predisposed him to *M. haemophilum* infection. However, his lesions did not appear until he received alemtuzumab. In patient 2, the immunosuppression associated with his transplant may have predisposed the patient to *M. haemophilum* infection.

This report identifies *M. haemophilum* as an opportunistic pathogen in patients who have received alemtuzumab. We recommend that all patients who have received at least 1 dose of alemtuzumab, and who have undiagnosed tender skin lesions located over the extremities, be evaluated by using appropriate techniques to isolate *M. haemophilum.* Communication with microbiology laboratory staff concerning appropriate methods for detection of the organism is crucial.

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Prior Evidence of Putative Novel *Rhinovirus* Species, Australia

To the Editor: Briese et al. (*1*) are to be congratulated for their delineation of the global geographic presence of human rhinovirus (HRV) strains similar to those reported in 2006 from one third of cases of an otherwise pathogen-negative respiratory outbreak in New York. Of equal importance is the temporal occurrence of these strains. Although it is intriguing to suggest, on the basis of limited sequence data, that these strains were circulating at least 2 centuries earlier (*1*), Briese et al. neglect to acknowledge empirical evidence that what we now call HRV-C strains circulated before 2004–2005. Unculturable PCR-positive rhinoviruses were reported in 1993; however, more compelling is the fact that subgenomic sequence and phylogenetic data were reported from Belgium (*2*), Australia (*3*), and then New York (*4*). The Belgium noncoding sequences were reported in 2006 but originated from specimens collected in 1998–1999. Australian coding sequences from 2003 to 2004 were assigned, for the first time, to a novel clade called HRV-A2, reflecting both their phylogenetic isolation and branching from the known HRV-A strains (*3*).

It can be deduced that NY-041 and NY-060, strains from the 2004 New York winter outbreak, are variants (>98% amino acid identity) of the first characterized HRV-A2 strain, HRV-QPM (*4*,*5*). More recently, we proposed that the HRV-A2 strains diverged sufficiently to meet several of the International Committee on Taxonomy of Viruses criteria for classifying a putative new species, HRV-C (*6*).

It is an exciting time for those interested in rhinoviruses. With increased implementation of multiplexed screening approaches (such as the MassTag PCR), or by simply including a specific

and sensitive PCR for all known strains (*7*), further details of the geographic and temporal extent of the neglected rhinoviruses should soon be available. Better identification may finally enable accurate characterization of the clinical, economic, and social impact (*8*) of HRV infection.

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In Response: We appreciate the enthusiasm for our recent publication highlighting the global distribution of a long-unrecognized third clade of rhinoviruses. Robust, sequence-based clock estimates with associated confidence limits indicate that these viruses have been circulating for hundreds of years (*1*), consistent with the presence of such viruses in historic samples. As isolates from various collections are analyzed in informative regions (e.g., virus protein [VP] 4/2 or VP1), we will undoubtedly find examples in which human rhinoviruses (HRVs) could have been classified as members of the new species HRV-C but were not because the characteristics that define HRV-C were not yet appreciated or because only noncoding sequences had been analyzed. Indeed, we anticipate that waxing interest in HRVs may well lead to the discovery of additional clades.

There has been discussion in the field as to whether the novel sequences represent a sublineage HRV-A2 of the classified species HRV-A (*2*,*3*), as Mackay et al. had proposed, or whether they should be considered as representatives of a third species of HRV (*4*,*5*). The International Committee on Taxonomy of Viruses (ICTV) is charged with the recognition and naming of taxonomic entities. Thus, we provisionally designated our sequences as a novel clade distinct from HRV-A and HRV-B (*4*) and submitted a proposal to ICTV with data supporting the recognition of HRV-C as a third species of rhinovirus. The proposal was recently approved by the ICTV Study Group on Picornaviruses (Europic May 2008 meeting in Sitges, Spain). Irrespective of taxonomic discourse, we agree with Mackay and colleagues that molecular analyses of as-yet-uncultured HRVs are fascinating and have potential to reveal unexpected insights into the role of HRVs in disease.

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Errata—Vol. 14, No. 9

In Forest Fragmentation as Cause of Bacterial Transmission among Primates, Humans, and Livestock, Uganda (T.L. Goldberg et al.), 2 errors occurred. In Table 3, the numerical values are not in the right positions. The corrected table is available from www.cdc.gov/EID/content/14/09/1375-T3.htm. In the same article, Figures 3 and 4 were inadvertently reversed. This has also been corrected in the online version of the article (available from www.cdc.gov/EID/content/14/9/1375.htm).

In Neurobrucellosis in Stranded Dolphins, Costa Rica (G. Hernández-Mora et al.), the name of co-author Elías Barquero-Calvo was misspelled. Several other editing changes to the online version of the article (available from www.cdc.gov/eid/content/14/9/1430.htm) have also been made upon the authors' request.

