# EMERGING INFECTIOUS DISEASES®

May 2009

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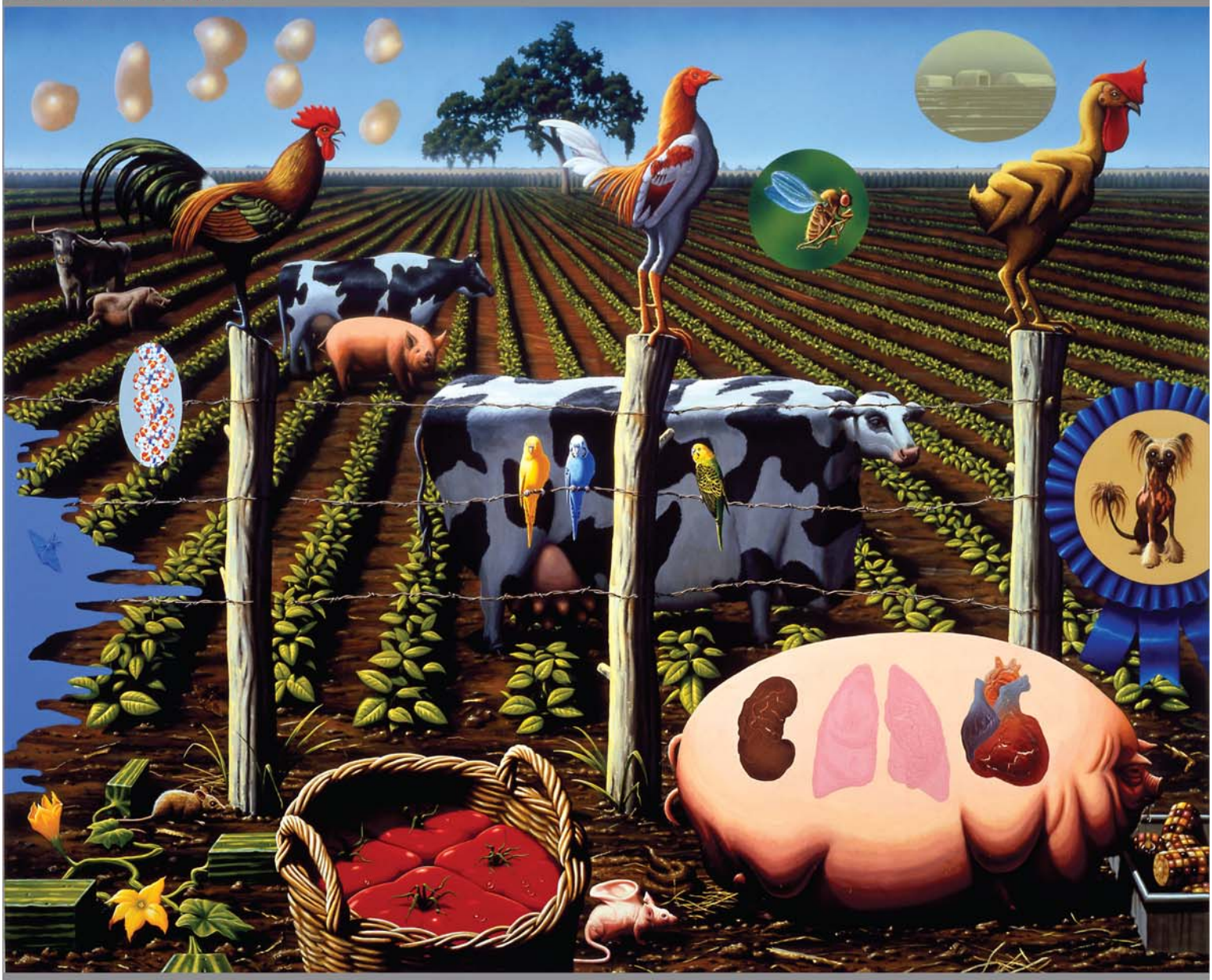

## Foodborne Infections

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# EMERGING INFECTIOUS DISEASES

May 2009

## On the Cover

Alexis Rockman (b. 1962)
The Farm (2000) (detail)
Acrylic on wood
(243.8 cm × 304.8 cm)
Collection of JGS Inc.,
New York. Courtesy of the artist.

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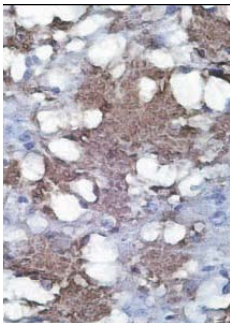
## Synopsis



## Research





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CME ACTIVITY

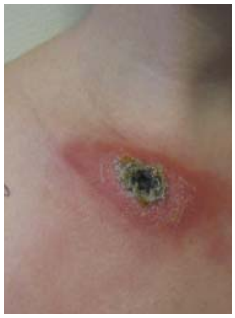
EMERGING INFECTIOUS DISEASES
May 2009

## Dispatches



## Another Dimension





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## Letters



## Book Review



## News and Notes

# Use of Unstructured Event-Based Reports for Global Infectious Disease Surveillance

**Mikaela Keller, Michael Blench, Herman Tolentino, Clark C. Freifeld, Kenneth D. Mandl, Abla Mawudeku, Gunther Eysenbach, and John S. Brownstein**

Free or low-cost sources of unstructured information, such as Internet news and online discussion sites, provide detailed local and near real-time data on disease outbreaks, even in countries that lack traditional public health surveillance. To improve public health surveillance and, ultimately, interventions, we examined 3 primary systems that process event-based outbreak information: Global Public Health Intelligence Network, HealthMap, and EpiSPIDER. Despite similarities among them, these systems are highly complementary because they monitor different data types, rely on varying levels of automation and human analysis, and distribute distinct information. Future development should focus on linking these systems more closely to public health practitioners in the field and establishing collaborative networks for alert verification and dissemination. Such development would further establish event-based monitoring as an invaluable public health resource that provides critical context and an alternative to traditional indicator-based outbreak reporting.

International travel and movement of goods increasingly facilitates the spread of pathogens across and among nations, enabling pathogens to invade new territories and adapt to new environments and hosts (*1*–*3*). Officials now need to consider worldwide disease outbreaks when determining what potential threats might affect the health and welfare of their nations (*4*). In industrialized countries, unprecedented efforts have built on indicator-based public health surveillance, and monitoring of clinically relevant data sources now provides early indication of outbreaks (*5*). In many countries where public health infrastructure is rudimentary, deteriorating, or nonexistent, efforts to improve the ability to conduct electronic disease surveillance include more robust data collection methods and enhanced analysis capability (*6*,*7*). However, in these parts of the world, basing timely and sensitive reporting of public health threats on conventional surveillance sources remains challenging. Lack of resources and trained public health professionals poses a substantial roadblock (*8*–*10*). Furthermore, reporting emerging infectious diseases has certain constraints, including fear of repercussions on trade and tourism, delays in clearance through multiple levels of government, tendency to err on the conservative side, and inadequately functioning or nonexistent surveillance infrastructure (*11*). Even with the recent enactment of international health regulations in 2005, no guarantee yet exists that broad compliance will be feasible, given the challenges associated with reporting mechanisms and multilateral coordination (*12*).

In many countries, free or low-cost sources of unstructured information, including Internet news and online discussion sites (Figure), could provide detailed local and near real-time data on potential and confirmed disease outbreaks and other public health events (*9*,*10*,*13*–*18*). These event-based informal data sources provide insight into new and ongoing public health challenges in areas that have limited or no public health reporting infrastructure but have the highest risk for emerging diseases (*19*). In fact, event-based informal surveillance now represents a critical source of

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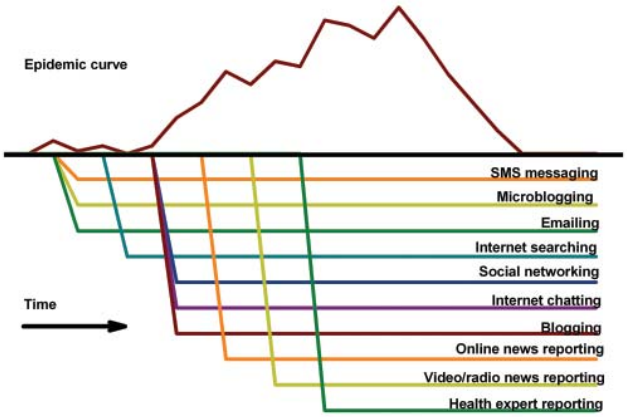


Figure. Hypothetical timing of informal electronic sources available during an outbreak. SMS, short message service.

epidemic intelligence—almost all major outbreaks investigated by the World Health Organization (WHO) are first identified through these informal sources (*9*,*13*).

With a goal of improving public health surveillance and, ultimately, intervention efforts, we (the architects, developers, and methodologists for the information systems described herein) reviewed 3 of the primary active systems that process unstructured (free-text), event-based information on disease outbreaks: The Global Public Health Intelligence Network (GPHIN), the HealthMap system, and the EpiSPIDER project (Semantic Processing and Integration of Distributed Electronic Resources for Epidemics [and disasters]; www.epispider.net). Our report is the result of a joint symposium from the American Medical Informatics Association Annual Conference in 2007. Despite key differences, all 3 systems face similar technologic challenges, including 1) topic detection and data acquisition from a high-volume stream of event reports (not all related to disease outbreaks); 2) data characterization, categorization, or information extraction; 3) information formatting and integration with other sources; and 4) information dissemination to clients or, more broadly, to the public.

Each system tackles these challenges in unique ways, highlighting the diversity of possible approaches and public health objectives. Our goal was to draw lessons from these early experiences to advance overall progress in this recently established field of event-based public health surveillance. After summarizing these systems, we compared them within the context of this new surveillance framework and outlined goals for future development and research.

## The GPHIN Project

### Background

GPHIN took early advantage of advancements in communication technologies to provide coordinated, near real-time, multisource, and multilingual information for monitoring emerging public health events (*20*,*21*). In 1997, a prototype GPHIN system was developed in a partnership between the government of Canada and WHO. The objective was to determine the feasibility and effectiveness of using news media sources to continuously gather information about possible disease outbreaks worldwide and to rapidly alert international bodies of such events. The sources included websites, news wires, and local and national newspapers retrieved through news aggregators in English and French. After the outbreak of severe acute respiratory syndrome (SARS), a new, robust, multilingual GPHIN system was developed and was launched November 17, 2004, at the United Nations.

### Data Acquisition

#### Automated process

The GPHIN software application retrieves relevant articles every 15 minutes (24 hours/day, 7 days/week) from news-feed aggregators (Al Bawaba [www.albawaba.com] and Factiva [www. factiva.com]) according to established search queries that are updated regularly. The matching articles are automatically categorized into ≥1 GPHIN taxonomy categories, which cover the following topics: animal, human, or plant diseases; biologics; natural disasters; chemical incidents; radiologic incidents; and unsafe products.

Articles with a high relevancy score are automatically published on the GPHIN database. The GPHIN database is also augmented with articles obtained manually from open-access web sites. Each day, GPHIN handles ≈4,000 articles. This number drastically increases when events with serious public health implications, such as the finding of melamine in various foods worldwide, are reported.

#### Human Analysis Process

Although the GPHIN computerized processes are essential for the management of information about health threats worldwide, the linguistic, interpretive, and analytical expertise of the GPHIN analysts makes the system successful. Articles with relevancy below the "publish" threshold are presented to a GPHIN analyst, who reviews the article and decides whether to publish it, issue an alert, or dismiss it. Additionally, the GPHIN analyst team conducts more in-depth tasks, including linking events in different regions, identifying trends, and assessing the health risks to populations around the world.

### Data Dissemination

#### Machine Translation

English articles are machine-translated into Arabic, Chinese (simplified and traditional), Farsi, French, Rus-

sian, Portuguese, and Spanish. Non-English articles are machine-translated into English. GPHIN has adopted a best-of-breed approach in selecting engines for machine translation. The lexicons associated with the engines are constantly being improved to enhance the quality of the output. As such, the machine-translated outputs are edited by the appropriate GPHIN analysts. The goal is not to obtain a perfect translation but to ensure comprehensibility of the essence of the article.

#### Information Access

Users can view the latest list of published articles or query the database by using both Boolean and translingual metadata search capabilities. In addition, notifications about events that might have serious public health consequences are immediately sent by email to users in the form of an alert.

#### Project Results

As an initial assessment of data collected during July 1998 through August 2001, WHO retrospectively verified 578 outbreaks, of which 56% were initially picked up and disseminated by GPHIN (*9*). Outbreaks were reported in 132 countries, demonstrating GPHIN's capacity to monitor events occurring worldwide, despite the limitation of predominantly English (with some French) media sources.

One of GPHIN's earliest achievements occurred in December 1998, when the system was the first to provide preliminary information to the public health community about a new strain of influenza in northern People's Republic of China (*20*). During the SARS outbreak, declared by WHO in March 2003, the GPHIN prototype demonstrated its potential as an early-warning system by detecting and informing the appropriate authorities (e.g., WHO, Public Health Agency of Canada) of an unusual respiratory illness outbreak occurring in Guangdong Province, China, as early as November 27, 2002. GPHIN was further able to continuously monitor and provide information about the number of suspected and probable SARS cases reported worldwide on a near real-time basis. GPHIN's information was ≈2–3 days ahead of the official WHO report of confirmed and probable cases worldwide.

In addition to outbreak reporting, GPHIN has also provided information that enabled public health officials to track global effects of the outbreak such as worldwide prevention and control measures, concerns of the general public, and economic or political effects. GPHIN is used daily by organizations such as WHO, the US Centers for Disease Control and Prevention (CDC), and the UN Food and Agricultural Organization.

### The HealthMap Project

#### Background

Operating since September 2006, HealthMap (*22*,*23*) is an Internet-based system designed to collect and display information about new outbreaks according to geographic location, time, and infectious agent (*24–26*). HealthMap thus provides a structure to information flow that would otherwise be overwhelming to the user or obscure important elements of a disease outbreak.

Healthmap.org receives 1,000–10,000 visits/day from around the world. It is cited as a resource on sites of agencies such as the United Nations, National Institute of Allergy and Infectious Diseases, US Food and Drug Administration, and US Department of Agriculture. It has also been featured in mainstream media publications, such as Wired News and Scientific American, indicating the broad utility of such a system that extends beyond public health practice (*24*,*26*). On the basis of usage tracking of HealthMap's Internet site, we can infer that its most avid users tend to come from government-related domains, including WHO, CDC, European Centre for Disease Prevention and Control, and other national, state, and local bodies worldwide. Although the question of whether this information has been used to initiate action will be part of an in-depth evaluation, we know from informal communications that organizations (ranging from local health departments to such national organizations as the US Department of Health and Human Services and the US Department of Defense) are leveraging the HealthMap data stream for day-to-day surveillance activities. For instance, CDC's BioPHusion Program incorporates information from multiple data sources, including media reports, surveillance data, and informal reports of disease events and disseminates it to public health leaders to enhance CDC's awareness of domestic and global health events (*27*).

#### Data Acquisition

The system integrates outbreak data from multiple electronic sources, including online news wires (e.g., Google News), Really Simple Syndication (RSS) feeds, expert-curated accounts (e.g., ProMED-mail, a global electronic mailing list that receives and summarizes reports on disease outbreaks) (*18*), multinational surveillance reports (e.g., Eurosurveillance), and validated official alerts (e.g., from WHO). Through this multistream approach, HealthMap casts a unified and comprehensive view of global infectious disease outbreaks in space and time. Fully automated, the system acquires data every hour and uses text mining to characterize the data to determine the disease category and location of the outbreak. Alerts, defined as information on a previously unidentified outbreak, are geocoded to the

country scale with province-, state-, or city-level resolution for select countries. Surveillance is conducted in several languages, including English, Spanish, Russian, Chinese, and French. The system is currently being ported to other languages, such as Portuguese and Arabic.

### Data Dissemination

After being collected, the data are aggregated by source, disease, and geographic location and then overlaid on an interactive map for user-friendly access to the original report. HealthMap also addresses the computational challenges of integrating multiple sources of unstructured information by generating meta-alerts, color coded on the basis of the data source's reliability and report volume. Although information relating to infectious disease outbreaks is collected, not all information has relevance to every user. The system designers are especially concerned with limiting information overload and providing focused news of immediate interest. Thus, after a first categorization step into locations and diseases, a second round of category tags is applied to the articles to improve filtering. The primary tags include 1) breaking news (e.g., a newly discovered outbreak); 2) warning (initial concerns of disease emergence, e.g., in a natural disaster area; 3) follow-up (reference to a past outbreak); 4) background/context (information on disease context, e.g., preparedness planning); and 5) not disease-related (information not relating to any disease [2–5 are filtered from display]). Duplicate reports are also removed by calculating a similarity score based on text and category matching. Finally, in addition to providing mapped content, each alert is linked to a related information window with details on reports of similar content as well as recent reports concerning either the same disease or location and links for further research (e.g., WHO, CDC, and PubMED).

### Project Results

HealthMap processes an average of 133.5 disease alerts/day (95% confidence interval [CI] 124.1–142.8); ≈50% are categorized as breaking news (65.3 reports/day). Looking 30 days back (default display), the system displays >800 breaking news alerts for any given day. From October 2006 through November 20, 2007, HealthMap had processed >35,749 alerts across 171 disease categories and 202 countries or semiautonomous or overseas territories. Most alerts come from news media (92.8%), followed by ProMED (6.5%) and multinational agencies (0.7%).

## The EpiSPIDER Project

### Background

The EpiSPIDER project was designed in January 2006 to serve as a visualization supplement to the ProMED-mail reports. Through use of publicly available software, EpiSPIDER was able to display topic intensity of ProMED-mail reports on a map. Additonally, EpiSPIDER automatically converted the topic and location information of the reports into RSS feeds. Usage tracking showed, initially, that the RSS feeds were more popular than the maps. Transforming reports to a semantic online format (W3C Semantic Web) makes it possible to combine emerging infectious disease content with similarly transformed information from other Internet sites such as the Global Disaster Alert Coordinating System (GDACS) website (www.gdacs.org). The broad effects of disasters often increase illness and death from communicable diseases, particularly where resources for healthcare infrastructure have been lacking (*28*,*29*). By merging these 2 online media sources (ProMED-mail and GDACS), EpiSPIDER demonstrates how distributed, event-based, unstructured media sources can be integrated to complement situational awareness for disease surveillance.

### Data Acquisition and Dissemination

EpiSPIDER connects to news sites and uses natural language processing to transform free-text content into structured information that can be stored in a relational database. For ProMED reports, the following fields are extracted: date of publication; list of locations (country, province, or city) mentioned in the report; and topic. EpiSPIDER parses location names from these reports and georeferences them using the georeferencing services of Yahoo Maps (http://maps.yahoo.com), Google Maps (http://maps.google.com), and Geonames (www.geonames.org).

Each news report that has location information can be linked to relevant demographic- and health-specific information (e.g., population, per capita gross domestic product, public health expenditure, and physicians/1,000 population). EpiSPIDER extracts this information from the Central Intelligence Agency (CIA) Factbook (www.cia.gov/library/publications/the-world-factbook/index.html) and the United Nations Development Human Development Report (http://hdr.undp.org/en) Internet sites. This feature provides different contexts for viewing emerging infectious disease information. By using askMEDLINE (*30*), EpiSPIDER also provides context-sensitive links to recent and relevant scientific literature for each ProMED-mail report topic. After EpiSPIDER extracts the previously described information, it automatically transforms it to other formats, e.g., RSS, keyhole markup language(KML; http://earth.google.com/kml), and JavaScript object notation (JSON, a human-readable format for representing simple data structures; www.json.org). Publishing content using those formats enables the semantic linking of ProMED-mail content to country information and facilitates EpiSPIDER's redistribution of structured data to services that can consume them. Continuing along this transformation chain, the SIMILE Exhibit

API (http://simile.mit.edu) that consumes JSON-formatted data files enables faceted browsing of information by using scatter plots, Google Maps, and timelines.

Recently, EpiSPIDER began outsourcing some of its preprocessing and natural language processing tasks to external service providers such as OpenCalais (www.opencalais.com) and the Unified Medical Language System (UMLS) web service for concept annotation. This action has enabled the screening of noncurated news sources as well.

### Project Results

Built on open-source software components, EpiSPIDER has been operational since January 2006. In response to feedback from users, additional custom data feeds have been incorporated, both topic oriented (by disease) and format specific (KML, RSS, GeoRSS), as has semantic annotation using UMLS concept codes. For example, the EpiSPIDER KML module was developed to enable the US Directorate for National Intelligence to distribute avian influenza event-based reports in Google Earth KML format to consumers worldwide and also to enable an integrated view of ProMED and World Animal Health Information Database reports.

EpiSPIDER is used by persons in North America, Europe, Australia, and Asia, and it receives 50–90 visits/hour, originating from 150–200 sites and representing 30–50 countries worldwide. EpiSPIDER has recorded daily visits from the US Department of Agriculture, US Department of Homeland Security, US Directorate for National Intelligence, US CDC, UK Health Protection Agency, and several universities and health research organizations. In the latter half of 2008, daily access to graphs and exhibits surpassed access to data feeds. EpiSPIDER's semantically linked data were also used for validating syndromic surveillance information in OpenRODS (http://openrods.sourceforge.net) and populating disease detection portals, like www.intelink.gov and the Research Triangle Institute (Research Triangle Park, NC, USA).

## Discussion

Despite their similarities, the 3 described event-based public health surveillance systems are highly complementary; they monitor different data types, rely on varying levels of automation and human analysis, and distribute distinct information. GPHIN, being the longest in use, is probably the most mature in terms of information extraction. In contrast, HealthMap and EpiSPIDER, being comparatively recent programs, focus on providing extra structure and automation to the information extracted. Their differences and similarities, summarized in the Table, can be analyzed according to multiple characteristics: What data sources do they consider? How do they extract information from those sources? And in what format is the information redistributed and how?

For completeness, the broadest range of sources is critical. GPHIN's data comes from Factiva and Al Bawaba, which are subscription-only news aggregators. Their strategy is to rely on companies that sell the service of collecting event information from every pertinent news stream. In contrast, HealthMap's strategy is to rely on open-access news aggregators (e.g., GoogleNews and Moreover) and curated sources (e.g., ProMED and EuroSurveillance). EpiSPIDER, until recently, has concentrated on curated sources only (e.g., ProMED, GDACS, and CIA Factbook). This distinction between free and paid sources raises the question of whether the systems have access to the same event information.

After the data sources have been chosen, the next step is to extract useful information among the incoming reports. First, at the level of the report stream, the system must filter out reports that are not disease related and categorize the remaining (disease-related) reports into predefined sets. Then, at a second level of triage, the information within each retrieved alert (e.g., an event's location or reported disease) is assessed. GPHIN does this data characterization through automatic processing and human analysis, whereas HealthMap and EpiSPIDER rely mainly on automated techniques (although a person per-

Table. Characteristics of 3 primary systems that process event-based informal data sources*

| System | Data sources (languages) | Data characterization | Information formatting | Data dissemination | | |
|---|---|---|---|---|---|---|
| | | | | Access | User interface | Format |
| GPHIN | Factiva, Al Bawaba (9 languages) | Automatic and human | Categorization, machine translation, geocoded | Subscription only | Boolean and metadata query system (native) | Email alert |
| HealthMap | Google News, Moreover, ProMED, WHO, EuroSurveillance (4 languages) | Automatic | Categorization, geocoded, time coded, extra information | Open | Mapping, faceted browsing (native) | RSS feed |
| EpiSPIDER | ProMED, GDACS, CIA Factbook (English only) | Automatic | Categorization, geocoded, time coded, extra information | Open | Web exhibits, faceted browsing (imported) | RSS, JSON, KML feeds |

*GPHIN, Global Public Health Intelligence Network; WHO, World Health Organization; RSS, Really Simple Syndication; EpiSPIDER, Semantic Processing and Integration of Distributed Electronic Resources for Epidemics (and disasters); GDACS, Global Disaster Alert Coordinating System; CIA, Central Intelligence Agency; JSON, JavaScript object notation; KML, keyhole markup language.

forms a daily scan of all HealthMap alerts and a sample of EpiSpider alerts).

After a report in the data stream is determined to be relevant, it is processed for dissemination. GPHIN automatically translates the reports into different languages and grants its clients access to the database through a custom search engine. GPHIN also decides which reports should be raised to the status of alerts and sent to its clients by email. HealthMap provides a geographic and temporal panorama of ongoing epidemics through an open-access user interface. It automatically filters out the reports that do not correspond to breaking alerts. The remaining alerts are prepared for display (time codes and geocodes as well as disease category and data source) to allow faceted browsing and are linked to other information sources (e.g., the Wikipedia definition of the disease). These data are also provided as daily email digests to users interested in specific diseases and locations. Although GPHIN and HealthMap provide their own user interface, EpiSPIDER explores conventional formats for reports, adding time-coding, geocoding, and country metadata for automatic integration with other information sources and versatile browsing by using existing open-source software. These reports are displayed under the name of Web Exhibits and include, for example, a mapping and a timeline view of the reports and a scatter plot of the alerts with respect to the originating country's human development index and gross domestic product per capita.

A division arises between the HealthMap and EpiSPIDER strategies and the GPHIN strategy regarding the level of access granted to users. This division is due in part to the access policies of the data sources used by the systems, as discussed previously.

A discrepancy also exists in the amount of human expertise, and thus in the cost, required by the systems. These differences also raise the question of whether information from one system is more reliable than that of the others. Undertaking an evaluation of the systems in parallel is a critical next step. Also, all 3 systems are inherently prone to noise because most of the data sources they use or plan to use (Figure) for surveillance are not verified by public health professionals, so even if the system is supervised by a human analyst, it might still generate false alerts. False alerts need to be mitigated because they might have substantial undue economic and social consequences. Event-based disease surveillance may also benefit from algorithms linked by ontology (formal representation of a set of concepts within a domain and the relationships between those concepts) detecting precursors of disease events. Measurement and handling of input data's reliability is a critical research direction.

Future development should focus on linking these systems more closely to public health practitioners in the field and establishing collaborative networks for alert verification and dissemination. Such development would ensure that event-based monitoring further establishes itself as an invaluable public health resource that provides critical context and an alternative to more traditional indicator-based outbreak reporting.

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# Chronic Wasting Disease Prions in Elk Antler Velvet

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Chronic wasting disease (CWD) is a contagious, fatal prion disease of deer and elk that continues to emerge in new locations. To explore the means by which prions are transmitted with high efficiency among cervids, we examined prion infectivity in the apical skin layer covering the growing antler (antler velvet) by using CWD-susceptible transgenic mice and protein misfolding cyclic amplification. Our finding of prions in antler velvet of CWD-affected elk suggests that this tissue may play a role in disease transmission among cervids. Humans who consume antler velvet as a nutritional supplement are at risk for exposure to prions. The fact that CWD prion incubation times in transgenic mice expressing elk prion protein are consistently more rapid raises the possibility that residue 226, the sole primary structural difference between deer and elk prion protein, may be a major determinant of CWD pathogenesis.

Chronic wasting disease (CWD) of deer, elk, and moose is the only recognized prion disease of wild animals. To date, 15 US states and 2 Canada provinces have reported CWD in wild and/or farm-raised cervids. Outbreaks have also occurred in South Korea as a result of importation of subclinically infected animals (*1*,*2*). The unparalleled efficiency of prion transmission in cervids by a largely undefined mechanism, combined with high deer densities in certain areas of North America, complicates strategies for controlling CWD as it continues to emerge in new locations.

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Growing antlers of male cervids are covered by a highly innervated and vascularized apical skin layer, referred to as velvet, which is shed after an increase in testosterone and ossification of antlers. Our study objective was to assess whether velvet from CWD-infected elk contains prion infectivity. Our rationale was 2-fold. First, the annual shedding of this material raises the possibility that it may play a role in CWD transmission. Second, although the most likely means of human exposure to CWD prions is consumption of contaminated venison (*3*), the substantial market for velvet in traditional Asian medicine also warrants concern.

We used CWD-susceptible transgenic (Tg) mice as a sensitive means to detect prions in antler velvet. Bioassays in Tg mice expressing deer prion protein (PrP) (*4*) and newly created Tg mice expressing elk PrP, demonstrated low levels of CWD prions in antler velvet. We also show that the associated protease-resistant PrP could be amplified in vitro (for detection by Western blot) by protein misfolding cyclic amplification (PMCA). Finally, comparative CWD transmissions in Tg mice indicated that the glutamine (Q) to glutamic acid (E) variation at residue 226, which is the sole primary structural difference between deer and elk PrP, may be a major determinant of CWD pathogenesis in these 2 species.

## Materials and Methods

### Production of Transgenic Mice

Tg(CerPrP)1536$^{+/-}$ mice expressing deer PrP have been described previously (*4*). To generate Tg(CerPrP-E226) mice expressing the elk PrP coding sequence, codon 226 of the deer PrP gene (*PRNP*) (GenBank accession no.

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AF009180) was mutated from Q to E by site-directed mutagenesis (Quick Change; Stratagene, La Jolla, CA, USA). The resulting expression cassette, CerPrP-E226, highlights the single amino acid difference between deer and elk PrP at this position (*5*). The coding sequence was inserted into the MoPrP.Xho expression vector, and the purified transgene was microinjected into pronuclei of fertilized FVB/*Prnp*$^{0/0}$ oocytes. Transgenic founders were identified by PCR screening of genomic DNA. Three Tg(CerPrP-E226) founders (Tg5029, Tg5034, Tg5037) were mated to FVB/*Prnp*$^{0/0}$ mice to produce hemizygous transgenic lines. Because of equivalent levels of CerPrP-E226 expression, studies were not duplicated in the Tg5034$^{+/-}$ and Tg5037$^{+/-}$ lines. Estimates of the levels of PrP expression in 3 different Tg5037$^{+/-}$ mice and 5 different Tg5029$^{+/-}$ mice were accomplished by immuno-dot blotting and Western blotting using monoclonal antibody (MAb) 6H4 (Prionics, Schlieren, Switzerland).

### Preparation of Prion Inocula

Antler velvet and matching brain samples were obtained from 4 elk from Canada that were naturally affected with CWD. Elk 01-0306 had severe CWD-specific neuropathologic changes in the obex and neurologic signs indicative of CWD; elk 02-0306 had moderate neuropathologic changes in the obex and was clinically normal. Although information about the clinical status of elk 03-0306 and 04-0306 was not available, these elk had mild and severe neuropathologic changes, respectively, in the obex. Brain samples were also obtained from CWD-affected mule deer D10 and D92 at the Colorado Division of Wildlife, Wildlife Research Center, and from CWD-affected elk 7378 and 99W12389 at the Wyoming Game and Fish Department's Sybille Wildlife Research Unit. Homogenates of brain and antler velvet (10% wt/vol) were prepared in sterile phosphate-buffered saline (PBS) lacking $Ca^{2+}$ and $Mg^{2+}$ ions.

### Measurement of Incubation Times

Groups of 5-week-old Tg mice were given general anesthesia and inoculated with 30 μL of brain or antler velvet homogenate through a 27-gauge needle inserted into the right parietal lobe of the brain. Mice were observed 3 times a week for clinical signs indicative of prion infection, e.g., ataxia, weight loss, hyperactivity, flattened posture, absent extensor reflex, or kyphosis. The incubation period is the time between inoculation and the first day on which subsequently progressive clinical signs were identified. For endpoint titration, groups of 8 Tg mice were inoculated with $10^{-1}$ to $10^{-10}$ dilutions of a 10% brain homogenate of D92 prepared in PBS lacking $Ca^{2+}$ and $Mg^{2+}$ ions.

### Western Blotting

Protein content in 10% brain homogenates was determined by bicinchoninic acid assay. Total protein (50 μg) was then digested with 40 μg/mL proteinase K (PK) (Roche, Mannheim, Germany) in the presence of 2% sarkosyl for 1 h at 37°C. Digestion was terminated with phenylmethylsulfonyl fluoride at a final concentration of 5 μM. Proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride Immobilon-FL membranes (Millipore, Billerica, MA, USA), which were immunoprobed with MAb 6H4 followed by horseradish peroxidase–conjugated antimouse secondary antibody. Proteins were visualized by using ECL Plus (GE Healthcare, Piscataway, NJ, USA) and an FLA-5000 scanner (Fujifilm Life Science, Woodbridge, CT, USA).

### Histoblotting

Coronal cryostat sections (10 μm) were transferred to nitrocellulose and probed with MAb 6H4 after PK digestion as described previously (*6*). Images were photographed with a NikonDMX 1200F (Tokyo, Japan) digital camera in conjunction with Metamorph software (Molecular Devices, Sunnyvale, CA, USA).

### Immunohistochemical Analysis

Sections (8 μm) of formalin-fixed, paraffin-embedded mouse brains on positively charged microscope slides were deparaffinized and subjected to immunohistochemical analysis to deter the disease-associated form of PrP ($PrP^{Sc}$) as described previously (*7*). $PrP^{Sc}$ was detected with MAb 6H4 after hydrolytic autoclaving for 15 min in 10 mmol/L HCl. Biotinylated secondary antibody in conjunction with 3,3′-diaminobenzidine was used to visualize $PrP^{Sc}$.

### PMCA

Healthy Tg(CerPrP)1536$^{+/-}$ mice were perfused with PBS/5 mM EDTA. Brain homogenates (10% wt/vol) were prepared in conversion buffer consisting of PBS containing 150 mmol/L NaCl, 1.0% Triton X-100, and Roche's Complete Protease Inhibitor Cocktail. Samples were clarified by centrifugation at 500 rpm for 60 s. Each PMCA cycle consisted of 30 min incubation at 37°C followed by a 20 s sonication pulse at setting 7 using a Misonix 3000 Sonicator (Misonix, Farmingdale, NY, USA). After 96 cycles, 6 μL of the 60-μL reaction was diluted into 54 μL of fresh Tg(CerPrP)1536$^{+/-}$ substrate for a subsequent round of PMCA. Amplification products were digested with 50 μg/mL PK at 50°C for 75 min.

## Results

### CWD Prions in Elk Antler Velvet

Mean incubation periods for mice inoculated with CWD prions from elk brains were more uniform (225–

335 days) than were those for mice inoculated with CWD prions from antler velvet. Not all Tg(CerPrP)1536$^{+/-}$ mice inoculated with antler velvet developed disease; incubation times for those that did were relatively long and highly variable (Table). Clinical signs did develop in Tg(CerPrP)1536$^{+/-}$ mice challenged with antler velvet from elk 01-0306 and 03-0306; mean incubation periods were ≈440 d and ≈460 d and attack rates were 75% and 66%, respectively. Tg(CerPrP)1536$^{+/-}$ mice inoculated with antler velvet from elk 02-0306 and 04-0306 remained healthy until the mice were euthanized at ≈600 d postinoculation.

We also tested the susceptibility of Tg mice expressing elk PrP. Mean incubation times after inoculation of Tg(CerPrP-E226)5037$^{+/-}$ mice with CWD prions from brains of elk 01-0306 and 04-0306 were 174 ± 7 d and 224 ± 6 d, respectively (Table). Of the Tg(CerPrP-E226)5037$^{+/-}$ mice inoculated with antler velvet from elk 01-0306, 29% died of prion disease in <400 d, and antler velvet from elk 04-0306 failed to induce disease (Table); this finding confirmed the antler velvet transmission results in Tg(CerPrP)1536$^{+/-}$ mice.

Tg(CerPrP-E226)5037$^{+/-}$ mice express PrP at ≈5-fold the level of PrP in the brains of wild type mice, similar to transgene expression levels in Tg(CerPrP)1536$^{+/-}$ mice; the level of expression in Tg(CerPrP-E226)5029$^{+/-}$ mice was approximately equal to that in wild type mice (Figure 1). Induction of disease by CWD prions in brain from diseased elk and deer was consistently and significantly more rapid in Tg(CerPrP-E226)5037$^{+/-}$ than in Tg(CerPrP)1536$^{+/-}$ mice (Table). Mean incubation times of the D92 isolate were equivalent in Tg(CerPrP)1536$^{+/-}$ and Tg(CerPrP-E226)5029$^{+/-}$ mice (Table), which have a 5-fold difference in transgene expression levels (Figure 1).

### PrP$^{Sc}$ Accumulation and Neuropathologic Changes in Diseased Tg Mice

Diagnoses for all Tg(CerPrP)1536$^{+/-}$ and Tg(CerPrP-E226)5037$^{+/-}$ mice were confirmed by the presence or absence of protease-resistant PrP$^{Sc}$ in brains, according to Western blotting (Figure 2) and histoblotting (Figure 3) and finding of disease-specific neuropathologic changes (Figure 4). PrP$^{Sc}$ immunostaining in histoblots of diseased Tg(CerPrP)1536$^{+/-}$ and Tg(CerPrP-E226)5037$^{+/-}$ mice was punctate (Figure 3). Although cortical florid plaques were observed in the brains of diseased Tg(CerPrP)1536$^{+/-}$ mice (Figure 4, panels A and B), PrP$^{Sc}$ accumulation in Tg(CerPrP-E226)5037$^{+/-}$ mice was more diffuse and granular. The extensive loss of cerebellar granular cells and accompanying PrP$^{Sc}$ deposition that characterized disease in Tg(CerPrP-E226)5037$^{+/-}$ mice (Figure 4, panels G and H) was not noted for Tg(CerPrP)1536$^{+/-}$ mice (Figure 4, panels C and D).

### Estimates of CWD Prion Titers in Antler Velvet

A brainstem preparation from CWD-affected mule deer D92, which contained high levels of PrP$^{Sc}$ according to Western blot (data not shown), was selected for endpoint titration of CWD prions in Tg(CerPrP)1536$^{+/-}$ mice. Disease developed in all mice inoculated with the 3 lowest dilutions; mean incubation periods ranged from 268 to 390 d (Figure 5). Disease did not develop in any of the mice inoculated with the $10^{-4}$ dilution, but disease did develop after 471 d in 1 mouse from the $10^{-5}$ dilution group. The remaining mice in the $10^{-5}$ dilution group and all mice inoculated with higher dilutions remained free of disease and were euthanized after 560–645 d. The disease status of all mice was confirmed by Western blotting (data not shown). We estimated the titer of CWD prions in D92 brain tissue

Table. Transmission of chronic wasting disease prions to transgenic mice*

| Isolate from animal no. | Mice expressing deer PrP$^{C}$ | | Mice expressing elk PrP$^{C}$ | | | |
|---|---|---|---|---|---|---|
| | Tg(CerPrP)1536$^{+/-}$ | | Tg(CerPrP-E226)5037$^{+/-}$ | | Tg(CerPrP-E226)5029$^{+/-}$ | |
| | Antler velvet | Brain | Antler velvet | Brain | Brain | % Decrease† |
| 01-0306 | 442 ± 16 (6/8) | 322 ± 9 (8/8) | 305 ± 86 (2/7)‡ | 174 ± 7 (8/8) | | 46 (<0.0001) |
| 02-0306 | >594 (0/5) | 225 ± 3 (7/7) | | | | |
| 03-0306 | 463 ± 23 (2/3)§ | 335 ± 5 (7/7) | | | | |
| 04-0306 | >601 (0/6) | 281 ± 5 (7/7) | >505 (0/5) | 224 ± 6 (7/7) | | 20 (<0.0001) |
| 7378 | | 235 ± 2 (8/8) | | 177 ± 15 (7/7) | | 25 (0.0061) |
| 99W12389 | | 230 ± 9 (8/8) | | 158 ± 13 (7/7) | | 31 (0.0004) |
| D10 | | 225 ± 1 (8/8) | | 201 ± 8 (8/8) | | 11 (0.013) |
| D92 | | 268 ± 15 (7/7) | | 201 ± 15 (8/8) | 263 ± 10 (7/7) | 25 (0.0061) |
| PBS | | >510 (0/6) | | | | |
| None | | | | >592 (0/4)¶ | | |

*Incubation times are given as mean ± SEM days. Values in parentheses are no. diseased mice/no. inoculated mice. PrP$^{C}$, cellular prion protein; Tg, transgenic; PBS, phosphate-buffered saline.
†Values represent the percent decrease in mean incubation time of isolates in Tg(CerPrP-E226)5037$^{+/-}$ mice compared with Tg(CerPrP)1536$^{+/-}$ mice. p values, calculated by the Student *t*-test, are shown in parentheses.
‡The remaining 5 of 7 inoculated mice remained free of disease until 525 d postinoculation, at which time they were euthanized.
§Mice inoculated with the antler velvet preparation from elk 03-0306 had an unusually high rate of intercurrent illnesses, resulting in early euthanasia of 5 inoculated mice.
¶Four uninoculated Tg(CerPrP E2226) 5037$^{+/-}$ mice were euthanized at 437 d.

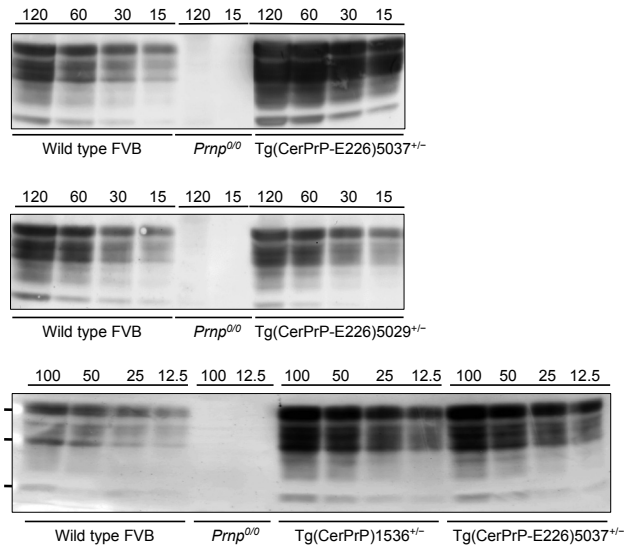


Figure 1. Levels of transgene expression in transgenic (Tg) mice expressing deer or elk cellular prion protein ($PrP^C$). Representative Western blot analysis of $PrP^C$ expression from different total protein loads in brain extracts from Tg mice Tg(CerPrP)1536$^{+/-}$, Tg(CerPrP-E226)5029$^{+/-}$, and Tg(CerPrP-E226)5037$^{+/-}$ compared with wild type and *Prnp*$^{0/0}$ mice (knock-out mice for PrP gene).

to be 6 log i.c. $ID_{50}$/g (i.c. $ID_{50}$ refers to the dose of CWD prions that produces infection in 50% of the intracerebrally inoculated Tg mice) (*8*).