In Texas Isolates Closely Related to *Bacillus anthracis* Ames (L.J. Kenefic et al.), 3 author names were inadvertently omitted from the submitted article. They are Carla P. Trim, Jodi A. Beaudry, and James M. Schupp; each is from Northern Arizona University, Flagstaff, Arizona, USA. The complete author list as it should have appeared on the article: Leo J. Kenefic, Talima Pearson, Richard T. Okinaka, Wai-Kwan Chung, Tamara Max, Carla P. Trim, Jodi A. Beaudry, James M. Schupp, Matthew N. Van Ert, Chung K. Marston, Kathy Gutierrez, Amy K. Swinford, Alex R. Hoffmaster, and Paul Keim. The corrected article is available online from www.cdc.gov/EID/content/14/9/1494.htm.

In Clindamycin-Resistant Clone of *Clostridium difficile* PCR Ribotype 027, Europe (D. Drudy et al.), the Figure contained errors. The correct version appears in the online version of this article (available from www.cdc.gov/EID/content/14/9/1485.htm) and is reprinted below.

We regret any confusion these errors may have caused.

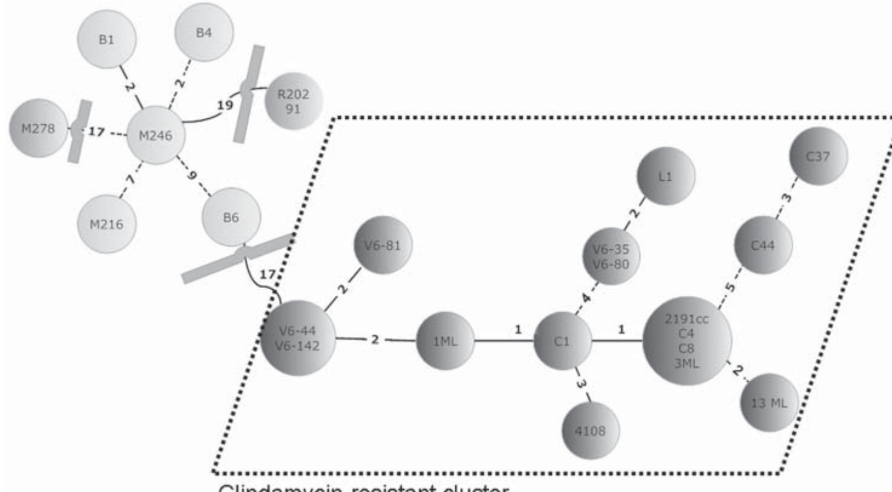

Figure. Minimal spanning tree of 23 *Clostridium difficile* isolates. In the circles, the individual isolates are mentioned. The numbers between the circles represent the summed tandem repeat differences (STRDs) between multiple-locus variable-number tandem-repeat analysis types. Straight lines represent single-locus variants, dashed lines double-locus variants. Curved lines represent triple-locus variants. Two related clusters can be discriminated: the light gray cluster (isolates B1, B4, M246, B6, and M216) and the cluster within dotted lines (isolates V6–44, V6–142, V6–81, 1ML, C1, 4108, V6–35, V6–80, L1, 2191cc, C4, C8, 3ML, C44, C37, and 13ML) The isolates in the light gray cluster are sensitive to clindamycin; isolates in the cluster surrounded by dashed lines are resistant. Two isolates (M278 and R20291) did not belong to a cluster but were more related to the sensitive cluster than to the resistant cluster. Genetically related clusters were defined by an STRD ≤10.

Food-Borne Viruses: Progress and Challenges

Marion P.G. Koopmans, Dean O. Cliver, and Albert Bosch, editors

American Society for Microbiology Press, Washington, DC, USA, 2008
ISBN: 978-1-55581-464-9
Pages: 245; Price: US $99.95

One question that is well known to persons working with food virology, and especially noroviruses, is "How important are these viruses, actually?" Frequently you feel somewhat uneasy when you start to reply, talking about the "trivial illness" of gastroenteritis, continuing on to the economic impact caused by the huge number of cases, and finally ending up admitting that you really do not know the answer. This book does not provide an answer to this difficult question either, but it does give you, among other things, an introduction to the pitfalls in trying to "estimate the burden of an underreported disease." One conclusion is that we probably underestimate its importance.