The inefficient transmission of prions from antler velvet samples (Table) is consistent with low levels of CWD prions. Because the incubation times of CWD prions in antler velvet from elk 01-0306 and 03-0306 were outside the linear range of dose and incubation time (Figure 5), we were unable to assign a definitive titer. The infection attack rates <100% suggested that CWD prion titers in these 1% inocula were close to, or at, the end point of the bioassay (≤3.5 log i.c.$ID_{50}$ units).

### Amplification of $PrP^{Sc}$ in Antler Velvet Preparations

When Western blot, ELISA, and immunohistochemical analyses failed to detect $PrP^{Sc}$ in antler velvet samples from 14 CWD-affected elk, including the 4 samples analyzed by bioassay (data not shown), we attempted to amplify $PrP^{Sc}$ in antler velvet samples by using serial PMCA (Figure 6). Whereas $PrP^{Sc}$ in brain homogenates of elk 01-0306, 03-0306, and 04-0306 was efficiently amplified, even at high dilution, after 1 or 2 rounds of PMCA, $PrP^{Sc}$ in antler velvet homogenates of elk 01-0306 and 03-0306 was not amplified until either round 3 or 4. $PrP^{Sc}$ was not amplified in antler velvet from elk 04-0306 or after PMCA of negative-control preparations. $PrP^{Sc}$ amplification correlated with the transmission efficiency of CWD prions in antler velvet. The antler velvet sample from elk 01-0306, which produced detectable amplification products after 3 rounds of PMCA, caused disease in Tg(CerPrP)1536$^{+/-}$ mice (mean incubation time 442 d, attack rate 75%) and in Tg(CerPrP-E226)5037$^{+/-}$ mice. The velvet sample from elk 03-0306, which produced detectable amplification products after 4 rounds of PMCA, and resulted in a 463-d mean incubation time and a 66% attack rate. In contrast, the antler velvet sample from elk 04-0306, which failed to amplify after 4 rounds of PMCA, also failed to transmit prions to either Tg mouse line.

## Discussion

The transmission of CWD prions in antler velvet from 2 naturally affected elk to mice in 2 Tg models demonstrates that this tissue contains low, but detectable, amounts of CWD prions. In addition, serial PMCA amplified otherwise undetectable levels of $PrP^{Sc}$ in antler velvet.

We characterized CWD prion infectivity by end-point titration. The ≈6 log i.c.$ID_{50}$/g CWD prion titer estimated by this method contrasts with ≈9 log i.c.$ID_{50}$/g titers of mouse-adapted scrapie prions in rodent brains (*9*) and ≈7–7.7 log i.c.$ID_{50}$/g titers of BSE prions estimated by bioassay in transgenic mice (*10*,*11*). The linear relationship between dose and incubation time (*12*) provides an opportunity to estimate the level of prions in materials containing an unknown amount of infectivity. The attack rates of <100% after inoculation with antler velvet preparations from elk 01-0306 and 03-0306 and the failure to transmit disease from the remaining antler velvet samples suggest

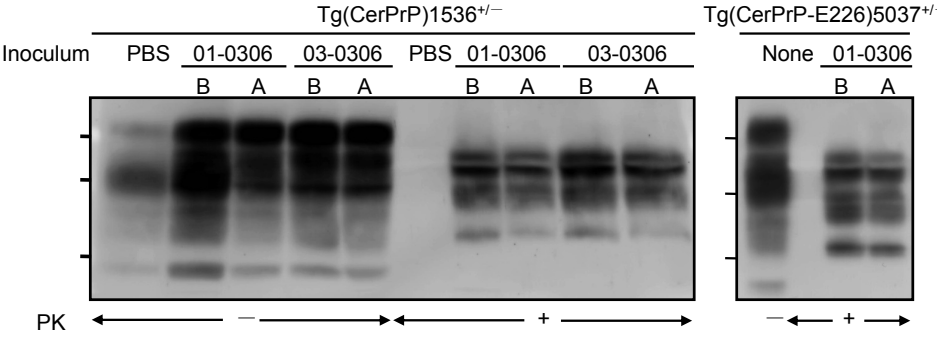


Figure 2. Accumulation of $PrP^{Sc}$ (disease-associated form of prion protein) in diseased transgenic (Tg) mice. Tg(CerPrP)1536$^{+/-}$ and Tg(CerPrPE226)5037$^{+/-}$ mice inoculated with phosphate-buffered saline (PBS), elk brain (B), or antler velvet (A) were treated with or without proteinase K (PK). Membranes were probed with monoclonal antibody 6H4. Molecular weights indicated are 37, 29, and 20 kD.

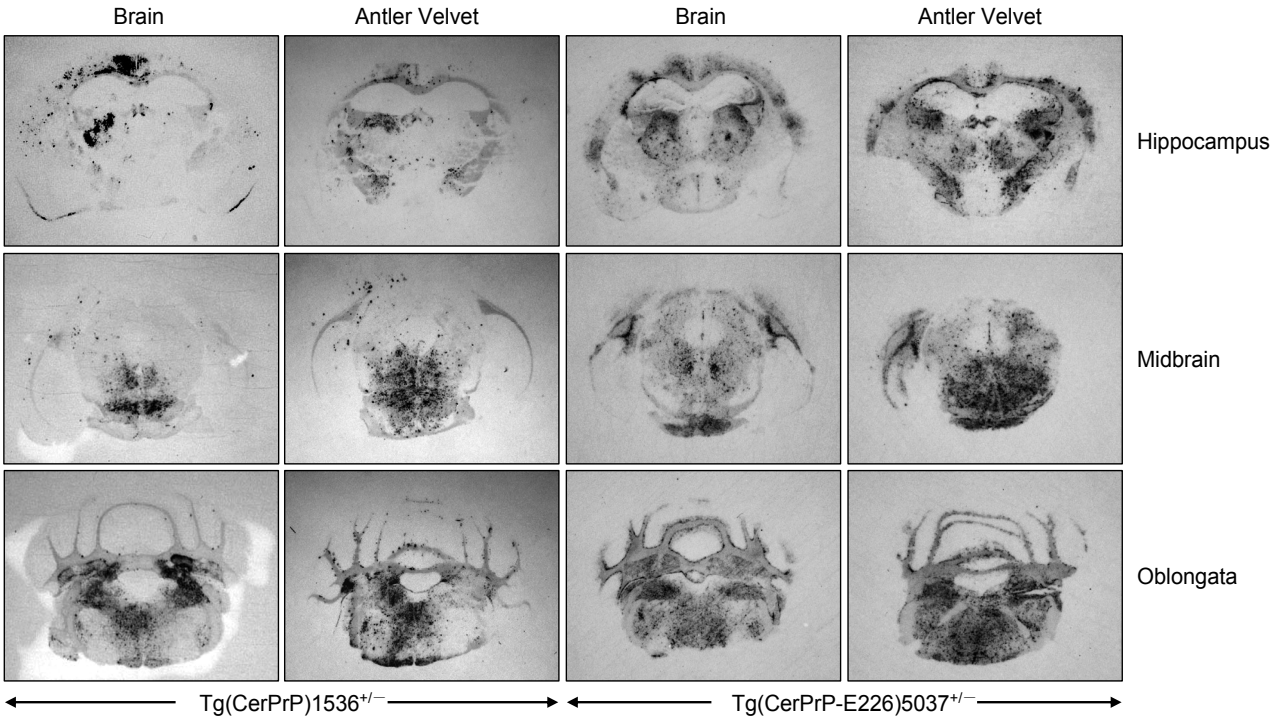


Figure 3. Distribution of $PrP^{Sc}$ (disease-associated form of prion protein) in brains of diseased mice. Histoblots of mice inoculated with 01-0306 brain or antler velvet material were treated with proteinase K and probed with monoclonal antibody 6H4. Tg, transgenic.

that CWD prion titers are close to, or at, the end point of the Tg(CerPrP)$1536^{+/-}$ bioassay. Although we are aware of the limitations of comparing levels of prions in tissues from different CWD-affected cervids, we estimated the end point of the CWD prion titration using D92 to be ≤3.5 log $i.c.ID_{50}$ units. Other factors could also influence levels of infectivity in the 4 tested samples, e.g., the portion of the antler processed and the age of the antler when harvested. Histologic evaluation indicated that the velvet samples used in these transmission studies came from elk antlers in the

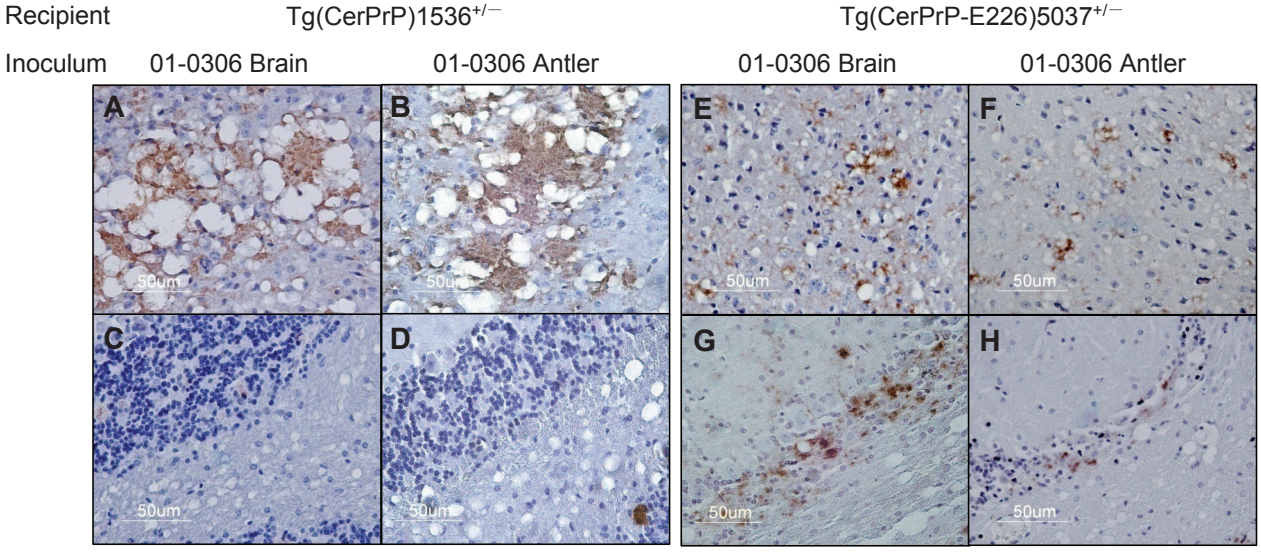


Figure 4. $PrP^{Sc}$ (disease-associated form of prion protein)–specific immunohistochemistry in the brains of diseased mice. Transgenic (Tg) mice Tg(CerPrP)$1536^{+/-}$ inoculated with brain (A) and antler velvet (B) preparations from elk 01-0306 exhibit florid $PrP^{Sc}$-reactive plaques in the cerebral cortex at the level of the thalamus but retain integrity of cerebellar granular cells (C and D). Tg(CerPrP-E226)$5037^{+/-}$ mice inoculated with brain (E) and antler velvet (F) preparations from elk 01-0306 display small plaques and diffuse granular staining in the cerebral cortex, $PrP^{Sc}$ deposition, and marked cerebellar neuronal loss (G and H).

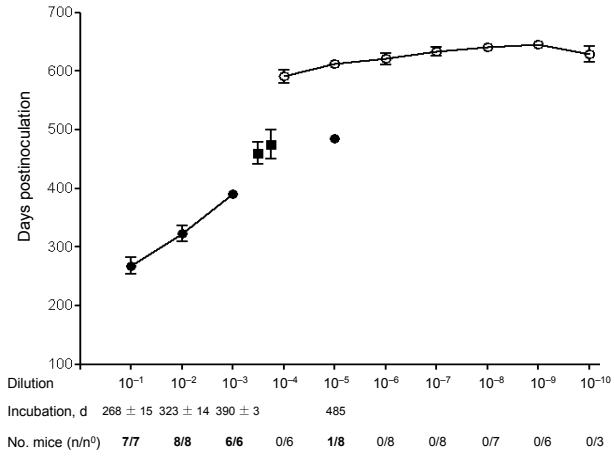


Figure 5. Quantification of chronic wasting disease prions. Diseased transgenic (Tg) mice Tg(CerPrP)1536$^{+/-}$ inoculated with dilutions of brain homogenate are indicated by filled symbols Asymptomatic mice are indicated by open circles; time at which asymptomatic aged mice were either euthanized or died of illnesses unrelated to prion disease is shown. Error bars indicate SEM. n/n$^0$ refers to the number of mice developing prion disease divided by the number of inoculated mice. Also shown are the mean incubation times of diseased Tg(CerPrP)1536$^{+/-}$ mice inoculated with antler velvet preparations (filled squares).

early stages of seasonal growth (data not shown). Whether CWD prion titers in antler velvet vary according to the state of antler growth remains to be determined. Whether prion infectivity is derived from nervous system tissue, blood (*13*), or another component of velvet, is also unclear.

### Implications for Horizontal CWD Transmission and Human Exposure

Our studies indicate that antler velvet represents a previously unrecognized source of CWD prions in the environment. Whereas oral transmission of rodent-adapted scrapie prions is known to be ≈5 orders of magnitude less efficient than transmission by intracerebral inoculation (*14*,*15*), the relative efficiency of oral CWD prion transmission is unknown. Multiple exposures to low levels of CWD prions in the environment (*16*,*17*), as well as increased infectivity when prions are bound to soil minerals (*18*), are factors that may influence transmission.

The appearance of variant Creutzfeldt-Jakob disease in humans exposed to bovine spongiform encephalopathy (BSE) (*19*,*20*) and the demonstration of CWD prions in muscle (*3*) placed the human species barrier to CWD prions at the forefront of public health concerns. Our studies indicate that antler velvet represents an additional source for human exposure to CWD prions. Widely used in traditional Asian medicine to treat a variety of ailments including impotence, arthritis, and high blood pressure, antler velvet can be readily purchased in caplet form and its usage has increased worldwide.

Fortunately, to date there is no epidemiologic evidence that rates of CJD in the CWD-endemic region (Colorado, USA) have increased (*21*,*22*). Also reassuring is the inefficient in vitro conversion of human PrP to protease-resistant PrP by CWD (*23*). Two studies have shown that CWD prions failed to induce disease in Tg mice expressing human PrP (*24*,*25*). However, the failure of BSE to be transmitted to Tg mice expressing human prion protein (HuPrP) was cited as early evidence of a BSE transmission barrier in humans (*26*); subsequent studies demonstrated a strong effect of the codon 129 polymorphism on transmissibility of BSE prions (*27*). To date, only mice expressing HuPrP with methionine at 129 have been challenged with CWD. In support of the argument that humans might be susceptible to CWD, intracerebral inoculation of squirrel monkeys produced disease after >30 months (*28*). Prion strain properties are also critical when considering the potential for interspecies transmission. The existence of multiple CWD strains has been suggested by several studies (*4*,*25*,*29*,*30*), but strain isolation and host range characterization have not been reported. Finally, it is worth considering that if CWD were to cross the species barrier into humans, this transmission source might not be recognized if the disease profile overlapped with one of the forms of sporadic CJD reported in North America.

### Possible Role for Residue 226 in CWD Pathogenesis

Previous studies that demonstrated more rapid CWD prion incubation times in Tg mice expressing elk PrP (*24*,*29*) than in Tg(CerPrP)1536$^{+/-}$ mice (*4*) raised the possibility that the single amino acid difference at residue 226 between elk and deer PrP (*5*) may influence CWD pathogenesis (*29*). However, when the transmission characteristics of CWD isolates were directly compared in Tg mice expressing differing levels of deer or elk PrP, Tamgüney et al. concluded that CWD incubation times were related solely to the level of PrP transgene expression (*25*). We compared CWD transmission in Tg(CerPrP-E226)5037$^{+/-}$ and Tg(CerPrP)1536$^{+/-}$ mice, which express PrP at levels ≈5-fold higher than PrP levels in wild type mouse brain (Figure 1A), and found that CWD transmission was consistently and substantially more rapid in Tg(CerPrP-E226)5037$^{+/-}$ mice. Our results appear compatible with more efficient CWD prion propagation by elk cellular prion protein (CerPrP$^C$) containing E at residue 226 than by deer CerPrP$^C$ containing Q at this position. Consistent with this interpretation, despite 5-fold lower levels of transgene expression in Tg(CerPrP-E226)5029$^{+/-}$ mice than in Tg(CerPrP)1536$^{+/-}$ mice, mean incubation times of the D92 isolate were equivalent in these 2 lines (Table). Nonetheless, undetected differences in CerPrP$^C$ expression, for example in particular cell types, might result in more rapid

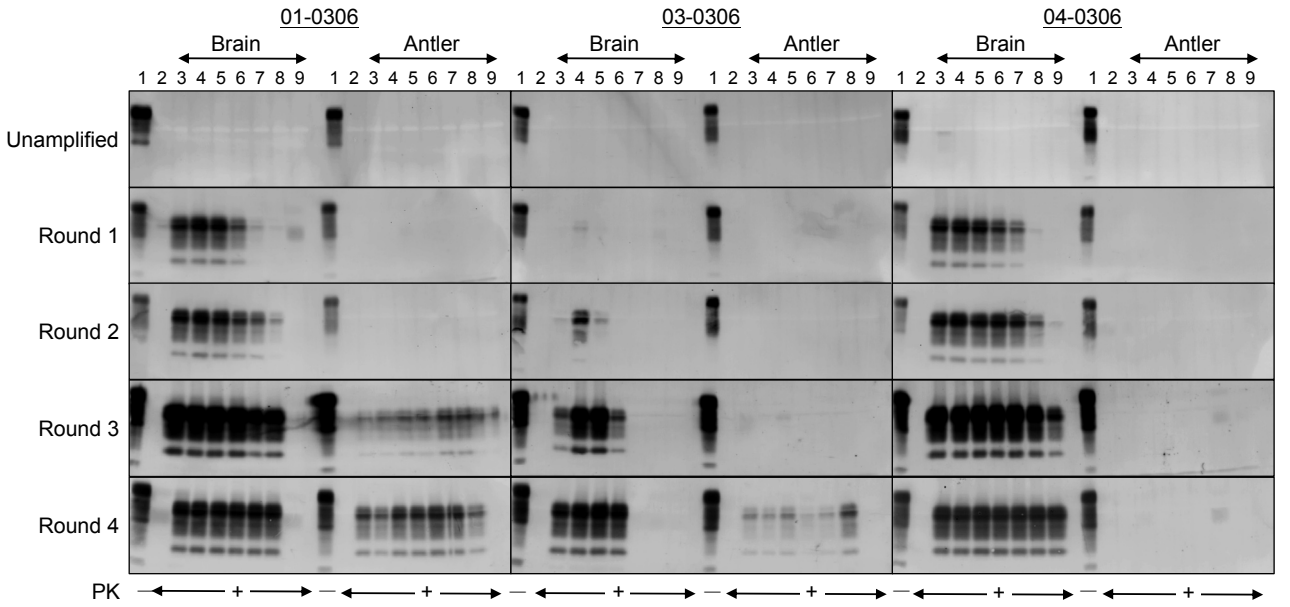


Figure 6. Detection of CerPrP$^{Sc}$ (disease-associated form of cervid prion protein [PrP]) in brain and antler velvet from chronic wasting disease (CWD)–affected elk after serial protein misfolding cyclic amplification (PMCA). Western blots demonstrate amplification of protease-resistant prion protein (PrP) after serial PMCA when seeded with brain or velvet antler material from CWD-affected elk. Brain samples: lane 1, Tg(CerPrP)1536$^{+/-}$ brain material not treated with proteinase K (PK); lane 2, Tg(CerPrP)1536$^{+/-}$ brain material used as a negative control seed for the PMCA reactions; lanes 3–9, $10^{-2}$ to $10^{-8}$ dilutions of 10% elk brain homogenate. Antler velvet samples: lane 1, Tg(CerPrP)1536$^{+/-}$ brain material not treated with PK; lane 2, Tg(CerPrP)1536$^{+/-}$ brain material used as a negative control seed for the PMCA reactions; lanes 3–9, replicate $10^{-2}$ dilutions of 10% antler velvet homogenate. Samples were either treated or not treated with PK as indicated. Membranes were probed with monoclonal antibody 6H4. Tg, transgenic.

disease and/or altered pathologic changes. The generation of transgenic mice expressing elk and deer coding sequences using gene replacement strategies would seem to be an excellent approach for resolving this issue.

The different responses to CWD in Tg mice also appear to recapitulate aspects of CWD pathogenesis in the natural hosts. Previous limited comparative transmission studies indicated that CWD developed ≈25% more rapidly in orally challenged elk than deer (*31*). Although plaques were not detected in brains of CWD-affected elk, florid plaques have been observed in the brains of diseased deer (*32*,*33*). Similar differences in pathologic changes were observed in Tg(CerPrP-E226)5037$^{+/-}$ and Tg(CerPrP)1536$^{+/-}$ mice (Figure 4). Structural analyses suggest that residue 226 is located within a region of PrP$^{C}$ proposed to interact with a factor (*34*), possibly equivalent to the postulated protein X (*35*). Although mutation of the equivalent residue from Q to lysine (K) in epitope-tagged mouse PrP had no effect on PrP$^{Sc}$ formation in transfected chronically infected ScN2A cells, the effects of the Q-to-E substitution were not assessed (*36*).

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# Virulent Strain of Hepatitis E Virus Genotype 3, Japan

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Hepatitis E virus (HEV) genotype 3, which usually causes asymptomatic infection in Japan, induced severe hepatitis in 8 patients. To better understand genetic features of HEV associated with increased virulence, we determined the complete or near-complete nucleotide sequences of HEV from these 8 patients and from 5 swine infected with genotype 3 strain swJ19. Phylogenetic analysis showed that the isolates from the 8 patients and the 5 swine grouped separately from the other genotype 3 isolates to create a unique cluster, designated JIO. The human JIO-related viruses encoded 18 amino acids different from those of the other HEV genotype 3 strains. One substitution common to almost all human HEV strains in the JIO cluster was located in the helicase domain (V239A) and may be associated with increased virulence. A zoonotic origin of JIO-related viruses is suspected because the isolates from the 5 swine also possessed the signature V239A substitution in helicase.

Hepatitis E virus (HEV) infection is relatively common. Anti-HEV antibodies are found in 10%–20% of the general population in Japan and most Asian countries (*1*,*2*); however, only a small fraction of these infections induce overt hepatitis. Although the mechanisms underlying induction of liver damage by HEV have not been well characterized, HEV genotypes seem to have distinct disease-inducing potential. HEV sequences have been classified into 4 genotypes (*3*). Genotype 1 consists of epidemic strains in developing countries of Asia and Africa. Genotype 2 is represented by the prototype sequences from an epidemic in Mexico, which have also recently been detected in Africa. Genotypes 3 and 4 are distributed worldwide and have been implicated in sporadic cases of acute hepatitis E in humans and domestic pigs. HEV genotypes 3 and 4 are found in Japan, but fulminant or severe acute hepatitis develops more frequently in persons infected with genotype 4 (*4*–*6*). The severity of liver disease may therefore be influenced by the HEV genotype with which the patient is infected as well as host factors such as age, gender, and pregnancy status.

In 1997, we identified a strain of HEV from a patient in Japan who had acute hepatitis (designated JIO) that clustered with genotype 3 sequences. From 2004 through 2006, JIO-related viruses were isolated from 7 additional patients who had acute or severe hepatitis. To better understand genetic features of HEV associated with severe hepatitis, we compared the complete or near-complete sequence of JIO isolates from these 8 patients with other well-characterized genotype 3 and 4 isolates. To determine whether these human genotype 3 sequences were zoonotic in origin, we sequenced full-length viral genomes from 5 swine infected with the swJ19 strain of HEV. These 5 animals were part of a larger outbreak of HEV infection that occurred in swine at a single farm in southern Japan during 2000–2002. The GenBank/EMBL/DDBJ accession numbers for nucleotide sequences of HEV isolates are AB291951–7/AB291960 (for the human isolates) and AB443623-7 (for the swine isolates).

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## Methods

We enrolled 8 patients who were infected with HEV genotype 3 and had clinical features of hepatitis (Table 1). A zoonotic source of HEV infection was identified for 3 of these patients: pig liver for patient 4, pig meat for patient 6, and wild boar meat for patient 7. Prothrombin time, a surrogate marker of hepatic insufficiency, averaged 63.9% (± 29.1%) of the reference range among the 8 HEV genotype 3–infected patients. Hepatitis was particularly severe in patients 3, 5, 7, and 8; at the peak of disease, prothrombin times for these patients ranged from 27% to 46% of the reference range. These sporadic HEV cases were not clustered geographically; they were distributed across several regions of Japan, including southern (Okinawa) and northern (Saitama) prefectures (Figure 1). Informed consent was obtained from all patients after the nature and purpose of the study was explained to them.

To assess possible zoonotic origins of these human infections, we sequenced HEV strain swJ19 isolates from 5 of 11 swine with previously documented infections (*7*). These animals had been raised commercially at a farm in the southern part of Miyazaki Prefecture where HEV infections were detected during 2000–2002. All animals received humane care, and the study was approved by the institutional review committee of Toshiba General Hospital, Tokyo, Japan.

To determine whether infections could be linked to a common genotype 3 virus, we compared the genetic structure and sequence homology of 8 human and 5 swine HEV strains. The entire or near-complete nucleotide sequences of the 8 JIO strain isolates from the human patients and the swJ19 strain isolates from the 5 swine were determined by a method reported previously (*8*,*9*), with some modifications. In brief, nucleic acids were extracted from serum with the QIAamp MinElute Virus Spin Kit (QIAGEN GmbH, Hilden, Germany). HEV RNA genomes were reverse transcribed, and cDNA was amplified by PCR with primers specific for 23 overlapping regions of the HEV genome. Reverse transcription and first-round PCR were conducted by using the SuperScript III One-Step RT-PCR System (Invitrogen Corporation, Carlsbad, CA, USA); second-round PCR was conducted with the Platinum Taq DNA polymerase (Invitrogen). The 5′- and 3′-terminal sequences were amplified by using the SMART RACE cDNA Amplification Kit (Clontech Laboratories Inc., Mountain View, CA, USA) and Oligo (dt)20 Primer (Invitrogen), respectively. The sequences enriched in G-C were amplified with the TaKaRa LA Taq in GC Buffer (TaKaRa Shuzo Co. Ltd., Shiga, Japan). The sequences not amplifiable by the above PCR methods were subjected to PCR with primers deduced from adjacent 5′ and 3′ sequences. The final products were sequenced in the 377 DNA Sequencer with use of the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Genetic analyses of HEV sequences were conducted by the unweighted pair-grouping method with arithmetic means by using computer software GENTYX-MAC Version 13 (Genetyx Corporation, Tokyo, Japan).

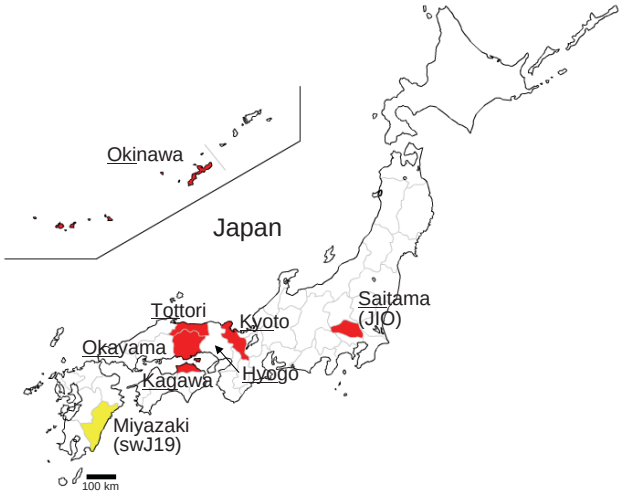


Figure 1. Map of Japan showing prefectures where human cases of hepatitis E virus have been found. Underlining indicates part of prefecture name included in isolate name; yellow indicates cases in swine; red indicates cases in humans.

Table 1. Profiles of 8 patients infected with hepatitis E virus, JIO strain, Japan*

| Patient no. | Age, y/sex | Residence | Month of disease onset | Diagnosis | Nadir PT, % | Presumed route of transmission | Isolate name |
|---|---|---|---|---|---|---|---|
| 1 | 50/M | Saitama | 1997 Apr | Acute hepatitis | 100 | Unknown | JIO-Sai97L |
| 2 | 76/M | Tottori | 2004 Jan | Acute hepatitis | 92 | Unknown | JYM-Tot04L |
| 3 | 63/M | Okinawa | 2004 May | Acute hepatitis | 46 | Unknown | JYU-Oki04L |
| 4 | 71/F | Okayama | 2004 Dec | Acute hepatitis | 75 | Pig liver | JSS-Oka04L |
| 5 | 65/M | Tottori | 2005 Jun | Acute severe hepatitis | 34 | Unknown | JIY-Tot05L |
| 6 | 78/M | Okinawa | 2005 Jul | Acute hepatitis | 92 | Pig meat | JSO-Oki05L |
| 7 | 63/M | Kagawa | 2006 Mar | Acute hepatitis | 45† | Wild boar meat | JTK-Kag06C |
| 8 | 79/M | Kyoto | 2006 Sep | Fulminant hepatitis | 27 | Unknown | JSW-Kyo-FH06L |

*PT, prothrombin time.
†Only 1 determination was made.

## Results

The prototypical isolate, JIO-Sai97L, had a genome length of 7,215 nt that contained a 5′ untranslated region (UTR), 3 open reading frames (ORFs), a 3′ UTR, and a poly-A tail. Lengths of HEV genomes from 6 other patients (JYM-Tot04L, JYU-Oki04L, JSS-Oka04L, JIY-Tot05L, JSO-Oki05L and JSW-Kyo-FH05L) were identical to that of JIO-Sai97L. An exception was the HEV isolate JTK-Kag06C from patient 7, which was slightly longer (7,236 nt). The 5 HEV isolates from swine (swJ19-1, swJ19-2, swJ19-5, swJ19-7, and swJ19-8) had genomes of 7,210 nt. The 3 ORFs of all swine and human HEV genomes had identical protein coding capacity. HEV isolates from all human patients had 97.9%–98.6% sequence homology with the prototypical JIO-Sai97L strain from patient 1. The 5 swine swJ19 isolates had 98.3%–99.9% sequence homology when compared with each other and 98.0%–99.8% homology when compared with the JIO strain from human patients.

Comparison of nucleotide sequences of the 13 human and swine HEV isolates in this study with those of published HEV genotype 3 sequences showed that the 13 complete and near-complete sequences described in this study closely matched those of 2 well-characterized genotype 3 viruses: JRA1 (89.4%–89.7% nucleotide identity) and swJ570 (88.9%–89.0% nucleotide identity). The 13 human and swine genotype 3 isolates displayed weak homology with other HEV genotypes. The B1 isolate of genotype 1 (GenBank accession no. M73218) was only 74.1%–74.7% similar to these genotype 3 viruses, the M1 isolate of genotype 2 (accession no. M74506) was only 73.6%–74.0% similar, and the T1 isolate of genotype 4 (accession no. AJ272108) was only 75.6%–76.0% similar.

Using the 13 complete or near-complete genomic sequences of HEV genotype 3 isolates described in this study (Figure 2), we constructed a phylogenetic tree. HEV sequences from the 8 patients (JTK-Kag06C, JYU-Oki04L, JSS-Oka04L, JIO-Sai97L, JSO-Oki05L, JSW-Kyo-FH06L, JIY-Tot05L, JYM-Tot04L) clustered on a branch separate from the other genotype 3 sequences, forming a distinct grouping related to the prototypical JIO strain. The swJ19 HEV sequences from the 5 swine (swJ19-1, swJ19-2, swJ19-7, swJ19-5, and swJ19-8) clustered closely with the JIO-related viruses from the human patients, indicating that the human and swine HEV isolates were highly similar (Figure 2, panel A). Another 18 swine isolates, from farms other than the 1 involved in the swJ19 outbreak, were phylogenetically distinct from those of the outbreak farms (Figure 2, panel B).

Another genotype 3 cluster was formed by 6 isolates from Hyogo Prefecture in western Japan (Figure 2, panel A). In this cluster were 5 HEV isolates from persons in whom hepatitis developed after they ate uncooked deer meat (*10*) and from serum from a local boar and a deer (*11*). Unlike the JIO-related viruses, which were broadly distributed from the most southern to northern Japanese prefectures, HEV strains responsible for the infections in Hyogo Prefecture were not commonly found in other parts

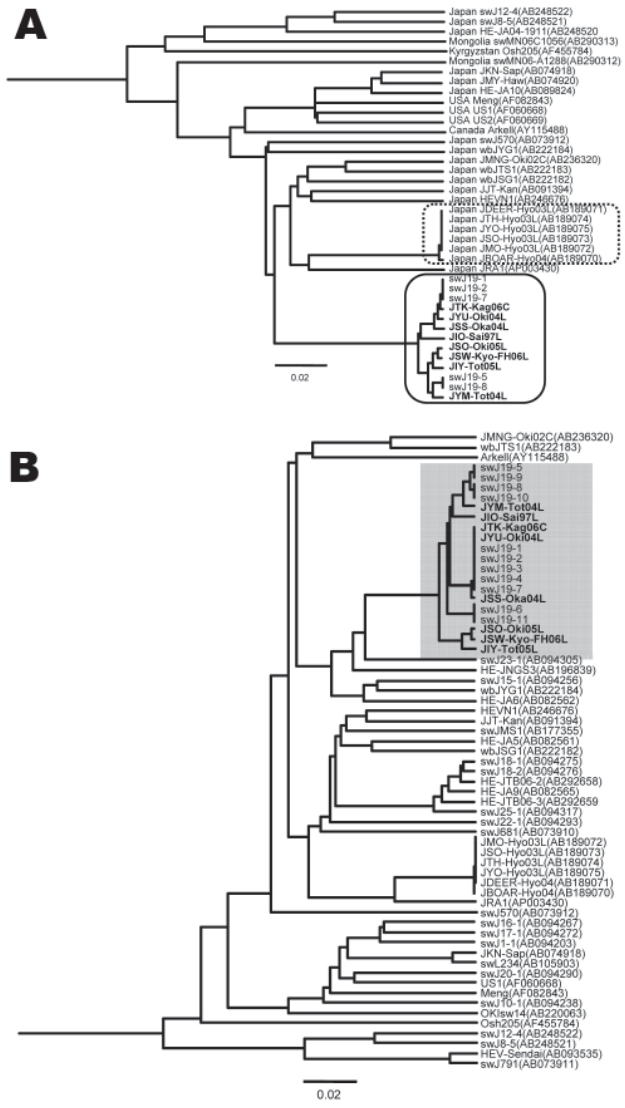


Figure 2. A) Phylogenetic tree (unweighted pair-grouping method with arithmetic means) constructed on the complete or near-complete nucleotide sequences of hepatitis E virus (HEV) genotype 3 isolates. Clustering of nucleotide sequences of 8 human patients infected with JIO strain of HEV and 5 swine infected with swJ19 strain of HEV is boxed by a solid line. Another clustering of local genotype 3 isolates from Hyogo Prefecture, Japan, is boxed by a dotted line. B) Phylogenetic tree (unweighted pair-grouping method with arithmetic means) constructed on a partial sequence of 412 nt in open reading frame (ORF) 2 (nt 5994-6405 of the US2 genome) of HEV genotype 3. Partial nucleotide sequences of 8 human JIO and 11 swine HEV swJI9 isolates (accession nos. AB094279–AB094289) are shown (shading). Analyses of full sequences of 5 of these 11 swine isolates were performed (swJ19-1, swJ19-2, swJ19-5, swJ19-7, and swJ19-8). Scale bars indicate nucleotide substitutions per site; **boldface** indicates isolates from humans.

of the country. Broad distribution of the JIO-related viruses seems to be unique in HEV epidemiology. In 2 (25%) of these 8 patients, pig liver or meat had been implicated in HEV infection.

Comparison of the 13 JIO-related viruses (Figure 2, panel A) with the other genotype 3 strains also showed 18 aa differences: 12 in ORF1, 3 in ORF2, and 3 in ORF3 (Table 2). Three mutations in the JIO strain were characteristic of genotype 4 viruses, which are typically more pathogenic than other HEV genotypes. ORF1 differences were found at amino acids 605 (serine to proline, S605P), 978 (isoleucine to valine, I978V), and 1213 (valine to alanine, V1213A). The V1213A substitution is potentially most relevant because it was not found in the prototypical isolate from patient 1 (JIO), who had mild clinical disease when infected in 1997, but was present in highly related isolates from the other 7 patients who had more severe hepatitis during 2004–2006. V1213A in ORF1 corresponds to V239A of the helicase domain, and its surrounding sequences were well conserved in HEV isolates of genotypes 3 and 4 (online Appendix Figure, available from www.cdc.gov/EID/content/15/5/704-appF.htm). Because V239A is common in genotype 4 isolates, we analyzed genomes of the genotype 3 JIO-related viruses for evidence of intergenotypic recombination. Comparison of 28 genotype 4 sequences with those of the JIO-related isolates showed no obvious signs of recombination (data not shown), which suggests that the V293A substitution arose independently in this genetically unique cluster of genotype 3 viruses. Notably, all 5 isolates recovered from swine on the Miyazaki Prefecture farm during the outbreak of 2000–2002 possessed the V239A substitution.

## Discussion

Circumstantial evidence indicates that HEV genotype influences the severity of liver disease. Remarkably, HEV seroprevalence studies in Egypt found no clinical illness in any person, including pregnant women, although most (67.7%–84.3%) had been exposed to HEV genotype 3 (*13,14*). In contrast, results of a survey of 254 patients with HEV infection in Japan showed hepatitis associated with genotype 4 to be more severe than that associated with genotype 3 (*4*). Our results showed a clustering of 8 HEV isolates of JIO strain, genotype 3, recovered from patients with clinical hepatitis.

Despite the high disease-inducing activity of the HEV JIO strain, the 8 patients infected with this strain were distributed widely over Japan. This distribution is at odds with a local cluster of genotype 3 infections restricted to persons with hepatitis and to wild animals living in Hyogo Prefecture, Japan (Figure 2, panel A) (*11*). Wide regional distribution has also been reported for some HEV genotype 4 isolates (*15*). Why JIO strains caused more severe hepatitis than might be expected for a genotype 3 virus is

Table 2. Amino acid residues in 8 human and 5 swine hepatitis E virus isolates compared with those in the other genotype 3 isolates*

| Amino acid position† | Conserved in genotype 3 | Human no. | | | | | | | | Swine no. | | | | | Conserved in genotype 4 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 1 | 2 | 3 | 4 | 5 | |
| ORF1 | | | | | | | | | | | | | | | |
| 154 | A | A | T | A | A | T | T | A | T | A | A | T | A | T | T |
| 547 | R | Q | Q | Q | Q | Q | Q | Q | Q | Q | Q | Q | Q | Q | R |
| 598 | R | Q | Q | Q | Q | Q | Q | Q | Q | Q | Q | Q | Q | Q | K |
| **605** | S | P | P | P | P | P | P | P | P | P | P | P | P | P | P |
| 721 | A | T | T | T | T | T | T | T | T | T | T | T | T | T | A |
| 807 | A | S | S | S | S | S | S | S | S | S | S | S | S | S | A |
| **978** | I | V | V | V | V | V | V | V | V | V | V | V | V | V | V |
| 979 | S | K | K | K | K | K | K | K | K | K | K | K | K | K | E |
| 1135 | I | T | T | T | T | T | T | T | T | T | T | T | T | T | V |
| **1213‡** | V | V | A | A | A | A | A | A | A | A | A | A | A | A | A |
| 1246 | Q | H | H | H | H | H | H | H | H | H | H | H | H | H | D |
| 1469 | C | S | S | S | S | S | S | S | S | S | S | S | S | S | C |
| ORF2 | | | | | | | | | | | | | | | |
| 98 | P | S | S | P | P | S | S | P | P | P | P | S | P | S | A |
| 113 | V/I | T | T | T | T | T | T | T | T | T | T | T | T | T | V |
| 660 | S | S | S | S | F | F | F | S | F | S | S | S | S | S | Y |
| ORF3 | | | | | | | | | | | | | | | |
| 91 | S | N | N | N | N | N | N | N | N | N | N | N | N | N | S |
| 97 | A | A | V | V | V | V | V | V | V | V | V | V | V | V | A |
| 98 | P | Q | Q | Q | Q | Q | Q | Q | Q | Q | Q | Q | Q | Q | P |

*Eighteen amino acids of 8 human isolates (JIO strain) and 5 swine isolates (swJ19 strain) not shared by other genotype 3 isolates. The 3 at positions 605, 978, and 1213 (**boldface**) were the same as the corresponding residues in genotype 4 isolates.
†Corresponds to the position in hepatitis E virus (HEV)-US2 (GenBank/EMBL/DDBJ accession no, AF060669) (*12*).
‡V1213A in the open reading frame (ORF) 1 polyprotein corresponds to V239A in the HEV-US2 genotype 3 isolate helicase domain within ORF1 (online Appendix Figure, available from www.cdc.gov/EID/content/15/5/704-appF.htm).

not clear, but the reason may depend on the magnitude of virus replication. Alternatively, recombination between divergent HEV strains (*16*) may have played a role. This possibility prompted us to look for any recombination of JIO strains with genotype 4 strains that cause severe hepatitis in Japan. However, we found no evidence of recombination between the JIO strain of genotype 3 HEV with which the 8 persons were infected and 28 isolates of genotype 4 retrieved from the public and our own databases. The 18 aa substitutions were unique to the 8 human JIO and 5 swine sw19 isolates and not present in other genotype 3 viruses. Three differences in ORF1 (S605P, I978V, and V1213A) were common in wild type genotype 4 but not in genotype 3 isolates (Table 2). Because S605P and I978V are located in an ORF1 region that has high sequence divergence, they are unlikely to be responsible for an enhanced disease-inducing capacity. In contrast, V1213A changes at amino acid 239 of helicase, an enzyme capable of enhancing the efficiency of viral replication (*17*), were detected in 7 of the 8 patients (online Appendix Figure). Indeed, the helicase region of the prototypical JIO-Sai97L isolated in 1997 did not contain this amino acid polymorphism. Remarkably, all 5 swine isolates recovered in Miyazaki Prefecture during 2000–2002 belonged to the JIO strain and possessed V1213A (*hel*V239A). Taken together, the evidence strongly suggests a zoonotic origin for the 8 human HEV infections with JIO-related viruses.

Experimental and circumstantial evidence suggests that *hel*V239A may have enhanced the helicase activity of the genotype 3 JIO strain to levels comparable with those of the more pathogenic genotype 4 viruses. However, the role of *hel*V239A in enhancing helicase activity should be evaluated in vitro in future studies; its role in inducing hepatitis is yet to be confirmed. In addition, the effect of other mutations of JIO strains need to be fully explored before a conclusion can be drawn regarding the hepatitis-inducing capacities of this strain of HEV.

Findings from this study have public health implications. Because farm swine constitute a melting pot for generating various HEV mutants, at least in Japan where virtually all swine become infected with HEV within 4 months of birth, it is conceivable that virulent HEV mutant(s) arise on pig farms. Such occurrence has been described for influenza, for which point mutations are associated with increased virulence (*18*,*19*); for example, mutant influenza viruses that arose on chicken farms in Hong Kong in 1997 were transmitted to humans and had fatal consequences (*20*,*21*). In addition, although a vaccine against HEV has recently been developed (*22*), a vaccination strategy for humans and animals has yet to be defined. The results of our study indicate that selective vaccination of farm swine bearing HEV isolates of high virulence, such as those of the JIO strain in Miyazaki Prefecture, should be recommended to decrease the incidence of fulminant or severe acute hepatitis E in Japan and elsewhere in the world.

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# A Case–Control Study on the Origin of Atypical Scrapie in Sheep, France

**Alexandre Fediaevsky, Eric Morignat, Christian Ducrot, and Didier Calavas**

A matched case–control study (95 cases and 220 controls) was designed to study risk factors for atypical scrapie in sheep in France. We analyzed contacts with animals from other flocks, lambing and feeding practices, and exposure to toxic substances. Data on the *prnp* genotype were collected for some case and control animals and included in a complementary analysis. Sheep dairy farms had a higher risk for scrapie (odds ratio [OR] 15.1, 95% confidence interval [CI] 3.3–69.7). Lower risk was associated with organic farms (OR 0.15, 95% CI 0.02–1.26), feeding corn silage (OR 0.16, 95% CI 0.05–0.53), and feeding vitamin and mineral supplements (OR 0.6, 95% CI 0.32–1.14). Genetic effects were quantitatively important but only marginally changed estimates of other variables. We did not find any risk factor associated with an infectious origin of scrapie. Atypical scrapie could be a spontaneous disease influenced by genetic and metabolic factors.

Atypical scrapie is a transmissible spongiform encephalopathy (TSE) of small ruminants; it was recently defined by the European Food Safety Authority according to phenotypic features (*1*). This disease was identified in 1998 (*2*), and little is known about its etiology and epidemiology (*3*), which contrasts with the etiology and epidemiology of classical scrapie (*4*). Diagnosis of atypical scrapie is impaired by discrepant clinical diagnostic results (*5*) of rapid diagnostic tests and variable accumulation of scrapie prion protein ($PrP^{Sc}$) in the brainstem (*3*). In France, the average apparent prevalence of atypical scrapie detected by active surveillance with tests recommended by the European Food Safety Authority (*6*,*7*) for the detection of this disease in brainstem samples was 6 cases/10,000 tested animals during 2002–2006. This prevalence was comparable elsewhere in Europe (*8*).

The origin of atypical scrapie is still unclear, and whether the disease has an infectious origin remains a major question. This disease has been transmitted experimentally to Tg-mice (*9*) and sheep (*10*), but histopathologic features of atypical scrapie have suggested similarities with human spontaneous TSE (Gerstmann-Sträussler-Scheinker syndrome) (*2*,*11*). The few reports on >1 case of atypical scrapie in the same flock provide insufficient information to draw conclusions on natural transmission of this disease (*3*). If atypical scrapie had an infectious origin, it could be influenced by risk factors associated with a pattern of infectious disease transmission as described for classical scrapie (*12*–*14*). In 2006, a case–control study of atypical scrapie in Norway did not detect such factors, but it showed that the removal of the placenta at lambing could have a protective effect (*15*). Feeding of vitamin and mineral supplements showed an adverse effect, which was interpreted as interaction of some minerals with cellular prion protein (*16*,*17*), rather than a feed contamination. Such an effect warrants confirmation.

Genetic factors should be considered when investigating risk factors for atypical scrapie because some mutations of the *prnp* gene, which codes for prion protein (PrP), modify the risk for this disease (*2*,*18*,*19*). Because all described genotypes of the *prnp* gene confer susceptibility to sheep, a purely genetic origin is unlikely but a confounding effect could occur.

Other possible origins for atypical scrapie could involve exposure to toxic substances, particularly pesticides, which were shown to be involved with other neurodegenerative diseases involving protein disorders such as Par-

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kinson disease and Alzheimer disease (*20*,*21*). Some biochemical mechanisms for these diseases could be similar. To confirm the findings of Hopp et al. (*15*) and to explore further hypotheses on risk factors for atypical scrapie, we conducted a case–control study of sheep in France.

## Materials and Methods

### Study Design

The epidemiologic unit was the animal, and most of the data collected concerned its birth cohort, assuming that in each flock all animals born during the same birth campaign ($C_0$, defined from July 1 of year n – 1 to June 30 of year n) shared the same exposure. Cases and controls were matched by frequency matching on their birth cohort ($C_0$) so that their distributions were similar over the birth campaigns.

Cases were recruited among cases detected by the active surveillance program during January 2006–March 2007. The index case had to be a female that was born and reared all its life in the same flock, with a known $C_0$. A total of 137 cases met these criteria.

Two controls per case were selected. The control animal was an animal born in $C_0$, kept until birth campaign 2006 ($C_{2006}$) in the same flock, and originated from a control flock randomly selected among the list of flocks from which ≥1 sheep had been tested in 2006 with a test recommended for the detection of atypical scrapie. All results from TSE rapid tests on sheep from control flocks had to be negative for atypical scrapie and classical scrapie. Each control had 9 replacement animals randomly selected from sheep flocks from the same county (French département).

Flocks of case and control animals were required to have no history of scrapie and >20 ewes kept for reproduction. Males were not included in the study because they have a low incidence of atypical scrapie and because farming practices used with rams are different from those used with ewes.

### Data Collection

Four persons interviewed farmers during the summer of 2007. The questionnaire, which was available in French on request, was divided in 5 parts: 1) 13 questions on structure and economic context of the flock, 2) 7 questions on purchase of sheep and contacts with other flocks, 3) 3 questions on lambing management, 4) 16 questions on feeding practices including list of feed, and 5) 8 questions on exposure to toxic products, including the list of products used. Questions related to structure of the flock were asked for $C_0$ and $C_{2006}$ to check if changes had occurred. Questions related to exposure during the first months of life were asked only for $C_0$, questions related to general feed exposure were asked for the period between $C_0$ and the 2 subsequent reproduction campaigns ($C_0$–$C_{0+2}$) and questions related to exposure to toxic products and mineral feeding were asked for $C_0$–$C_{2006}$. For each flock, the number of animals tested with a recommended test for atypical scrapie during active surveillance programs during 2002–2006 was extracted from the Base Nationale des Encéphalopathies Spongiformes Transmissibles Animales.

*Prnp* genotypes at codons 136, 141, 154, and 171 were determined by Labogena (Jouy en Josas; France). For cases, material examined consisted of a sample of soft tissue (muscle or ear) or brainstem. For controls, the matching constraint was relaxed to enable interviewers to sample some hair from any ewe born during $C_0$–$C_{0+2}$.

### Data Management

Data were entered into a Microsoft (Redmond, WA, USA) Access 2000 database. All statistical analyses were performed by using R 2.6.1 for Windows (*22*).

Three types of toxic exposure were assessed: pesticides on crops, insecticides on premises, and antiparasitic treatments. For each category, active components of products reported were identified from databases (*23*–*26*). The Direction des Végétaux et de l'Environnement from the Agence Française de Sécurité Sanitaire des Aliments specified the known or suspected neurotoxic components and their mechanism of action. Three categorical variables were created and were assigned a value of 1 for a case of exposure to any neurotoxic product of the category of concern during $C_0$–$C_{2006}$ and a value of 0 otherwise. Missing values from the questionnaire were imputed by using available covariates as predictors after verifying that the missingness pattern was compatible with random missing (*27*).

Genotypes were linearly classified into 5 levels of risk on a log scale (Table 1) according to the odds ratio (OR) estimated for the sheep population in France (*19*). Many genotypes were missing, mainly for controls because of

Table 1. Genotypes grouped by levels of genetic risk for atypical scrapie in sheep, France*

| Group | Genotypes of *prnp* gene | Coded level |
|---|---|---|
| 1 | ALRR-ALRQ, ALRR-VLRQ, ALRQ-ALRQ, ALRQ-ALRH, ALRQ-VLRQ | 0 |
| 2 | ALRR-ALRR, ALRR-ALRH, VLRQ-VLRQ | 1 |
| 3 | ALHQ-ALRH, ALHQ-VLRQ, AFRQ-ALRH, ALRH-ALRH, AFRQ-VLRQ, ALRH-VLRQ | 2 |
| 4 | ALRR-ALHQ, ALRR-AFRQ, ALHQ-ALRQ, AFRQ-ALRQ | 3 |
| 5 | ALHQ-ALHQ, ALHQ-AFRQ, AFRQ-AFRQ | 4 |

*Groups showed homogeneous odds ratios for atypical scrapie. The level of risk is the value of the corresponding log linear variable introduced into the multivariate model. *prnp*, prion protein.

difficulties in extracting DNA from hair samples (n = 117) and for a few cases because of unsuitable samples (n = 13). Missing values for controls were randomly imputed by using distribution of genotypes per breed. From the distribution of all available genotypes of cases of atypical scrapie in France, 20 datasets were imputed. To account for geographic distribution of flocks, France was divided into 9 sheep production areas according to sheep farming density and production patterns.

### Univariate and Multivariate Analyses

Analyses were conditional to the matching variable and based on univariate and multivariate generalized linear mixed models with the logit link function for the outcome and $C_0$ as a random coefficient (*28*,*29*). ORs and their 95% confidence intervals were derived from the coefficient estimates and variance parameters. When variables could not be introduced simultaneously in the multivariate analysis because they were collinear, the most biologically sound variable was selected.

Variables for the multivariate model were selected according to the recommendations of Hosmer and Lemeshow (*28*). Candidate variables for the multivariate model were backward selected according to the log-likelihood ratio test. Candidate variables with a p value <20% in univariate analyses were tested before other variables were tested. The effect of variables with a p value ≥20% on the coefficient parameter of the selected variables was then verified 1 at a time. Best parameterization of continuous variables and statistical significance of interactions terms were then checked. A false discovery rate (FDR) (*30*) was calculated by using p values of the log-likelihood ratio tests for tested variables and interaction terms.

A complementary model was used to assess if genetics influenced stability of the final model. For each of the datasets imputed, level of genetic risk was introduced in the final model as an ordinal covariate; coefficients, standard errors, and Wald test p values of different variables were inferred according to the method of Little and Rubin (*27*).

### Sensitivity Analysis

The national database used to sample controls did not enable us to take into account the size of the flocks. Therefore, counties with a large percentage of small flocks (<20 ewes) may have been overrepresented. To assess the influence of geographic selection bias, we conducted a sensitivity analysis by using 2 methods: 1) weighting of controls in the final model with weights being defined for each county as the ratio of the percentage of flocks >20 ewes in the county divided by the percentage of flocks >20 ewes at the national scale, and 2) introduction of sheep production areas as random coefficients in the final model.

## Results

### Study Population

Among 137 selected farms containing cases, 11 did not satisfy the selection criteria. In addition, 11 farmers refused to participate and 20 could not be reached. A total of 95 cases were included in the study. For controls, 1,131 farmers were contacted to participate in the study; 621 controls did not satisfy the selection criteria (374 because flocks had <20 ewes, 41 because matching criteria could not be satisfied, 20 because flocks had <20 ewes and matching criteria were not satisfied, and 186 because of other reasons)**.** In addition, 124 farmers refused to participate and 161 could not be reached. A total of 225 controls were included in the study.

The 95 cases and 225 controls were located throughout France (Figure 1). Case animals were born during 1994–2005. Because of exclusion of cases independent of the selection of their matched controls, the average ratio of controls per case was 2.4 instead of 2 and varied according to $C_0$ (Figure 2). There were a few missing values for questionnaire variables (0.4%).

### Univariate Analyses

Flocks containing cases were larger than flocks containing controls, had more animals tested for TSEs, and were present more often on sheep dairy farms (Table 2). Moreover, these 3 variables showed a significant correlation (Pearson coefficient of correlation between size of flock and number of animals tested $\rho = 0.67$, Spearman coefficients of correlation between size of flocks and dairy production $\rho = 0.23$ and between number of animals tested

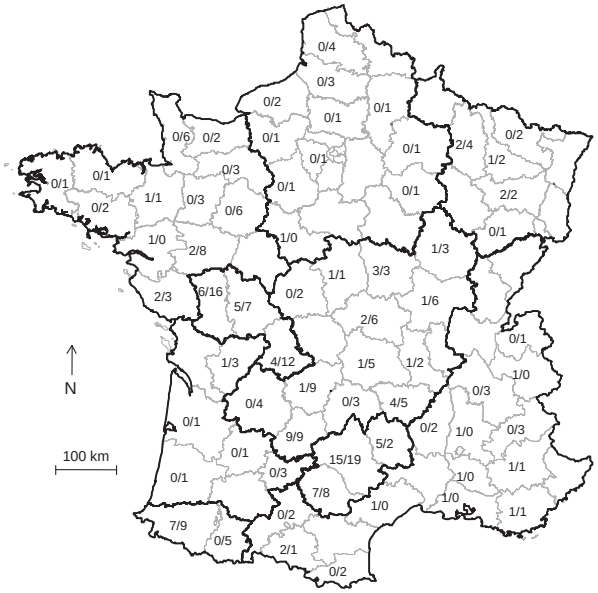


Figure 1. Distribution of cases of atypical scrapie and controls (no. cases/no. controls) in sheep, France, 2007. Sheep production areas are outlined in black, and counties are outlined in gray.

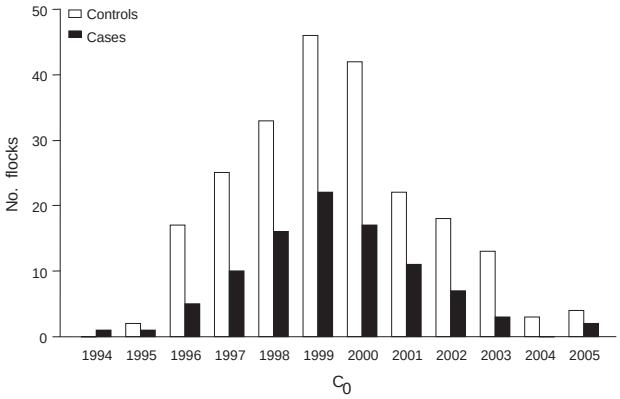


Figure 2. Distribution of $C_0$ for cases of atypical scrapie and controls in sheep, France, 1994–2005. $C_0$, birth cohort assuming that in each flock all animals born during the same birth campaign (defined from July 1 of year n – 1 to June 30 of year n) shared the same exposure.

and dairy production $\rho = 0.20$, $p<0.001$). None of the variables associated with a hypothesis of infectious origin was associated with atypical scrapie (Table 3).

Variables associated with feeding practices were not associated with increased risk for atypical scrapie (Table 4). Feeding milk replacers, which was negatively correlated with dairy production (Spearman coefficient of correlation $\rho = –0.41$, $p<0.001$), and feeding corn silage showed an inverse association with atypical scrapie.

Pesticides and insecticides on the premises correlated with an increased risk for atypical scrapie (Table 5). These 2 variables correlated with dairy production (Spearman coefficients of correlation $\rho = 0.33$ and $\rho = 0.39$, respectively, $p<0.001$).

### Multivariate Analyses

The set of candidate variables included 36 categorical variables and 1 continuous variable (Tables 6, 7). The final

Table 2. Univariate analyses of farm structure variables conditional to $C_0$ for atypical scrapie in sheep, France*

| Variable | Controls | Cases | Odds ratio | p value |
|---|---|---|---|---|
| Mean ± SE no. animals tested during 2002–2006 | 15 ± 17 | 32 ± 29 | 1.04 | <0.001 |
| Sheep dairy farm | | | | |
| No | 196 | 64 | 3.3 | <0.001 |
| Yes | 29 | 31 | | |
| Flock of familial origin | | | | |
| No | 70 | 24 | | |
| Yes | 155 | 71 | 1.3 | 0.29 |
| Flock of external origin | | | | |
| No | 126 | 58 | | |
| Yes | 99 | 37 | 0.8 | 0.41 |
| Member of a producer organization during $C_0$–$C_{2006}$ | | | | |
| No | 104 | 35 | | |
| Yes | 121 | 60 | 1.5 | 0.12 |
| Follow-up of farm results during $C_0$–$C_{2006}$ | | | | |
| No | 112 | 36 | | |
| Yes | 113 | 59 | 1.6 | 0.05 |
| Organic farm during $C_0$–$C_{2006}$ | | | | |
| No | 211 | 94 | | |
| Yes | 14 | 1 | 0.2 | 0.08 |
| Sent flock animals to breeding centers during $C_0$–$C_{2006}$ | | | | |
| No | 192 | 63 | | |
| Yes | 33 | 32 | 3.0 | <0.001 |
| Presence of cows during $C_0$–$C_{0+2}$ | | | | |
| No | 115 | 67 | 0.4 | <0.001 |
| Yes | 110 | 28 | | |
| Presence of goats during $C_0$–$C_{0+2}$ | | | | |
| No | 200 | 81 | 1.4 | 0.36 |
| Yes | 25 | 14 | | |
| Presence of pigs during $C_0$–$C_{0+2}$ | | | | |
| No | 216 | 93 | 0.5 | 0.40 |
| Yes | 9 | 2 | | |
| Presence of poultry during $C_0$–$C_{0+2}$ | | | | |
| No | 209 | 89 | 0.9 | 0.80 |
| Yes | 16 | 6 | | |

*$C_0$, birth cohort assuming that in each flock all animals born during the same birth campaign (defined from July 1 of year n – 1 to June 30 of year n) shared the same exposure; SE, standard error; $C_{2006}$, birth campaign 2006; $C_0$–$C_{0+2}$, period between $C_0$ and the 2 subsequent reproduction campaigns.

Table 3. Univariate analyses of contact with sheep from other flocks and afterbirth exposure variables conditional to $C_0$ for atypical scrapie in sheep, France*

| Variable | Controls | Cases | Odds ratio | p value |
|---|---|---|---|---|
| Contact with other flocks during $C_0$–$C_{2006}$ | | | | |
| No | 189 | 79 | 1.1 | 0.85 |
| Yes | 36 | 16 | | |
| Purchase of rams during $C_0$–$C_{2006}$ | | | | |
| No | 30 | 17 | 0.7 | 0.29 |
| Yes | 195 | 78 | | |
| Purchase of ewes during $C_0$–$C_{2006}$ | | | | |
| No | 139 | 68 | 0.6 | 0.10 |
| Yes | 86 | 27 | | |
| No. flocks of origin of ewes purchased during $C_{2005}$–$C_{2006}$ | | | | |
| 0 | 139 | 68 | 1.0 | 0.22 |
| 1 | 35 | 13 | 0.8 | |
| 2 | 25 | 9 | 0.7 | |
| ≥4 | 26 | 5 | 0.4 | |
| Disposal of placenta in $C_0$ | | | | |
| Never | 82 | 37 | 1.0 | 0.51 |
| Sometimes | 36 | 19 | 1.2 | |
| Always | 107 | 39 | 0.8 | |
| Use of adoption cases in $C_0$ | | | | |
| No | 41 | 12 | 1.5 | 0.22 |
| Yes | 184 | 83 | | |

*$C_0$, birth cohort assuming that in each flock all animals born during the same birth campaign (defined from July 1 of year n – 1 to June 30 of year n) shared the same exposure; $C_{2006}$, birth campaign 2006; $C_{2005}$, birth campaign 2005.

model included 5 variables and 1 interaction term (Table 6). The random coefficient had a null variance, and the scale parameter was close to 1, which indicated an absence of shrinkage.

No variable associated with a hypothesis of infectious origin was present in the final model. The number of animals tested and sheep dairy farming were associated with disease (Table 7). Organic farms, feeding corn silage, and use of vitamin and mineral supplements showed an inverse association with disease. Use of these supplements, which was not significant by univariate analysis, was significant after adjustment for sheep dairy farming, and the 2 variables had a significant interaction term. The uncontrolled FDR for our analysis was 33%, which indicated that one third of the variables in the final model were spurious.

After we introduced the genetic effect, estimates of other variables did not vary by >25% of their initial values (Table 8). In addition, genetics showed a strong effect; OR for atypical scrapie ranged from 2.6 for genotypes in group 2 to 48.4 for genotypes in group 5 (Table 1).

### Sensitivity Analysis

Sensitivity analysis to check possible geographic selection bias led to the same results as analysis without taking into account geographic selection bias when either weighting of samples (Figure 3) or adjusting for sheep production areas (Figure 1) was used. These results indicate that putative bias had no statistical effect on the results.

Table 4. Univariate analyses of feeding component variables during the specified period and conditional to $C_0$ for atypical scrapie in sheep, France*

| Variable | Controls | Cases | Odds ratio | p value |
|---|---|---|---|---|
| Lambs fed milk replacers in $C_0$ | | | | |
| No | 68 | 42 | 0.5 | 0.02 |
| Yes | 157 | 53 | | |
| Corn silage in $C_0$ | | | | |
| No | 195 | 90 | 0.4 | 0.04 |
| Yes | 30 | 5 | | |
| Beet root in $C_0$ | | | | |
| No | 185 | 86 | 0.5 | 0.06 |
| Yes | 40 | 9 | | |
| Straw in $C_0$ | | | | |
| No | 77 | 23 | 1.6 | 0.08 |
| Yes | 148 | 72 | | |
| Oil cake in $C_0$ | | | | |
| No | 164 | 73 | 0.8 | 0.47 |
| Yes | 61 | 22 | | |
| Compound feed in $C_0$ | | | | |
| No | 78 | 28 | 1.3 | 0.37 |
| Yes | 147 | 67 | | |
| Grass silage in $C_0$ | | | | |
| No | 195 | 77 | 1.5 | 0.20 |
| Yes | 30 | 18 | | |
| Grain in $C_0$ | | | | |
| No | 45 | 18 | 1.1 | 0.83 |
| Yes | 180 | 77 | | |
| Molasses in $C_0$ | | | | |
| No | 212 | 88 | 1.3 | 0.59 |
| Yes | 13 | 7 | | |
| Vitamin and mineral supplements in $C_0$ | | | | |
| No | 102 | 48 | 0.8 | 0.40 |
| Yes | 123 | 47 | | |
| Salt licks (pure salt) during $C_0$–$C_{2006}$ | | | | |
| No | 7 | 2 | 1.5 | 0.62 |
| Yes | 218 | 93 | | |
| Salt licks with minerals during $C_0$–$C_{2006}$ | | | | |
| No | 46 | 28 | 0.6 | 0.08 |
| Yes | 179 | 67 | | |
| Other ruminants feed during $C_0$–$C_{0+2}$ | | | | |
| No | 205 | 90 | 0.6 | 0.27 |
| Yes | 20 | 5 | | |
| Other ruminants minerals during $C_0$–$C_{0+2}$ | | | | |
| No | 203 | 85 | 1.1 | 0.84 |
| Yes | 22 | 10 | | |
| Pig feed during $C_0$–$C_{0+2}$ | | | | |
| No | 209 | 89 | 0.9 | 0.80 |
| Yes | 16 | 6 | | |
| Poultry feed during $C_0$–$C_{0+2}$ | | | | |
| No | 193 | 80 | 1.1 | 0.71 |
| Yes | 32 | 15 | | |

*$C_0$, birth cohort assuming that in each flock all animals born during the same birth campaign (defined from July 1 of year n – 1 to June 30 of year n) shared the same exposure; $C_{2006}$, birth campaign 2006; $C_0$–$C_{0+2}$, period between $C_0$ and the 2 subsequent reproduction campaigns.

Table 5. Univariate analyses of exposure to toxic product variables during the specified period and conditional to $C_0$ for atypical scrapie in sheep, France*

| Variable | Controls | Cases | Odds ratio | p value |
|---|---|---|---|---|
| Use of mineral drugs during $C_0$–$C_{2006}$ | | | | |
| No | 99 | 38 | 1.2 | 0.50 |
| Yes | 126 | 57 | | |
| Pesticides containing neurotoxic components used on crops during $C_0$–$C_{2006}$ | | | | |
| No | 155 | 51 | 1.9 | 0.009 |
| Yes | 70 | 44 | | |
| Insecticides containing neurotoxic components used on premises during $C_0$–$C_{2006}$ | | | | |
| No | 169 | 55 | 2.2 | 0.002 |
| Yes | 56 | 40 | | |
| Antiparasitic treatments containing neurotoxic components during $C_0$–$C_{2006}$ | | | | |
| No | 100 | 47 | 0.8 | 0.42 |
| Yes | 125 | 48 | | |

*$C_0$, birth cohort assuming that in each flock all animals born during the same birth campaign (defined from July 1 of year n – 1 to June 30 of year n) shared the same exposure; $C_{2006}$, birth campaign 2006.

## Discussion

There was no evidence of a relationship between risk for atypical scrapie and factors related to an infectious origin of the disease in France. Our results are consistent with those of Hopp et al (*15*). The only difference in the study by Hopp et al. was that removal of the placenta was associated with a decreased risk for disease. Other studies of atypical scrapie also suggested that the disease could have a noninfectious origin (*3*). The variables in our dataset were not associated with disease, and their corresponding ORs in univariate and multivariate analyses, regardless of the parameterization of the variables, were ≈1, which suggests that they did not tend to be risk factors.

These results contrasts with those of case–control studies on classical scrapies, which found relationships between risk for disease and introduction of ewes (*12*–*14*), grazing on the same pasture as other flocks (*14*), exposure to placenta (*13*), and feeding concentrates (*12*). Green et al. reported that flocks in which cases of scrapie (atypical or classical) were detected were larger and had more exchange of animals between flocks than control flocks (*31*). When they studied movement of sheep between flocks of equivalent status, flocks positive for classical scrapie were interconnected but flocks positive for atypical scrapie were not. These authors indicated that their results were compatible with atypical scrapie not being infectious.

Our results showed the influence of nutritional and metabolic factors. Although sheep dairy farming covers a broad category of farms with many factors, sheep dairy farms often use more sophisticated technology, and dairy ewes are more exposed to metabolic disorders because of high levels of exported nutrients, including minerals, during milk production. Thus, some feed components such as vitamin and mineral supplements or corn silage could alleviate the risk for disease. This lessening of risk may also occur with less harsh farming conditions found on organic farms.

There is evidence suggesting that minerals, especially copper, manganese, and zinc, could play a role in the physiopathology of prion diseases (*16*,*17*). In contrast with our results, Hopp et al. (*15*) found that feeding of vitamin and mineral supplements was associated with atypical scrapie. The difference in the association between atypical scrapie and vitamin and mineral supplements in the 2 studies could be explained by differences in local conditions or roles of some minerals (*17*). Proper balance of minerals is complex because many interactions occur between mineral intake, diet of the animals, and physiologic conditions. Results

Table 6. Multivariate analyses coefficient parameters of the final model for atypical scrapie in sheep, France*

| Variable | Coefficient (β) | Standard error (β) | p value† |
|---|---|---|---|
| Random coefficient | 0 | $2.00 \times 10^{-5}$ | |
| Intercept | −1.51 | 0.24 | $2 \times 10^{-10}$ |
| No. animals tested during 2002–2006 | 0.04 | 0.01 | $6 \times 10^{-10}$ |
| Sheep dairy farm | 2.71 | 0.78 | $2 \times 10^{-5}$ |
| Organic farm | −1.88 | 1.08 | 0.03 |
| Corn silage in $C_0$ | −1.81 | 0.59 | $5 \times 10^{-4}$ |
| Vitamin and mineral supplements in $C_0$ | −0.51 | 0.33 | 0.02 |
| Interaction term between sheep dairy farm and vitamin and mineral supplements in $C_0$ | −1.69 | 0.88 | 0.04 |

*For categorical variables, the reference value was no. $C_0$, birth cohort assuming that in each flock all animals born during the same birth campaign (defined from July 1 of year n – 1 to June 30 of year n) shared the same exposure.
†By log likelihood ratio test.

Table 7. Adjusted odds ratios of atypical scrapie associated with variables computed from the final model in sheep, France*

| Variable | Adjusted odds ratio | 95% CI |
|---|---|---|
| No. animals tested increased by 5 | 1.22 | 1.11–1.35 |
| Sheep dairy farm when vitamin and mineral supplements not given | 15.06 | 3.25–69.73 |
| Sheep dairy farm when vitamin and mineral supplements given | 2.77 | 1.21–6.37 |
| Organic farm | 0.15 | 0.02–1.26 |
| Corn silage | 0.16 | 0.05–0.53 |
| Vitamin and mineral supplements used on sheep dairy farms | 0.18 | 0.03–1.04 |
| Vitamin and mineral supplements not used on sheep dairy farms | 0.6 | 0.32–1.14 |

*CI, confidence interval.

of the few epidemiologic studies conducted on this topic (*32*–*35*) were rarely conclusive but such associations are difficult to demonstrate with observational data.

Among different mechanisms that should be explored to understand occurrence of atypical scrapie, we believe that toxic exposure should not be overlooked. In our study, organic farms were less at risk, and univariate analysis showed that pesticides on crops and insecticides on the premises were risk factors for disease. Multivariate analysis showed a confounding effect of dairy farming but this finding should not rule out a possible effect of pesticides. Organophosphates and pyrethroids were frequently identified among reported products; these 2 groups of active components, which may be associated with Parkinson disease (*21*,*36*), interfere with the mitochondrial chain, which is a possible pathway for prion diseases (*37*). Another study on toxic exposures and gene–environment interaction could be more conclusive regarding this issue (*38*). In that regard, flocks recently exposed to insecticide treatment for control of bluetongue vectors could constitute interesting cohorts to follow up for atypical scrapie.

Genetic factors had no confounding effect on variables in the final model, and associated ORs were high (≤48 for animals with the highest genetic risk). This high magnitude of risk, compared with other factors, suggests that genetic factors play a role in the epidemiology of atypical scrapie. However, exploring the role of genetic factors in the origin of atypical scrapie would require estimation of penetrance of different mutations, but this estimate is not currently available. In addition, genetic factors are known to be a major risk factor for classical scrapie despite a contagious origin (*4*).

In an exploratory study such as ours, interpretation of variables in the final model is not straightforward. Particular caution should be given to the risk for purely statistical associations and to selection, classification, or confusion biases. The risk for purely statistical associations increases with the number of variables tested. An FDR estimates the rate of such spurious associations (*30*), which was high in our final model (FDR 33%). To limit the FDR to a low value (5%), one would only consider as confident results variables with a p value $<6 \times 10^{-4}$, which are the number of animals tested, dairy farming, and use of corn silage. However, a high FDR should not prevent discussion of findings. Rather, this value provides a safeguard against overinterpretation of results when testing many hypotheses.

We identified a geographic selection bias in selection of controls. However, sensitivity analysis indicated that this bias did not influence the results. Misclassification problems for some variables could not be excluded, especially those regarding recall bias and memory failure. To minimize these problems, farming documents and account books were used when available, and some data were directly obtained from national databases.

Year of birth could have been a confounding factor because cases of atypical scrapie are usually found in old animals, and exposure to risk factors could be time-dependant. We matched controls on the birth cohort and accounted for

Table 8. Multivariate analysis including genetic risk from multiple imputation parameters for atypical scrapie in sheep, France*

| Variable | Coefficient (β) | Standard error (β) | p value† |
|---|---|---|---|
| Intercept | −3.03 | 0.37 | $7 \times 10^{-16}$ |
| Level of genetic risk‡ | 0.97 | 0.13 | $1 \times 10^{-13}$ |
| No. animals tested during 2002–2006 | 0.03 | 0.01 | $5 \times 10^{-5}$ |
| Sheep dairy farm | 2.52 | 0.96 | $8 \times 10^{-3}$ |
| Organic farm | −2.38 | 1.29 | 0.07 |
| Corn silage in $C_0$ | −1.48 | 0.68 | 0.03 |
| Vitamin and mineral supplements in $C_0$ | −0.40 | 0.40 | 0.31 |
| Interaction term between sheep dairy farm and vitamin and mineral supplements in $C_0$ | −1.99 | 1.09 | 0.07 |

*Genetic risk from multiple imputation parameters was estimated by the method of Little and Rubin (*27*). $C_0$, birth cohort assuming that in each flock all animals born during the same birth campaign (defined from July 1 of year n – 1 to June 30 of year n) shared the same exposure.
†By Wald test.
‡Because the variable is ordinal, the odds ratio (OR) for a given level of genetic risk is the exponential of the coded level (see Table 1) multiplied by the estimated coefficient β. The ORs for groups 2–5 are 2.6, 7.0, 18.4, and 48.4, respectively.

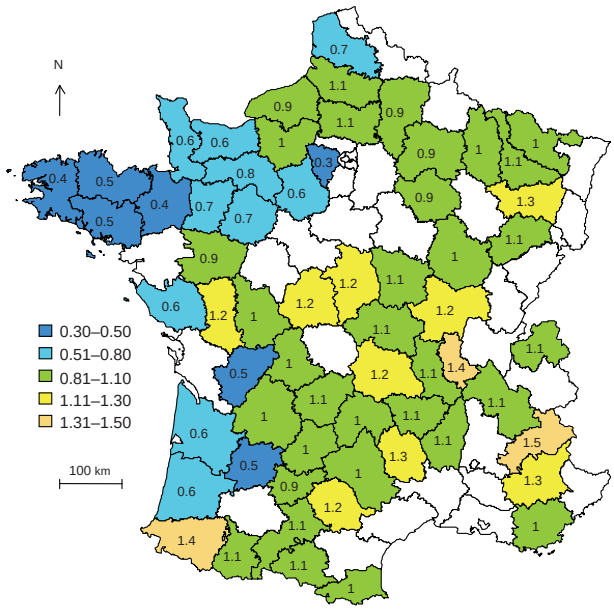


Figure 3. Distribution of control weightings calculated as the ratio of the percentage of flocks with >20 ewes in the county over the average percentage of flocks with >20 ewes for atypical scrapie in sheep, in France, 2007. Ranges represent classes of weightings.

year of birth as a random coefficient in a generalized linear mixed model that is recommended with this design (*28*,*29*). Null variance of the random coefficient indicated that there was no variation of risk between different birth cohorts.

In several studies on scrapie (atypical or classical), size of the flock was a risk factor for disease (*12–14*,*31*,*39*,*40*). There are many possible relationships between size and status of the animals. In the hypothesis of infectious origin, larger flocks are more exposed because of increased contacts, whereas in the hypothesis of spontaneous disease, expectancy of the number of cases increases with the size of the flock. Farming practices could also affect risk for developing disease and could be linked to the size of the flock. Moreover, larger flocks have higher number of animals tested as part of active surveillance, which increases the probability of detecting an animal with disease. The number of animals tested is determined by a combination of many structural factors that involve the size of the flock, local conditions of implementation of the surveillance program, mortality and culling rates, and use of a TSE qualification program. We could not simultaneously adjust for size of the flock and number of animal tested, and we prioritized control of surveillance bias. However, the 2 options were numerically equivalent.

Genetic analysis suggested no confounding effect but a strong association with the disease. However, results must be interpreted with caution because sensitivity analysis was conducted after imputing missing data for 53% of the controls and 13% of the cases.

Our final model suggested that atypical scrapie in sheep could be a spontaneous disease with a genetic determinant and possible influence of environmental and metabolic factors. On the basis of our results, there was no risk factor linked to an infectious origin. In particular, atypical scrapie is unlikely to originate from purchase of sheep. Other epidemiologic approaches such as spatial analyses or surveys on occurrence of secondary cases could help substantiate these findings. If infectious origin is confirmed, this finding would indicate that movement limitations of animals from flocks positive for atypical scrapie would not be a key measure in controlling the disease.

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# New Respiratory Enterovirus and Recombinant Rhinoviruses among Circulating Picornaviruses

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Rhinoviruses and enteroviruses are leading causes of respiratory infections. To evaluate genotypic diversity and identify forces shaping picornavirus evolution, we screened persons with respiratory illnesses by using rhinovirus-specific or generic real-time PCR assays. We then sequenced the 5′ untranslated region, capsid protein VP1, and protease precursor 3CD regions of virus-positive samples. Subsequent phylogenetic analysis identified the large genotypic diversity of rhinoviruses circulating in humans. We identified and completed the genome sequence of a new enterovirus genotype associated with respiratory symptoms and acute otitis media, confirming the close relationship between rhinoviruses and enteroviruses and the need to detect both viruses in respiratory specimens. Finally, we identified recombinants among circulating rhinoviruses and mapped their recombination sites, thereby demonstrating that rhinoviruses can recombine in their natural host. This study clarifies the diversity and explains the reasons for evolution of these viruses.

Human rhinoviruses (HRVs) and enteroviruses (HEVs) are leading causes of infection in humans. These 2 picornaviruses share an identical genomic organization, have similar functional RNA secondary structures, and are classified within the same genus (www.ictvonline.org/virusTaxonomy.asp) because of their high sequence homology (*1*). However, despite their common genomic features, these 2 groups of viruses have different phenotypic characteristics. In vivo, rhinoviruses are restricted to the respiratory tract, whereas enteroviruses infect primarily the gastrointestinal tract and can spread to other sites such as the central nervous system. However, some enteroviruses exhibit specific respiratory tropism and thus have properties similar to rhinoviruses (*2*–*5*). In vitro, most HRVs and HEVs differ by their optimal growth temperature, acid tolerance, receptor usage, and cell tropism. The genomic basis for these phenotypic differences between similar viruses is not yet fully understood.

HRVs and HEVs are characterized by ≈100 serotypes. Recently, molecular diagnostic tools have shown that this diversity expands beyond those predefined serotypes and encompasses also previously unrecognized rhinovirus and enterovirus genotypes. As an example, a new HRV lineage named HRV-C was recently identified and now complements the 2 previously known A and B lineages (*6*–*8*) (N.J. Knowles, pers. comm.). The C lineage has not only a distinct phylogeny (*9*–*16*) but is also characterized by specific cis-acting RNA structures (*17*).

In this study, we screened a large number of persons with acute respiratory diseases by using assays designed to overcome the diversity of both rhinoviruses and enteroviruses circulating in humans. Whenever possible, we systematically sequenced 5′ untranslated region (UTR), capsid protein VP1, and protease precursor 3CD regions of strains. Our goals were 1) to characterize the diversity of circulating rhinoviruses and, to a lesser extent, enteroviruses, to identify putative new picornavirus variants, and 2) to assess whether recombination may drive HRV evolution, which has not been shown in natural human infections (*18*).

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## Materials and Methods

### RNA Extraction, Reverse Transcription-PCR, and Real-Time PCR

Reverse transcription–PCR (Superscript II; Invitrogen, Carlsbad, CA, USA) was performed on RNA extracted by using the HCV Amplicor Specimen Preparation kit (Roche, Indianapolis, IN, USA), TRIzol (Invitrogen), or the QIAamp Viral RNA Mini kit (QIAGEN, Valencia, CA, USA). Real-time PCR specific for HRV-A, HRV-B, and HEV (*19*), and a generic panenterhino real-time PCR (forward primer 5′-AGCCTGCGTGGCKGCC-3′, reverse primer 5′-GAAACACGGACACCCAAAGTAGT-3′, and probe 5-FAM-CTCCGGCCCCTGAATGYGGCTAA-TAMRA-3′), were performed in several cohort studies (Table).

### Clinical Specimens

Picornavirus-positive samples were detected from patients enrolled in cohort studies in different regions of Switzerland during 1999–2008. The main characteristics of these populations, type of respiratory specimens, and screening methods are shown in the Table. The rhinovirus serotypes used for 3CD sequencing were obtained from the American Type Culture Collection (Manassas, VA, USA).

### PCR and Sequencing

Sequencing was performed directly from the clinical specimen except for samples selected by routine isolation methods on human embryonic (HE) primary fibroblast cell lines (Table) or for HRV reference serotypes. Primers used to amplify the 5′-UTR and the VP1 and 3CD regions are listed in online Technical Appendix 1 Table 1A (available from www.cdc.gov/EID/content/15/5/719-Techapp1.pdf).

Full-length genome sequences of CL-1231094, a related clinical strain of enterovirus, and partial sequences of CL-Fnp5 and CL-QJ274218 were obtained as follows. RNA extracted by using the QIAamp Viral RNA Mini kit (QIAGEN) plus DNase treatment or with Trizol was reverse transcribed with random-tagged primer FR26RV-N and amplified with the SMART RACE cDNA Amplification kit (Clontech, Mountain View, CA, USA) with a specific forward primer and FR20RV reverse primer (online Technical Appendix 1 Table 1B) (*23*). Amplification products were separated by electrophoresis on agarose gels and fragments (0.6–2.5 kb) were extracted by using the QIAquick Gel Extraction kit (QIAGEN). Purified products were cloned by using the TOPO TA cloning kit (Invitrogen).