The book consists of 10 chapters written by persons well known to food virologists as experts in their fields. It is well organized, well written, and presents the history of foodborne viruses, the state of the art, and a glance into the future. The history of food virology is given by Dean O. Cliver, one of the pioneers in this area, who spices up his contribution by sharing interesting personal experiences with the reader. Most of the focus in the book is, naturally, directed toward norovirus and hepatitis A virus. These viruses are covered by up-to-date presentations on molecular biology, clinical disease and diagnostics, pathogenesis and immunity, epidemiology, and detection in food matrixes. Recommendations are also given about which foods should be tested and under which circumstances. Although protocols are not presented, many references are listed.

The molecular revolution has improved the detection of viruses that are impossible or difficult to propagate in cell culture, such as norovirus and hepatitis A virus, and reverse transcription–PCR has become the standard method for virus detection in food. However, sensitive detection methods are still lacking. This is one of the recurrent topics in the book because it affects outbreak investigation, risk assessment, and risk management.

The need for standardization for virus detection in food is stated, as is the proper use of controls. Nobody will argue against detection of false-negative and false-positive results, but some thoughts on the cost-benefit aspects regarding accurate quantification of virus genomes in foods would have been interesting.

The book provides a broad overview of the present situation and gives a good introduction to the topic. Factors that may contribute to the emergence of new viral diseases are also discussed, including demographic and pathogen-related changes. This is interesting reading because virus families and viral evolution are discussed within the context of foodborne transmission. The editors encourage the reader to enjoy the book, and, most of the time, I did.

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Searching Eyes: Privacy, the State, and Disease Surveillance in America

Amy L. Fairchild, Ronald Bayer, and James Colgrove

University of California Press, Berkeley, California, USA, 2007
ISBN-10: 978-0-520-25202-8
ISBN-13: 978-0-520-25325-4
Pages: 342; Price: US $19.95 (soft cover)

Searching Eyes is a history of privacy, a value central to the American democratic way of life, and disease surveillance, a core activity critical to the public health mission of intervening as appropriate to protect the populace from preventable causes of illness and death. Public health surveillance is framed as a social practice that is embedded within particular contexts rather than as a purely technical undertaking insulated from politics, law, economics, ethics, and societal forces. The authors cite encounters with tuberculosis (TB), syphilis, HIV/AIDS, and immunization registry efforts to illustrate the pervasive tension in disease surveillance activities that has existed between privacy and the welfare of society since the inception of surveillance in the 19th century.

Although public health officials take for granted the long-established disease surveillance system that enables them to monitor the public's health, such practices are not viewed as positively by the populace who contest the feared intrusion into what is perceived as an American's right to privacy. Even the medical community, our strongest ally in public health activities, has feared the intrusion and encroachment of the doctor–patient relationship. The well-publicized AIDS struggle in the early 1980s captured our attention as we strived to respond to the political and ethical ques-

tions that, up until that time, had only received an episodic focus.

One drawback of the book is that the authors cover a wide gamut of topics. Consequently, disease-specific topics are presented without adequately providing the necessary background on the disease entity to the reader. Not all readers may fully understand the necessary disease-specific background that would make the discussions of public health intervention understandable. For instance, the chapter on TB presents a history of the disease from the turn of the 20th century. But if the reader were unaware of the public health threat posed by the undiagnosed or nonadherent TB patient, the reader would understandably question the authority of public health practitioners to occasionally take extraordinary steps to ensure that infection is not transmitted within the community.

Even without a comprehensive background on specific diseases, this book will interest a wide audience, not only public health practitioners but the medical and legal community with whom we partner. Searching Eye tackles a topic that deserves more of our respective attention, for as noted by the authors, "The vitality of democratic communities necessitates an ongoing effort to negotiate and renegotiate the boundaries between privacy, society's limiting principle, and public health, which at its best has sought to expand the role of government as a guardian against disease and suffering." I congratulate the authors on their well-researched and thorough discourse on this core public health activity.

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Emerging Pests and Vector-borne Diseases in Europe

Willem Takken and Bart G.J. Knols, editors

Wageningen Academic Publishers, Wageningen, the Netherlands, 2007
ISBN-10: 9086860532
ISBN-13: 978-90-8686-053-1
Pages: 500; Price: US $141.00

Today's hot topic is the risk of introducing new vector-borne diseases and harmful ectoparasites into Europe, or of the geographic extension of existing ones. The recent outbreaks of bluetongue virus infection in northwestern Europe and of chikungunya infection in Italy are real reminders of the vulnerability of this ill-prepared continent, where the idea prevails that these things cannot happen there but are merely problems that occur on other continents.

This book is the first volume of a new series, "Ecology and Control of Vector-Borne Diseases," written by more than 70 European scientists who are recognized experts in their specific fields. The cover text states that in 24 chapters "this book provides examples of the most likely pests and diseases affecting man and animals in Europe, with emphasis on ecological factors favoring these diseases and methods for prevention and intervention." Indeed, the raised expectations are largely fulfilled.