Minipreps were prepared from individual colonies and clones with the largest inserts were chosen for sequencing. Sequences obtained were used to design a new forward primer (online Technical Appendix 1 Table 1) to advance toward the 3′ end of the genome. PCR products of 3′ genomic ends were obtained by using the BD Smart

Table. Characteristics of screened study populations and respiratory samples, Switzerland*

| Type of study (no. enrolled) | Age group | Patient characteristics | Years of study | Type of specimens | PCR | No. (%) positive | Reference |
|---|---|---|---|---|---|---|---|
| Respiratory infection in newborns (243) | <1 y | Nonhospitalized children with initial respiratory episode with cough | 1999–2005 | NPS | HRV-A and HRV-B specific real time for the first 203 and panenterhino for 40 | 36 (15) | (*20*) |
| Lower respiratory tract infection in hospitalized patients (147) | Adults | Mainly immunocompromised patients with lower respiratory tract complications and comorbidities | 2001–2003 | BAL, NPS | HRV-A and HRV-B specific real time | 16 (11) | (*21*) |
| Acute respiratory tract infection in children (653) | <17 y | Nonhospitalized children with AOM or pneumonia | 2004–2007 | NPS | Panenterhino | 121 (18) | (*22*) and ongoing study |
| Lower respiratory tract infection in hospitalized patients (485) | Adults | Mainly immunocompromised patients with lower respiratory tract complications and concurrent illnesses | 2003–2006 | BAL, NPS | Panenterhino | 52 (11) | (*21*) and ongoing study |
| Acute respiratory tract infection in children (64) | <12 y | Children at an emergency department with fever and acute respiratory symptoms treated with antimicrobial drugs | 2006–2007 | NPS | Panenterhino | 23 (36) | NP |
| Isolation in routine procedures (NA) | Children and adults | Hospitalized patients | 1999–2008 | BAL, NPS | HE culture isolation | NA | NP |

*NPS, nasopharyngeal samples; HRV, human rhinovirus; BAL, bronchoalveolar lavage; AOM, acute otitis media; NP, not published; NA, not available; HE, human embryonic primary fibroblast cell line.

Race cDNA amplification kit (Becton Dickinson, Franklin Lakes, NJ, USA) according to manufacturer's instructions. All PCR products were purified by using microcon columns (Millipore, Billerica, MA, USA) and sequenced by using the ABI Prism 3130XL DNA Sequencer (Applied Biosystems, Foster City, CA, USA). Chromatograms were imported for proofreading with the vector NTI Advance 10 program (Invitrogen). Overlapping fragments were assembled with the contigExpress module of the vector NTI Advance 10.

### Sequence Analysis, Phylogeny, and Bootscanning of Recombinants

Alignments were constructed by using MUSCLE (*24*) with a maximum of 64 iterations. (For detailed analyses, see http://cegg.unige.ch/picornavirus.) Multiple FastA was converted into PHYLIP format (for tree building) with the EMBOSS program Seqret (*25*). Trees were built with PhyML (*26*) by using the general time reversible model, BIONJ for the initial tree, and optimized tree topology and branch lengths. Trees with <50 species and larger trees used 16 and 8 rate categories, respectively. Transition/transversion ratios, proportions of invariant sites, and shape parameters of the γ distribution were estimated.

To investigate the hypothesis of recombination and map the breakpoints, we adapted the bootscanning method (*27*) as follows. The alignment was sliced into windows of constant size and fixed overlap and a 100-replicate maximum-likelihood (using HRV-93 as an outgroup) was computed for each window. From each tree, the distance between the candidate recombinant and all other sequences was extracted. This extraction yielded a matrix of distances for each window and for each alignment position. A threshold was defined as the lowest distance plus a fraction (15%) of the difference between the highest and lowest distances. The nearest neighbors of the candidate recombinant were defined as sequences at a distance smaller than this threshold. This distance ensured that the nearest neighbor, as well as any close relative, was always included. Possible recombination breakpoints thus corresponded to changes of nearest neighbors. Serotypes included in this analysis represented serotypes close to CL-013775 and CL-073908 on the basis of 5′-UTR and VP1 phlyogenetic trees (online Technical Appendix 2 Figure 1, panels A, B, available from www.cdc.gov/EID/content/15/5/719-Techapp2.pdf), as well as serotypes close to CL-135587 on the basis of VP1 and 3CD phlyogenetic trees (online Technical Appendix 2 Figure 1, panels B, C) and whose full-length sequence was available.

Distance matrices were computed from alignments with the distmat program in EMBOSS (http://bioweb2.pasteur.fr/docs/EMBOSS/embossdata.html) by using the Tamura distance correction. This method uses transition and transversion rates and takes into account the deviation of GC content from the expected value of 50%. Gap and ambiguous positions were ignored. Final values were then converted to similarity matrices by subtracting each value from 100.

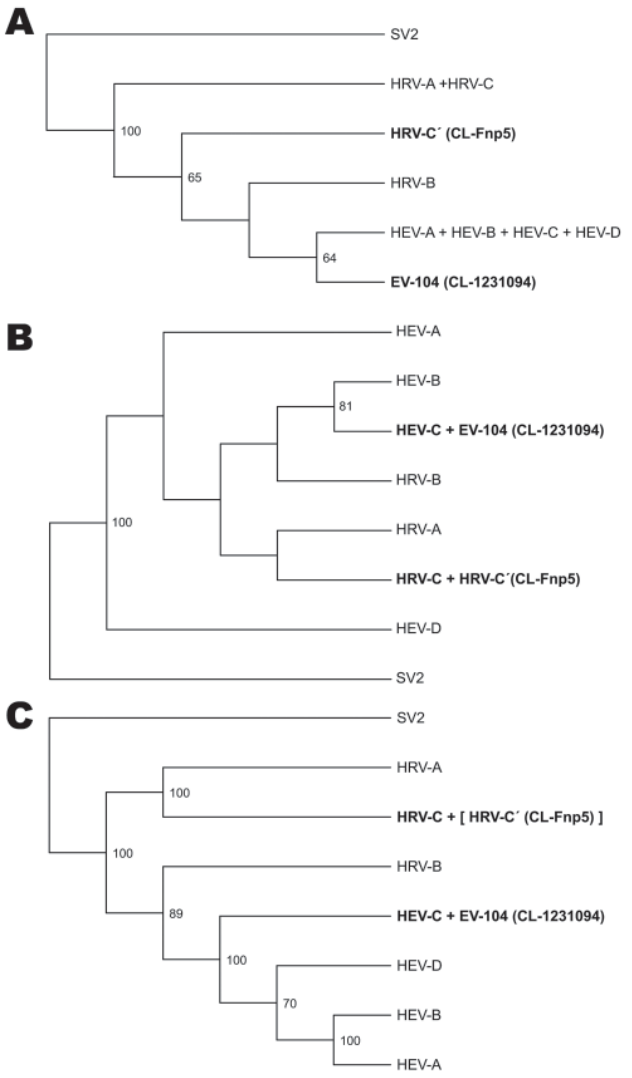


Figure 1. 5′ untranslated region (A), capsid protein VP1 (B), and complete genome (C) phylogeny of the virus clades studied. Trees were produced by condensing the full phylogeny shown in online Technical Appendix 2 Figure 1, panels A, B, and D (available from www.cdc.gov/EID/content/15/5/719-Techapp2.pdf). Human rhinovirus C′ (HRV-C′) includes the divergent rhinoviruses described in 2007 (*13*) and a related clinical strain (CL-Fnp5). HRV-C includes the new clade described since 2006 (*9–14,16*). Enterovirus 104 (EV-104) and the related strain CL-1231094 refer to a previously unknown enterovirus clade described in this study. In panel C, HRV-C′ is shown in brackets to indicate its expected location (based on VP1 and 3D sequences). Simian picornavirus 1 (SV2) was used as an outgroup. HEV, human enterovirus. Bootstrap support values <50 are not shown in the trees. New viruses are shown in **boldface**.

## Results

### Screening of Persons with Respiratory Tract Infections

Persons enrolled in several cohorts of children and adults with respiratory infections (Table) were screened for picornavirus by culture isolation on HE cell lines, real-time PCR specific for HRV-A and HRV-B (*19*), or by a panenterhino real-time PCR designed to theoretically detect all rhinoviruses and enteroviruses with publicly available sequences. Of 1,592 respiratory samples tested by real-time PCR, 248 were virus positive (Table). The 5′-UTR sequences were obtained for 77 real-time PCR or culture-positive samples and VP1 and 3CD sequences for 48 of these (Table; online Technical Appendix 1 Table 2). In parallel, the 3CD sequences were identified for all reference serotypes. The results of this screening are summarized in online Technical Appendix 1 Table 2, and all sequences are available from GenBank (accession nos. EU840726–EU840988).

On the basis of these results, respiratory infections caused by HRV-B might be less frequent than those caused by HRV-A, and HRV-A infections are distributed among the whole library of reference serotypes. A specific real-time PCR used to detect enteroviruses in respiratory specimens from some of the cohorts studied indicated that these viruses are rare in children (2.5% vs. 6.3% for HRV) and even rarer or absent in adults (0% vs. 24% for HRV) (*28*).

### Phylogeny and Molecular Epidemiology of 5′-UTR

To include all 99 HRV reference strains and new divergent rhinoviruses described recently by Lee et al. (*13*), we reconstructed a phylogenetic tree (online Technical Appendix 2 Figure 1, panel A) on the basis of a sequence of 280 nt in the 5′-UTR. This sequence provided a correct clustering of HRV-A, HRV-B, and HEV strains according to the accepted whole-genome phylogeny (online Technical Appendix 2 Figure 1, panel D) (*15*) but did not resolve appropriately the phylogeny of the 4 HEV species and the HRV-A and HRV-C viruses. The condensed tree version (Figure 1, panel A) enabled us to identify 2 groups phylogenetically distant from all previously known HRVs and HEVs. The first group, referred to as HRV-C′, contained some of our clinical samples and rhinoviruses sequenced by Lee et al. (*13*). The second group was a new clade and was named EV-104. This clade included 8 clinical samples collected in different regions of Switzerland without direct epidemiologic links (online Technical Appendix 1 Table 2).

### Identification of HRV-C Viruses by Sequencing of HRV Viruses with Divergent 5′-UTRs

Characterization of HRVs newly identified during 2006–2008 showed that they all belong to the same HRV-C species (*9*–*16*). Recently, Lee et al. (*13*) identified another cluster of viruses (HRV-C′; Figure 1, panel A) and suggested that this group was phylogenetically distinct from all other HRVs on the basis of analysis of their 5′-UTR sequences. To define the phylogeny, we adapted a previously described method (*23*) to complete the genome sequence directly from our clinical strains (CL-Fnp5 and CL-QJ274218) that showed a similar divergent 5′-UTR (online Technical Appendix 2 Figure 1, panel A). A condensed version (Figure 1, panel B) of the phylogenetic tree based on VP1 sequences (online Technical Appendix 2 Figure 1, panel B) indicated that CL-Fnp5 clustered with the new HRV-C clade, a finding further confirmed by CL-QJ 274218 partial sequences. This finding supports the view that new HRVs variants described since 2006 (*9*–*16*) all belong to the same lineage.

### New Divergent Lineage of HEV Species C

As shown in Figure 1, panel A, the panenterhino real-time PCR enabled detection of a new HEV strain phylogenetically distinct from all previously known HEV species and associated with respiratory diseases. Enterovirus-specific real-time PCRs or reference VP1 primer sets routinely used to type enteroviruses (primers 222 and 224 and nested primers AN88 and 89) (*29*,*30*) did not amplify this new genotype. We could not grow this virus on HeLa and HE cell lines. Consequently, we applied the method described above to complete the genome sequence directly from the CL-1231094 (EU840733) clinical specimen. VP1 and full-length genome sequences showed that, albeit divergent at the 5′-UTR level, this new variant belonged to the HEV-C species (Figure 1, panels B, C). Full-length genome phylogenetic tree (Figure 2) and VP1 protein identity plots (online Technical Appendix 2 Figure 2) with all members of the HEV-C species indicated that this virus represents a new HEV-C genotype that shares 68%, 66%, and 63% nucleotide and 77%, 75%, and 68% amino acid sequence identity, respectively, with coxsackieviruses A19 (CV-A19), A22, and A1, the closest serotypes. This new virus was named EV-104 (www.picornastudygroup.com/types/enterovirus_genus.htm).

Specific primers (Ent_P1.29/P2.13 and Ent_P3.30/P3.32; online Technical Appendix 1 Table 1C) were then designed to amplify the VP1 and 3D regions of the 7 other samples of this cluster collected from children with acute respiratory tract infections and otitis media. VP1 nucleotide homology among these strains was 94%–98%, except for 1 distantly related sample (74%–76%), which may represent an additional genotype. Additional sequencing is ongoing to verify this assumption.

At the 5′-UTR level, the strain described by Lee et al. (*13*) and EV-104 diverged from other members of HRV-C and HEV-C species, respectively. Thus, the 5′-UTR–based

phylogeny was inconsistent with that based on VP1 sequences and suggested possible recombination events (Figure 1, panels A, B). Because the 5′-UTR is the target of most molecular diagnostic assays, this sequence divergence needs to be taken into account in future studies.

### Recombination Events between 5′-UTR, VP1, and 3CD Genome Regions

Other studies have provided sequences of clinical strains, but genetic characterization was often limited to 1 genomic region. Our goal was to sequence 3 genomic regions for each analyzed strain to determine definitively whether recombination events could represent a driving force for the evolution of rhinoviruses in their natural environment. Although recombination events have been suggested for reference serotypes, they have never been shown for circulating clinical strains (*18*,*31*,*32*). In contrast, recombination is well established as a driving force of enterovirus evolution. Thus, we completed the 5′-UTR, VP1, and 3CD sequences of 43 clinical strains by using a pool of adapted and degenerated primers (online Technical Appendix 1 Table 1A).

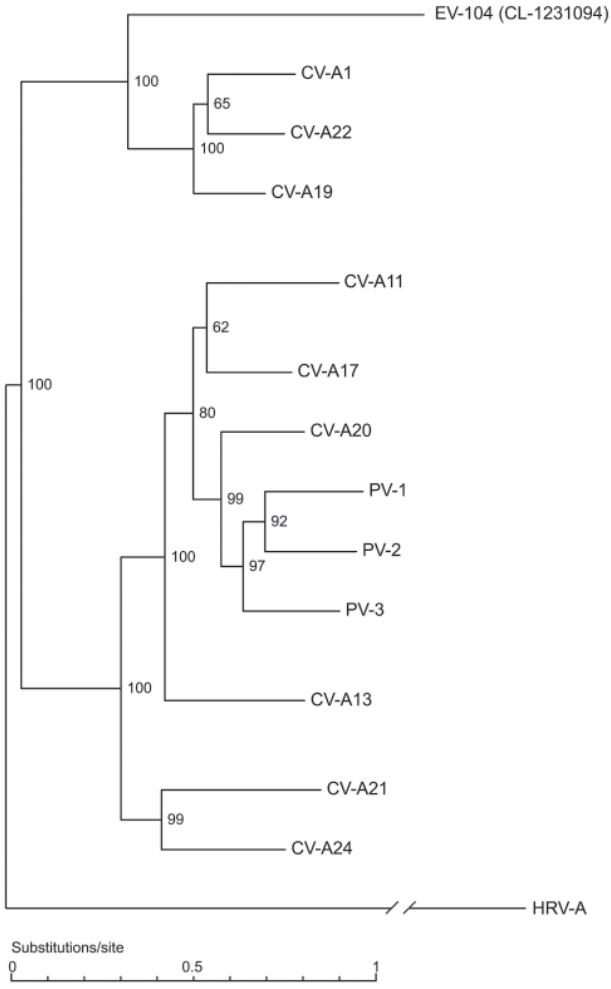


Figure 2. Full genome phylogenetic tree of enterovirus 104 (EV-104), representative strain CL-1231094, and members of the human enterovirus C (HEV-C) species. Human rhinovirus A (HRV-A) (GenBank accession no. DQ473509) was used as outgroup. Coxsackievirus A1 (CV-A1) (AF499635), CV-A21 (AF546702), CV-A20 (AF499642), CV-A17 (AF499639), CV-A13 (AF499637), CV-A11 (AF499636), CV-A19 (AF499641), CV-A22 (AF499643), CV-A24 (D90457), poliovirus 1 (PV-1) (V01148), PV-2 (X00595), and PV-3 (X00925) sequences were obtained from GenBank.

Independent phylogenetic trees (online Technical Appendix 2) and similarity matrices were constructed for the 3 genomic regions. Since the last common ancestor and as depicted on the distance matrices and highlighted by boxplots of maximum-likelihood branch length distributions (online Technical Appendix 2 Figure 3), there are more mutations fixed in the VP1 region than in the 3CD region, and more in the 3CD region than in 5′-UTR, which is indicative of a variable rate of evolution in these regions. Accordingly, VP1 sequences enabled genotyping of all but 3 clinical strains analyzed (online Technical Appendix 2, Figure 1, panel B). These strains may represent rhinovirus genotypes only distantly related to predefined reference serotypes. In contrast, genotyping based on 3CD and 5′-UTR was less accurate, as expected. These results confirmed that molecular typing of rhinoviruses, similarly to other picornaviruses, must use capsid sequences.

Phylogeny of the 5′-UTR, VP1, and 3CD of reference serotypes showed many incongruities caused by insufficient tree resolution or recombinant viruses as previously proposed (*18*,*31*). As an example, 2 VP1 clusters including HRV-85/HRV-40 and HRV-18/HRV-50/HRV-34 (online Technical Appendix 2 Figure 1, panel B) were reorganized as HRV-85/HRV-18/HRV-40 and HRV-50/HRV-34, respectively, on 3CD (online Technical Appendix 2 Figure 1, panel C). The differential cosegregations between these virus strains suggested recombination events. When available, full-length genome sequence bootscanning applied to all serotypes will give an estimate of the number of reference strains with mosaic genomes.

Similarly, the noncoding region, VP1, and 3CD trees showed major phylogenetic incongruities for 3 clinical isolates (online Technical Appendix 2 Figure 1). Two of these isolates (CL-013775 and CL-073908) were typed as HRV-67 on the basis of VP1 sequence and were closest to this serotype in 3CD, whereas the 5′-UTR cosegregated with HRV-36 (see 5′-UTR recombinant; online Technical Appendix 2 Figure 1, panels A–C). These viruses were isolated by cell culture from 2 epidemiologically linked cases and thus represented transmission of the same virus. To confirm the recombination, we completed the sequencing by obtaining the 5′-UTR, VP4, and VP2 sequences (EU840918 and EU840930) and compared them with HRV-36, HRV-67, and other closely related serotypes. Bootscanning analysis

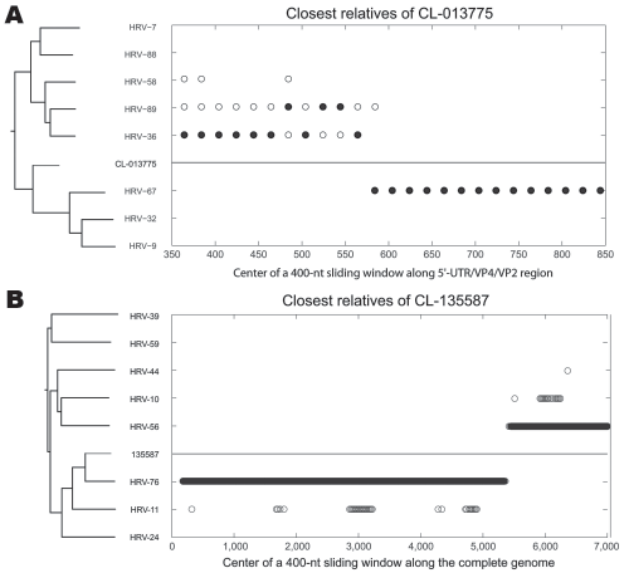


Figure 3. Nearest-neighbor relatedness of rhinovirus CL-013775 (and CL-073908) along the 5′ untranslated region/VP4/VP2 region (A), and nearest-neighbor relatedness of rhinovirus CL-135587 along the complete genome (B), identified by bootscanning. At each position of a sliding window, the solid circles indicate the closest relative within a defined threshold of the phylogenetic distance to CL-013775 (A) and CL-135587 (B). Both panels show phylogenetic trees of analyzed serotypes over the entire scanned region. Human rhinovirus 7 (HRV-7), -9, -10, -11, -24, -32 (accession nos. EU096019, AF343584), -36, -39, -44, -56, -58 (EU096045, AY040236), -59, -67 (EU096054, AF343603, and DQ473505), -76, -88, and -89 sequences were obtained from GenBank (see online Technical Appendix 2 Figure 1, available from www.cdc.gov/EID/content/15/5/719-Techapp2.pdf, for full-length genome accession numbers).

(Figure 3, panel A) enabled mapping of the recombination site within the 5′-UTR, just before the polyprotein start codon. Sequence alignment mapped recombination breakpoints more precisely between positions 524 and 553 with reference to HRV-2 (X02316).

The other incongruent isolate (CL-135587) was typed as HRV-76 on the basis of VP1 sequence and was closest to this serotype in the 5′-UTR, but 3CD cosegregates with HRV-56 (3C recombinant; online Technical Appendix 2 Figure 1, panels B, C). Similarly, we completed the full-length sequence of this isolate (EU840726) and HRV-56 (EU840727). The same approach enabled mapping of the recombination site at the N terminus of protein 3C between positions 1511 and 1523 with reference to HRV-2 (Figure 3, panel B). These results demonstrate that recombination occurs among clinical rhinoviruses. In our analysis of 40 rhinovirus-positive samples collected over 9 years (3 additional samples were duplicates of 2 different viruses; online Technical Appendix 1 Table 2) for 3 genomic regions, 2 of the analyzed viruses appeared to be recombinants. The 2 documented recombinations occurred in members of the HRV-A species. The design of this study and technical issues (e.g., inability to sequence low viral loads) limited the ability to calculate a recombination rate, particularly for HRV-B and HRV-C.

## Discussion

Our genomic analysis of picornaviruses associated with upper or lower respiratory diseases in adults and children indicates that rhinoviruses circulating in the community are widely diverse. The large number of circulating genotypes supports the view that rhinoviruses do not circulate by waves or outbreaks of a given dominant genotype, which might explain the high frequency of reinfection during short periods. As expected, the observed variability is higher for surface capsid proteins, the targets of most immune pressure, and this region remains the only accurate one for genotyping and defining phylogeny. Technical constraints such as the limited amount of clinical specimens, the use of different screening methods, and the need to sequence an unknown target of extreme variability might have limited the representativeness of our sequence collection. Therefore, our study should not be considered as an exhaustive epidemiologic analysis of rhinoviruses and enteroviruses associated with respiratory diseases.

By using a systematic approach, we have identified a new enterovirus genotype (EV-104) that has a divergent 5′-UTR. Undetectable by conventional methods, EV-104 could be detected by using a more generic real-time PCR assay designed to match all known available rhinovirus and enterovirus sequences. Such diagnostic tools have and will lead to constant discovery of new picornavirus genotypes (*9–14*,*16*,*33–36*). These genotypes may represent viruses, in most instances, that have remained undetected because of insensitive cell cultures or overly restrictive molecular tools. In addition, enterovirus genotypes causing respiratory infections, such as EV-68 and CV-A21, might be underrepresented because enteroviruses are usually searched for in fecal specimens (*37*).

EV-104 belongs to the HEV-C species: CV-A19, CV-A22, and CV-A1 are its closest serotypes. These HEV-C subgroup viruses are genetically distinct from all other serotypes of the species. These viruses show no evidence of recombination with other HEV-C strains and, similar to EV-104, do not grow in cell culture (*29*). On the basis of our epidemiologic data, we conclude that EV-104 was found in 8 children from different regions of Switzerland who had respiratory illnesses such as acute otitis media or pneumonia. Future studies using adapted detection tools will provide more information on the range of this virus. On the basis of its genomic features and similarities with coxsackieviruses and poliovirus, EV-104 could theoreti-

cally infect the central nervous system (*2*,*38*). Detection of new subtypes of picornaviruses indicates that viruses with new phenotypic traits could emerge, and conclusions on tropism of new strains should be substantiated by extensive experimental or clinical investigations (*39*).

By completing the sequence of a seemingly divergent rhinovirus (*13*), we assigned this virus to the new HRV-C species, thus limiting currently to 3 the number of HRV species. For the sake of simplicity, we propose to consider this virus as a member of the HRV-C clade.

Finally, we demonstrated that rhinovirus evolves by recombination in its natural host. Known to be a driving force of enterovirus evolution, rhinovirus recombination among clinical strains has never been observed. Two clinical isolates of 40 viruses analyzed resulted from recombination events and their breakpoints were identified within the 5′-UTR sequence and the N terminus of protein 3C, respectively. These findings are consistent with the fact that recombination breakpoints in picornaviruses are restricted to nonstructural regions of the genome or between the 5′-UTR and the capsid-encoding region (*40*). Our observations provide new insight on the diversity and ability of rhinovirus to evolve in its natural host. The fact that only 2 of 40 analyzed viruses over a 9-year period were recombinants is suggestive of a lower recombination frequency in rhinoviruses than in other picornaviruses (*32*,*40*) and might be related, but not exclusively, to the short duration of rhinovirus infection (*18*,*31*,*32*). Recombination events occurred between HRV-A genotypes, but whether they can occur in species B and C remains unknown. Interspecies recombination is rare in picornaviruses and is mainly the result of in vitro experiments. For rhinoviruses, the different location of *cre* elements in each species might be an additional limiting constraint (*17*).

In summary, we have highlighted the large genomic diversity of the most frequent human respiratory viral infection. Our phylogenetic analysis has characterized circulating strains relative to reference strains and has identified a previously unknown enterovirus genotype. We have shown that recombination also contributes to rhinovirus evolution in its natural environment.

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Dr Tapparel is a molecular virologist at the University Hospitals of Geneva. Her research interests are the molecular epidemiology of picornaviruses (rhinoviruses and enteroviruses), development of new diagnostic methods, and determination of fundamental aspects of these viruses.

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# Cross-Border Dissemination of Methicillin-Resistant *Staphylococcus aureus*, Euregio Meuse-Rhin Region

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Because the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) differs among the 3 countries forming the Euregio Meuse-Rhin (EMR) region (Belgium, Germany, and the Netherlands), cross-border healthcare requires information about the spread of MRSA in the EMR. We investigated the emergence, dissemination, and diversity of MRSA clones in the EMR by using several typing methods. MRSA associated with clonal complexes 5, 8, 30, and 45 was disseminated throughout the EMR. Dutch isolates, mainly associated with sequence types (ST) ST5-MRSA-II, ST5-MRSA-IV, ST8-MRSA-IV, and ST45-MSRA-IV had a more diverse genetic background than the isolates from Belgium and Germany, associated with ST45-MRSA-IV and ST5-MRSA-II, respectively. MRSA associated with pigs (ST398-MRSA-IV/V) was found in the Dutch area of the EMR. Five percent of the MRSA isolates harbored Panton-Valentine leukocidin and were classified as community-associated MRSA associated with ST1, 8, 30, 80, and 89.

Almost one third of the European population lives in a border region (Euregio). These border regions have collaborated since the late 1950s, especially in the field of healthcare (*1*). Cross-border patient mobility and free access to healthcare facilities within the European Union in general, and the Euregios in particular, are important for patients, medical doctors, healthcare facilities, and healthcare insurance companies. The Euregio Meuse-Rhin (EMR), an area totaling 4,973 square miles (12,882 km$^2$), is the border region of Belgium, Germany, and the Netherlands. The EMR comprises the Belgian provinces of Limburg and Liège, the German-speaking region of Belgium, the Aachen region in Germany, and the southern part of the Dutch province of Limburg. Each year, thousands of the 3.88 million inhabitants of the EMR cross the border to consult a medical specialist or a healthcare facility. Since 2003, hospitals in the EMR have built a strong collaboration. For example, the University Hospital Maastricht in the Netherlands and the University Hospital Aachen in Germany have an official agreement for the transfer of patients; consequently, dozens of patients are transferred each year between the 2 hospitals. The same applies for the University Hospital Maastricht in the Netherlands and the General Hospital Vesalius in Belgium, between which nearly 100 patients are transferred each year. In an official publication of the European Commission (D. Byrne, Maastricht Conference on Cross-Border Health Care, Maastricht, the Netherlands, June 8, 2004), the EMR was mentioned as a model region for the European Union in the field of cross-border healthcare and cross-border cooperation of hospitals. Furthermore, in July 2008, establishment of a pan-European university hospital was announced, a collaboration among the university hospitals of Maastricht in the Netherlands and Aachen in Germany.

Of particular concern is cross-border dissemination of multidrug-resistant bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA). The 3 countries forming the EMR differ considerably in the prevalence of hospi-

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tal-isolated MRSA (23.6%, 13.8%, and 0.6% in Belgium, Germany, and the Netherlands, respectively) (*2*). Consequently, cross-border transfer of patients may affect the dissemination and prevalence of MRSA, particularly when patients are transferred from countries with a relatively high prevalence to a country with a low prevalence.

A study of MRSA isolates from the EMR between December 1999 and February 2004 showed that isolates from clonal complex (CC) 5 and CC 8, which harbor the resistance elements staphylococcal cassette chromosome *mec* (SCC*mec*) types I–IV, had been disseminated in the EMR (*2*). Our aim was to investigate the emergence, dissemination, and diversity of MRSA clones in the EMR during a 10-month period in 2005 and 2006 and to compare the results with those of the previous study. We used sequencing of the short sequence repeat (SSR) region of the *S. aureus* protein A gene (*spa* typing), multilocus sequence typing (MLST), and SCC*mec* typing by PCR to investigate the genetic background of all MRSA isolates. The *spa* locus was typed to provide more detailed information about prevalent MRSA clones in the EMR, especially because the previous study used only MLST analyses on a small subset of isolates (*2*). Finally, because an increase of Panton-Valentine leukocidin (PVL)–positive MRSA isolates in the Netherlands has recently been observed (*3*), we investigated the possible spread of PVL-positive MRSA clones into hospitals in the EMR, as well as the prevalence of the virulence factors collagen adhesion (CNA) and toxic shock syndrome toxin-1 (TSST-1).

## Materials and Methods

### MRSA Isolates

We investigated 257 MRSA isolates, cultured during July 2005–April 2006 from 8 geographically closely related hospitals in the EMR. The hospitals included 1 hospital in Belgium (General Hospital Vesalius, Tongeren, 355 beds), 2 hospitals in Germany (General Hospital Dūren, 521 beds, and Marien Hospital, Aachen, 321 beds), and 5 hospitals in the Netherlands (Atrium Medical Center, Heerlen, 811 beds; Orbis Medical and Care Center, Sittard, 578 beds; Laurentius Hospital, Roermond, a 458-bed general hospital; University Hospital Maastricht, a tertiary hospital, 680 beds; and VieCuri Medical Center, Venlo, a 554-bed general hospital). The 257 MRSA isolates comprised 44 from Belgium, 92 from Germany, and 121 from the Netherlands. Isolates from the Belgian and German hospitals were from patients with MRSA infection; Dutch isolates were from patients carrying MRSA who were admitted to the Dutch hospitals. All isolates were identified as *S. aureus* by Gram stain, catalase, and coagulase testing. The presence of the *mecA* gene was determined as described previously (*2*).

### Antimicrobial Drug Susceptibility Testing

The susceptibility pattern of the MRSA isolates was determined according to the guidelines of the Clinical and Laboratory Standards Institute (*4*). Susceptibility to the following antimicrobial agents was determined as MIC: cefaclor, cefuroxime, clindamycin, ciprofloxacin, clarithromycin, gentamicin, linezolid, moxifloxacin, oxacillin, penicillin, rifampin, teicoplanin, tetracycline, trimethoprim/sulfamethoxazole, and vancomycin. The susceptibility to fucidic acid and mupirocin (Rosco, Taastrup, Denmark) was determined by using the disk-diffusion method (*5*,*6*). MRSA isolates resistant to clarithromycin were tested for inducible clindamycin resistance by using the D-test (*7*).

### Typing Methods

SCC*mec* typing was performed as described by Oliveira et al. (*8*) with the modification described previously (*2*). SCC*mec* type I elements that lack locus A (*pls* region) are indistinguishable (*9*) from SCC*mec* type IV elements when the method of Oliveira et al. is used (*8*). Furthermore, locus D (*dcs* region) is detected in both SCC*mec* types IV and VI (*10*). Therefore, SCC*mec* elements that were typed as SCC*mec* type IV using the method of Oliveira et al. (*8*) were further analyzed for presence of the *ccrAB* gene. SCC*mec* elements that could not be typed with the method of Oliveira et al (*8*) were further analyzed by using the methods of Ito et al. (*11*) and Zhang et al. (*12*).

Real-time amplification of the *spa* gene was performed as described previously, followed by sequencing of the SSR region (*13*). The *spa* types were clustered into *spa*-CCs using the algorithm Based Upon Repeat Pattern (BURP) with the Ridom StaphType version 1.4 software package (www.ridom.de). Because *spa* typing, together with the algorithm BURP, yields results concordant with typing results obtained by MLST and pulsed-field gel electrophoresis (*13*), the associated CCs, as determined with MLST, were allocated through the Ridom SpaServer (http://spaserver.ridom.de). To confirm the association between MLST and *spa* typing, in combination with BURP, MLST was performed on a representative set of 12 strains of each major *spa* type and *spa*-CC (*2*).The presence of CNA, PVL, and TSST-1 was determined with real-time PCR assays (*14*,*15*).

## Results

### Antimicrobial Drug Susceptibility Patterns

All 257 MRSA isolates were resistant to the β-lactam antimicrobial agents cefaclor, cefuroxime, oxacillin, and penicillin and were susceptible to linezolid, teicoplanin, and vancomycin. Most isolates were also resistant to ciprofloxacin (84%) and moxifloxacin (82%). The Dutch MRSA isolates were more often susceptible to ciprofloxacin and moxifloxacin than were the Belgian and German isolates

(Table 1) ($p<0.05$). Furthermore, 78% of the MRSA isolates were resistant to clarithromycin, and 62%, to clindamycin. Susceptibility for clarithromycin and clindamycin differed by country (Table 1). A total of 41 MRSA isolates (19 from Belgium, 5 from Germany, and 17 from the Netherlands) was resistant to clarithromycin and susceptible to clindamycin. The D-test showed that 31 (76%) of these 41 MRSA isolates had the inducible clindamycin resistant phenotype, including 15 from Belgium, 5 from Germany, and 11 from the Netherlands.

### Distribution of MRSA Clones

SCC*mec* type IV was predominant in MRSA isolates from Belgium (77%), whereas MRSA isolates from Germany harbored mainly SCC*mec* type II (82%). MRSA isolates from the Dutch region harbored both SCC*mec* type II and IV (27% and 65%, respectively). Although 25 (10%) of the 257 MRSA isolates harbored an SCC*mec* element that could not be typed with the method of Oliveira et al. (*8*), they could be typed with the other methods. Seven MRSA isolates from Belgium harbored a SCC*mec* type III element that lacked Tn*554*, which is usually characteristic for SCC*mec* type III. From the German region, 1 isolate that had a nontypeable SCC*mec* element harbored *ccrC*, locus E, and Tn*554*. The method of Zhang et al. (*12*) classified this element as SCC*mec* type III. In the Netherlands, 17 MRSA isolates contained a nontypeable SCC*mec* element as defined by Oliveira et al. (*8*). Ten of these were classified as SCC*mec* type IV, lacking locus D. The remaining 7 harbored *ccrC*, characteristic for SCC*mec* type V, and were classified as such with the method of Zhang et al. (*12*).

The 257 MRSA isolates were classified into 36 different *spa* types, and BURP analysis showed 6 *spa*-CCs, 4 singletons, and 3 *spa* types that were excluded from the analysis because the *spa* region was <5 *spa* repeats long (Table 2). MLST analyses showed 10 different STs among the 12 MRSA strains (Table 2). In the EMR, *spa*-CC 045 (MLST CC5; 21%) and *spa*-CC 038 (MLST CC45; 75%) were found predominantly among MRSA isolates from the Belgian region; *spa*-CC 045 (MLST CC5; 85%) was found among isolates from the German region. The Dutch MRSA isolates were grouped into *spa*-CC 045 (MLST CC5; 39%), *spa*-CC 019/012/318/011/108 (MLST CC30 and CC398; 15%), *spa*-CC 038 (MLST CC45; 15%), *spa*-CC with no founder 5 (MLST CC8; 16%), and *spa*-CC with no founder 6 (MLST CC 45; 5%).

The ST5-MRSA-II (New York/Japan) clone was found mainly in Germany and the Netherlands, and the ST45-MRSA-IV (Berlin) clone was found in Belgium and the Netherlands. Furthermore, the ST5-MRSA-IV (Pediatric) clone was found among the Dutch isolates. The MRSA isolates classified as CC30 (ST30-MRSA-IV and ST36-MRSA-II) were found only in the Netherlands. Most of the ST8-MRSA-IV (UK EMRSA-2/6) isolates were found in the Netherlands. Furthermore, several ST398-MRSA-IV and ST398-MRSA-V isolates were found in certain Dutch hospitals (Figure; Table 3).

### Prevalence of Virulence Factors

Eleven (5%) of the 257 MRSA isolates were PVL-positive. These isolates were associated with different genetic backgrounds, i.e., ST1-MRSA-V (1 Dutch isolate), ST8-MRSA-IV, ST30-MRSA-IV (2 Dutch isolates each), ST45-MRSA-IV (1 isolate from Germany), ST80-MRSA-IV (1 isolate from Germany and 2 from the Netherlands), ST89-MRSA-IV and ST89-MRSA-V (1 Dutch isolate each). Six of the PVL-positive isolates were positive for the *cna* gene, and none harbored the *tst* gene.

Nine (4%) of the 257 MRSA isolates were positive for the *tst* gene, 4 isolates were classified as ST22-MRSA-IV, 3 as ST36-MRSA-II, 1 as ST30-MRSA-IV, and 1 could not be classified as an MRSA clone (*spa* type t779). All isolates were from the Netherlands and were positive for the *cna* gene; none harbored PVL.

Ninety-five (37%) of the 257 MRSA isolates were positive for the *cna* gene (34 from Belgium, 9 from Germany, and 52 from the Netherlands). All MRSA isolates from CC30, CC45, and ST398 harbored the *cna* gene. Furthermore, 1 isolate from CC5, 1 from CC80, 6 classified as singletons (associated with ST22 and ST89), and 2 excluded from the BURP analyses were positive for the *cna* gene.

## Discussion

Because cross-border healthcare is an issue in the EMR, and the prevalence of MRSA differs among the

Table 1. Non–β-lactam antimicrobial drug resistance patterns of 257 MRSA isolates in the Euregio Meuse-Rhin region, July 2005–April 2006*

| Country | No. isolates | No. (%) resistant MRSA isolates | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | CIP | MXF | CLI | GEN | CLR | SXT | TET | RIF | MUP | FUC |
| Belgium | 44 | 44 (100) | 43 (98) | 5 (11) | 2 (5) | 24 (55) | 0 | 4 (9) | 0 | 4 (9) | 0 |
| Germany | 92 | 89 (97) | 89 (97) | 78 (85) | 5 (5) | 83 (90) | 0 | 3 (3) | 2 (2) | 1 (1) | 1 (1) |
| The Netherlands | 121 | 84 (69) | 79 (65) | 76 (63) | 11 (9) | 93 (77) | 3 (2) | 22 (18) | 0 | 8 (7) | 1 (1) |
| Total | 257 | 217 (84) | 211 (82) | 159 (62) | 18 (7) | 200 (78) | 3 (1) | 29 (12) | 2 (1) | 13 (5) | 2 (1) |

*MRSA, methicillin-resistant *Staphylococcus aureus*; CIP, ciprofloxacin; MXF, moxifloxacin; CLI, clindamycin; GEN, gentamicin; CLR, clarithromycin; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; RIF, rifampin; MUP, mupirocin; FUC, fucidic acid. No isolates showed resistance to linezolid, vancomycin, or teicoplanin.

Table 2. Composition of the *spa*-CCs of 257 MRSA isolates in the Euregio Meuse-Rhin region, July 2005–April 2006*

| *spa*-CC | No. (%) isolates | No. (%) *spa* types | *spa* types | ST | CC |
|---|---|---|---|---|---|
| 045 | 134 (52) | 9 (25) | t002, **t003**, t041, **t045**, t179, **t447**, t504, t838, t1107 | ST5/ST225† | CC5 |
| 019/012/318/011/108 | 19 (7) | 7 (19) | **t011**, **t012**, t019, t034, t108, t318, t582 | ST36/ST398‡ | CC30/CC398 |
| 038 | 58 (22) | 5 (14) | **t038**, t161, **t740**, t1288, t1310 | ST45 | CC45 |
| 044/042 | 4 (2) | 4 (11) | t042, **t044**, t131, **t345** | ST728/ST772§ | CC1/CC80 |
| No founder 5 | 22 (9) | 2 (6) | **t008**, t622 | ST8 | CC8 |
| No founder 6 | 8 (3) | 2 (6) | **t040**, t553 | ST45 | CC45 |
| Singletons | 8 (3) | 4 (11) | **t223**, t375, t682, t786 | ST217¶ | CC22/CC89 |
| Excluded# | 4 (2) | 3 (8) | t457, t779, t1011 | | |
| Total | 257 (100) | 36 (100) | | | |

*CC, clonal complex; MRSA, methicillin-resistant *Staphylococcus aureus*; ST, sequence type. **Boldface** indicates *spa* types on which multilocus sequence typing analysis was performed.
†The strains *spa* typed as t003 and t045 had ST225, a single-locus variant of ST5 at the *tpi* locus. The strain *spa* typed as t447 had ST5.
‡The strain *spa* typed as t011 had ST398, and the strain *spa* typed as t012 had ST36.
§The strain *spa* typed as t044 had ST728, a single-locus variant of ST80 at the *tpi* locus. The strain *spa* typed as t345 had ST772, a single-locus variant of ST1 at the *pta* locus.
¶The strain *spa* typed as t223 had ST217, a single-locus variant of ST22 at the *tpi* locus.
#*spa* types with <5 *spa* repeat.

countries forming the EMR, studying the possible emergence, spread, and diversity of MRSA clones within and among these countries is important (*2*). In addition to MRSA clones from CC5 and CC8, found previously in the EMR, we observed MRSA isolates from CC30 and CC45. Furthermore, the Dutch isolates had a more heterogeneous genetic background than did MRSA isolates from Belgium and Germany. The prevalence of PVL-positive MRSA isolates, belonging to ST1, 8, 30, 80 and 89, was higher than that found in the previous study (5% vs. 1.3%) (*2*).

The antimicrobial susceptibility of the MRSA isolates depends on the *S. aureus* lineage. The observation that the Dutch MRSA isolates were more often susceptible to ciprofloxacin and moxifloxacin than were isolates from Belgium and Germany can be explained by the fact that the isolates associated with ST5-MRSA-IV, ST22-MRSA-IV, and ST30-MRSA-IV, which were susceptible to ciprofloxacin and moxifloxacin, were mainly observed in the Netherlands. Although ST22-MRSA-IV is commonly susceptible to tetracycline, the ST22-MRSA-IV isolates in this study were resistant to tetracycline (*16*). *S. aureus* can harbor resistance genes on mobile genetic elements on the genome, such as Tn*554*, as well as on plasmids, and these can be exchanged among *S. aureus* lineages, possibly because of antimicrobial drug pressure (*17*).

Primarily because of the Dutch "search-and-destroy" policy, isolates derived from colonized persons were available from the Netherlands, whereas isolates from Belgium and Germany were derived from infections. However, nasal carriers are at increased risk of acquiring MRSA infection (*18*). Consequently, not preventing the spread of MRSA among nasal carriers could lead to MRSA infection among these persons. Furthermore, the molecular epidemiology of MRSA can vary widely among hospitals. In the Dutch hospitals of the EMR, MRSA clones in each hospital were diverse, whereas in the Belgian hospital and 2 German hospitals, 1 MRSA clone predominated, showing that the number of hospitals is unlikely to have biased the results of our study.

Most of the MRSA isolates from Belgium were associated with the Berlin clone (ST45-MRSA-IV). This clone has previously been found in Belgium, Germany, and the Netherlands (*19*). Most of the MRSA isolates from Germany were associated with the New York/Japan clone (ST5-MRSA-II), previously found in Belgium and Germany (*2*,*19*). Most of the Dutch MRSA isolates belonged to 5 MRSA clones (Table 3). Twenty-five percent of the Dutch isolates were associated with the New York/Japan clone (ST5-MRSA-II), which has not been previously found in the Netherlands. The Pediatric clone (ST5-MRSA-IV), which represented 14% of the Dutch isolates, has been found in Belgium but not in the Netherlands (*20*,*21*). The Berlin clone (ST45-MRSA-IV), comprising 21% of the Dutch isolates, and the UK EMRSA-2/-6 clone (ST8-MRSA-IV), comprising 16% of the Dutch isolates, have been described in all 3 EMR countries (*19*,*20*). In addition, some less prevalent MRSA clones were observed. Four *tst*-positive MRSA isolates were associated with the UK EMRSA-15 clone (ST22-MRSA-IV), previously found in Belgium and Germany but not in the Netherlands (*19*,*20*). Three Dutch MRSA isolates (*spa* type t012), harboring SCC*mec* type II, were associated with the CC30 lineage. These isolates might be derived from the UK EMRSA-16 (ST36-MRSA-II) clone (*spa* type t018) because *spa* types t012 and t018 differ in 1 *spa* repeat (r24) and are thus related. Furthermore, both clones harbor the *cna* and *tst* genes (*22*,*23*). The highly endemic UK EMRSA-16 clone has not been observed before in the Netherlands, although this clone has previously been found in Belgium (*24*). Seven and 5 of the Dutch MRSA isolates were associated with ST398-MRSA-

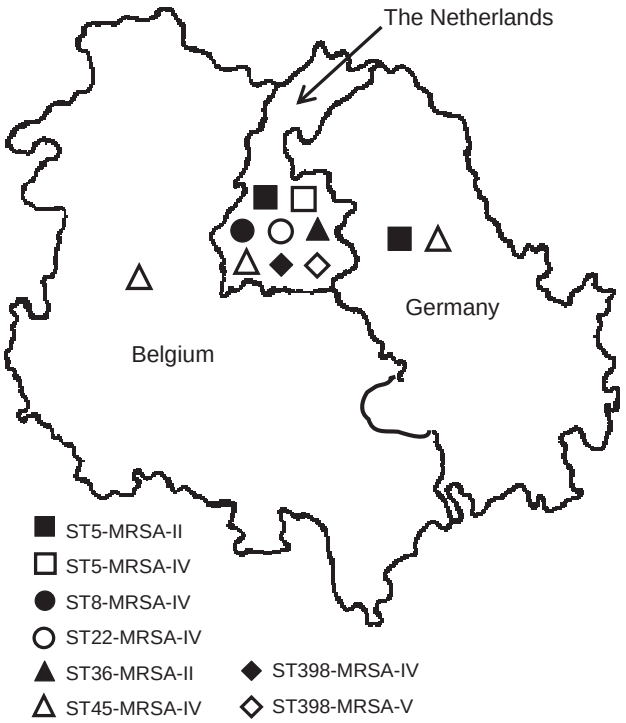


Figure. Distribution of the major methicillin-resistant *Staphylococcus aureus* (MRSA) clones in the Euregio Meuse-Rhin region, July 2005–April 2006.

IV and ST398-MRSA-V, respectively, MRSA clones usually observed in pigs and among screening samples from pig farmers (*25*). The ST398 clone is now observed among screening samples of veterinarians from many countries in Europe, including Belgium and Germany (*26*). However, ST398 also has been isolated from several forms of human infections in Germany (*27*). The ST398 isolates from our study were positive for the *cna* gene, suggesting a higher virulence than that of the CNA-negative German ST398 MRSA isolates (*27*). One Dutch MRSA strain was associated with the ST30-MRSA-IV clone, previously reported in Belgium, Germany, and France (*20*,*21*,*28*). The more diverse genetic background among MRSA isolates in the Dutch part of the EMR and the close cooperation of hospitals in the EMR might suggest that importation of MRSA from Belgium and Germany has occurred through cross-border healthcare (Table 4) (*2*). Other, less likely, explanations for the diversity of MRSA clones in the Netherlands are the spread of MRSA from countries other than Belgium or Germany (*19*) and the emergence of new MRSA clones in vivo through transfer of the SCC*mec* element from methicillin-resistant coagulase-negative staphylococci to methicillin-sensitive *S. aureus* strains (*29*).

We could not determine the SCC*mec* type for 10% of the MRSA isolates by using the method of Oliveira et al. (*8*). This percentage was similar to that found in other studies (*30*,*31*) but higher than the 3% previously found in the EMR (*2*). The relatively large number of nontypeable SCC*mec* types found in this study, probably formed by homologous recombination among SCC*mec* elements, supports the need for a new system for SCC*mec* typing and nomenclature (*19*).

The 7 Belgian MRSA isolates with the nontypeable SCC*mec* type III element were associated with CC5 and had the related *spa* types t045 and t1107 (http://spaserver.ridom.de). Although SCC*mec* type III usually is found in the CC8 genetic background, such as in the ST239-MRSA-III clone, an MRSA associated with CC5 (*spa* type t045) and harboring SCC*mec* type III recently was observed in Belgium (*32*). This might suggest that a new MRSA clone, ST5-MRSA-III, is beginning to emerge in Belgium.

The nontypeable SCC*mec* element of the German MRSA isolate harbored locus E and *ccr*C, specific for SCC*mec* type V (*2*), and Tn*554*, normally carried by SCC*mec* type II, III, and SCCmercury. Zhang et al. (*12*) classified this element as SCC*mec* type III, but the SCC*mec* type III-specific primers used by this method are situated near locus E on SCCmercury (*33*), indicating that this element could be a SCCmercury element containing *mecA*. Further investigation is needed into the structure of this element.

Previous studies have shown that MRSA isolates classified as community-associated usually harbor either SCC*mec*

Table 3. Distribution of MRSA clones in the Euregio Meuse-Rhin region, by country, July 2005–April 2006*

| MRSA clone | No. isolates | | | |
|---|---|---|---|---|
| | Belgium | Germany | The Netherlands | Total |
| ST1-MRSA-V | | | 1 | 1 |
| ST5-MRSA-I | 1 | 1 | 1 | 2 |
| ST5-MRSA-II | 1 | 75 | 30 | 106 |
| ST5-MRSA-IV | | 2 | 17 | 19 |
| ST8-MRSA-IV | 1 | 2 | 19 | 22 |
| ST22-MRSA-IV | | | 4 | 4 |
| ST30-MRSA-IV | | | 3 | 3 |
| ST36-MRSA-II | | | 3 | 3 |
| ST45-MRSA-IV | 33 | 8 | 25 | 66 |
| ST80-MRSA-IV | | 1 | 2 | 3 |
| ST89-MRSA-I | | | 1 | 1 |
| ST89-MRSA-V | | | 1 | 1 |
| ST398-MRSA-IV | | | 7 | 7 |
| ST398-MRSA-V | | | 5 | 5 |
| NT MRSA | 7† | 1‡ | 2§ | 17 |
| Excluded¶ | 1 | 2 | 1 | 4 |
| Total | 44 | 92 | 121 | 257 |

*MRSA, methicillin-resistant *Staphylococcus aureus*; ST, sequence type; NT, nontypeable. Based on *spa* and staphylococcus cassette chromosome (SCC) *mec* typing.
†These strains were classified into clonal complex (CC) 5 and had a nontypeable SCC*mec* type III element.
‡This strain had a nontypeable SCC*mec* element belonging to CC30.
§These strains harbored SCC*mec* type IV and could not be classified into a CC.
¶*spa* types with <5 *spa* repeats.

Table 4. Suggested cross-border dissemination of the major MRSA clones in the Euregio Meuse-Rhin region, July 2005–April 2006*

| MRSA clone | Previously observed in/possible dissemination from |
|---|---|
| ST5-MRSA-II | Belgium, Germany |
| ST5-MRSA-IV | Belgium |
| ST8-MRSA-IV | Belgium, Germany, the Netherlands |
| ST22-MRSA-IV | Belgium, Germany |
| ST30-MRSA-IV | Belgium, Germany |
| ST36-MRSA-II | Belgium |
| ST45-MRSA-IV | Belgium, Germany, the Netherlands |

*MRSA, methicillin-resistant *Staphylococcus aureus*; ST, sequence type.

type IV or V, and often PVL, but may differ in their genetic backgrounds (CC1, CC8, CC30, CC59 and CC80) (*34*). In the EMR, 5% of the MRSA isolates were positive for PVL, which is higher than the previously reported 1.3% (*2*). Thus, PVL-positive MRSA isolates with a heterogeneous genetic background are emerging in the EMR.

PVL-positive MRSA isolates associated with ST8-MRSA-IV, ST30-MRSA-IV, and ST80-MRSA-IV have been isolated in the Netherlands (*3*,*35*). In the present study, 2 of the PVL-positive MRSA isolates harbored SCC*mec* type V. The different genetic background of these isolates, i.e., ST89 and ST772, a single-locus variant of ST1 at the *pta* locus, might suggest that SCC*mec* type V was introduced on different occasions into different *S. aureus* lineages. A PVL-positive ST772-MRSA-V has been observed in Germany (*36*). One of the PVL-positive isolates harbored SCC*mec* type I, and such isolates with a ST30 and ST37 genetic background have been described in the Netherlands (*3*). Although a recent study suggested that CNA and PVL combined contribute to virulence, only 6 of the 11 PVL-positive MRSA isolates from the EMR harbored the *cna* gene (*37*). Further studies are needed to investigate the contribution of the combination of CNA and PVL to virulence.

The genetic background of 1 PVL-positive ST45-MRSA-IV isolate from Belgium was similar to that of the Berlin clone. Hitherto, only PVL-negative isolates with this background have been found in EMR countries (*19*,*20*). PVL-positive MRSA isolates, associated with the major CA-MRSA clones (ST8-MRSA-IV, ST30-MRSA-IV, and ST80-MRSA-IV) have been reported from Belgium (*38*). Because PVL is situated on a phage, the genes encoding PVL might have been transferred to *S. aureus* with a CC45 genetic background (*34*).

Our study found a PVL-positive MRSA isolate from Germany with *spa* type t042 (*spa* repeat pattern r26r23r12r34r34r33r34). This *spa* type is strongly related to *spa* types t044 and t131 (*spa* repeat patterns r07r23r12r34r34r33r34 and r07r23r12r34r33r34, respectively), which are usually associated with the CA-MRSA ST80-MRSA-IV clone found in Germany (*34*).

The *cna* gene has been previously observed among MRSA isolates from CC22, CC30, and CC45 (*23*,*29*). Therefore, the presence of the *can* gene might, together with *spa* typing, be used as a marker for different genetic backgrounds.

MRSA clones associated with the hospital associated-MRSA CCs 5, 8, 22, 30, and 45, the PVL-positive CA-MRSA CCs 1, 8, 30, 80, and 89, as well as MRSA related to pigs (ST389-MRSA-IV/V) were observed in the EMR. Dissemination of these clones is possible because of the introduction of new MRSA clones associated with travel; with patients who have previously been admitted to a hospital abroad (cross-border healthcare); or with other high-risk patients, such as pig-farmers and their families. Therefore, a cross-border search-and-contain policy may help control the further spread of MRSA and reduce the financial cost to hospitals, nursing homes, and the community in the EMR.

The study was partly performed within the framework of the Interreg-III project "Cross-Border Health Care in the Euregio Meuse-Rhin."

Dr Deurenberg is a postdoctoral fellow at the Department of Medical Microbiology at the University Hospital Maastricht. His research interests focus on molecular diagnostics, mechanisms of antimicrobial drug resistance, and the molecular epidemiology of *S. aureus*.

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# Chloroquine-Resistant Haplotype *Plasmodium falciparum* Parasites, Haiti

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*Plasmodium falciparum* parasites have been endemic to Haiti for >40 years without evidence of chloroquine (CQ) resistance. In 2006 and 2007, we obtained blood smears for rapid diagnostic tests (RDTs) and filter paper blots of blood from 821 persons by passive and active case detection. *P. falciparum* infections diagnosed for 79 persons by blood smear or RDT were confirmed by PCR for the small subunit rRNA gene of *P. falciparum.* Amplification of the *P. falciparum* CQ resistance transporter (*pfcrt*) gene yielded 10 samples with amplicons resistant to cleavage by *Apo*I. A total of 5 of 9 samples had threonine at position 76 of *pfcrt*, which is consistent with CQ resistance (haplotypes at positions 72–76 were CVIET [n = 4] and CVMNT [n = 1]); 4 had only the wild-type haplotype associated with CQ susceptibility (CVMNK). These results indicate that CQ-resistant haplotype *P. falciparum* malaria parasites are present in Haiti.

The island of Hispaniola is the only area in the Caribbean Sea where *Plasmodium falciparum* malaria is endemic (*1*). It has been reported that up to 75% of the population of Haiti lives in malarious areas, especially at altitudes <300 m above sea level (*2*,*3*). *P. falciparum* is the only malaria parasite species that causes malaria in Haiti. The last confirmed endogenous case of *P. vivax* malaria was in 1983 (*4*); 6 cases of *P. malariae* malaria were reported recently in Haitian refugees in Jamaica (*5*).

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Haiti has been a remarkable outlier as a country in which *P. falciparum* malaria is endemic without evidence of chloroquine (CQ) resistance (*3*,*6*–*8*). Even though Haiti has had no comprehensive national malaria control program for 20 years (*9*), several reports have found no evidence of CQ resistance in Haiti (*3*,*6*–*8*). Those reports are consistent with the conclusions of domestic and international health agencies, which recommend CQ for the prevention of malaria in Haiti and the treatment of patients with malaria acquired in Haiti (*8*–*10*).

Accordingly, the original objectives of this research focused not on CQ resistance but on quantifying *P. falciparum* infection, including the heterogeneity and multiplicity of infection, and on identifying factors associated with low-intensity transmission in the Artibonite Valley of Haiti (*11*,*12*). We describe secondary analyses of blood samples for CQ-resistant *P. falciparum* haplotypes from samples collected in 2006 and 2007 that previously tested positive (*11*–*13*).

## Materials and Methods

### Ethical Approval

The protocols for these studies were reviewed and approved by the Institutional Review Boards of Tulane University and the Hôpital Albert Schweitzer (Deschapelles, Haiti). All samples were collected after obtaining informed consent.

### Study Site

Studies were performed in the low-lying Artibonite Valley. The valley has abundant rainfall and is heavily farmed; 80% is irrigated for the cultivation of rice and other crops. The major peak in malaria cases (>99% caused by *P. falciparum*) (*11*,*14*–*16*) is during November–January (*11*,*12*,*17*).

The population of the Artibonite Valley relies primarily on subsistence farming and informal trade (barter) for income. This population is poor; only 18% of households have electricity and just 12% have piped water (*12*). As a result, members of the population rarely travel outside the study area, and international travel to other malaria-endemic countries is uncommon. The primary malaria control activities currently being implemented include improvement of microscopy at Hôpital Albert Schweitzer, a facility that is supported by the Global Fund (www.theglobalfund.org/en/worldmalariaday/2007) and vector control (*10*).

Hôpital Albert Schweitzer was the base of operations for the household surveys, passive case detection, and laboratory studies (i.e., thick and thin blood smears, antigen testing by using rapid diagnostic tests [RDTs], clinical examinations, and clinical and laboratory follow-up of patients). This hospital provides comprehensive inpatient care at its 100-bed facility and delivers preventive and primary health services to a population of 300,000 through a network of health centers, dispensaries, and workers in the community. Data from Hôpital Albert Schweitzer indicate that malaria transmission in this area of Haiti varies annually according to rainfall. For example, 157 of 2,739 suspected cases were confirmed by microscopy and treated with CQ in 2005 (smear positivity rate 5.7%), and only 29 of 1,307 suspected cases were confirmed and treated in 2006 (smear positivity rate 2.2%). The prevalence of *P. falciparum* infection in this area of Haiti is estimated to be 3.1% (*13*).

### Household Survey in 2006 (Active Case Detection)

A 2-stage cluster design, in which probability was proportional to cluster size, was used to generate a sample of 200 households within the study area, as described elsewhere (*11*,*12*). Thick and thin blood films and 4 blots of blood on filter paper for PCR were collected from 714 persons >1 month of age within selected households. All smear-positive case-patients were treated with CQ.

### Passive Case Detection in 2006 and 2007

#### Data for 2006

Four blots of blood on filter paper (each containing 50 μL) and axillary temperatures were obtained from 55 persons (age range 11–80 years) with clinically suspected cases of malaria who came to Hôpital Albert Schweitzer during December 2006. All 55 samples were tested for *P. falciparum* infection by using PCR.

#### Data for 2007

As part of pilot studies of a passive case detection system to identify households with malaria, 4 blots of blood on filter paper and axillary temperatures were obtained before treatment with CQ. Forty-seven smear-positive persons 2–84 years of age were seen and treated at Hôpital Albert Schweitzer or a nearby satellite clinic in Liancourt from November 5 through December 3, 2007. A data collection team was sent to households of 45 positive case-patients within 3 days for blood sample collection from all household residents >1 month of age. Thick and thin blood films, a drop of blood for an RDT (OptiMAL-IT; DiaMed AG, Cressier sur Morat, Switzerland), 4 blots of blood on filter paper, and axillary temperatures were obtained from 249 household members 2–85 years of age. Five of these persons (age range 5–37 years) had positive results for *P. falciparum* by RDT and were treated with CQ. Fifty-two samples from persons who had either a positive smear at Hôpital Albert Schweitzer or a positive RDT result at home were then examined for *P. falciparum* infection by using PCR.

### Diagnosis of Malaria by Blood Smear or RDT and Species-Specific PCR for *P. falciparum* Small Subunit rRNA Gene

Thick and thin Giemsa-stained blood smears were examined for malaria parasites at Hôpital Albert Schweitzer by trained laboratory technologists by using standard methods (*18*,*19*). Filter paper blots were transported from Haiti to New Orleans where parasite DNA was extracted (*20*,*21*), and microscopy results were confirmed by using a PCR for the *P. falciparum* small subunit (SSU) rRNA gene (*22*). DNA was extracted from filter paper blots by using the Charge Switch Forensic DNA Purification Kit (catalog no. CS 11200; Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. This extraction yielded 150 μL of DNA in buffer (10 mmol/L Tris, pH 8.5, 1 mmol/L EDTA) from each specimen.

PCR for the *P. falciparum* SSU rRNA gene used a *P. falciparum*–specific forward primer (which hybridizes only with *P. falciparum* DNA) and a genus-specific reverse primer (which hybridizes with DNA from all 4 *Plasmodium* spp. that infect humans: *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*) (*22*) (Table 1). To perform this PCR, 4 μL of DNA extracted from filter paper blots was added to 19 μL of PCR master mixture (Promega, Madison, WI, USA) and 1 μL of each primer. Parasite DNA was amplified after an initial denaturation at 95°C for 15 min; 43 cycles of denaturation at 95°C for 45 s and annealing at 60°C for 90 sec; and a final extension at 72°C for 5 min in an i-Q thermocycler (Bio-Rad, Hercules, CA, USA). Positive controls for these assays contained DNA from in vitro culture of the Haiti I/CDC strain of *P. falciparum* (*26*). Resulting amplicons were visualized by electrophoresis on 1% agarose gels stained with ethidium bromide (*27*,*28*). Amplicon sizes were estimated by using a 100–600-bp DNA ladder (catalog no. 15628–019; Invitrogen).

Table 1. Primers used to amplify *Plasmodium falciparum* DNA during study in Haiti*

| Primers (5′ → 3′) | Amplicon, bp | Tm, °C | Reference |
|---|---|---|---|
| Primers for *P. falciparum* species-specific SSU rRNA gene | 276 | | (*22*) |
| Forward: AACAGACGGGTAGTCATGATTGAG | | 56.5 | |
| Reverse: GTATCTGATCGTCTTCACTCCC | | 54.5 | |
| Primers for single-step *pfcrt* gene PCR | 170 | | (*23*) |
| Forward: TgTgCTCATgTGTTTAAACTT | | 50.6 | |
| Reverse: AATAAAgTTgTgAgTTTCggA | | 49.8 | |
| Primers for nested (2-step) *pfcrt* gene | 573 | | (*24*,*25*) |
| First round of amplification | | | |
| Forward (CRTP1): CCGTTAATAATAAATACACGCAG | | 49.9 | |
| Reverse (CRTP2): CGGATGTTACAAAACTATAGTTACC | | 51.5 | |
| Second round of amplification | | | |
| Forward (CRTD1): TGTGCTCATGTGTTTAAACTT | 134 | 50.6 | |
| Reverse (CRTD2): CAAAACTATAGTTACCAATTTTG | | 46.1 | |

*Nucleotides in upper case letters were conserved in 100% of sequences at those positions, and nucleotides in lower case letters were conserved in most (e.g., >50%) sequences at those positions. Tm, melting (annealing) temperature; SSU, small subunit; *pfcrt*, *P. falciparum* chloroquine resistance transporter.

### Amplification of *P. falciparum pfcrt* Gene from Specimens Positive for *P. falciparum* SSU DNA

Two protocols were used to amplify the *P. falciparum* CQ resistance transporter (*pfcrt*) gene responsible for CQ resistance (*23*–*25*). The first protocol (single-step PCR) was used to screen 79 smear-positive and RDT-positive specimens that were positive for *P. falciparum* SSU DNA (*23*). In this assay, 4 μL of DNA extracted from filter paper blots was mixed with 21 μL of PCR master mixture (Promega) plus 2 μL of primers (Table 1) and amplified by an initial denaturation at 95°C for 7 min; 40 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec, and extension at 72°C for 30 sec; and a final extension at 72°C for 10 min in an i-Cycler thermocycler (Bio-Rad).

The second protocol (nested PCR) (*24*,*25*) was used to retest 58 specimens positive for SSU DNA that were negative in the single-step PCR for *pfcrt*. The nested PCR protocol used primers CRTP1 and CRTP2 for the first round of amplification and primers CRTD1 and CRTD2 for the second round (*24*,*25*). Samples in the first round were amplified by an initial denaturation at 94°C for 3 min; 45 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, and extension at 60°C for 1 min; and a final extension at 60°C for 3 min. Samples in the second round were amplified by an initial denaturation at 95°C for 5 min; 30 cycles of denaturation at 92°C for 30 sec, annealing at 48°C for 30 sec, and extension at 65°C for sec; and a final extension at 65°C for 3 min (Table 1).

### Digestion of Amplicons from *pfcrt* with *Apo*I

For each sample positive for SSU DNA, an aliquot (10 μL) of the *pfcrt* gene PCR product was digested with 10 U of *Apo*I (New England Biolabs, Beverly, MA, USA) according to the manufacturer's instructions. Briefly, 10 U of *Apo*I in 1× NE buffer 3 (100 mol/L NaCl, 50 mmol/L Tris-HCl, 10 mmol/L $MgCl_2$, 1 mmol/L dithiothreitol) and bovine serum albumin (100 μg/μL) were incubated overnight with 10 μL of the PCR product at 50°C (*23*–*25*). DNA fragments from samples and positive and negative controls were resolved by electrophoresis on 3% agarose gels stained with ethidium bromide.

*Apo*I digests most wild-type *pfcrt* genes (with CVMNK haplotype sequences at positions 72–76) but not the CQ-resistant mutant gene (i.e., K76, not T76) (*23*–*25*). On the basis of a single-step PCR for *pfcrt,* which yields an amplicon of 170 bp, amplicons with a lysine at position 76 (K76) are digested into 2 fragments (98 bp and 72 bp). Amplicons from CQ-resistant parasites (i.e., parasites with CVIET and CVMNT sequences at positions 72–76) are not digested by *Apo*I, resulting in an unchanged amplicon of 170 bp. The nested PCR product is slightly smaller (134 bp vs. 170 bp). As with the single-step PCR, most amplicons from CQ-susceptible parasites are digested by *Apo*I (in this instance to 30-bp and 104-bp fragments); amplicons from CQ-resistant parasites are not digested (unchanged amplicons of 134 bp; *24*,*25*).

### Amplification, Cloning, and Sequencing of *pfcrt* Genes Not Digested by *Apo*I

Samples not digested by *Apo*I for which DNA was available (9 of 10) were reamplified under the conditions described above for nested *pfcrt* PCR, cloned into the pCRII-TOPO vector, and transfected into the TOP10 strain of *Escherichia coli* by using the TOPO TA Cloning Kit (Invitrogen) according to the manufacturer's instructions (*29*,*30*). Cloned *pfcrt* amplicons were sequenced in both directions by using CRTD1 and CRTD2 primers at an automated DNA sequencing facility (Davis Sequencing, Davis, CA, USA). Data for ≥3 clones sequenced in both directions were compared by using the multiple sequence alignment function in Lasergene version 7.2 software (DNASTAR, Madison, WI, USA) (*31*,*32*).

## Results

We identified 79 *P. falciparum* infections in 821 persons by using PCR for the *P. falciparum* SSU rRNA gene (Table 2) (*23*–*25*). The 51 persons identified by passive case detection were thought to have malaria because their temperatures were ≥37.5°C. In contrast, only 9 (39%) of 23 infected persons identified by active case detection in the 2006 household survey had temperatures ≥37.5°C (*11*). The *pfcrt* gene was amplified from these 79 samples by using either single-step (n = 21) or nested PCR (n = 58). After digestion by *Apo*I, 10 samples did not yield the 100-bp and 34-bp fragments characteristic of the CQ-susceptible *pfcrt* gene (Figure). PCR-amplified *pfcrt* DNA from 9 of these samples (no DNA was available for the 10th sample) was cloned into the TOPO TA vector, transfected into the TOP10 strain of *E. coli*, grown on selective medium, and sequenced. Sequences from 5 of 9 samples had *pfcrt* haplotypes associated with CQ resistance (5/79 [6%]; 4 CVIET and 1 CVMNT); 4 of these 5 samples were mixed infections that also had CQ-susceptible haplotype sequences (CVMNK). The remaining 4 samples had only sequences associated with CQ susceptibility (CVMNK) (Table 2). Although CQ treatment failures have not been reported in Haiti, no follow-up information was available for the 5 persons with CQ-resistant haplotype parasites.

## Discussion

For as long as CQ has been available, *P. falciparum* has been endemic to Haiti without evidence of CQ resistance. During the past 20 years, several reports have noted the continued susceptibility of *P. falciparum* to CQ in Haiti (*3*,*6*–*9*), although Haiti had no comprehensive national malaria control program (*10*). Our results indicate that CQ-resistant haplotype *P. falciparum* parasites are now present in Haiti.

Our study has several limitations. First, because data on CQ-resistant parasites were not obtained from probability-based sampling, we were unable to estimate the potential effect and distribution of CQ resistance in the general population of Haiti. We can only report the presence of CQ-resistant haplotype parasite sequences in this area of Haiti. Second, we have not performed in vivo studies of treatment with CQ in Haiti to confirm molecular evidence for CQ resistance. Lastly, because these studies were based on results of filter paper blots, we have not yet been able to examine live *P. falciparum* parasites from the study area to test the effects of CQ on those parasites in vitro.

Beginning with studies of Djimde et al. (*24*) and Fidock et al (*34*), several studies have established a cause-and-effect relationship between the K76T point mutation (lysine → threonine at position 76 of *pfcrt*) and CQ resistance (*23*,*25*,*35*). In addition, studies in Southeast Asia, South America, and Africa have shown that persons who do not clear *P. falciparum* parasitemias after treatment with CQ have parasites that contain the K76T point mutation (*36*–*39*). Thus, *P. falciparum* parasites with CQ-resistant haplotypes that we identified in Haiti are likely to reduce the efficacy of CQ in Haiti as they have in sub-Saharan Africa, South America, and Southeast Asia (*36*–*39*).

Because the frequency of CQ-resistant *P. falciparum* in Haiti may be low, we suggest continuing CQ chemoprophylaxis for travelers to Haiti as currently recommended (*14*,*40*). We also suggest continuing to treat patients with uncomplicated *P. falciparum* infections acquired in Haiti with CQ in the absence of CQ chemoprophylaxis. However, if the presence of CQ-resistant *P. falciparum* in Haiti is confirmed by in vivo studies of resistance in humans or in vitro studies of parasite resistance to CQ, tourists and other nonimmune persons who acquire *P. falciparum* infections in Haiti or after travel to Haiti despite CQ chemoprophylaxis should be treated with alternative antimalarial drugs (mefloquine, atovaquone plus proguanil [Malarone], or sulfadoxine-pyrimethamine [Fansidar]), as they would be treated in other regions of the world where CQ resistance is present.

There are at least 2 potential explanations for CQ-resistant haplotype parasites in Haiti. First, CQ-resistant parasites may have been imported into Haiti by persons who acquired CQ-resistant *P. falciparum* in areas with established resistance, such as South America, sub-Saharan Africa, or Southeast Asia, where CVMNT and CVIET haplotypes circulate on a regular basis. Although this hypothesis could explain the presence of CVIET haplotype

Table 2. Samples from household surveys (active case detection) and hospital outpatients (passive case detection) tested by small subunit PCR for *Plasmodium falciparum*, by year of collection, Haiti

| Characteristic | 2006 | 2007 | Total |
|---|---|---|---|
| Samples from household surveys, no. positive/no. tested (%) | 23/714 (3.2) | 5/5 (100) | 28/719 (4) |
| Samples from hospital outpatients, no. positive/no. tested (%) | 9/55 (16) | 42/47 (89) | 51/102 (50) |
| Total, no. positive/no. tested (%) | 32/769 (4.2) | 47/52 (90.4) | 79/821 (9.6) |
| Molecular studies | | | |
| Resistance to *Apo*I digestion, no. positive/no. tested (%) | 6/32 (19) | 4/47 (9) | 10/79 (13) |
| Haplotype, no. samples | | | |
| CVIET | 4 | 0 | 4 |
| CVMNT | 0 | 1 | 1 |
| CVMNK | 2 | 3 | 5 |

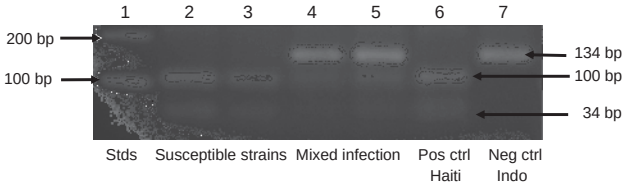


Figure. Agarose gel electrophoresis of amplicons for the *Plasmodium falciparum* chloroquine (CQ) resistance transporter gene digested with *Apo*I. Lane 1, DNA molecular mass standards (Stds) (Invitrogen, Carlsbad, CA, USA); lanes 2 and 3, amplicons susceptible to cleavage by *Apo*I, showing 2 fragments of 100 and 34 bp, consistent with infection by only CQ-susceptible haplotype parasites; lanes 4 and 5, amplicons partially resistant to cleavage by *Apo*I, showing 3 fragments of 134, 100, and 34 bp, consistent with mixed infections by CQ-resistant and CQ-susceptible haplotype parasites; lane 6, positive control (Pos ctrl), amplicon from CQ-susceptible Haiti I/CDC strain (*26*), showing 2 fragments of 100 and 34 bp; lane 7, negative control (Neg ctrl), amplicon from CQ-resistant Indochina (Indo) I/CDC strain (*33*), showing 1 fragment of 134 bp.

parasites in Haiti, it would require an initial importation by persons with greater financial resources than the residents of the Artibonite Valley. Second, CQ-resistant CVMNT haplotype parasites may have arisen by a single point mutation at position 76 in the *pfcrt* gene among naturally infected persons in Haiti, a mutation that could convert the predominant CQ-susceptible CVMNK haplotype to a CQ-resistant CVMNT haplotype. Defining the origin of these haplotypes will require additional sequencing within the *pfcrt* gene (beyond the 134-bp amplicon we studied) and at other loci.

At the Hôpital Albert Schweitzer and across Haiti, no clinical failures with CQ have been reported, and fatal cases of malaria are extremely rare. However, because CQ remains the first-line antimalarial drug in Haiti, selection for CQ-resistant parasites will continue and is likely to decrease the efficacy of CQ. Therefore, we suggest that now would be an opportune time to eliminate malaria from the island of Hispaniola before CQ resistance becomes broadly established, renders CQ ineffective, and makes elimination more much difficult. A commitment to eliminate malaria on Hispaniola would also provide an opportunity to test strategies being considered for malaria elimination on an island close to the US mainland and its resources, and in an area with a relatively low level of malaria transmission.

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# CTX-M β-Lactamases in *Escherichia coli* from Community-acquired Urinary Tract Infections, Cambodia

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Despite the recent global spread of CTX-M β-lactamases in *Escherichia coli* isolates from community-acquired urinary tract infections (CA-UTIs), their dissemination has been little studied in developing countries. In a 2-year prospective study, we documented the prevalence of extended-spectrum β-lactamases (ESBLs) in *E. coli* that were responsible for CA-UTIs in Phnom-Penh, Cambodia. Ninety-three *E. coli* strains were included. We observed a high prevalence of resistance to amoxicillin (88.2% of strains), cotrimoxazole (75.3%), ciprofloxacin (67.7%), gentamicin (42.5%), and third-generation cephalosporins (37.7%). A total of 34 strains carried ESBLs, all of which were CTX-M type. CTX-M carriage was associated with resistance to fluoroquinolones and aminoglycosides. Using repetitive extragenic palindromic–PCR, we identified 4 clusters containing 9, 8, 3, and 2 strains. The prevalence of CTX-M β-lactamases has reached a critical level in Cambodia, which highlights the need for study of their spread in developing countries.

*Escherichia coli* is the bacterium most frequently isolated in community- and hospital-acquired urinary tract infections (CA-UTIs and HA-UTIs, respectively). Despite possessing the gene encoding cephalosporinase, *ampC* (*1*), wild strains of *E. coli* are susceptible to most β-lactams because of the absence of an efficient *ampC* promoter region. The extensive use of β-lactam antimicrobial drugs has led to the emergence of resistant strains worldwide. β-lactam resistance is mostly mediated through acquisition of β-lactamase genes located on mobile genetic elements such as plasmids or transposons. Most β-lactamases found in *E. coli* belong to Ambler class A and can be further divided into narrow-spectrum β-lactamases (e.g., TEM-1, TEM-2, and SHV-1) and extended-spectrum β-lactamases (ESBLs) (e.g., TEM-3, SHV-5, and CTX-M-like) (*1–3*). ESBLs confer resistance to extended-spectrum cephalosporins, widely used to treat *E. coli* infections.

CTX-M–type β-lactamases (CTX-Ms) are broad-spectrum β-lactamases derived from the chromosomally encoded β-lactamases of *Kluyvera* sp. (*4–6*). So far, >70 CTX-M types have been isolated (www.lahey.org/studies, updated October 2008); these have been divided into 5 clusters on the basis of amino acid sequence: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25. Native CTX-Ms are cefotaximases that usually hydrolyze cefotaxime rather than ceftazidime. However, point mutations can extend their target spectrum to ceftazidime. Thus, CTX-M-15 and CTX-M-27 are derived by a single Asp240Gly substitution from CTX-M-3 and CTX-M-14, respectively (*7*,*8*).

Until recently, most ESBLs in clinical samples came from a hospital environment, belonged to the TEM or SHV β-lactamase family, and were produced by *Klebsiella* spp., *Enterobacter* spp., and *E. coli* (*9*). Within the past few years, the nature of ESBL dissemination has changed: *E. coli* is now the most frequently isolated ESBL-carrying bacterium, and CTX-Ms have become the most frequently isolated ESBLs (*10*). Moreover, in the last few years CTX-M–type ESBLs have emerged within the community, particularly among *E. coli* isolated from UTIs (*11–16*). Risk factors for CTX-M carriage in the community are largely unknown. CTX-Ms have been isolated from patients with CA-UTIs who have neither had recent antimicrobial drug treatment nor been admitted to a hospital or long-term-care facility (*17–19*). Resistance to fluoroquinolones, aminoglycosides,

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and cotrimoxazole is often associated with CTX-M production (*16*,*17*), thereby limiting the choice of effective antimicrobial drugs to carbapenems or colistin.

Some studies have shown high prevalence of ESBLs among various *Enterobacteriaceae* in hospitals in developing countries (*20*–*22*). However, little information is available regarding prevalence within the community. Few bacteriologic data are available for Cambodia, and no studies of resistance of *Enterobacteriaceae* to antimicrobial agents have been reported. We investigated *E. coli* CA-UTIs in a 2-year prospective study. Our aims were 1) to establish the prevalence of community-acquired urinary *E. coli* resistance to a wide range of antimicrobial drugs and 2) to characterize the mechanisms underlying *E. coli* resistance to β-lactams.

## Methods

### Clinical Isolates from Patient Samples

Our laboratory at the Institut Pasteur in Phnom Penh, Cambodia, is a community laboratory, involved in various biological analyses. We receive biological samples from outpatients and hospitals that do not have bacteriology laboratories. Included in the study were patients who visited our laboratory with a suspected UTI from January 2004 through December 2005. Currently hospitalized patients were excluded, yet previous hospitalization was not an exclusionary criterion. We collected basic clinical data for each patient, including age and gender, whether antimicrobial drugs had been taken within the month preceding the sample submission, UTI history, and recent hospital visit. A UTI was defined by $\geq 10^4$ leukocytes/mL urine and $\geq 10^5$ CFU/mL urine. We used CLED (cystine-lactose-electrolyte deficient) agar (Dynamic Pharma, Phnom-Penh, Cambodia) for culture. Bacteria were identified with the API20E identification gallery (bioMérieux, Marcy l'Etoile, France). Strains were named (CEC [Cambodian *Escherichia coli*]) and numbered independently from isolation date.

### Antimicrobial Drug Susceptibility Testing and ESBL Confirmatory Testing

We determined antimicrobial drug susceptibility by the disk-diffusion method on Mueller-Hinton agar plates (Bio-Rad, Marnes-la-Coquette, France), as recommended by the French Society for Microbiology (www.sfm.asso.fr). We tested the following antimicrobial agents: amoxicillin, amoxicillin/clavulanic acid (coamoxiclav), ticarcillin, ticarcillin/clavulanic acid, piperacillin, piperacillin/tazobactam, cefalothin, cefoxitin, cefotaxime, ceftazidime, cefepime, imipenem, moxalactam, aztreonam, nalidixic acid, norfloxacin, ciprofloxacin, gentamicin, tobramycin, netilmicin, amikacin, nitroxolin, fosfomycin, and sulfamethoxazole/trimethoprim (cotrimoxazole). We detected ESBLs by using the double-disk synergy test (clavulanic acid and cefotaxime, ceftazidime, cefepime and aztreonam) performed on Mueller-Hinton media. We determined MICs for cefoxitin, cefotaxime, ceftazidime, and cefepime by using the agar-dilution method for ESBL-carrying strains and strains with decreased susceptibility to cefoxitin (as interpreted by the disk-diffusion method) as recommended by the French Society for Microbiology. For cefoxitin, strains were considered susceptible if MICs were ≤8 mg/L; intermediate susceptible if MICs were 16–32 mg/L, and resistant if MICs were >32 mg/L. For cefotaxime, strains were considered susceptible if MICs were ≤1 mg/L; intermediate susceptible if MICs were 2 mg/L, and resistant if MICs were >2 mg/L. For ceftazidime and cefepime, strains were considered susceptible if MICs were ≤4 mg/L, intermediate susceptible if MICs were 8 mg/L, and resistant if MICs were >8 mg/L.