The book is divided into 6 sections preceded by an appealing introductory chapter—Alarm Bells Ringing: More of the Same, and New and Novel Diseases and Pests—and followed by a poignant epilogue, which summarizes the different problem areas discussed in the book as well as concise actions proposed to reduce the threats.

In the first section, pertinent questions are answered on the possible return of malaria in Europe and on the problem of leishmaniasis in southern Europe. Avian malaria, a rather unknown but interesting paradigm, is discussed as well. In the second section, different arboviruses such as bluetongue, West Nile, chikungunya, and dengue are discussed in addition to the rather unknown Usutu virus. Readers can download the well-written chapter on chikungunya and dengue in southern Europe from www.wageningenacademic.com. The third section gives an overview on the current problems of tick-borne encephalitis and Lyme disease in Europe; discussion is limited to the Baltic States and the Netherlands, respectively. The fourth section discusses strategies on the following emerging arthropod pests and problems: psoroptic mang, the establishment and spread of the *Aedes albopictus* mosquito in Europe, bed bugs, houseflies, head lice, and *Culicoides* midges. The authors highlight the most recent information on pest control with an emphasis on tools other than classic chemical control to manage these infestations because these pests are likely to escape these classic chemical tools in the future. In the fifth section, Surveillance, Protection and Control, the authors discuss monitoring systems for adult insect pests and disease vectors, personal protection against European disease vectors, and mosquito control in Europe. The last section, Nature Conservation, Wildlife Management and Human Activities as Drivers, covers subjects such as changes in global scale land use and its implications for nature conservation and emerging vector-borne diseases, wildlife and the risk of vector-borne viral diseases, and invasions of vector-borne diseases driven by transportation and climate change.

Throughout the book, the role of the different factors that drive the changing epidemiology of diseases and pests is described in a well-balanced manner. This changing epidemiology is recognized as a possible consequence of the complex interplay of factors such as climate change (whether or not it is anthropogenic),

human-made environmental change (e.g., agricultural activities), increasing international trade and traffic (e.g., long-distance tourism), changes in human behavior (e.g., more outdoor activities), and the development of insecticide resistance.

It was a rewarding task, as well as a pleasant challenge for us, a physician and a biologist/entomologist, to read the book from cover to cover. The volume is not intended to be read as a novel, but every chapter is certainly well worth reading. The book is written for the nonexperienced (as an introductory text) and for the experienced scientist (as a refresher regarding knowledge in a specific domain). Because it contains chapters on human and animal diseases and on pests, it can help broaden the horizon for every concerned scientist who might not be accustomed to the problems outside of a particular discipline.

The information on the different topics has increased since the authors finished writing it (end of September 2007), but this outstanding book is a useful beginning for the novice who wants to acquire, in a relatively short period, a nearly complete insight on the existing information concerning pests and vector-borne diseases in Europe. Although this book does not cover everything (e.g., rickettsial diseases were only superficially discussed), it nevertheless deserves a place on the bookshelf of every concerned infectious diseases specialist or epidemiologist, scientist or student.