### PCR Amplifications

Template DNA was prepared by boiling. Briefly, 5 colonies were suspended thoroughly in 1 mL DNase- and RNase-free water and boiled for 10 min. After centrifugation, supernatant was used as template DNA. We amplified the *ampC* upstream region, $bla_{TEM}$, $bla_{SHV}$, $bla_{CTX\text{-}M}$, $bla_{VEB}$, $bla_{OXA\text{-}1}$, and $bla_{CMY}$ by PCR, using specific oligodeoxynucleotides (Table 1). PCR was performed in a 25-μL mixture of 1× buffer (supplied with *Taq* polymerase), 2.5 mmol/L $MgCl_2$, 2.5 U of FIREPol DNA polymerase (Solis BioDyne, Tartu, Estonia), 200 μmol/L of each deoxynucleoside triphosphate, and 25 pmol of each primer. The PCR mixture was subjected to a 5-min denaturation step at 94°C, followed by 30 cycles of 45 s at 94°C, 45 s at 55°C, and 60 s at 72°C, and a final elongation step of 5 min at 72°C. PCR products were separated by 100-V electrophoresis in a 2% agarose gel for 30 min, after which they were stained with ethidium bromide.

### PCR Product Sequencing

Amplification products were purified with Montage PCR Filter Units (Millipore, Billerica, MA, USA). Sequencing reactions were performed in a PTC-225 Peltier Thermal Cycler (MJ Research, Waltham, MA, USA) by using an ABI PRISM BigDye Terminator Cycle Sequencing Kit with AmpliTaq DNA polymerase (Applied Biosystems, Branchburg, NJ, USA), according to the manufacturer's instructions. Each template was sequenced with the appropriate primer. Fluorescence-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Table 1. Primers used to study CTX-M β-lactamases in *Escherichia coli,* Cambodia, 2004–2005

| Gene detected | Primer name | Primer sequence (5′ → 3′) | Reference |
|---|---|---|---|
| $bla_{TEM}$ | C | TCG GGG AAA TGT GCG CG | (*23*) |
| | D | TGC TTA ATC AGT GAG GCA CC | |
| $bla_{SHV}$ | OS-5 | TTA TCT CCC TGT TAG CCA CC | (*24*) |
| | OS-6 | GAT TTG CTG ATT TCG CTC GG | |
| $bla_{CTX-M}$ | MA-1 | SCS ATG TGC AGY ACC AGT AA | (*23*) |
| | MA-2 | CCG CRA TAT GRT TGG TGG TG | |
| $bla_{CTX-M}$ group 9 | M9U | ATG GTG ACA AAG AGA GTG CA | (*23*) |
| | M9L | CCC TTC GGC GAT GAT TCT C | |
| $bla_{CTX-M}$ group 1 | M13U | GGT TAA AAA ATC ACT GCG TC | (*23*) |
| | M13L | TTG GTG ACG ATT TTA GCC GC | |
| $bla_{ampC}$ | AmpC1 | AAT GGG TTT TCT ACG GTC TG | (*25*) |
| | AmpC2 | GGG CAG CAA ATG TGG AGC AA | |
| $bla_{VEB}$ | casF | CGA CTT CCA TTT CCC GAT GC | (*26*) |
| | casB | GGA CTC TGC AAC AAA TAC GC | |
| $bla_{OXA-1}$ | OXA-1up | TAT CAA CTT CGC TAT TTT TTT A | (*27*) |
| | OXA-1low | TTT AGT GTG TTT AGA ATG GTG A | |
| $bla_{CMY}$ | CF1 | ATGATGAAAAAATCGTTATGC | (*28*) |
| | CF2 | TTGTAGCTTTTCAAGAATGCGC | |
| *chuA* | ChuA.1 | GAC GAA CCA ACG GTC AGG AT | (*29*) |
| | ChuA.2 | TGC CGC CAG TAC CAA AGA CA | |
| *ygaA* | YgaA.1 | TGA AGT GTC AGG AGA CGC TG | (*29*) |
| | YgaA.2 | ATG GAG AAT GGG TTC CTC AAC | |
| *TspE4C2* | TspE4C2.1 | GAG TAA TGT CGG GGC ATT CA | (*29*) |
| | TspE4C2.2 | CGC GCC AAC AAA GTA TTA GC | |

### Phylogenetic Group Determination

To determine phylogenetic group (i.e., A, B1, B2, and D), we performed triplex PCR for all strains (n = 93) as described previously (*29*). We used *chuA* and *yjaA* genes and an *E. coli* DNA fragment, TSPE4.C2.

### Repetitive Extragenic Palindromic PCR

The clonality of ESBL-positive strains was assessed by repetitive extragenic palindromic (rep)–PCR. DNA was extracted by using the QIAGEN Mini kit (QIAGEN, Courtaboeuf, France). REP-PCRs were performed for all strains in the same batch with primers rep-1R and rep-2T, as described previously (*28*). The resulting products were separated by 70-V electrophoresis in a 1% agarose gel for 3 h, after which they were stained with ethidium bromide. Strains with suspected similar migration profiles were then migrated together with the ESBL-positive strains to assess their similarity. Photographs of the gels (Figure) have been harmonized to be more informative.

### Statistical Analysis

Data were analyzed by using Epi Info version 3.2 (www.cdc.gov/epiinfo). Risk factors for ESBL-producing *E. coli* were assessed by using univariate analysis with the $\chi^2$ or Fisher exact tests; however, we used analysis of variance to determine whether age had an association with ESBL-producing *E. coli*. Associations between ESBL type, co-resistance, mutations in *ampC* promoter regions, and phylogenetic groups were separately tested by using the $\chi^2$ or Fisher exact tests. The significance threshold was 0.05.

## Results

### Population Characteristics

Of the 861 urine samples, 194 were positive for UTI and 163 contained *E. coli.* Samples from hospitalized patients and samples isolated from the same patient in a short period were excluded from the study. Overall, 93 patients with *E. coli*–related CA-UTIs were recruited for the study, all of whom were living in Phnom Penh. Their mean age was 43 years (range 1–88 years, median 40 years), and the M:F ratio was 0.21. No pregnant women were included. Twenty-eight (30.1%) patients had a history of UTI (with no details available), 24 (25.8%) had taken antimicrobial drugs, and 9 (9.7%) had sought consultation at a hospital in the month preceding the urine sampling. Patients with a history of UTI had taken significantly more antimicrobial drugs than had persons without UTIs ($p<0.0001$).

### Prevalence of Antimicrobial Drug Resistance in *E. coli* Strains

Disk-diffusion susceptibility testing indicated high prevalence of resistance to various antimicrobial agents: 88 (94.6%) strains were resistant to amoxicillin and ticarcillin, and 20 (21.5%) were immediate susceptible to cefoxitin. We observed synergy between clavulanic acid, oxyimino-

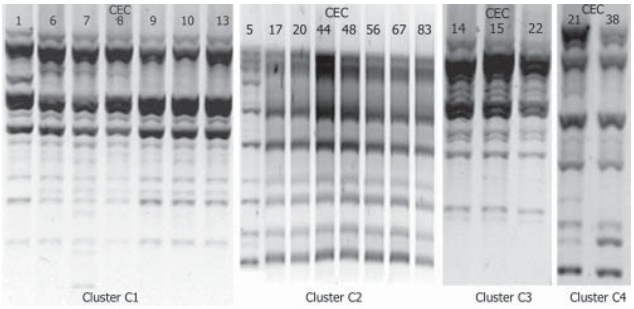


Figure. Results of repetitive extragenic palindromic–PCR of *Escherichia coli* isolates belonging to the 4 clusters, Cambodia, 2004–2005. CEC, Cambodian *E. coli*.

cephalosporins, and aztreonam for 34 strains (36.6%). Decreased susceptibility to cefoxitin was significantly associated with ESBL expression (p<0.001). All strains (n = 93) were susceptible to imipenem. One strain (CEC93) showed a high level of resistance to cefoxitin, cefotaxime, and ceftazidime but not to cefepime; double-disk synergy test results were negative for clavulanate. We observed a substantial level of resistance to quinolones; 63 strains (67.7%) had an intermediate level of resistance or were fully resistant to nalidixic acid, norfloxacin, and ciprofloxacin. Quinolone resistance was strongly associated with ESBL expression (p<0.0001). Of the 34 ESBL-carrying strains, only 2 were susceptible to quinolones. Cotrimoxazole resistance was observed in 70 strains (75.3%) but was not significantly associated with ESBL expression (p = 0.06). Aminoglycoside resistance was also significantly associated with ESBL expression (p<0.01). Thirty-nine strains (41.9%) were resistant to at least 1 of the 4 tested aminoglycosides. The most frequently observed phenotypic profile included resistance to gentamicin, tobramycin, and netilmicin (37.6% of the strains). None of the strains were resistant to fosfomycin or nitrofurantoin (Table 2).

### Risk Factors for ESBL Carriage

Univariate analysis showed no risk factors for ESBL carriage. Carriage was not significantly associated with gender, age, previous hospitalization (within the past month), antimicrobial drug treatment (within the last month), or history of UTI.

### β-Lactamase Characterization

Thirty-four strains were tested for ESBL identification. They were all positive for $bla_{\text{CTX-M}}$ and negative for $bla_{\text{VEB}}$ and $bla_{\text{SHV}}$. We detected $bla_{\text{TEM}}$ in 26 (76.4%) of the ESBL-carrying strains. CTX-M-14 was the most frequently isolated ESBL (n = 15), followed by CTX-M-27 (n = 12) and CTX-M-15 (n = 5). One strain (CEC7) was carrying both $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-15}}$. Strain CEC14 was carrying a $bla_{\text{CTX-M-14}}$ variant, which differed from the parental enzyme by a single transversion (C825G) leading to a Ser273Arg amino acid change. $bla_{\text{TEM-1}}$ was detected in 24 (96%) of the $bla_{\text{TEM}}$-carrying strains. One strain (CEC23) had a DNA sequence that differed from TEM-1 by 4 nucleotides. Only 1 of these mutations resulted in an amino acid change (Met182Thr). Amino acid sequence comparison (www.lahey.org/studies/webt.asp) showed that this TEM had the same amino acid sequence as TEM-135.

Strain CEC93, exhibiting a phenotypic profile indicative of a high level of cephalosporinase expression, was positive for $bla_{\text{CMY}}$. Sequencing confirmed the presence of $bla_{\text{CMY-2}}$ in this strain. We detected $bla_{\text{OXA-1}}$ in 4 CTX-M-15 –producing strains: CEC15, CEC48, CEC68, and CEC89.

### *ampC* Sequencing Results

Strains with different levels of resistance to cefoxitin, ranging from intermediate (cefoxitin MIC = 8 mg/L) to full resistance (cefoxitin MIC >8 mg/L), had different mutations within the region upstream from *ampC* (Table 3). Only 1 strain did not display any mutations. Most mutations occurred within the transcriptional attenuator region; the most frequently observed profile were +22 C>T, +26 T>G, +27 A>T, +32 G>A, and +70 C>T (11 strains). The other strains each exhibited a different profile. Strain CEC57 had the profile –32 T>A with an additional nucleotide change, which significantly increases *ampC* expression (*31*). Strain CEC92 had the combination –42 C>T and –28 G>A, which also increases *ampC* expression (*25*).

Table 2. Resistance to antimicrobial agents among ESBL-positive and ESBL-negative *Escherichia coli,* Cambodia, 2004–2005*

| Antimicrobial agent | Resistance ratios, % (no. resistant strains) | | | p value |
|---|---|---|---|---|
| | ESBL+, n = 34 | ESBL–, n = 59 | Total, n = 93 | |
| Fluoroquinolones | 94 (32) | 53 (31) | 68 (63) | <0.001 |
| Cotrimoxazole | 85 (29) | 69 (41) | 75 (70) | 0.06 |
| Aminoglycosides | 65 (22) | 27 (16) | 42 (39) | <0.01 |
| Co-amoxiclav | 94 (32) | 19 (11) | 46 (43) | <0.001 |
| Cefoxitin | 41 (13) | 11 (7) | 22 (20) | <0.001 |
| Nitrofurantoin | 0 | 0 | 0 | NS |
| Fosfomycin | 0 | 0 | 0 | NS |

*ESBL, extended-spectrum β-lactamase; NS, not significant.

Table 3. Mutations detected in the *ampC* promoter region of CTX-M β-lactam–resistant *Escherichia coli**

| Isolate | CEF MIC, mg/L | CTX-M | Phylogenetic group | Mutation at position† | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | –42 | –32 | –28 | –18 | –1 | +22 | +26 | +27 | +32 | +58 | +70 | +81 |
| *E. coli* K12 (U00096) | – | – | – | C | T | G | G | C | C | T | A | G | C | C | G |
| CEC1 | 16 | Yes | D | | | | | | T | G | T | A | | T | |
| CEC3 | 32 | No | D | | | | | | T | G | T | A | | T | |
| CEC4 | 32 | No | D | | | | A | T | | | | | T | | |
| CEC6 | 32 | Yes | D | | | | | | T | G | T | A | | T | |
| CEC8 | 32 | Yes | D | | | | | | T | G | T | A | | T | |
| CEC9 | 32 | Yes | D | | | | | | T | G | T | A | | T | |
| CEC10 | 32 | Yes | D | | | | | | T | G | T | A | | T | |
| CEC13 | 32 | Yes | D | | | | | | T | G | T | A | | T | |
| CEC14 | 16 | Yes | D | | | | | | | | | | | T | |
| CEC15 | 16 | Yes | D | | | | | | | | | | | T | |
| CEC19 | 32 | No | D | | | | | | T | G | T | A | | T | |
| CEC21 | 16 | Yes | D | | | A | | | | | | | T | | A |
| CEC22 | 16 | Yes | D | | | | | | | | | | | T | |
| CEC31 | 16 | Yes | D | | | A | | | | | | | T | | A |
| CEC57 | 32 | No | D | | A | | | | T | G | T | A | | T | |
| CEC88 | 32 | No | B2 | | | A | | | | | | | | | A |
| CEC89 | 32 | Yes | D | | | | | | T | G | T | A | | T | |
| CEC92 | >128 | Yes | D | T | | | A | T | | | | | T | | |
| CEC93 | >128 | No | D | | | | | | T | G | T | A | | T | |

*CEF, cefoxitin; CEC, Cambodian *E. coli*.
†According to numbering of Jaurin and Grundström (*30*).

### Phylogenetic Groups

Most of the strains studied belonged to groups B2 (53%; n = 49) and D (28%; n = 26), as do most pathogenic *E. coli* (*32*). Twelve strains belonged to group A (13%) and 6 to group B1 (6%). Among ESBL-carrying strains, 53% belonged to group B2 (n = 18), 38% to D (n = 13), 6% to A (n = 2), and 3% to B1 (n = 1). Resistance to cotrimoxazole was less prevalent in B2 strains than among strains from the other groups ($p<0.05$). Resistance to quinolones was more prevalent among group D strains than in strains from other groups ($p<0.005$). Furthermore, a strong association ($p<0.0001$) was found between decreased susceptibility to cefoxitin and group D strains. No association was shown between phylogenetic group and other characteristics such as ESBL carriage and aminoglycoside resistance. Among ESBL-carrying strains, none of the 4 groups were associated with 1 particular type of CTX-M.

### Rep-PCR Findings

Twenty-one ESBL positive strains and the CMY-2–producing strain were divided into 4 clusters; the remaining strains were genetically unrelated (online Appendix Table, available from www.cdc.gov/EID/content/15/5/741-appT.htm; Figure). Cluster C1 consisted of 8 strains with an identical *ampC* mutation profile, all belonging to phylogenetic group D. However, β-lactamase content was variable: 6 strains harbored CTX-M-27 and TEM-1, 1 strain harbored only CMY-2, and 1 harbored only CTX-M-15. Strain CEC7 (belonging to group B2, carrying CTX-M-14 and CTX-M-15, with no *ampC* mutations) exhibited a REP-PCR profile similar to that of strains in cluster C1. Cluster C2 consisted of 8 strains belonging to group B2 with no *ampC* mutations and carrying various β-lactamases; 6 strains produced CTX-M-14 (2 of which co-produced TEM-1), 1 produced CTX-M-27/TEM-1, and 1 produced CTX-M-15/TEM-1/OXA-1. Cluster C3 consisted of 3 strains from group D, all with the same *ampC* mutation profile, but with 3 distinct β-lactamase profiles (CTX-M-14 and TEM-1; CTX-M-14-like and TEM-1; and CTX-M-15 and TEM-1). Cluster C4 consisted of 2 strains from group D, each with the same *ampC* mutation profile but different β-lactamase profiles (CTX-M-14 and TEM-1; and CTX-M-27 only).

## Discussion

We surveyed antimicrobial drug resistance in Cambodia. Our prospective study in this developing country focused on CTX-Ms CA-UTIs caused by *E. coli*. Although the patients included came from the community, antimicrobial drug resistance was prevalent among UTI-causing strains, particularly to β-lactams (including extended-spectrum cephalosporins). Our findings suggest that CTX-M-type β-lactamases are widespread in Cambodia. CTX-M production was significantly associated with resistance to quinolones and aminoglycosides and with decreased susceptibility to cefoxitin, leading to a high prevalence of multiresistant strains. The spread of CTX-M in the community has already been described through prospective studies in industrialized countries such as Canada (*33*), France (*34*),

and the United Kingdom (*12*); however, we found higher prevalence of CTX-Ms in Cambodia than that reported in these previous studies.

We propose 3 possible explanations for the situation in Cambodia. First, although no reliable data were available, it is well known that many persons in the community self-medicate or obtain prescriptions for non-adapted drugs (drugs taken without any antibiotic susceptibility testing of the strain that causes the infections); these practices suggest that uncontrolled consumption of antimicrobial agents is likely to play a major role. This factor, together with the likely substandard quality of some drugs, undoubtedly contributes to the high prevalence of resistance. Second, hygiene in Cambodia is poor, and the population density in Phnom Penh is high. The saturated sewer system, particularly during the rainy season, likely facilitates efficient propagation and spread of bacteria within the community. Grenet et al. proposed that that poor hygiene in a French-Guyanese Indian community led to the spread of resistant bacteria despite low antibiotic pressure (*35*). Similarly, poor hygiene conditions may have led to the spread of resistant strains in the Cambodian community studied here. The TEM-type and SHV-type ESBL—until recently the predominant ESBL family subtypes—have never been implicated in the spread of ESBL in the community. Why CTX-M strains are the only ESBL types to spread in the community is not clear. CTX-M strains may have a particularly high capacity to disseminate or an ecologic advantage over other ESBLs and thus persist in the community, whereas other ESBL may be progressively eliminated with decreasing antibiotic pressure.

One limitation to our study is that patients at our institute are not necessarily representative of the Cambodian population. For most persons in Cambodia, antimicrobial agents and biologic analyses are expensive. Therefore, our patients may have been wealthier and may have taken more courses of antimicrobial drug treatment than other Cambodians.

REP-PCR yielded 4 clusters of strains, consistent with their identical *ampC* mutational profiles, yet with various contents of β-lactamases. Because all included patients were living in Phnom Penh, mediocre hygiene might have favored the diffusion of clones within an urban area, a phenomenon that had previously been observed in large-scale studies undertaken in the United Kingdom (*12*), Canada (*36*), Italy (37), and Brazil (*15*) but not in Hong Kong (*13*). In contrast to our results, clones identified in those studies harbored the same β-lactamases. Further investigation using multilocus sequence typing would be necessary to identify the molecular determinants of the CTX-M–carrying *E. coli* pandemic in Cambodia.

Branger et al. have observed that group D *E. coli* were more frequently resistant to quinolones (*38*) than were non-D *E. coli*; these findings are consistent with our results. Moreover, we found a strong association ($p<0.001$) between group D strains and decreased susceptibility to cefoxitin (secondary to the effect of mutations in the *ampC* promoter region). This association was also present when all strains were taken into account ($p<0.001$). However, although CTX-M carriage was more frequently observed for group D strains than for non-D strains, the association was not significant. Given that quinolone resistance and *ampC* hyperexpression involve several mutations, a possible explanation for this association with group D strains may be a stronger mutation capacity for these strains than for strains belonging to other groups. Further investigations will be required to explain this phenomenon.

Community-isolated ESBL-carrying strains are an emerging challenge for community practitioners and hospitals. Information is not readily available in either developing countries or in industrialized countries, and UTI treatment guidelines remain unchanged. In Cambodia, and probably in many other developing countries, resistant *E. coli* strains are endemic to the community. Investigating the current situation in Cambodia may improve our understanding of the situation in industrialized countries, where ESBLs are no longer uncommon in the community. According to our experience in Cambodia, measures should focus on improving hygiene and appropriate prescribing of antimicrobial agents. In conclusion, we suggest that this high prevalence of β-lactam resistance in Cambodia is due to the intrinsic capacity of CTX-M–encoding genes to disseminate through communities where hygiene and living conditions are poor and antimicrobial drug consumption is uncontrolled.

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# Increased Risk for Severe Malaria in HIV-1–infected Adults, Zambia

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## CME ACTIVITY

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**Learning Objectives**

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

- Describe the impact of HIV infection on malaria infection
- Identify different levels of severity of malaria infection
- Describe the presentation of severe malaria in patients infected with HIV-1 in one region in Zambia
- Compare the risk for severe malaria in relation to CD4 count in patients infected with HIV-1

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To determine whether HIV-1 infection and HIV-1–related immunosuppression were risk factors for severe malaria in adults with some immunity to malaria, we conducted a case–control study in Luanshya, Zambia, during December 2005–March 2007. For each case-patient with severe malaria, we selected 2 matched controls (an adult with uncomplicated malaria and an adult without signs of disease). HIV-1 infection was present in 93% of case-patients, in 52% of controls with uncomplicated malaria, and in 45% of asymptomatic controls. HIV-1 infection was a highly significant risk factor for adults with severe malaria compared with controls with uncomplicated malaria (odds ratio [OR] 12.6, 95% confidence interval [CI] 2.0–78.8, p = 0.0005) and asymptomatic controls (OR 16.6, 95% CI 2.5–111.5, p = 0.0005). Persons with severe malaria were more likely to have a CD4 count <350/μL than were asymptomatic controls (OR 23.0, 95% CI 3.35–158.00, p<0.0001).

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The geographic overlap between HIV-1 infection and malaria, particularly in eastern and southern Africa, has caused concern since the 1980s. The degree of interaction between HIV-1 infection and malaria emerged during 1999–2009 and has been extensively reviewed for both nonpregnant and pregnant adult women (*1*,*2*). The effect of HIV-1 on malaria seems to be driven mainly by the incapacity of the immune system to control parasite load, leading to a higher prevalence of infection (*3*), a higher incidence of clinical malaria (*4*,*5*), and a risk for treatment failure (*6*) in immunosuppressed HIV-1 patients.

Reports of HIV-1 infection as a risk factor for hyperparasitemia or severe malaria are few and limited. In urban Burkina Faso, >30% of adults with severe malaria were also infected with HIV-1, whereas HIV-1 prevalence in the general adult population was ≈5%–14% (*7*). In South Africa, in an area of low malaria transmission (<1 case/1000/

year), HIV-1 infection was associated with severe falciparum malaria (*8–10*). Similarly, in Mumbai, India, where malaria transmission is low, HIV-1 prevalence was higher in persons with severe malaria than in the general population (*11*). Therefore, in areas of low malaria transmission, HIV-1 infection seems to be an important risk factor for severe malaria and death. In areas of high malaria transmission (>1/case/1000/year), the relationship between HIV-1 infection and severe malaria is less well established, with a small hospital-based study in Zimbabwe reporting higher risk for severe malaria and related death in HIV-1–infected adults than in HIV-1–uninfected adults (*12*). In all these studies, either the number of cases was small or HIV-1 testing was not performed, and CD4 cell count was not routinely done. The effect of both HIV-1 infection and malaria is important in several African countries with high malaria endemicity. We report the results of a matched case–control study exploring whether HIV-1 is an important risk factor for severe malaria in adults living Luanshya, Zambia, an area of high malaria transmission (>200/cases/year).

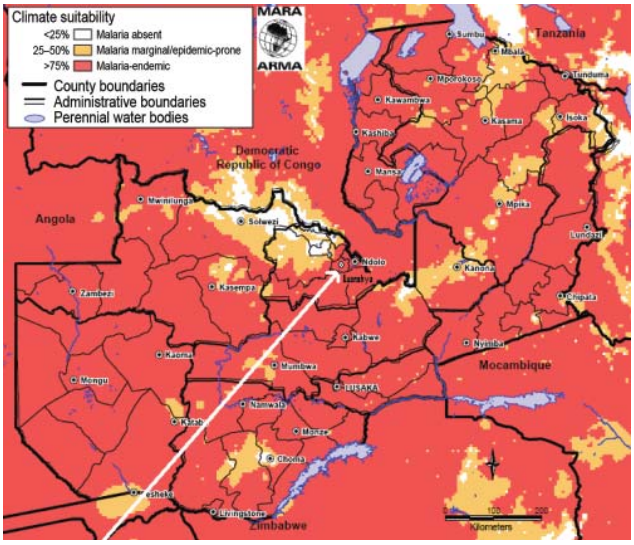


Figure. Malaria endemicity in Zambia. This map is a product of the Mapping Malaria Risk in Africa/Atlas du Risque de la Malaria en Afrique (MARA/ARMA) collaboration (www.mara.org.za), July 2005. Malaria distribution was obtained from Craig et al. (*14*). Topographic data were obtained from African Data Sampler, World Resources Institute (www.igc.org/wri/sdis/maps/ads/ads_idx.htm).

## Methods

### Study Design

We chose a case–control design for this study because severe malaria is relatively rare in areas of stable transmission (*13*). Each adult with severe malaria was matched with 2 controls: 1 adult who had uncomplicated malaria and 1 asymptomatic adult in the community. We matched for major confounding variables: age group (15–19, 20–29, 30–39, and 40–49 years), sex, area of residence, and seasonal variation. Controls were recruited within 4 weeks after identification of case-patients.

### Study Site and Patients

The study was conducted at Thompson Hospital, a government district hospital serving Luanshya district in the Copperbelt province of Zambia. In this district, 99% of the population is at risk for malaria (Figure), and 30% of women attending voluntary counseling and testing at the antenatal care department are infected with HIV-1 (*15*). Luanshya is not a site of sentinel surveillance for HIV, but sentinel surveillance conducted during late 2004 and early 2005 in the adjacent district of Ndola showed an HIV prevalence rate of 30% (*16*). From October 2005 through April 2007, all patients 15–49 years of age who sought treatment for symptoms and signs of febrile illness were screened for falciparum malaria by thick and thin blood smear. Patients in whom severe malaria was diagnosed were enrolled after informed consent was given by the patient or by his or her legally authorized representative. We defined severe malaria (*16*) as fever (body temperature ≥37.5°C ) or history of fever in the previous 48 hours, *Plasmodium falciparum* monoinfection with a density of at least 100 parasites per 200 leukocytes (assumed to be ≥4,000 parasites/μL) in the absence of any other evident causes of illness, and at least 1 of the following signs: impaired consciousness (Glasgow Coma Score ≤10), multiple grand mal convulsions, jaundice, hypoglycemia (glycemia ≤2.5 mmol/L), hyperparasitemia (parasite density >100,000/μL), renal impairment, and cardiorespiratory distress. We collected information about anemia but did not include anemia in the case definition because it can be HIV-1 related, resulting in possible bias, and because uncomplicated and severe malaria episodes can be misclassified in HIV-1–infected persons.

Patients with severe malaria were treated according to the national guidelines for severe malaria in Zambia (i.e., quinine 10 mg/kg/8 h for 7 d), with adequate supportive treatment (*17*). On the basis of information about areas of unstable malaria and of HIV surveillance data in Zambia, we predicted that HIV-1 increased the risk for severe malaria 5-fold and assumed a 30% HIV-1 prevalence (primary exposure) among controls. Using a 1:1 case:control ratio, we determined that we needed to recruit 30 case–control pairs to detect this risk with 80% power. Recruitment of cases continued until the required sample size was reached.

Controls with uncomplicated malaria were selected from the clinic closest to the homes of the case-patients. The first patient attending the clinic and exhibiting fever or having a history of fever in the previous 48 hours, without any other disease, with a positive rapid diagnostic test, residing in the catchment area of the health center, matching sex and

age group of the case-patient, and willing to participate was recruited as a control. Pregnant women were excluded.

Field trips were organized to case-patients' residences, where, following the random sampling method, a community control was identified and independently assessed. The first healthy asymptomatic adult fulfilling the matching criteria and willing to participate was recruited as a community control. Pregnant women were excluded. All community controls were assessed for possible confounding factors and screened for falciparum malaria by thick and thin blood smear. We sought to identify controls within 4 weeks after we recruited a case-patient.

### Laboratory Tests

Laboratory technicians were blinded to patient identity and to patient-related parameters. We used microscopy to screen case-patients and community controls for malaria using Giemsa staining 10% for 5 min. A thin blood film was examined to identify type of parasite; a thick film was taken for quantification. Computation of parasite density took into consideration the actual leukocyte count. In asymptomatic controls, we screened for *P. falciparum* infection by using a rapid diagnostic test, Malaria Pf immunochromatographic test (ICT) (ICT Diagnostics, Cape Town, South Africa). For each participant, a blood sample (0.5 mL) was collected for HIV-1 testing and CD4 cell count, and for hemoglobin (1 mL), by venipuncture. Neither the study staff nor the patient had access to the HIV-1 test results. HIV-1 testing followed an unlinked anonymous procedure: blood samples were sent to the hospital laboratory, where they were processed anonymously. Determine HIV1/2/O (Abbott Laboratories, Abbott Park, IL, USA) was the first test. If test results were negative, the patient was considered not infected. If results were positive or indeterminate, the blood sample was tested with Unigold Recombinant HIV-1/2 (Trinity Biotech PLC, Bray, Ireland); if results were positive, the patient was classified as HIV-1 infected. If results were negative, the outcome was considered indeterminate, and the sample was further tested with ELISA and Western blot. Patients, after recovering from malaria, and controls were counseled about HIV-1 and offered the opportunity for voluntary counseling and testing.

CD4 counts were determined by flow cytometry on a CyFlow (Cyflow Counter, Partec, Germany) within 5 hours after collection, and multiset software was used to obtain the absolute counts and the CD4+ lymphocyte ratio (*18*). A FACSCount machine (Becton Dickinson, Sparks, MD, USA) was used as a quality control to validate the accuracy of the Cyflow data over time and served as backup.

### Ethics and Consent

The trial was approved by the ethical committee of the Institute of Tropical Medicine, Antwerp, Belgium, and by the Research Ethics Committee of the Tropical Disease Research Centre, Ndola, Zambia. The written informed consent signed by study participants or their legally authorized representatives described the purpose of the study, procedures followed, and risks and benefits of participation. The consent form was in both English and the local language. Patients were counseled on HIV and offered the opportunity to undergo voluntary counseling and testing in conjunction with the study; this procedure was followed to ensure adherence to the standard of counseling and testing stipulated in the guidelines for HIV/AIDS counseling in Zambia (*17*).

### Statistical Analysis

Crude associations between the primary outcome measures and potential risk factors, including HIV-1 status and HIV-1–related immunosuppression (HIV-1+ with CD4 $\geq$350/μL and HIV-1+ with CD4 <350/μL), were described by using summary statistics, means, and count and were tested by using Wilcoxon signed rank test for matched variables.

## Results

From December 2005 through March 2007, we recruited 30 case-patients with severe malaria, 30 controls with uncomplicated malaria, and 30 asymptomatic community controls who fulfilled all inclusion criteria. Because of the low malaria incidence among adults, 5 controls with uncomplicated malaria who met the matching criteria could not be identified within 28 days after admission of the case-patient to the hospital. One case-patient and corresponding matched controls were omitted because of missing laboratory results. One case-patient was recruited during March–October 2006, the low malaria transmission season. Median age was 33 years, and men and women were equally represented (Table 1). Eighty-six percent of participants lived in concrete houses. Households contained an average of 4.7 inhabitants, few of whom slept under a bed net (1.5 persons per household, 32%). Only 24% of case-patients (7/29) had slept under a bed net before admission.

Impaired consciousness and hypoglycemia were the most common signs of severe malaria in HIV-1–infected case-patients (Table 2). Nineteen percent of patients, all HIV-1 infected, had anemia. All parasite densities were above the fever threshold (2,088/μL–635,500/μL), conservatively set at 2,000 parasites/μL. Seven of 13 patients with a parasite density below the geometric mean were treated with quinine only; others conservatively received some antimicrobial drugs. Six case-patients, all HIV-1–infected, had hyperparasitemia, 5 with >200,000 parasites/μL. None of the case-patients were receiving antiretroviral drugs or cotrimoxazole prophylaxis. Fifteen case-patients received concomitant antimicrobial drug treatment(s) determined

Table 1. Risk factors for severe malaria in case–control study, Luanshya, Zambia

| Risk factors | Case-patients, n = 29 | Controls with uncomplicated malaria, n = 29 | Asymptomatic controls, n = 29 | p value |
|---|---|---|---|---|
| Demographic characteristics* | | | | |
| GM† age, y (range) | 33 (18–50) | 33 (20–49) | 33 (18–50) | |
| No. (%) male | 14 (48) | 14 (48) | 14 (48) | |
| Living conditions† | | | | |
| No. (%) living in mud/clay hut | 4 (14) | 4 (14) | 5 (17) | 0.91 |
| No. (%) living in concrete house | 25 (86) | 25 (86) | 24 (83) | |
| GM no. persons living in house* (95% CI) | 4.7 (3.7–6.0) | 3.6 (2.9–4.5) | 4.7 (3.8–5.8) | 0.76 |
| Mean no. sleeping under bed net (95% CI) | 1.5 (0.7–2.3) | 1.5 (0.7–2.3) | 1.4 (0.5–2.3) | 0.85 |
| No. (%) sleeping under bed net | 7 (24) | 9 (31) | 9 (31) | 0.80 |
| No. (%) using antimalarial drug during previous week | 13 (45) | 8 (28) | 5 (17) | 0.13 |
| No. using quinine | 2 | 2 | 1 | |
| No. using sulfadoxine pyrimethamine | 8 | 5 | 3 | |
| No. using artemether–lumefantrine | 5 | 1 | 0 | |
| No. (%) with HIV-1 | 27 (93) | 15 (52) | 13 (45) | 0.03 |
| GM CD4 count (95% CI)‡ | 173 (125–240) | 205 (112–377) | 677 (427–1074) | |
| No. (%) CD4 count <200/μL‡ | 11/23 (48) | 5/14 (36) | 1/12 (8) | <0.001 |
| No. (%) CD4 count <350/μL‡ | 19/23 (83) | 11/14 (79) | 1/12 (8) | <0.001 |

*Matching variables.
†GM, geometric mean; CI, confidence interval.
‡Not measured in 4 case-patients, 1 control with uncomplicated malaria, and 1 asymptomatic control because of technical constraints.

by clinical symptoms: penicillin (8 patients), amoxicillin (1), gentamicin (2), metronidazole (1), fluconazole (2), chloramphenicol (3), ciprofloxacin (1), and cotrimoxazole (4). Clinical history, symptoms, outcome, illness duration, and other relevant parameters were similar in case-patients who received concomitant antmicrobial drugs and in those who received quinine only. Five (19%) patients died, 4 within 2 days after admission. Median length of hospital stay for successfully treated patients was 5.5 days. Case-patients were more likely to have used antimalarial treatment (45%) during the week before admission than were controls with uncomplicated malaria (28%) and asymptomatic controls (17%) (Table 1). Three case-patients used antimicrobial agents the week before admission, compared with 2 controls with uncomplicated malaria and 1 asymptomatic control.

Table 2. Clinical features of case-patients with severe malaria in case–control study, by HIV-1 infection status, Luanshya, Zambia

| Clinical features | HIV-1 infected, n = 27 | HIV-1 uninfected,* n = 2 |
|---|---|---|
| Signs and symptoms, no. (%) | | |
| Fever (>37.5°C) | 20 (74) | 1 (50) |
| History of fever | 25 (93) | 2 (100) |
| Impaired consciousness (Glasgow Coma Score ≤10) | 15 (56) | 0 (0) |
| Severe anemia (<7 g/dL)† | 5 (19) | 0 (0) |
| Convulsions | 6 (22) | 0 (0) |
| Jaundice | 3 (11) | 1 (50) |
| Hypoglycemia (<2.5 mmol/L) | 11 (41) | 0 (0) |
| Hyperparasitemia (>100,000 parasites/μL) | 6 (22) | 0 (0) |
| Renal impairment | 0 (0.0) | 0 (0) |
| Leukocyte count, mean 1,000/μL (SD)‡ | 6.9 (3.9) | 4.2 and 8.7 |
| Lymphocytes, % (SD)‡ | 25 (11) | 18 |
| Monocytes, % (SD)‡ | 11 (6) | 5.2 |
| Granulocytes, % (SD)‡ | 62 (20) | 76.8 |
| Parasite density, geometric mean/UL (95% confidence interval) | 43,314 (25,467–81,145) | 11,745 and 38,942 |
| Concomitant antimicrobial drugs | 15 (56) | 0 |
| Outcome | | |
| No. (%) discharged | 24 (82) | 2 (100) |
| Median time hospitalized, d (range) | 5.5 (1–31) | 4 and 17 |
| No. deceased (case-fatality ratio) | 5 (19) | 0 |
| Median length of illness before death, d (range) | 2 (1–11) | – |

*Because only 2 results were HIV negative, no SD or confidence interval was specified.
†Anemia was not an inclusion criterion.
‡Not measured in 4 patients because of technical constraints.

### Risk Analysis

Case-patients differed from controls in use of other drugs during the previous week, HIV-1 infection, and CD4 count. No parasitemia was detected in any asymptomatic controls. Because of the matching, controls with uncomplicated malaria and asymptomatic controls were similar to case-patients for all other assessed risk factors (Table 1).

HIV-1 infection was detected in 45% of asymptomatic controls, 52% of controls with uncomplicated malaria, and 93% of case-patients. HIV-1 infection was not a risk factor for uncomplicated malaria (odds ratio [OR] 1.3, 95% confidence interval [CI] 0.5–3.7, p = 0.59) (Table 3). Case-patients were more likely to be infected with HIV-1 than were controls with uncomplicated malaria and asymptomatic controls (OR 12.6, 95% CI 2.0–78.8, p = 0.0005, and OR 16.6, 95% CI 2.5–111.8, p = 0.0005, respectively).

Eighty-three percent of case-patients had a CD4 count <350/μL, compared with 79% of controls with uncomplicated malaria (p = 0.76) and 8% of asymptomatic controls (p<0.0001). Controls with uncomplicated malaria were more likely than asymptomatic controls to have a CD4 count <350/μL (OR 7.67, 95% CI 1.78–33.01, p = 0.001). Case-patients were more likely to have a CD4 count <350/μL than were asymptomatic controls (OR 23.00, 95% CI 3.35–158.00, p<0.0001) but not controls with uncomplicated malaria (OR 3.00, 95% CI 0.83–10.83, p = 0.32).

The extremely high proportion of low CD4 count in both case-patients and controls with uncomplicated malaria might be confounded by a temporary malaria-induced reallocation of specific T-cells (*19*). Therefore, 28 days after successful treatment, the absolute CD4 count was measured in 10 HIV-infected case-patients. During this period, the mean CD4 count increased >2-fold, from 142 (95% CI 76–269) to 320 CD4/μL (95% CI 169–607) (data not shown). However, the proportion of case-patients with CD4 count <350/μL remained substantial (70%), although slightly less than at admission (90%).

## Discussion

In Luanshya, Zambia, an area where malaria is mesoendemic, HIV-1 infection is an important risk factor for severe malaria in adults, primarily in those with a low CD4 count. The increased risk for severe malaria in HIV-1–infected persons already has been reported from areas of low and unstable transmission (*8–11*) but never has been firmly established in areas of stable malaria transmission. The paucity of information results from the difficulty of obtaining it. Studies collecting relevant information retrospectively are vulnerable to considerable bias, and prospective studies are difficult to carry out where adults have acquired immunity and severe malaria is rare (*20*). In our study, recruitment of 30 persons with severe malaria from a population of 10,000 persons took 2 years. However, even prospectively, diagnosing severe malaria with certainty might be difficult, particularly in the presence of HIV-1 co-infection, because several opportunistic infections of AIDS patients could have clinical presentations similar to those of severe malaria. Persons with severe malaria could have had a concomitant *Streptococcus pneumoniae* or *Salmonella enterica* serovar Typhimurium infection (*21*) or HIV-1 immunosuppression-related meningitis, such as cryptococcal meningitis. Although, none of the case-patients we recruited were identified by the treating physicians as having clinical AIDS, in the absence of a blood culture, physicians conservatively decided to concomitantly prescribe antimicrobial drugs in 15 cases. More detailed review of patients' files showed that most of these persons had hypoglycemia or hyperparasitemia, and none were in septic shock. Moreover, these patients recovered rapidly after treatment, and all but 1 were recruited during the high malaria transmission season, which support the argument that these persons had true cases of severe malaria.