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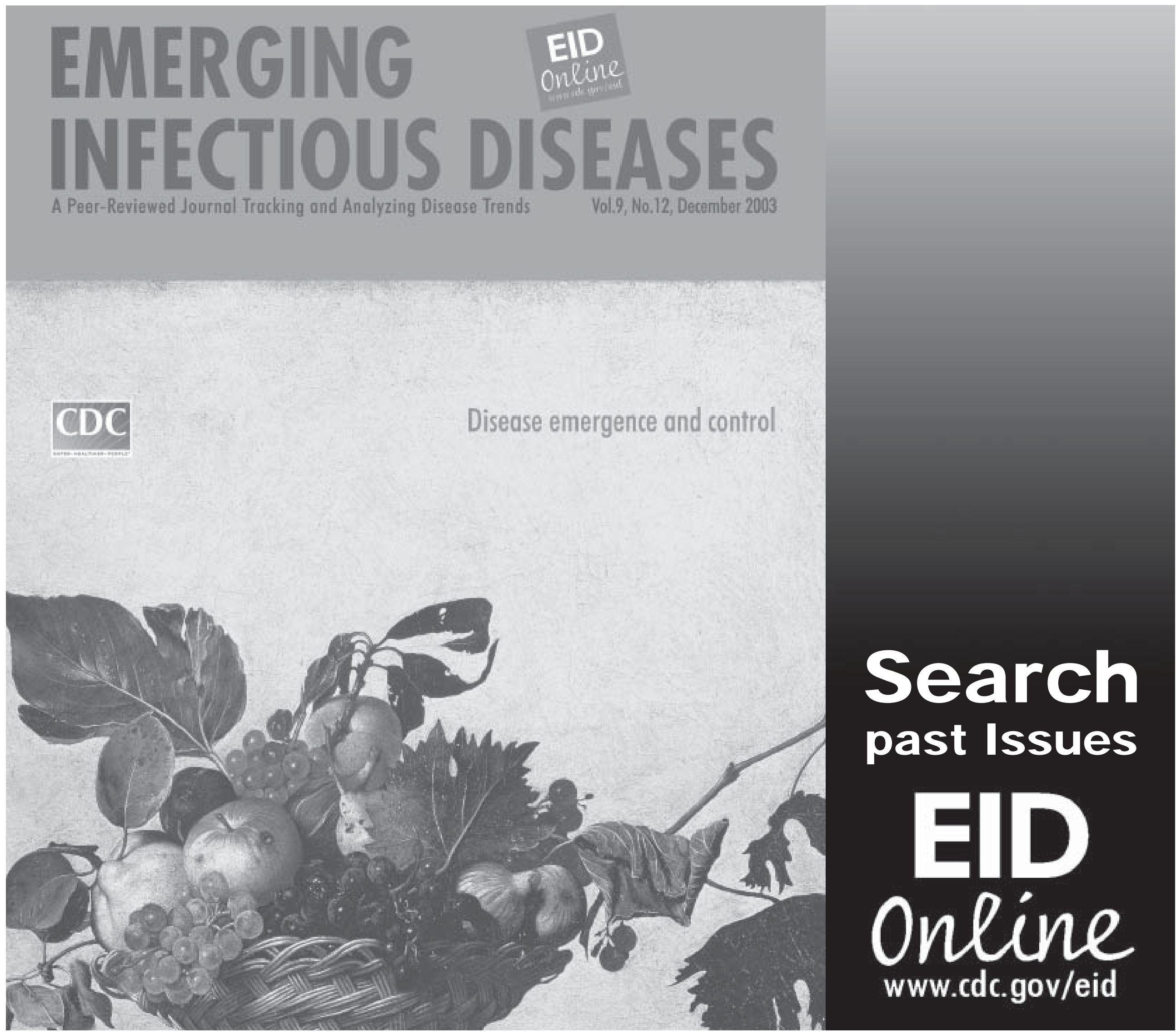

Giuseppe Arcimboldi or Arcimboldo (1527–1593) Vertumnus (1590–91) Oil on wood (71 cm × 57.5 cm) Skoklosters slott, Balsta, Sweden

The Extraordinary Nature of Illusion

Polyxeni Potter

"When our first encounter with some object surprises us and we find it novel or very different from what we formerly knew or from what we supposed it ought to be, this causes us to wonder and be astonished at it," wrote 17th-century philosopher René Descartes in his Passions of the Soul. Indeed, astonishment awaits anyone who views for the first time the work of Giuseppe Arcimboldi, Milanese painter extraordinaire, portraitist of emperors, and master of illusion (*1*).

Arcimboldi grew up in a distinguished family. He associated with philosophers and other scholars and knew the son of Bernardino Luini, a student of Leonardo, who had notes and sketchbooks given to him when the master left Milan, site of most of his experiments (*2*). In writing about the family, historian Paolo Morigia described Giuseppe as "a trustworthy gentleman with an impeccable lifestyle," who started his artistic career at age 22 designing tapestry and stained glass with his father, also an artist (*3*). This early work at Milan Cathedral already contained elements found in his unique later style.

In the beginning of the 16th century, partly because of a plague epidemic, Milan's position as leader in the arts was declining. Nonetheless, Arcimboldi's reputation was strong. "This is a painter with a rare talent, who is also extremely knowledgeable in other disciplines," wrote Morigia about Arcimboldi's acceptance of the invitation of Emperor Ferdinand I to go to Prague. "And having proved his worth as an artist and as a bizarre painter, not only in his own country but also abroad, he has been given the highest praise" (*3*).

Arcimboldi flourished during his tenure with Ferdinand. "He was liked and treated well and received with great kindness, and the Emperor gave him a good salary worthy of his merits" (*3*). He came to know the works of such greats as Hieronymus Bosch, Peter Bruegel the Elder, and Albrecht Altdorfer. He painted portraits of the imperial family and the first series of his Four Seasons, composite heads with allegorical meanings. His work continued for succeeding Holy Roman Emperors Maximilian II and Rudolph II. In their courts, "This noble and inspired man fashioned a great number of rare and delicate works of art which caused considerable amazement" (*3*).

Apart from painting, Arcimboldi had other duties. He was designer of costumes, masques, and disguises worn during festivals by impersonators of ancient gods, the liberal arts, or anything else living or mythical. He served as architect, stage designer, engineer, and advisor to Maximilian, who delighted in animals and believed in the healing power of plants. He started a museum to house rare specimens and artifacts from as far away as the newly discovered

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continent of America. Later, under Rudolph, this museum would become the Art and Wonder Chambers. Arcimboldi traveled to Germany to buy art objects and exotic birds for it, which he studied from nature and incorporated in his works.

Rudolph was interested in all disciplines, from mathematics to gardening. Many scholars and artists, among them botanist Charles de l'Écluse, astronomers Tycho Brahe and Johannes Kepler, and painters Joris Hoefnagel and Roelandt Savery, were brought to the court (*4*). Prague became a major European cultural center with a characteristic flavor of science and the occult, which found its way into Arcimboldi's work. He painted the Four Seasons twice during this time. Humanist Giovanni Fonteo composed verses, published in a separate booklet, expanding on the resemblances between the staying power of imperial rule and the cycles of nature.

Arcimboldi's work has been ascribed to mannerism, the art of his times, known for its aesthetic quality, exaggeration, and emphasis on emotion. But his creative imagination moved in an entirely original direction. He turned elements from nature or everyday life into images of his own invention, transforming fruits, vegetables, flowers, animals, or books into enigmatic portraits. The parts were known and clearly understood, but the whole was new and elusive. For these elaborate illusionist tricks or "hieroglyphic wit," poet and theologian Gregorio Comanini called Arcimboldi a "learned Egyptian" (*5*).

"For his long and conscientious service" (*3*), the painter was given permission to return to Milan, where he continued to work for Rudolph and completed his most famous works, Flora and the Nymph and Vertumnus—both of which he sent to Prague. He received the emperor's highest orders for these paintings and died a year later.

Despite fame during his lifetime, Arcimboldi was soon forgotten to be rediscovered in our times. The century of Albert Einstein and Sigmund Freud took a closer look at the philosophy, symbolism, and sheer magic of his work. He was dubbed "the arch-father of surrealism" (*6*) for exerting influence on André Breton and Salvador Dalí. Pablo Picasso, who owned a copy of his Portrait of a Librarian (c. 1565), was inspired by it to paint the cubist Portrait of Daniel-Henry Kahnweiler (1910).

Arcimboldi's Vertumnus, on this month's cover, was the most famous work of art in Rudolph's Prague. In this portrait, the emperor was shown as the Roman god of seasons, gardens, and plants, Vertumnus, who could change at will and was notorious for his disguises (Ovid Metamorphoses, Book XIV). The portrait was eulogized and explicated in a poem by Comanini: "If in looking you don't admire/The ugliness that makes me handsome,/It's that you don't know how/ugliness surpasses/Every beauty."

To deify Rudolph as lord of the seasons embodying the fruits of the world in a perpetual golden age, Arcimboldi rearranged nature's bounty into a brand new ensemble. And by composing the emperor out of familiar parts, fruits and flowers, the painter reinvented him: "I vary from myself,/ And thus, so varied, I am/One only, and from various things/ With my varied countenance/I portray resemblances."

"Look at the apple and the peach—/Round, red, and fresh—/That form both cheeks;/Turn your mind to my eyes—/One is a cherry,/The other a red mulberry," exclaimed Vertumnus in Comanini's poem, as if surprised by his own fantastic appearance. Composite creatures have fascinated throughout the ages. Hellenic mythology proposed Chimera, which appeared on pottery 2,500 years ago and was described by Homer in the Iliad (Book VI) as "a thing of immortal make, not human, lion-fronted and snake behind, a goat in the middle, and snorting out of breath of the terrible flame of bright fire."

A tempting metaphor, Chimera has been adopted by many civilizations and, more recently, by various disciplines, among them genetics, molecular biology, and virology. Composites abound in nature. Those in the microbial world have gained notoriety in the face of emerging disease, one that Arcimboldi would have delighted in immortalizing. For this complex illusion, instead of fruits or flowers, he would have portrayed MRSA, avian influenza (H5N1), West Nile virus, *E. coli* O157:H7, and other monsters of emergence: ordinary parts rearranged in a new context. Its specter would have gone beyond astonishment to other common reactions evoked by the master's unpredictable work: unease and foreboding.