The increased risk for uncomplicated malaria in HIV-1–infected patients with a low CD4 count is consistent with information in several cohort studies (*1*). Although the extremely high proportion of a low CD4 count in patients

Table 3. HIV-1 infection and HIV-1–related immunosuppression as risk factors for nonsevere and severe malaria, case–control study, Luanshya, Zambia*

| Participant characteristics | No. (%) | OR (95% CI) | p value | OR (95% CI) | p value |
|---|---|---|---|---|---|
| HIV-1 infected† | | | | | |
| Asymptomatic controls | 13/29 (45) | 1 | – | | |
| Controls with uncomplicated malaria | 15/29 (52) | 1.3 (0.5–3.7) | 0.59 | 1 | – |
| Case-patients (severe malaria) | 27/29 (93) | 16.6 (2.5–111.8) | 0.0005 | 12.6 (2.0–78.8) | 0.0005 |
| CD4 cell count <350/μL‡ | | | | | |
| Asymptomatic controls | 1/12 (8) | 1 | – | | |
| Controls with uncomplicated malaria | 11/14 (79) | 7. 67 (1.78–33.01) | 0.001 | 1 | – |
| Case-patients (severe malaria) | 19/23 (83) | 23.00 (3.35–158.00) | <0.0001 | 3.00 (0.83–10.83) | 0.08 |

*OR, odds ratio; CI, confidence interval.
†p value obtained by using matched-pairs signed-ranks test.
‡CD4 count not measured in 4 HIV-1–infected case-patients, 1 control with uncomplicated malaria, and 1 asymptomatic control because of technical constraints. p value obtained by using Wilcoxon signed-ranks test and Wilcoxon rank-sum test.

in our study might have been confounded by a temporary malaria-induced reallocation of specific T cells; 70% had a CD4 count <350/μL 1 month after successful treatment. Therefore, immunosuppression is likely an additional risk, but absolute CD4 count cannot be interpreted during a severe malaria episode.

Almost half of malaria cases throughout the world occur in areas where the disease is holoendemic (*22*). HIV-1 program managers working in areas where both diseases are prevalent should be aware that HIV-1 infection—and certainly HIV-1–related immunosuppression—are important risk factors for severe malaria. Early detection of HIV-1 infection is extremely important because comprehensive measures to prevent malaria and chemoprophylaxis with cotrimoxazole could be promptly implemented to protect against uncomplicated and severe malaria, a disease with a high fatality rate (*23*). Clinicians in such settings also should be aware of the strong association between severe malaria and HIV-1 so they can assess patients for other underlying diseases and offer the opportunity for voluntary counseling and testing for HIV-1 when patients have recovered from malaria.

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Dr Chalwe is a medical scientist with the Tropical Diseases Research Centre, Ndola, Zambia. His research interests are poverty-related diseases, particularly malaria and HIV/AIDS.

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# Seroprevalence of Antibodies to Avian Influenza Virus A (H5N1) among Residents of Villages with Human Cases, Thailand, 2005[1]

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In 2005, we assessed the seroprevalence of neutralizing antibodies to avian influenza virus A (H5N1) among 901 residents of 4 villages in Thailand where at least 1 confirmed human case of influenza (H5N1) had occurred during 2004. Although 68.1% of survey participants (median age 40 years) were exposed to backyard poultry and 25.7% were exposed to sick or dead chickens, all participants were seronegative for influenza virus (H5N1).

Three apparent waves of highly pathogenic avian influenza virus A (H5N1) infection in humans occurred in Thailand from early 2004 through 2006; these waves, which corresponded to influenza (H5N1) outbreaks in poultry, resulted in 25 confirmed human cases and 17 deaths (*1–4*). However, the frequency of asymptomatic and clinically mild cases of influenza (H5N1) infection was unknown in areas where these outbreaks occurred. In 2005, we conducted a cross-sectional seroprevalence study of influenza virus (H5N1) antibodies among residents of 4 rural villages in Thailand where at least 1 human influenza (H5N1) case had occurred in 2004. Backyard poultry farming is common in these villages, but the villages have no live poultry markets.

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## Methods

The study was conducted during October 11–27, 2005, among residents of 4 rural villages in central and northern Thailand where influenza (H5N1) outbreaks in poultry and human influenza (H5N1) cases had occurred: village A in Prachin Buri Province (1 confirmed case), village B in Kamphaeng Phet Province (1 probable case, 1 confirmed case), village C in Sukhothai Province (1 confirmed case), and village D in Phetchabun Province (1 confirmed case) (Figure). Illness onset in these case-patients occurred from August 31 through October 8, 2004. Residents of any of the villages for at least 2 weeks before and after illness onset of the respective case-patient in each village were eligible to participate in the study. Participants were enrolled by random selection from lists of village residents or by convenience sampling. Village residents were excluded if they had influenza (H5N1) diagnosed from August 17 through October 22, 2004, were ≤18 years of age and did not have parental consent, had an underlying coagulopathy, or were taking anticoagulant drugs within 2 weeks of enrollment. Written informed consent was obtained from all study participants or their proxies.

Using a standard questionnaire, trained interviewers collected demographic and exposure data through brief face-to-face interviews with study participants. Exposure was defined as either direct contact (touching) or close contact (within 1 m without direct contact) with chickens or other poultry or with a person with confirmed influenza (H5N1) infection. A 5-mL blood specimen was collected

[1]Preliminary findings from this study were presented on June 18, 2007, at the Options for the Control of Influenza Conference VI, Toronto, Ontario, Canada.

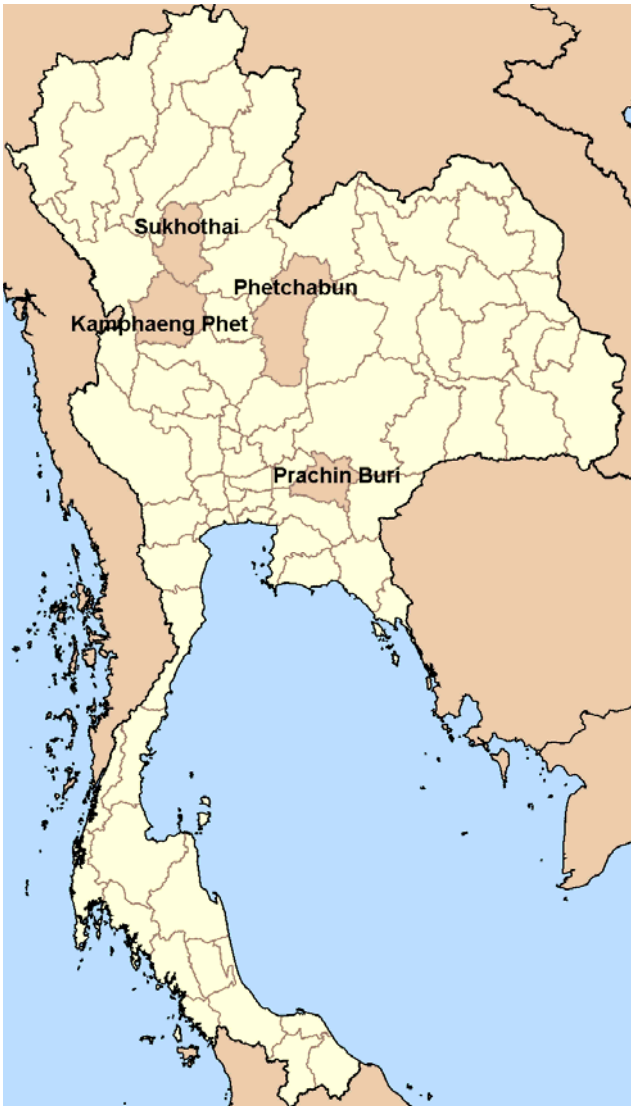


Figure. Province location of study villages with laboratory-confirmed avian influenza A (H5N1) cases in humans, Thailand, 2004. (Adapted from http://commons.wikimedia.org/wiki/Image:BlankMap_Thailand.png.)

from participants ≥5 years of age, and a 3-mL specimen was collected from those <5 years of age. Serum samples were separated at a local hospital and transported on wet ice to a laboratory within 48 h after collection. Serologic testing by microneutralization (MN) assay was performed in an enhanced biosafety level-3 containment facility in accordance with a slightly modified version of a protocol described previously (*5*–*7*). Influenza virus A/Thailand/1(KAN-1)/2004 (H5N1) was selected for the MN assay because of its antigenic similarity to influenza virus (H5N1) isolates from humans in Thailand (*2*). Immunofluorescence with use of 293T cells transfected with hemagglutinin H5N1 recombinant plasmid as the test antigen was used to confirm MN assay results. In accordance with our modified protocol, we considered an influenza virus (H5N1) neutralizing antibody titer ≥40 (equivalent to ≥80 in other protocols) to be a positive result (*5*–*7*).

Epi Info 2002 (Centers for Disease Control and Prevention, Atlanta, GA, USA) was used to enter and analyze study data. Mean, median, and proportion values were calculated for variables and compared by using bivariate analysis. The $\chi^2$ test was used for most analyses, analysis of variance was used to compare means from the convenience sample with those from the random sample, and the Fisher exact test was used if expected cell values were <5. Differences between the 2 sample groups were considered significant at $p<0.05$. The study was approved by the Ethical Review Committee for Research in Human Subjects, Thai Ministry of Public Health.

## Results

The study population consisted of 901 participants: 228 from village A (28.1% of village residents), 203 from village B (28.4%), 209 from village C (30.5%), and 260 from village D (19.6%). Their median age was 40 years (range 2–101 years), and 42.4% were male. The 901 participants were enrolled in 2 ways: 131 (14.5%) by random selection (out of 838 randomly selected villagers: 15.6% participation), and 770 (85.5%) by convenience sampling. The 2 groups of study participants did not differ significantly by demographic characteristics, history of illness, or exposure to poultry (Table 1). Most participants (68.1%) reported direct or close contact with backyard poultry, 25.7% reported direct or close contact with sick or dead chickens, and 7.1% reported close contact with a person with confirmed influenza (H5N1) infection (Table 1). Of 110 participants who reported a history of acute respiratory symptoms, 74.5% reported direct or close contact with backyard poultry, 31.8% reported direct or close contact with sick or dead chickens, and 13.6% reported close contact with a person with confirmed influenza (H5N1) infection (data not shown). All participants were seronegative for influenza virus (H5N1) neutralizing antibodies (Table 2).

## Discussion

Participants in this study were from villages in central and northern Thailand where widespread, confirmed outbreaks of influenza (H5N1) infection in poultry and at least 1 human influenza (H5N1) case had occurred during 2004. A substantial proportion of participants reported exposure to backyard poultry, including contact with sick or dead chickens, the primary risk factor for influenza (H5N1) infection (*8*,*9*). Nevertheless, we found no serologic evidence of mild or subclinical influenza (H5N1) infection, suggesting that clade 1 influenza virus A (H5N1) strains circulat-

Table 1. Characteristics of the study population, overall and by method of selection, Thailand, 2005

| Characteristic | Random sample (n = 131), no. (%) | Convenience sample (n = 770), no. (%) | p value | Total sample (N = 901), no. (%) |
|---|---|---|---|---|
| Age group, y | | | 0.30 | |
| 1–14 | 19 (14.5) | 178 (23.1) | | 197 (21.9) |
| 15–29 | 14 (10.7) | 99 (12.9) | | 113 (12.5) |
| 30–44 | 37 (28.2) | 177 (23.0) | | 214 (23.8) |
| 45–59 | 32 (24.4) | 175 (22.7) | | 207 (22.9) |
| 60–74 | 24 (18.3) | 109 (14.2) | | 133 (14.8) |
| 75–89 | 4 (3.1) | 28 (3.6) | | 32 (3.6) |
| 90–104 | 1 (0.8) | 4 (0.5) | | 5 (0.5) |
| Occupation | | | 0.04 | |
| Plant farmer | 59 (45.0) | 236 (30.6) | | 295 (32.7) |
| Animal farmer | 3 (2.3) | 11 (1.4) | | 14 (1.6) |
| Farmer (plant and animal) | 2 (1.5) | 7 (1.0) | | 9 (0.10) |
| Merchant | 2 (1.5) | 13 (1.7) | | 15 (1.7) |
| Government officer | 0 | 3 (0.4) | | 3 (0.4) |
| Other (employee, housekeeper) | 42 (32.1) | 346 (44.9) | | 388 (43.0) |
| Missing | 23 (17.6) | 154 (20.0) | | 177 (19.6) |
| Sex | | | 0.70 | |
| Male | 58 (44.3) | 324 (42.1) | | 382 (42.4) |
| Female | 73 (55.7) | 446 (57.9) | | 519 (57.6) |
| Risk factors | | | 0.60 | |
| Direct or close contact with backyard poultry (including chickens) | 89 (67.9) | 525 (68.2) | | 614 (68.1) |
| Direct or close contact with backyard chickens | 86 (65.6) | 519 (67.4) | | 605 (67.1) |
| Direct or close contact with dead/sick chicken | 36 (27.5) | 196 (25.5) | | 232 (25.7) |
| Close contact with a person with a confirmed case of avian influenza A | 13 (9.9) | 51 (6.6) | | 64 (7.1) |
| Acute respiratory symptoms* | | | 0.31 | |
| Symptoms | 12 (9.2) | 98 (12.7) | | 110 (12.2) |
| No symptoms | 119 (90.8) | 672 (87.3) | | 791 (87.89) |
| Influenza-like illness† | | | 0.39 | |
| Symptoms | 7 (5.3) | 61 (7.9) | | 68 (7.5) |
| No symptoms | 124 (94.7) | 709 (92.1) | | 833 (92.5) |

*Acute respiratory symptoms were rhinorrhea, cough, sore throat, or dyspnea.
†Influenza-like illness was defined as a temperature ≥38.0°C in conjunction with any of the following: rhinorrhea, cough, sore throat, or dyspnea.

ing in Thailand among backyard poultry during 2004 did not transmit easily to our study population.

Our findings differ from those from a study of poultry workers in Hong Kong, among whom the estimated seroprevalence of influenza virus (H5N1) neutralizing antibodies was 10% during the 1997 outbreak (*10*). The Hong Kong poultry workers, however, likely had much greater intensity of exposure to poultry infected with influenza virus (H5N1) than our study population had. Furthermore, the clade 1 influenza virus (H5N1) strains that infected poultry and humans in Thailand during 2004 were antigenically and genetically distinct from the clade 0 influenza virus (H5N1) strains that caused the 1997 outbreak in Hong Kong (*11*). Our finding of no serologic evidence of asymptomatic or mild influenza (H5N1) infection among Thai villagers is consistent with findings from smaller influenza virus (H5N1) seroprevalence studies among workers in live poultry markets in the People's Republic of China (*12*), among villagers exposed to backyard poultry infected with clade 1 influenza virus (H5N1) in rural Cambodia (*13*), among poultry workers exposed to poultry infected with clade 2.2 influenza virus (H5N1) in northern Nigeria (*14*), and among poultry farmers exposed to poultry infected with clade 1 influenza virus (H5N1) in Thailand (*7*). Results of studies conducted since 2004 thus suggest that the risk for influenza (H5N1) infection is low among persons exposed to infected poultry; however, our finding of no serologic evidence of asymptomatic or mild influenza (H5N1) infection among Thai villagers suggests that the high case-fatality proportion in Thailand (17 deaths among 25 persons with confirmed infection) may accurately reflect the severity of the infection in Thailand.

Our study had 3 notable limitations. First, because study participants, most of whom were enrolled by convenience sampling, were generally older than the populations of the villages in which they resided (Thai Ministry of Public Health, unpub. data), our findings may not be generalizable to these villages' populations. Second, because the study was conducted in 2005, some participants may not have accurately recalled relevant exposures or symp-

Table 2. Avian influenza virus A (H5N1) neutralizing antibody titers among study participants (N = 901), as determined by microneutralization assay, Thailand, 2005

| Village | No. residents | No. residents by antibody titer | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | <5 | 5 | 10 | 20 | 40 | 80 | >80 |
| A | 228 | 227 | 1 | 0 | 0 | 0 | 0 | 0 |
| B | 204 | 202 | 2 | 0 | 0 | 0 | 0 | 0 |
| C | 209 | 202 | 6 | 0 | 1* | 0 | 0 | 0 |
| D | 260 | 257 | 2 | 1† | 0 | 0 | 0 | 0 |
| Total | 901 | 888 | 11 | 1 | 1 | 0 | 0 | 0 |

*Serum obtained from a 52-year-old woman (farmer) in village C without history of respiratory symptoms who reported contact with a sick/dead chicken and live poultry.
†Serum obtained from an 18-year-old man in village D without history of respiratory symptoms who reported contact with a sick/dead chicken and live poultry.

toms from 2004 when the influenza (H5N1) cases occurred. Third, some participants with mild or asymptomatic influenza (H5N1) infection in 2004 may not have generated an antibody response strong enough or durable enough to be detected in serum samples collected in October 2005.

Further data are needed on the natural history and kinetics of the immune response to influenza (H5N1) infection over time among severely ill persons who survived, as well as among those with clinically mild illness. Such prospective serial data may help researchers interpret the significance of low levels of influenza virus (H5N1) neutralizing antibody titers, as well as the results of additional seroprevalence studies. In addition, because influenza virus (H5N1) strains continue to evolve, additional seroprevalence studies to estimate human risk for infection are needed worldwide among populations exposed to the virus, including poultry workers, rural residents, market workers, farm workers, healthcare workers, and family members of infected persons.

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# Hospitalizations and Deaths Associated with *Clostridium difficile* Infection, Finland, 1996–2004[1]

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To determine whether the rate of *Clostridium difficile*–associated disease (CDAD) and CDAD-related deaths were increasing in Finland, we analyzed registry data from 1996 through 2004. We determined the number of hospital discharges that had a diagnosis code specific for CDAD from the International Classification of Diseases, 10th revision: "enterocolitis due to *Clostridium difficile*" (A04.7) and "pseudomembranous enterocolitis associated with antimicrobial therapy" (K52.8), listed as any diagnosis in the National Hospital Discharge Registry. CDAD-related deaths were identified from death certificates. Those discharged with a CDAD diagnosis doubled from 810 (16/100,000 population) in 1996 to 1,787 (34/100,000) in 2004. The increase was most prominent for patients >64 years of age but concerned only those discharged with diagnosis code A04.7. The number of those discharged with diagnosis code K52.8 remained stable. The age-standardized mortality rate associated with CDAD increased from 9/million in 1998 to 17/million in 2004; the increase was limited to persons >64 years of age.

Toxin-producing *Clostridium difficile* is the most frequent cause of antimicrobial drug–associated diarrhea. The clinical spectrum of *C. difficile*–associated disease (CDAD) ranges from mild diarrhea to severe life-threatening pseudomembraneous enterocolitis. Several reports from hospitals in the United States, Canada, and Europe suggest that the incidence and severity of CDAD are increasing (*1*). This increase is assumed to be associated with higher toxin production by a fluoroquinolone-resistant strain belonging to pulsed-field gel electrophoresis type NAP1, PCR ribotype 027, and toxinotype III (*2*,*3*). However, the same virulence factors may also be present in other *C. difficile* strains. Since 2003, PCR ribotype 027 has been detected in several European countries, including Finland in 2007 (*4*,*5*). Little information from nationwide, population-based studies on CDAD epidemiology existed before the emergence of this strain.

Various methods and strategies are used to diagnose *C. difficile* infection, which makes it difficult to accurately compare the epidemiology of CDAD over time and place (*6*). Toxin detection, along with culture, is considered the most accurate method of CDAD diagnostics, and it also allows the isolates to be typed in epidemiologic studies.

In Finland, CDAD has not continuously been a notifiable disease. To determine whether the rate of CDAD and CDAD-related deaths were increasing in Finland, we analyzed data from 2 national registers, the hospital discharge and death registers, from 1996 through 2004. We also conducted a national survey to evaluate the methods used in clinical microbiology laboratories in Finland to diagnose infection with *C. difficile*.

## Methods

In Finland (population 5.3 million), the national healthcare system is organized into 20 geographically and administratively defined healthcare districts; populations range from 67,800 to 1.7 million. Fifteen healthcare districts have only secondary and primary care hospitals, and 5 also provide tertiary care services.

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### Data Sources

The National Hospital Discharge Registry (HILMO) contains comprehensive healthcare records on inpatients, provided by all hospitals and municipal health centers in Finland. Each report to HILMO includes the patient's national identity code, admission and discharge dates, healthcare provider, type of service, medical specialty, the place (home or institution) from which the patient was transferred to the facility, and data on surgical procedures and discharge diagnoses. HILMO data were used to determine the number of discharges with a code specific for CDAD from the International Classification of Diseases, revision 10 (ICD-10): "enterocolitis due to *Clostridium difficile"* (A04.7) and "pseudomembranous enterocolitis associated with antimicrobial therapy" (K52.8), listed as the first or any discharge diagnosis. CDAD-related deaths were identified from death certificates of the National Population Information System in which underlying and immediate causes of death and contributory factors are currently coded according to ICD-10. In addition, for patients with CDAD identified from HILMO, we obtained death certificate data by using the national identity code, and, correspondingly, for persons with CDAD identified from death certificates, we obtained their discharge register data in HILMO for the preceding year.

### Incidence, Mortality Rates, Case-Fatality Rates, and Statistical Analysis

Data from the National Population Information System for years 1996–2004 were used as denominators to calculate age- and sex-specific incidence and mortality rates. The average annualized incidence rates during the surveillance period in different healthcare districts were calculated by using the total number of discharges within the total population during 1996–2004. For age standardization, we used the European Standard Population as appropriate (*7*). To evaluate secular trends, we calculated the rates for different groups, classified by age and sex, for each 12-month period from January 1996 through December 2004. Poisson regression was used to assess whether the observed changes in the rates were statistically significant. We calculated case-fatality proportions by dividing all deaths due to any cause within 30 days after the last CDAD-related discharge, including CDAD-related deaths, by the total number of patients discharged with a CDAD-related diagnosis. Appropriate permissions to use the data from the national hospital discharge and death registers were acquired from Statistics Finland and the National Research and Development Center for Welfare and Health.

### Laboratory Survey

In June 2006, we sent a standardized questionnaire to 32 clinical microbiology laboratories, which represented all tertiary (n = 5) and secondary (n = 16) care hospital laboratories and most private laboratories in Finland. The questionnaire covered different aspects pertaining to CDAD diagnosis, such as requests and criteria used for undertaking *C. difficile* investigations, current methods used for the diagnosis, and changes in diagnostic procedures during 1996–2005 as well as the total number of investigations that identified *C. difficile* in 2005.

## Results

We found 7,946 and 10,958 discharges from 1996 through 2004, for which CDAD was listed as either the primary diagnosis or as any diagnosis, respectively. Among 8,093 individual patients, 5,239 (65%) were >64 years of age and 5,005 (62%) were female. The average annualized age-specific incidence rate was >6-fold higher for patients >64 years of age than for those 45–64 years of age (108 vs. 16 hospital discharges/100,000 population). The age-standardized rate was similar for female and male patients (19/100,000 population versus 18/100,000).

Discharges for which CDAD was listed as any diagnosis doubled from 810 (16/100,000 population) in 1996 to 1,787 (34/100,000 population) in 2004 (Figure 1). The number of instances in which a patient was given a first diagnosis of CDAD also increased. The increases only concerned discharges with code A04.7; the number of discharges with code K52.8 remained at the same level. The increase was most prominent for patients >64 years of age, from 63/100,000 population to 162/100,000 (Figure 2). A slight increase was detected for those 45–64 years of age but none for children or those 15–44 years of age. The numbers of CDAD-related discharges increased in most healthcare districts (80%, 16/20) and in all tertiary care regions (5/5); the trend was statistically significant in 13 of the 20 (65%) healthcare districts ($p<0.05$ by Poisson regression). The average annualized age-standardized incidence rates varied greatly between the healthcare districts, from 9/100,000 population to 28/100,000.

A total of 761 CDAD-related deaths were identified (range by year, 18–143); most (733/761, 96%) occurred in persons >64 years. For 81 patients (11%) CDAD was considered as the underlying cause of death (range by year, 7–13). During 1996–1997, the annual number of CDAD-related deaths was ≈20, and thereafter the number increased from 70 to a peak of 140. During 1998–2004, the age-standardized mortality rate associated with CDAD increased from 9 per million population to 17 per million. The increase in mortality rate was limited to persons >64 years of age; during 1998–2004, the age-specific mortality rate of CDAD for persons >64 years of age doubled, from 76 per million population to 146 per million (Figure 3).

Of the 8,402 patients discharged with a CDAD-related diagnosis, 1,196 (14.2%) died within 30 days. The overall

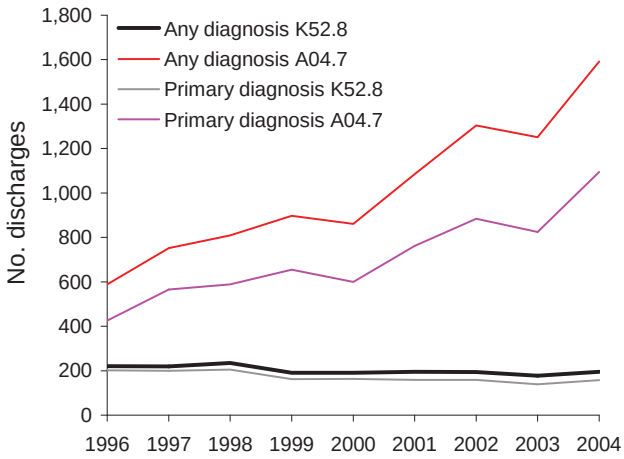


Figure 1. Number of hospital discharges with *Clostridium difficile* listed as any and primary diagnoses, 1996–2004, Finland. International Classification of Diseases, 10th revision, codes K52.8, "pseudomembranous enterocolitis associated with antimicrobial drug therapy," and A04.7, "enterocolitis due to *Clostridium difficile*."

case-fatality proportion was similar for female and male patients (14.1% vs. 14.4%) but varied by age group; the case-fatality proportion was highest for patients >64 years (20.5%) and lowest for those 15–44 years (0.8%). From 1996 through 2004, the case-fatality proportion doubled, from 7.6% to 15.5%.

Of the 32 clinical microbiology laboratories invited to take the survey on diagnostics, 28 laboratories (88%) responded, including all healthcare districts except 1 (19/20, 95%). Twenty-four (86%) laboratories, in 18 different healthcare districts, reported routinely performing *C. difficile* diagnostic tests. All laboratories had specific requests for *C. difficile* investigation or for investigating cases of antimicrobial drug–associated diarrhea. The methods used included toxin detection (88%) and culture (79%); most laboratories currently detect both toxins A and B by commercial kits, and 3 detect only toxin A. In 16 laboratories, a positive result reported to clinicians was based on both a positive culture and a toxin test result; in 5, on a positive toxin test result, and in 3, on a positive culture alone. In most laboratories, the change from toxin A detection only to testing for both toxins A and B occurred after the year 2000. In 2005, diagnostic sampling activity varied nearly 10-fold between healthcare districts, from 209 to 1,845 tests per 100,000 population. The laboratories also had wide variation in the proportion of samples positive for *C. difficile* (1%–28%), as well as in those positive for toxin (range 3%–15%).

## Discussion

Our nationwide population-based study shows that the rates of CDAD and CDAD-related deaths among elderly patients are increasing in Finland. However, on the basis of ICD-10 codes, we did not detect an increase in the severe form of CDAD, pseudomembranous enterocolitis. Although the rates of CDAD and CDAD-related deaths in Finland are still lower than the population-based estimates reported from the United States (*8*) and the United Kingdom (*9*), clinicians in Finland were reminded about the increasing risk for CDAD, especially among elderly patients.

A similar population-based study has been performed in the United States, where the number of hospital patients discharged with CDAD listed as any diagnosis doubled from 1996 through 2003 (*8*). The most pronounced increase occurred during 2000–2003. The US incidence increased to a considerably higher level than that in Finland (61 discharges/100,000 population vs. 34/100,000); the US increase was several-fold among elderly patients, as in Finland. A difference from our study was that ICD-9 codes were in use in the United States during the study period, and only code 008.45 (intestinal infection caused by *C. difficile*) was analyzed. A doubled rate was also found in another US study, which was limited to adults hospitalized with CDAD during 2000–2005 (*10*).

The CDAD-related mortality rate has been evaluated in England and Wales using death register data (*9*). Deaths associated with CDAD or pseudomembranous enterocolitis more than doubled from an age-standardized mortality rate of 11 per million population in 1999 to 24 per million in 2004. The increase was slightly greater than in Finland during the same period (from 9 to 17 per million population ), and the mortality rate increased to a somewhat higher level; on more than half of the death certificates in England and Wales, CDAD was the underlying cause of death. By 2006, the mortality rate had further increased by 72% to 64–65 per million population (*11*). A similar trend in CDAD-related mortality rates has also reported from the United States during 1999–2004 (*12*). However, those figures are not comparable to ours because the 2000 US population was used as a standard.

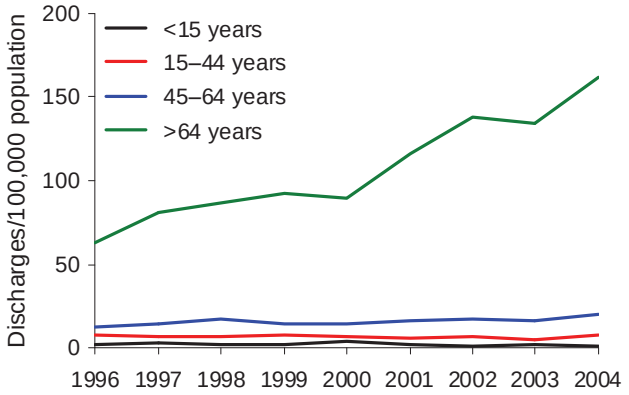


Figure 2. Rates of hospital discharges with *Clostridium difficile* listed as any diagnosis, by age, 1996–2004, Finland.

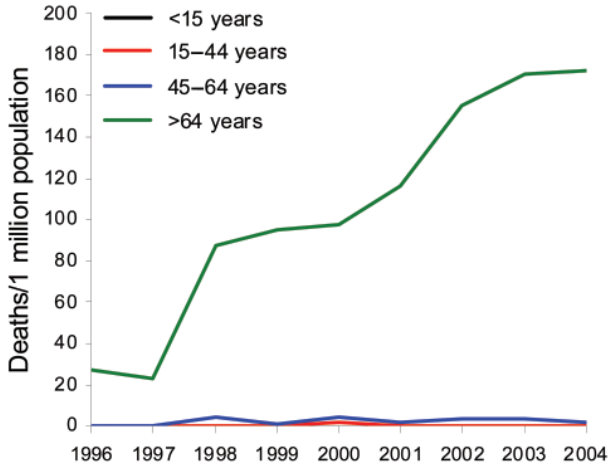


Figure 3. Mortality rates associated with *Clostridium difficile,* by age, 1996–2004, Finland.

In our study, the 30-day case-fatality proportion (deaths from all causes) was 14%, which is similar to findings from other studies that report case-fatality rates ranging from 11% to 25% during hospitalization (*3*,*13*–*16*). However, the results are not directly comparable because we analyzed the deaths within 30 days after the date of discharge with a CDAD-related diagnosis, not after the date of diagnosis, because the latter was not available.

During 1996–2005, considerable changes occurred in Finland in the diagnostic methods of *C. difficile,* which may have contributed to the increasing rate of CDAD. The regional variation in diagnostic activity may also have had an effect. Improvement in CDAD diagnostic procedures can still be made, if culture and toxin detection together are considered the most accurate method.

Our study, as other register-based studies, has some limitations. First, we only analyzed hospital discharge data, and the sensitivity and specificity of hospital discharge coding for CDAD are not well known. In the US study, the correlation between toxin assay result and ICD-9 code was good: the specificity of toxin assay results was excellent (99.7%), but the sensitivity was lower (78%) (*17*). Alternatively, it is very unlikely that the sensitivity would have remarkably changed during the study period, as stated in article about the US study. The validity of Finnish hospital discharge and death registers has been assessed only for diseases other than CDAD (*18*,*19*). The second code we used (K52.8, pseudomembranous enterocolitis associated with antimicrobial therapy) is not specific for *C. difficile,* and its use might have been inconsistent for the severe form of CDAD, which is likely to obscure a possibly increasing trend. Second, because the ICD codes were given at the time of hospital discharge, we could not assess which proportion of CDAD was related to ambulatory healthcare or to in-hospital care. In addition, the changes in laboratory diagnostics may have influenced the results. A considerable proportion of the microbiology laboratories changed from using immunoassays that detect only toxin A to using assays that detect both toxins A and B. That this would be the only reason for the increase in CDAD is unlikely because only a minor proportion of CDAD (5%) has been reported to be caused by strains producing only toxin B (*20*). No such information is available from Finland. In addition, trends in diagnostic activity could not be analyzed because the data were available only for 2005.

According to the first national prevalence survey performed in 2005 in 30 acute care hospitals in Finland, 5% of the healthcare-associated infections confirmed microbiologically were caused by *C. difficile* (*21*). CDAD was >10× more prevalent than infections due to methicillin-resistant *Staphylococcus aureus*. During 1995–1997, findings positive for *C. difficile* (positive culture and/or toxin production) were included in national laboratory-based surveillance reports in Finland. Because electronic reporting was not yet common, surveillance was halted due to the workload and difficulties in interpreting surveillance data because most of the positive findings were based on cultures only.

Our data from this study offer a comprehensive picture of the trends and the outcome of CDAD in a well-defined population during the period before the new virulent PCR ribotype 027 was detected in Finland. This information serves as a point of reference for Finland and other industrialized countries when assessing the effects of emerging, highly virulent clones on the epidemiology of CDAD. Notably, the results we detected in the epidemiology of CDAD are similar to those found in limited settings in many industrialized countries, even though the rates of CDAD and CDAD-related deaths were at a lower level in Finland. As elsewhere, the increase in incidence in Finland may be due to a growing population at risk, reflecting the increased prevalence of chronic disease and improved survival for patients with severe chronic underlying diseases as well as increasing use of antimicrobial drugs. The results of our study have been used to set up a national surveillance system for CDAD, including recommendations for CDAD diagnostic procedures.

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# Methicillin-Resistant *Staphylococcus aureus* ST398 from Human Patients, Upper Austria

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Methicillin-resistant *Staphylococcus aureus* (MRSA) clonal type ST398 is usually associated with animals. We examined 1,098 confirmed MRSA samples from human patients and found that 21 were MRSA ST398. Most (16) patients were farmers. Increasing prevalence from 1.3% (2006) to 2.5% (2008) shows emergence of MRSA ST398 in humans in Austria.

In the past few years, interest has focused on the emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) in animals and the potential for cross-transmission between humans and animals (*1*). MRSA isolates that are strongly associated with pigs or contact with pigs show at least 2 similarities: they are not typeable by pulsed-field gel electrophoresis (PFGE) because their DNA cannot be digested by the restriction enzyme *Sma*I (*2*), and most belong to the MRSA clonal lineage sequence type (ST) 398 (*1*).

## The Study

From January 2006 through May 2008, a total of 21 laboratories and/or hospitals in Upper Austria (project MRSA-Registry Upper Austria) sent us 1,210 suspected MRSA primary isolates consecutively collected from human patients. For quality control, all isolates were cultured and investigated for *mecA/femA* by PCR at the Austrian National Reference Centre (*3*). Of the 1,210 isolates, 1,098 (90.7%) were confirmed to be MRSA; the other 112 (9.3%) were either methicillin-sensitive *S. aureus* or were not *S. aureus* and therefore were excluded from the MRSA registry.

Most molecular biological investigations (DNA isolation, detection of the Panton-Valentine leukocidin [PVL] genes *lukS-lukF*, PFGE, *spa* typing) were performed as described (*3*). Determination of staphylococcal cassette chromosome *mec* (SCC*mec*) subtypes was performed by PCR according to Boye et al. (*4*). Multilocus sequence typing (MLST) PCR was performed according to Enright et al. (*5*). Sequence reactions were conducted by using BigDye fluorescent terminators (Applied Biosystems, Foster City, CA, USA). Sequencing was performed on the ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Sequence types were assigned according to the *S. aureus* MLST database with use of the typing software at www.mlst.net (*6*).

Etest showed resistance patterns of the MRSA ST398 isolates to the following antimicrobial agents: ciprofloxacin, clindamycin, erythromycin, fosfomycin, fusidic acid, gentamicin, moxifloxacin, mupirocin, rifampin, and vancomycin. Resistance to doxycycline was determined by using the disk diffusion test. Data were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute antimicrobial susceptibility testing standards, where available.

Demographic information and data from patient histories were systematically collected by using standardized questionnaires and were interpreted by physicians and infection control teams in hospitals as well as by physicians working in outpatient care. Infection and colonization were differentiated according to definitions from the Centers for Disease Control and Prevention (*7*) and interpreted by a physician.

Of the 1,098 primary MRSA isolates, 21 could not be digested by the restriction enzyme *Sma*I and were therefore investigated further (Table). All 21 patients (14 male, 7 female; median age 58 years, range 1–83 years) harbored MRSA of clonal lineage ST398. Although MRSA ST398 is suspected of being able to acquire virulence factor genes (*1*), only 5 patients were infected, whereas 15 were colonized. Status was unknown for 1. Of the 15 colonization cases, 12 were detected by screening. Regarding the infections, 4 cases were of minor clinical relevance, but 1 case (isolate no. 3332) showed progression of major clinical relevance: a 64-year-old pig farmer had received a prosthetic knee, and postoperative joint empyema with MRSA ST398 developed soon afterwards.

The first MRSA isolates in which ST398 was detected were collected in France during 1996–2002 (*8*). However, most publications concerning MRSA ST398 refer to samples collected since 2004 (*9*–*11*). In our institute, the first isolate belonging to ST398 was detected in January 2006, although we have investigated 2,657 MRSA isolates from persons all over Austria since 1996. Therefore, we assume that the emergence of MRSA ST398 in Austria is rather recent. Until now, this MRSA strain seemed to be restricted to Upper Austria, although pig farming is equally common in Lower Austria and in Styria. It might be interesting to investigate the reason for the varying prevalence of MRSA ST398 in different regions of Austria.

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Our results correspond well with published data. SCC*mec* type V was predominant in our isolates, as has been found in Germany (*9*). One isolate harbored SCC*mec* type IV, but no type III, as has been reported in the Netherlands (*12*). None of our isolates harbored PVL genes, which confirms that the *lukF/lukS* genes are not necessarily present in community-acquired MRSA. Apparently, some community-acquired MRSA types (e.g., ST30, ST80, ST152) are PVL positive in most cases, whereas others (e.g., ST398) are not. However, although all the MRSA ST398 isolates found in Europe to date were PVL negative, a PVL-positive MRSA ST398 strain was recently detected in China (*13*).

The *spa* types among our isolates were t011, t034 (both commonly found in ST398), and t2346. They are closely

Table. Clinical MRSA isolates of clonal lineage ST398, Upper Austria, January 2006–May 2008*

| Isolate | *spa* type | SCC*mec* group | Resistance | Patient age, y/ sex | Sample source | Diagnosis | Infected or colonized | Hospitalized | Profession, direct animal contact |
|---|---|---|---|---|---|---|---|---|---|
| 2753 | t2346 | V | CLI, DOX, ERY | 1/M | Throat | Pyodermatitis | I (SST) | Yes | Grandson of pig farmer (son of 2754), yes |
| 2064 | t011 | V | CLI, DOX, ERY | 54/M | Wound smear | Gouty tophus | I (SST) | Yes | Pig farmer, yes |
| 3190 | t011 | V | CLI, DOX, ERY | 48/M | Wound smear | Decubitus ulcers | I (SST) | Yes | Construction worker, no |
| 3332 | t011 | V | DOX | 64/M | Wound smear, joint puncture fluid | Joint empyema | I (BJ) | Yes | Pig farmer, yes |
| 3509 | t011 | V | DOX | 80/M | BAL | COPD | I (pneumonia) | Yes | Caster, unknown |
| 1960 | t011 | V | DOX | 62/M | Screening conjunctival swab | Paroxysmal tachycardia | C | Yes (ICU) | Farmer, yes |
| 2023 | t011 | V | CLI, DOX, ERY | 68/M | Screening wound smear | Peripheral arterial occlusive disease | C | Yes | Pig farmer, yes |
| 2167 | t011 | V | DOX | 47/M | Screening tracheal secretion | Epidural hematoma | C | Yes (ICU) | Agricultural worker, yes |
| 2256 | t011 | V | DOX | 73/M | Screening wound smear | Ulcus cruris | C | Yes | Cattle and pig farmer, yes |
| 2726 | t034 | IV | – | 50/M | Screening nose swab | Pneumothorax | C | Yes | Unknown |
| 2754 | t2346 | V | CLI, DOX, ERY | 33/F | Screening nose, throat swab | Premature delivery | C | No | Daughter of pig farmer, yes |
| 2770 | t011 | V | CLI, DOX, ERY | 81/F | Screening wound smear | Ulcus cruris | C | No | Farmer, yes |
| 2832 | t011 | V | CLI, DOX, ERY | 79/F | Screening wound smear | Ulcus cruris | C | No | Pig farmer, yes |
| 2909 | t011 | V | DOX | 79/M | Screening wound smear | Ulcus cruris | C | No | Pig farmer, yes |
| 3078 | t011 | V | CLI, DOX, ERY | 42/M | Screening nose swab | Erysipelas, septicemia | C | Yes | Farmer, yes |
| 3195 | t011 | V | – | 69/M | Wound smear | Intertrigo | C | Yes | Retired farmer (hens), yes |
| 3335 | t011 | V | DOX | 65/F | Screening axilla, nose, groin | Screening | C | No | Wife of pig farmer 3332, yes |
| 3336 | t011 | V | DOX | 32/M | Nose, wound smear | Screening | C | No | Son of pig farmers 3332 and 3335, yes |
| 3391 | t011 | V | CLI, DOX, ERY | 83/F | Feces | Polytrauma | C | Yes | Retired, no |
| 3456 | t011 | V | DOX | 69/F | BAL | Hemoptysis, bronchitis | C | Yes | Cleaner, no |
| 3008 | t011 | V | DOX | 48/F | Unknown | Unknown | Unknown | Unknown | Farmer, yes |

*MRSA, methicillin-resistant *Staphylococcus aureus;* SCC, staphylococcal cassette chromosome; CLI, clindamycin; DOX, doxycycline; ERY, erythromycin; I, infected; SST, skin and soft tissue infection; C, colonized; BJ, bone and joint infection; BAL, bronchioalveolar lavage; COPD, chronic obstructive pulmonary disease; ICU, intensive care unit; –, no resistance found.

related to each other as well as to other types belonging to ST398.