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

Clinical Characteristics and Molecular Subtyping of *Vibrio vulnificus*, Israel

Genetic Characterization of Toggenburg Orbivirus, a New Bluetongue Virus, Switzerland

Surveillance of Coyotes to Detect Bovine Tuberculosis, Michigan

Highly Pathogenic Avian Influenza Virus (H5N1) in Red Foxes Fed Infected Bird Carcasses

Influenza Infection in Wild Raccoons

Introduction of African Swine Fever Virus Isolate, Georgia, 2007

Francisella novicida Bacteremia, Thailand

Human Illnesses Caused by *Opisthorchis felineus* Flukes, Italy

Rickettsia parkeri in Argentina

Transmission of Atypical Bovine Prion Disease to Mice Transgenic for Human Prion Protein

Outbreak of Trichinellosis Caused by *Trichinella papuae*, Thailand, 2006

Multiple *Francisella tularensis* Subspecies and Clades in Focal Outbreak of Tularemia

Novel Borna Virus in Psittacine Birds with Proventricular Dilatation Disease

Mycobacterium bovis Strains as Cause of Human Tuberculosis, Southwest Ireland

Mycobacterium bovis Infection in Holstein Friesian Cattle, Iran

Hemoplasma Infection in HIV-positive Patient, Brazil

Occupational Exposure to *Streptococcus suis* among Swine Workers, United States

Multicenter Study of Brucellosis in Egypt

New Rabies Virus Variant in Mexican Immigrant, California

Complete list of articles in the December issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

February 12–13, 2009
The International Symposium on the Asian Tiger Mosquito
Rutgers University
New Brunswick, NJ, USA
http://www.rci.rutgers.edu/~vbcenter/atmsymposium.php

February 13–16, 2009
International Meeting on Emerging Diseases and Surveillance (IMED 2009)
Hotel Hilton
Vienna, Austria
http://imed.isid.org

Announcements

To submit an announcement, send an email message to EIDEditor (eideditor@cdc.gov). In 50–150 words, describe timely events of interest to our readers. Include the date of the event, the location, the sponsoring organization(s), and a website that readers may visit or a telephone number or email address that readers may contact for more information.

Announcements may be posted on the journal Web page only, depending on the event date.

The Public Health Image Library (PHIL)

The Public Health Image Library (PHIL), Centers for Disease Control and Prevention, contains thousands of public health–related images, including high-resolution (print quality) photographs, illustrations, and videos.

PHIL collections illustrate current events and articles, supply visual content for health promotion brochures, document the effects of disease, and enhance instructional media.

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To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions and earn continuing medical education (CME) credit, please go to **http://www.medscape.com/cme/eid**. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.com. If you are not registered on Medscape.com, please click on the New Users: Free Registration link on the left hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/category/2922.html. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for *AMA PRA Category 1 Credits*™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit is acceptable as evidence of participation in CME activities. If you are not licensed in the US and want to obtain an AMA PRA CME credit, please complete the questions online, print the certificate and present it to your national medical association.

Article Title

Antimicrobial Drug Use and Resistance in Europe

CME Questions

1. Which of the following classes of antimicrobial drugs is least likely to be used in European countries?

A. Lincosamides
B. Fluoroquinolones
C. Cotrimoxazole
D. Macrolides

2. Which of the following regions in Europe has the highest outpatient utilization of antimicrobial drugs?

A. Southern
B. Northern
C. Eastern
D. Central

3. Which of the following is the most widely used antimicrobial drug class in Europe?

A. Macrolides
B. Penicillins
C. Nonpenicillin beta-lactams
D. Fluoroquinolones

4. Which of the following European countries showed both the greatest use of antimicrobial drugs in ambulatory care and the highest resistance proportions?

A. France
B. Croatia
C. Italy
D. Greece

5. Among European countries with high antimicrobial drug resistance rates, a robust and consistent association was most likely to be found between utilization and resistance for which of the following drugs?

A. Penicillins and macrolides
B. Lincosamides and macrolides
C. Penicillins and streptogramins
D. Penicillins and fluoroquinolones

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

EMERGING INFECTIOUS DISEASES www.cdc.gov/eid

JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- New infections resulting from changes or evolution of existing organisms.
- Known infections spreading to new geographic areas or populations.
- Previously unrecognized infections appearing in areas undergoing ecologic transformation.
- Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

   - Investigates factors known to influence emergence: 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.
   - Reports laboratory and epidemiologic findings within a broader public health perspective.
   - Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

   - Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   - Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
   - Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

EMERGING INFECTIOUS DISEASES®

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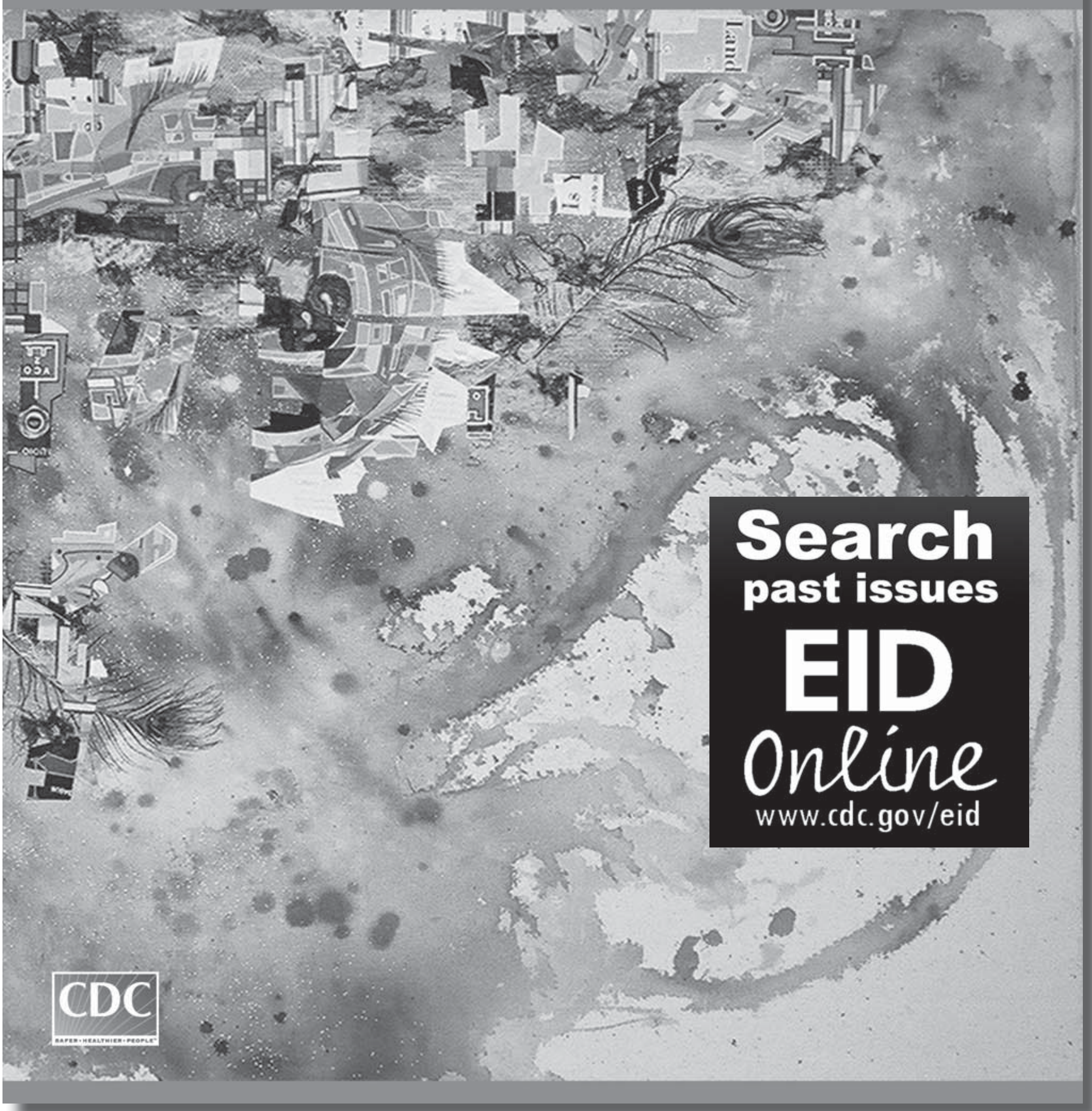
October 2008

Bacterial Infections



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 www.cdc.gov/eid/ncidod/ EID/instruct.htm.

Emerging Infectious Diseases is published in English. To expedite publication, we post articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (www.cdc. gov/ncidod/EID/trans.htm).

Instructions to Authors

Manuscript Preparation. For word processing, use MS Word. List the following information in this order: title page, article summary line, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, figure legends, appendixes, and figures. Each figure should be in a separate file.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Include up to 10 keywords; use terms listed in Medical Subject Headings Index Medicus.

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

Tables. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

Figures. Provide figures as separate files, not embedded in MS Word. Use Arial font for text content. Place keys within figure area. Provide footnotes and other information (e.g., source/copyright data, explanation of boldface) in figure legend. Submit figures with text content in native, editable, PC file formats (e.g., MS Excel/PowerPoint). Submit image files (e.g., electromicrographs) without text content as high-resolution (300 dpi/ppi minimum) TIFF or JPG files. Submit separate files for multiple figure panels (e.g., A, B, C). EPS files are admissible but should be saved with fonts embedded (not converted to lines). No PNG or BMP files are admissible. For additional guidance, contact fue7@cdc.gov or 404-639-1250.

Manuscript Submission. Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch) and verifying that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid).

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. 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. 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. 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 a 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. 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.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Name, publisher, number of pages, other pertinent details should be included.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted online 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.