Antimicrobial drug–susceptibility testing showed that 19 of 21 isolates were resistant to doxycyline, 9 of which were also resistant to clindamycin and erythromycin. All isolates were susceptible to the other drugs tested. Only 2 isolates were fully susceptible to all agents tested. These antimicrobial drug–resistance profiles might reflect the frequent use of tetracyclines in veterinary medicine; in Austria, two thirds of all antimicrobial drugs used in veterinary practice, especially in pig and poultry farming, are tetracycline derivatives (*14*), a situation similar to that in other European countries (*11*,*12*). Therefore, it is not surprising that our resistance profiles correspond well with those from the Netherlands, Germany, and France (*9*,*12*,*15*).

The only isolate harboring *spa* type t034 was also the only isolate harboring SCC*mec* IV and is 1 of the 2 isolates that were fully susceptible to all antimicrobial drugs tested. Among all MRSA isolates, the percentage of ST398 in Upper Austria was 1.3% (6/463) in 2006, 2.3% (9/392) in 2007, and 2.5% (6/243) in 2008 (January–May). These percentages agree with data from Witte et al., who reported that MRSA ST398 is not frequent among *S. aureus* in Germany or the United Kingdom (*9*). However, the proportion of MRSA isolates that are ST398 has slightly increased in Upper Austria.

Most patients discussed in this article had had contact with animals. MRSA ST398 is known to be associated with animal contact, especially with pigs and cows (*8*–*10*,*12*). In the Netherlands and in France, the MRSA carriage rate is substantially higher for pig farmers and veterinarians than for the general population (*11*,*15*). In our study, 10 patients were pig farmers or direct relatives of pig farmers, and 6 were farmers (raised hens or unknown animal species). The animal contact status of 2 was unknown. In 2008, 3 of our patients had no direct animal contact; possible MRSA transmission from healthcare workers or other sources was not investigated. Thus, the question arises as to whether these isolates might represent more spread of this sequence type strain outside pig farms.

## Conclusions

MRSA of clonal lineage ST398 has emerged in humans in Austria. Moreover, it is not confined to Europe but has also been detected in China (*13*), Thailand, and Canada (*11*). This finding indicates a great potential for spread, quantitatively as well as geographically. Because the international meat and livestock market is active, the stage is set for rapid spread. In addition, the largest exporter of live pigs in Europe is the Netherlands, and up to 39% of pigs from the Netherlands carry MRSA in their nares (*12*). Thus, we suggest intensified establishment of collaborations between laboratories from different countries.

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# Evaluation of Surveillance Methods for Staphylococcal Toxic Shock Syndrome

**Lindsey Lesher, Aaron DeVries, Richard Danila, and Ruth Lyn eld**

We compared passive surveillance and International Classification of Diseases, 9th Revision, codes for completeness of staphylococcal toxic shock syndrome (TSS) surveillance in the Minneapolis–St. Paul area, Minnesota, USA. TSS-specific codes identified 55% of cases compared with 30% by passive surveillance and were more sensitive ($p = 0.0005$, McNemar $\chi^2$ 12.25).

Staphylococcal toxic shock syndrome (TSS) is a severe illness associated with toxin-producing *Staphylococcus aureus*. First named in 1978, TSS has been associated with tampon use, intravaginal contraceptive devices, and skin infections, particularly after surgical procedures (*1*,*2*). In January 1980, the Minnesota Department of Health (MDH) initiated surveillance for TSS with active and passive components. The national incidence of TSS decreased during 1980–1996 (*3*,*4*) after removal of high-absorbency tampons from the market and public awareness campaigns. In subsequent years, surveillance methods in Minnesota were changed to a solely passive surveillance system that relied on clinicians to report cases. MDH uses the 1997 case definition of the Centers for Disease Control and Prevention (CDC) (Atlanta, GA, USA) to determine case criteria for any probable TSS case (≥4 clinical criteria and laboratory criteria) considered reportable (Table 1) (*5*). Given the complexity of the case definition, we suspected that TSS underreporting was likely.

Recently, several factors, including increasing prevalence of community-associated methicillin-resistant *S. aureus* that carries superantigens and the trend toward earlier menarche, suggested that the incidence of TSS might be increasing (*6*,*7*). Additionally, the number of requests for superantigen testing in the Minneapolis–St. Paul (MSP) area made to a reference microbiology laboratory that tests staphylococcal isolates from TSS cases increased during 2000–2003 (*8*). To determine the incidence of TSS, active surveillance was initiated at all MSP area hospitals using International Classification of Diseases, 9th Revision (ICD-9), codes assigned at hospital discharge. We compared passive surveillance reports with ICD-9 codes to determine an effective and efficient surveillance method for TSS.

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## The Study

The MSP area is composed of 7 counties with a population of 2,642,056 (2000 US Census) and 24 acute-care hospitals. Requests were sent to medical record departments of these hospitals for data on inpatients discharged from hospitals from January 1, 2000, through December 31, 2003, whose medical records indicated ≥1 of the select ICD-9 study codes. Medical records from all hospitalizations receiving the TSS-specific code (040.82 or 040.89) were reviewed (Figure), and a 20% random sample of medical records from hospitalizations that received ≥1 nonspecific TSS study code (Table 2) from within each hospital was reviewed. Each medical record was reviewed for TSS case criteria and pertinent epidemiologic and clinical information. Additionally, death certificates assigned the ICD-10 code for TSS (A48.3) and cases from the Minnesota Unexplained Critical Illness and Death of Possible Infectious Etiology project (UNEX) (*9*) during 2000–2003 were reviewed. TSS cases identified through ICD-9 code searches were compared with cases reported to MDH during 2000–2003. Data were analyzed with Stata version 9 (StataCorp, College Station, TX, USA). Statistical analyses included Pearson and McNemar $\chi^2$ tests.

Of 7,414 hospitalizations with ≥1 study code, 116 (1.6%) were assigned the TSS-specific code and were reviewed (Figure). Of the remaining 7,298 hospitalizations assigned ≥1 nonspecific TSS code, 1,575 (21.6%) randomly selected hospitalizations were reviewed. Of these 1,691 hospitalizations, 55 had 5 or 6 criteria for TSS, of which 12 (22%) met the CDC case definition for streptococcal TSS, and 7 were non-MSP residents. The remaining 36 cases were probable or confirmed TSS. No cases from UNEX or death certificate searches met the TSS case definition. Of the 36 TSS cases, 17 (47%) were reported to MDH by passive surveillance. Thirty-one (86%) cases were found by using TSS-specific ICD-9 codes. Five cases were found by using non-TSS–specific ICD-9 codes. After adjusting for 20% random sampling for cases identified by non-TSS–specific codes, we identified the estimated number of cases by using non-TSS–specific codes to be 25. This analysis resulted in 56 estimated TSS cases identified in the surveillance area during 2000–2003 by using ICD-9 codes.

The TSS-specific ICD-9 code search identified 31 of the 56 estimated TSS cases (sensitivity 55%, specificity

Table 1. Staphylococcal toxic shock syndrome case definitions*

| Criteria | Definition |
|---|---|
| Clinical | |
| Fever | Temperature ≥38.9°C (102.0°F) |
| Rash | Diffuse macular erythroderma |
| Desquamation | 1–2 weeks after onset of illness, particularly on the palms and soles |
| Hypotension | Systolic blood pressure ≤90 mm Hg for adults or <5th percentile by age for children <16 years of age; orthostatic decrease in diastloc blood pressure ≥5 mm Hg from lying to sitting, orthostatic syncope, or orthostatic dizziness |
| Multisystem organ involvement† | |
| Gastrointestinal | Vomiting or diarrhea at onset of illness |
| Muscular | Severe myalgia or creatine phosphokinase level at least twice the upper limit of normal |
| Mucous membrane | Vaginal, oropharyngeal, or conjunctival hyperemia |
| Renal | Blood urea nitrogen or creatinine at least twice the upper limit of normal for laboratory or urinary sediment with pyuria (≥5 leukocytes by high-power field) in the absence of urinary tract infection |
| Hepatic | Total bilirubin, alanine aminotransferase, or aspartate aminotransferase levels at least twice the upper limit of normal |
| Hematologic | Platelet counts ≤100 × $10^9$/L |
| Central nervous system | Disorientation or alterations in consciousness with focal neurologic signs when fever and hypotension are absent |
| Laboratory | |
| Culture | If obtained, negative results on blood, throat, or cerebrospinal fluid cultures (blood culture may be positive for *Staphylococcus aureus*) |
| Titer | If obtained, no increase in titer for Rocky Mountain spotted fever, leptospirosis, or measles |
| Case classification | |
| Probable | Meets laboratory criteria and in which 4 of 5 clinical findings described above are present |
| Confirmed | Meets laboratory criteria and in which all 5 of the clinical findings described above are present, including desquamation, unless the patient dies before desquamation occurs |

*From the US Centers for Disease Control and Prevention (*5*).
†Involving ≥3 organ systems.

99%). Seventeen cases were reported to MDH by passive surveillance (sensitivity 30%, specificity 99.9%); all were coded with the TSS-specific code. The TSS-specific ICD-9 code search was more sensitive than passive surveillance ($p = 0.0005$, McNemar $\chi^2$ 12.25). Of those cases reported to MDH, more were likely to be associated with menstruation (14/17 vs. 5/19; $p<0.001$) and to have had a positive test result for *S. aureus* (16/17 vs. 11/19; $p = 0.01$). Twenty-seven of 36 TSS cases detected had a bacterial culture positive for *S. aureus*. The 3 TSS cases with methicillin-resistant *S. aureus* isolates were not reported to MDH. The positive predictive value of being a case among those coded with the TSS-specific code was 27% (31/116). In 68 of 116 cases that received the TSS-specific code, there was clinical suspicion of TSS, but these cases did not meet the clinical case definition (<5 criteria): 10 were streptococcal TSS and 7 were in non-MSP residents. All 17 cases reported to MDH were detected through the ICD-9 code search.

## Conclusions

Surveillance for TSS is challenging given the lack of a diagnostic test and a case definition with multiple components. Under the current passive surveillance system, between one third and half of potential TSS cases were identified. Discrepancies were found in reporting, with menstruation-associated cases more likely to be reported to MDH than nonmenstrual-associated cases. This discrepancy was observed with prior active surveillance efforts (*10*).

Using ICD-9 codes, we found 12 TSS cases that were of streptococcal etiology. Accuracy may be improved by developing separate ICD-9 codes specific for staphylococ-

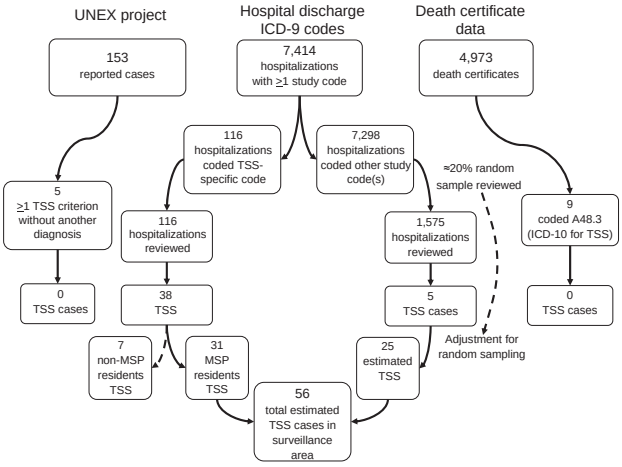


Figure. Flow diagram of toxic shock syndrome (TSS) case ascertainment. TSS cases were identified from International Classification of Diseases, 9th Revision (ICD-9), codes assigned at hospital discharge, cases reported to the Minnesota Unexplained Critical Illness and Death of Possible Infectious Etiology project (UNEX), and death certificate data by using International Classification of Diseases, 10th Revision (ICD-10), code A48.3. MSP, Minneapolis–St. Paul area.

Table 2. ICD-9 study codes used for staphylococcal toxic shock syndrome case ascertainment*

| Code | Associated diagnosis |
|---|---|
| Specific toxic shock syndrome code | |
| 040.89 or 040.82† | Toxic shock syndrome |
| Nonspecific toxic shock syndrome codes | |
| 038.11 | *Staphylococcus aureus* septicemia |
| 038.19‡ | Other staphylococcal septicemia |
| 038.9 | Unspecified sepsis |
| 785.50 | Shock without mention of trauma |
| 785.59 or 785.52† | Sepsis |

*ICD-9, International Classification of Diseases, 9th Revision.
†The code assigned to toxic shock syndrome changed from 040.89 to 040.82 on October 1, 2002, and the code assigned to sepsis changed from 785.59 to 785.52 on October 1, 2003. Although the codes changed, their associated diagnoses remained unchanged and are considered mutually exclusive.
‡Code eliminated after interim analysis of 627 medical records at 14 of 24 hospitals. Of 122 records receiving only this code, 40% had 0 case criteria, all had ≤3 criteria, and 88% had bacteremia with a staphylococcal species other than *S. aureus*.

cal, streptococcal, or unidentified TSS. In addition to the TSS-specific ICD-9 code, we selected 5 other ICD-9 codes on the basis of previous studies to address the concern that TSS cases may be classified under a staphylococcal infection or sepsis code, but not the TSS-specific code (*8–10*). These 5 additional non-TSS–specific ICD-9 codes required reviewing 1,575 medical records; only 5 (0.3%) additional TSS cases were identified. The non-TSS–specific ICD-9 codes detected 25 estimated cases. However, this detection required 8 trained staff and substantial resources with ≈40 minutes required per medical record review.

Passive surveillance requires fewer public health resources because it relies on clinicians to report cases. Active surveillance involves public health resources in identifying cases. The disadvantage of passive surveillance is the potential for missed cases. Despite possible inaccuracies associated with the assignment of ICD-9 codes, these codes represent a standardized data source that may be readily available. In the absence of a specific diagnostic test, ICD-9 codes represent an efficient method for surveillance and following trends.

Medical record abstraction per hospitalization was labor- and resource-intensive and is not feasible for most health departments. With increasing use of automated electronic reporting for disease surveillance (*11*), querying hospital discharge data for the TSS-specific ICD-9 code is a feasible adjunct to passive surveillance to detect TSS trends over time. Consequently, it is imperative that clinicians and coders are thorough to ensure that ICD-9 codes are accurate.

We found it useful to add regular ICD-9 code searches for TSS-specific codes as an active surveillance adjunct to our passive surveillance system. This addition increases sensitivity of TSS surveillance with a minimal increase in resources. Use of this more sensitive system increases the ability to detect trends in TSS, which may develop because of changes in bacterial virulence characteristics, host characteristics such as the use of new devices or products, changes in human behavior, or changes in host susceptibility. Evaluation of this approach in other areas to assess sensitivity of TSS surveillance would be useful because coding practices may differ.

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# Human *Streptococcus agalactiae* Isolate in Nile Tilapia (*Oreochromis niloticus*)

**Joyce J. Evans, Phillip H. Klesius, David J. Pasnik, and John F. Bohnsack**

*Streptococcus agalactiae*, the Lancefield group B streptococcus (GBS) long recognized as a mammalian pathogen, is an emerging concern with regard to fish. We show that a GBS serotype Ia multilocus sequence type ST-7 isolate from a clinical case of human neonatal meningitis caused disease and death in Nile tilapia (*Oreochromis niloticus*).

*Streptococcus agalactiae*, group B streptococcus (GBS), has a broad host range and is pathogenic to mammals, reptiles, amphibians, and fish (*1*). This organism has also been identified in aquatic mammals, both captive and wild bottlenose dolphins (*Tursiops truncatus*) (*2*,*3*). GBS causes mastitis in cattle and meningitis in human neonates (*4*). It also causes meningoencephalitis in fish (*5*,*6*). Since the first report of GBS in hatchery-reared freshwater fish in the United States in 1966 (*7*), reports of piscine GBS have increased. Piscine GBS, like human and bovine GBS, is found worldwide and affects a variety of freshwater and marine fish under a broad spectrum of environmental conditions (*6*,*8*).

Sustained emergence of human GBS neonatal disease for undetermined reasons has spurred many GBS genomic diversity studies. Acquisition of bovine GBS by humans has been proposed as 1 plausible explanation (*9*). Human and bovine GBS isolates have been considered genetically distinct populations (*10*) and as related populations arising from a common ancestor, presumably bovine (*1*,*9*).

Two lineages of bovine GBS (multilocus sequence type ST-23 and ST-61) appear to have a genetic relationship with human GBS. Human serotype Ia strains in the ST-23 lineage and human serotype III ST-17 strains are related to bovine ST-61 strains and frequently associated with neonatal infections (*10*,*11*). Phylogenetic studies have focused on homogeneity or heterogeneity of lineages and association with carried or invasive GBS. Many researchers believe that GBS serotype does not correlate with evolutionary relationships. Strains of different serotypes and sequence types often share more genes than strains of the same serotype and contain carried and invasive strains, traits suggestive of opportunistic pathogenicity (*9*,*11*,*12*).

Whether GBS is a zoonotic organism has not been adequately explored. Conclusions of genomic studies can only infer virulence, infectivity, and transfer between different animals on the basis of serologic, molecular, and computational analyses of GBS isolates. Bovine GBS isolates of unknown sequence type were not infective for Nile tilapia (*Oreochromis niloticus*) (*13*). However, potential for infection between homothermic hosts and poikilothermic animals has been demonstrated.

GBS transmission from mice to reptiles occurs by oral ingestion (*14*). In tilapia (mean weight 40 g) infectivity studies with a dolphin GBS isolate, disease signs and a mortality rate of 90% were noted within 6 days postinoculation with $10^7$ CFU/fish (*3*). Piscine, dolphin, and human GBS isolates have been reported (*15*) to share the same serotype (Ia) and sequence type (ST-7) as that reported from human GBS carried (strain from a person with no evidence of disease) and neonatal invasive strains from Japan (*4*) and North America (isolate A909) (*11*). This finding indicates that piscine and dolphin GBS isolates may have been derived from human sources and caused a fish epidemic in Kuwait and that serotype Ia ST-7 GBS may cause a zoonosis (*15*). Non–ST-7 fish isolates are widely divergent from other animal GBS isolates (*12*,*15*). We conducted a study of experimentally induced infection to determine whether a human serotype, Ia ST-7 GBS isolate, could cause disease signs and death in fish.

## The Study

Nile tilapia served as experimental fish because they could be held at warm water temperatures closer to the normal human body temperature of 37°C than other available fish species. Seven groups of 10 tilapia (mean ± SE weight 28.20 ± 0.51 g) each were housed in 57-L aquariums at the Aquatic Animal Health Research Laboratory (Chestertown, MD, USA). All tanks were supplied with flow-through dechlorinated tap water and 2 submersible heaters and air stones to maintain desired water temperature and dissolved oxygen (DO) levels. Water quality (mean ± SE temperature, DO, and ammonia concentration) was measured daily by using a YSI 85 meter (Yellow Springs Instrument Co., Yellow Springs, OH, USA) and a Fresh Water Aquaculture Kit (Model AG-2; LaMotte Company, Chestertown, MD, USA). Temperature was 32.1 ± 0.09°C, DO was 4.2 ± 0.14 mg/L, and am-

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monia concentration was 0.74 ± 0.08 mg/L. Fish were fed daily (4% of bodyweight) with Aquamax Grower 400 fish feed (Purina, Brentwood, MO, USA) and maintained and handled according to Institutional Animal Care and Use Committee–approved guidelines.

The serotype Ia ST-7 human GBS isolate (ID# 510012) was obtained from a patient in Japan who had neonatal meningitis. The isolate was cultured overnight on 5% sheep blood agar (SBA; Remel, Lenexa, KS, USA) at 32°C. Before the study, the isolate was passed through 5 Nile tilapia (weight 18.4 ± 0.48 g) 1 time each by intraperitoneal injection of $10^7$ CFU of GBS/fish. Specimens from a fish that died 3 days postchallenge were cultured on SBA, and GBS was recovered from nostrils, intestines, posterior kidney, and brain. One GBS colony isolated from brain was cultured on SBA, typed as GBS by Lancefield grouping (*6*), and used for experimental infection.

Serial dilutions of the GBS isolate were prepared in tryptic soy broth (TSB; Remel), and 10 fish (weight 28.2 ± 0.51 g) were each injected intraperitoneally with 0.1 mL of inoculum at $10^7$, $10^6$, $10^5$, $10^4$, $10^3$, or $10^2$ CFU/fish. Ten control fish were injected with 0.1 mL of TSB only. Fish were placed in separate 57-L aquariums at 32°C according to dose and monitored daily for signs of disease and death for 14 days postchallenge. Moribund fish were humanely euthanized by an overdose of tricaine methanesulfonate (MS-222; Argent Chemical Laboratories, Redmond, WA, USA).

Bacterial samples were obtained from nostrils and brains of all moribund or dead fish and cultured on SBA at 32°C for 24 h for GBS. Identification of GBS was performed by using methods of Evans et al. (*6*) and the BIOLOG MicroLog3 Microbial Identification System (BIOLOG, Hayward, CA, USA) according to the manufacturer's instructions. BIOLOG results were compared with a Microlog database (www.biolog.com/mID_product.html); a similarity index >0.50 and high probability (>90%) were considered a strong confirmation for GBS.

Within 7 days postchallenge, the $10^2$, $10^3$, and $10^7$ CFU/fish groups had a mean cumulative mortality rate of 11.7% (7/60) (Figure). Overall, deaths occurred on day 2 and the mortality rate reached 20% after 14 days of observation. Deaths after day 10 occurred in fish that received $10^6$ CFU. Sampled organs were negative for GBS and deaths were attributed to tank mate aggression to weakened fish. A linear dose response was not seen. Deaths occurred at low ($10^2$–$10^3$ CFU/fish) and high ($10^7$ CFU/fish) doses but not at median doses (Table). Disease signs in tilapia exposed to human GBS were lethargy, anorexia, dark coloration, opaque eyes, and remaining stationary at the bottom of the tank.

All sampled organs from fish dying within 7 days of infection contained β-hemolytic, gram-positive, oxidase-negative, catalase-negative bacteria. BIOLOG analysis confirmed identification as GBS (similarity index 0.79, probability 100%). None of the TBS-injected control fish showed signs of disease or died.

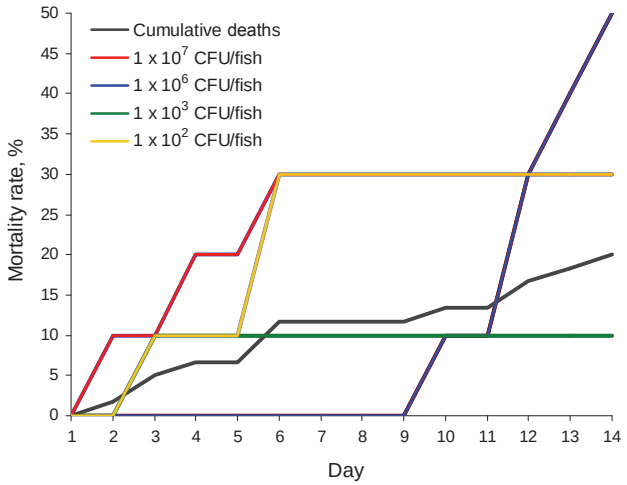


Figure. Mortality rates for 60 Nile tilapia at all doses (black line) and 10 tilapia each challenged with a human *Streptococcus agalactiae* isolate (#510012): $10^2$ (gray line), $10^3$ (green line), $10^6$ (red line), and $10^7$ (blue line) CFU/fish. No deaths occurred at $10^4$ and $10^5$ CFU/fish or in tryptic soy broth controls.

## Conclusions

Deaths among experimentally infected Nile tilapia indicate that an Ia ST-7 human GBS isolate can be pathogenic to fish. Such isolates have been associated with human (*4*,*12*) and fish disease as well as with a marine mammal sampled during a GBS fish kill (*15*). The human isolate was virulent in tilapia at $10^2$, $10^3$, $10^6$, and $10^7$ CFU/fish, and GBS was reisolated from diseased fish. These experimental findings suggest GBS transmission between mammals and fish and that GBS-induced fish epidemics can originate from mammalian sources. Although we studied only 1 human isolate, other isolates or isolates repeatedly passed

Table. Mortality rates among Nile tilapia (*Oreochromis niloticus*) infected with *Streptococcus agalactiae**

| Dose (CFU/fish) | No. (%)† deaths |
|---|---|
| Tryptic soy broth (control) | 0[a] |
| $10^2$ | 3 (30)[ab] |
| $10^3$ | 1 (10)[a] |
| $10^4$ | 0[a] |
| $10^5$ | 0[a] |
| $10^6$ | 5 (50)[b] |
| $10^7$ | 3 (30)[ab] |

*Ten fish per dose group were intraperitoneally injected with human *S. agalactiae*/fish or tryptic soy broth and observed for 14 days postchallenge at a water temperature of 32°C.
†Superscript letters indicate significant differences (p<0.05) in percentage mortality rates between groups, determined by using the SAS program (SAS Institute, Cary, NC, USA) lifetest procedure (Kaplan-Meier method).

through fish may be more virulent. Susceptibility to GBS may also be enhanced by suboptimal environmental conditions, such as low DO, high ammonia levels, euthrophication, harmful algae, and changing or extreme water temperatures (*5*,*6*). Future histopathologic investigations may characterize the distribution and nature of the host response to human GBS.

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# Cowpox Virus Transmission from Pet Rats to Humans, Germany

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We describe a cluster of cowpox virus (CPXV) infections in humans that occurred near Munich, Germany, around the beginning of 2009. Previously, only sporadic reports of CPXV infections in humans after direct contact with various animals had been published. This outbreak involved pet rats from the same litter.

Cowpox virus (CPXV) belongs to the family *Poxviridae*, genus *Orthopoxvirus* (OPV), and is closely related to other species, such as variola virus, vaccinia virus (VV), and monkeypox virus. Originally, cows were wrongly presumed to be CPXV reservoirs. Wild rodents are now considered to be the true reservoirs; cows, cats, zoo animals, and humans are only incidental hosts (*1*). Human CPXV infections are rare and usually cause localized skin lesions. However, in immunocompromised patients, severe generalized skin infections may occur (*2*). CPXV is transmitted to humans by direct contact with infected animals, mostly cats (*3*–*5*). In 2002, Wolfs et al. described a human CPXV infection transmitted by a wild rat (*6*). We report an epidemiologically linked cluster of 5 cases of human CPXV infection caused by contact with a litter of pet rats (*Rattus norvegicus*).

## The Study

Within 4 days, 5 patients with skin lesions suggestive of an OPV infection were reported to the Infectious Disease Task Force at the Bavarian Health and Food Safety Authority. Infected patients were from 2 unrelated families living in 2 different counties in the greater Munich area, Germany. The families had bought 1 and 2 rats, respectively, from the same pet shop on December 15 and December 17, 2008. Source tracing showed that the pet shop owner had sold a litter of 8 rats to 7 different households in the greater Munich area. The pet shop owner had purchased the litter from a Bavarian rat breeder 7 days before the last rat in the litter was sold. These rats had been kept in cages separate from animals of different species. No symptoms of OPV infection were reported in the rats or any another animal in the pet shop. Moreover, all pet shop workers remained free of signs and symptoms. The breeder denied purchasing any animals from abroad that could have been related to the 2003 US monkeypox outbreak (*7*), although he did acknowledge owning another breeding facility in the Czech Republic. Inspection of the breeding facility in Bavaria found 4 rats with crusts suspicious for OPV infection. Mice, hamsters, rabbits, and degus (*Octodon degus*) were also bred in the facility, but none had clinical signs of OPV. A total of 31 rats from the facility were tested for OPV infection by oral swabs and serology.

Members of 6 households were interviewed; 1 customer gave a wrong address. According to their owners, all pet rats were asymptomatic when purchased, but 2 rats (1 in each family with a human OPV infection) died after 9 and 14 days, respectively. One rat had distinct skin lesions on its extremities, mouth, and nose (Figure 1, panel A); the other had only 2 very small lesions on its front leg and nose. Two additional rats from the litter in 2 other households were euthanized due to clinical suspicion of OPV infection; 3 rats were assessed as healthy by their owners.

In households 1 and 2 (Table) with human cases of infection, 2 and 3 persons, respectively, reported only direct skin contact with their pet rats since the first day of purchase. All patients had circumscribed nodules with central necrosis and inflamed edges. Skin lesions were up to 1.5 cm in diameter. Notably, the onset and severity of symptoms were apparently associated with a patient's VV vaccination status: 2 girls (patients 2 and 5, each 16 years of age with no history of VV vaccination) had multiple lesions on the neck, chest, and abdomen (Figure 1, panel B) accompanied by fever and local lymphadenopathy. Incubation periods for these 2 patients were 3 and 5 days, respectively. In contrast, the incubation period for 2 VV-vaccinated mothers (patients 1 and 3, 42 and 40 years of age, respectively) and for the VV-vaccinated grandmother of 1 of the girls (patient 4, 60 years of age) was >7 days. All showed less severe symptoms (Figure 1, panel C) without fever or lymphadenopathy and only 1 small skin lesion on the neck or chest.

In household 3, one person was receiving cyclosporine therapy after a kidney transplantation. She already owned

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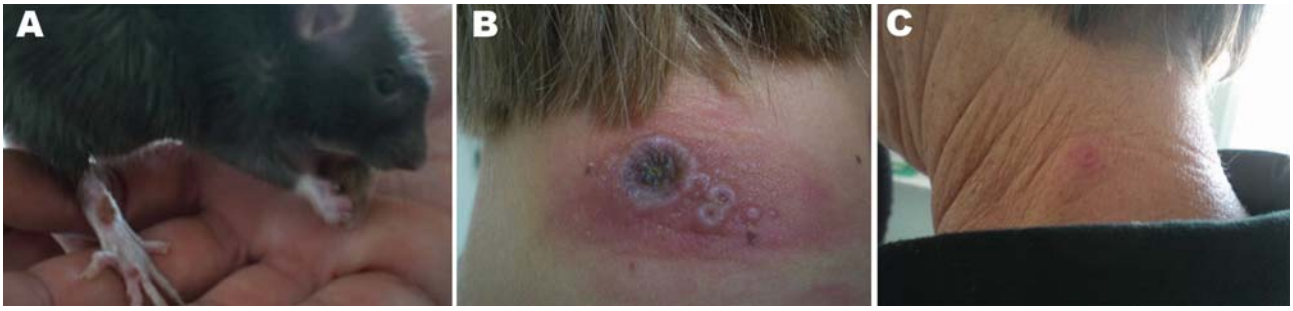


Figure 1. Figure 1. Cowpox lesions on rats and humans during an outbreak in Germany, 2009. A) Rat named Shiva (strain named after this rat) with lesions on the right hind limb; it died 1 day later. B) Neck lesions of a girl without previous vaccinia virus (VV) vaccination. C) Neck lesion of the girl's grandmother with a history of VV vaccination. Photographs taken by authors 13 days after purchase of the rats. Patient is the grandmother (patient no. 4); rat is rat no. 2.

4 rats before purchasing another rat from the infected litter. After 35 days, skin lesions developed in all of her rats, including the initially asymptomatic index rat. All were euthanized due to clinical suspicion of OPV. Fortunately, the kidney transplant patient without previous VV vaccination did not develop signs or symptoms suggestive of CPXV infection. Nevertheless, we collected a blood sample and swabs from her throat and a recent rat-bite finger wound.

Various specimens (skin biopsies, crusts, oral swabs, serum, and whole blood) obtained from 5 patients and from rats from 3 households and 31 other rats (9–39) from the local breeding facility in Bavaria were sent to the Bavarian Health and Food Safety Authority (Table). Depending on specimen type, various investigations were performed (Table). Skin biopsy and crust specimens were homogenized and inspected for typical OPV-like particles

Table. Summary of investigations for cowpox virus, by source, household, and individual (human or rat), Munich, Germany, 2009*

| Pet origins, households, and cases | Clinical findings | Specimens | Diagnostic methods | | | | |
|---|---|---|---|---|---|---|---|
| | | | Antibody titer | EM† | PCR† | Sequencing | Virus isolation |
| Pet shop | | | | | | | |
| Household 1 | | | | | | | |
| Human case 1 | Lesion | Skin biopsy, serum | 640 | ND | Positive | Cowpox virus | Positive |
| Human case 2 | Multiple lesions | Serum | 2,560 | ND | ND | ND | ND |
| Rat 1 | Lesions, fatal outcome | Crusts | ND | ND | Positive | Cowpox virus | ND |
| Household 2 | | | | | | | |
| Human case 3 | Lesion | Crust | ND | ND | Positive | Cowpox virus | Positive |
| Human case 4 | Lesion | NA | ND | ND | ND | ND | ND |
| Human case 5 | Multiple lesions | Crust, serum | 1,280 | Positive | Positive | Cowpox virus | Positive |
| Rat 2 and 3 | Rat 2: lesion, fatal outcome; rat 3: healthy (no symptoms) | Crusts, serum | Rat 2: 1,280 | Rat 2 positive | Rat 2 positive | Rat 2, cowpox virus | Rat 2 positive |
| Household 3 | | | | | | | |
| Human contact | None | Swabs, blood, serum | Negative | ND | Positive | ND | ND |
| Rat 4 plus 4 previously owned rats (40–43) | All rats: lesions, euthanized | Crusts | 3/3 positive: 160, 1,280, 2,560 | ND | 5/5 positive | Cowpox virus | ND |
| Households 4–6 | | | | | | | |
| Human contacts + rats 5–7 | All rats and human contacts with no clinical findings; 2 rats euthanized | NA | ND | ND | ND | ND | ND |
| Breeder | | | | | | | |
| Rats 9–39 | 4 rats with lesions (1 dead); all others with no clinical findings | Mouth swabs, serum | 4/30 positive (>40) | ND | 17/31 positive | Cowpox virus | ND |

*All obtained hemagglutinin open reading frames were 924 bp, and the respective sequences were 100% identical to each other (GenBank accession no. FJ654467). Rat 8 from the outbreak litter belonging to household 7 is missing because the rat owner could not be identified. EM, electron microscopy; ND, not done; NA, materials not available.
†For *Orthopoxvirus* spp.

by using electron microscopy. Virus isolation for these materials was performed using standard procedures. DNA from all samples was extracted using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. For OPV DNA detection, the RealArt Orthopoxvirus LC Kit (QIAGEN) was used. For species identification, the products of a second PCR, spanning the entire open reading frame of the hemagglutinin gene (*8*,*9*), were sequenced. Datasets were edited and aligned using BioEdit (*10*). BLAST search (www.ncbi.nlm.nih.gov/blast/Blast.cgi) was performed to confirm species identification of the isolated strain as well as similarity with published CPXV strains. A phylogenetic tree was constructed using the maximum parsimony method with the Phylogeny Inference Package version 3.68 (http://evolution.genetics.washington.edu/phylip.html) with 100 bootstraps; the tree was drawn with TreeView version 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) (Figure 2). OPV-specific serum antibody titers were determined using an immunfluorescence test based on VV-infected cells and either an antihuman or an antirat immunoglobulin G fluorescein-labeled conjugate (Dako, Hamburg, Germany).

## Conclusions

Besides molecularly proven wild rat-to-human CPXV transmission (*6*) an additional CPXV infection probably transmitted from a pet rat was reported (*11*). Recently, 4 human infections acquired from pet rats were reported to the reference laboratory for poxviruses at the Robert Koch Institute (*12*). We describe a CPXV outbreak among 5 patients caused by infected pet rats from the same litter. CPXV infections seem to be increasing (*13*), but because CPXV infections in humans and in most animals (e.g., cats) are not notifiable, this increase remains an assumption. One obvious reason for an increase might be the fading cross-protective immunity to OPV after the cessation of VV vaccination (*14*). In our small cluster, the onset and severity of symptoms seemed to be correlated with VV vaccination status; however, although patients reported similar contact with pet rats, patient age and manner of infection might confound this hypothesis (*15*).

The rising popularity of pet rats might also be a point of concern in a population with decreasing cross-protection to OPV and an increasing number of immunocompromised persons. Our findings emphasize the necessity to monitor OPV infections in humans and all animals (e.g., notification requirement) and to improve public awareness. Our outbreak investigation underlines the importance of close cooperation between human health and veterinary authorities in the management of zoonotic diseases.

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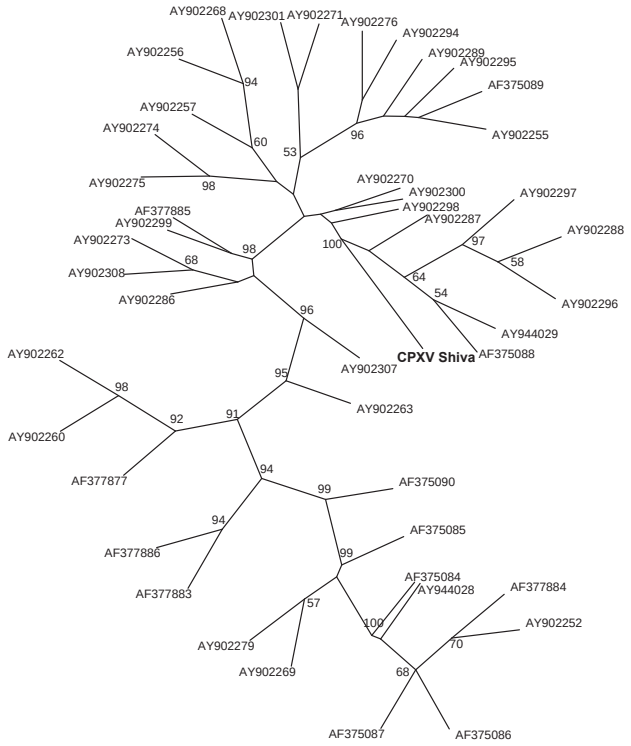


Figure 2. Phylogenetic tree of the isolated cowpox virus (CPXV) Shiva strain (in **boldface**; named after pet rat shown in Figure 1, panel A; GenBank accession no. FJ654467), constructed by the maximum-parsimony method based on the partial sequences method based on the hemagglutinin (HA) gene, unrooted. BLAST search (www.ncbi.nlm.nih.gov/blast/Blast.cgi) confirmed the identification of this strain as a CPXV strain with a unique HA gene sequence. The highest identity of 98.1% was found for strain cowHA72 (accession no. AY902300), a CPXV strain isolated from an elephant in the Netherlands. Bootstrap values >50% are shown. Additional unique CPXV strains shown for comparison, by accession number: AY902307 (cowHA35e), AY902301 (cowHA82), AY902299 (cowHA70), AY902298 (cowHA68), AY902297 (cowHA52), AY902296 (cowHA51), AY902295 (cowHA48), AY902294 (cowHA46), AY902289 (cowHA47), AY902288 (cowHA41), AY902287 (cowHA40), AY902286 (cowHA37), AY902279 (cowHA76), AY902276 (cowHA23), AY902308 (cowHA38), AY902275 (cowHA22), AY902274 (cowHA21), AY902273 (cowHA81), AY902271 (cowHA19), AY902270 (cowHA18), AY902269 (cowHA17), AY902268 (cowHA16), AY902263 (cowHA15), AY902262 (cowHA34), AY902260 (cowHA13), AY902257 (cowHA09), AY902256 (cowHA07), AY902255 (cowHA63), AY902252 (cowHA73), AY944029 (CPV90_ger2), AY944028 (CPV91_ger3), AF377886 (cowpox virus), AF377885 (cpv-922-99), AF377884 (cpv-867-99b), AF377883 (cpv-667-94b), AF377877 (cpv-1218-00), AF375090 (cpx-ep-2), AF375089 (cpx-brt), AF375088 (cpx-90-5), AF375087 (CPX-90-1), AF375086 (cpx-89-5), AF375085 (cpx-89-4), and AF375084 (cpx-89-1).

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Dr Campe is a clinical virologist with a strong interest in respiratory and enteric viruses, human herpesvirus Type 7, and viral disease in immunosuppressed patients.

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# Cowpox Virus Transmission from Pet Rats to Humans, France

**Laetitia Ninove, Yves Domart, Christine Vervel, Chrystel Voinot, Nicolas Salez, Didier Raoult, Hermann Meyer, Isabelle Capek, Christine Zandotti, and Remi N. Charrel**

In early 2009, four human cases of cowpox virus cutaneous infection in northern France, resulting from direct contact with infected pet rats (*Rattus norvegicus*), were studied. Pet rats, originating from the same pet store, were shown to be infected by a unique virus strain. Infection was then transmitted to humans who purchased or had contact with pet rats.

The recent trend of adopting wild animals as pets will inevitably create conditions favorable for emerging pathogens. Consequently, under the influence of increasing commercial enterprise, potentially highly pathogenic agents are likely to emerge and fuel unprecedented epidemic situations. Cowpox virus (CPXV) is a member of the family *Poxviridae*, genus *Orthopoxvirus*. In contrast to smallpox virus (exclusively human), the reservoir for CPXV, and possibly monkeypox virus, is believed to be rodents (*1*,*2*). CPXV is distributed in Europe, Russia, the western states of the former United Soviet Socialist Republic, and adjacent areas of northern and central Asia (*3*). Natural reservoir hosts of CPXV are wild rodents, such as bank voles and wood mice (*4*,*5*). Transmission to humans is through contact with infected animals, mostly domestic cats, which are occasional predators of wild rodents (*4*–*6*). We studied an outbreak of cowpox virus cutaneous infection among 4 human case-patients.

## The Cases

Case-patient 1 was an 18-year-old woman. She was scratched on the right arm by a pet rat while visiting a friend who had several domestic rats (*Rattus norvegicus*). One rat had been purchased at the end of December 2008 from a pet store. The rat became sick with sneezing, conjunctival hemorrhages, and epistaxis; it died 4 days after purchase. On January 4, 2009, the patient sought treatment at the emergency department of Compiègne Hospital. The lesion was excised and the patient was treated with amoxicillin-clavulanate. However, the wound did not heal. On January 11, ofloxacin was added to the treatment regimen. Eight days later, the patient was admitted to the hospital with a black necrotic scab on the internal surface of the right arm, regional lymphangitis, and axillar lymphadenopathies (Figure 1, panel A). After 3 weeks of unsuccessful antimicrobial drug treatment, she underwent surgery to remove the affected area. The outcome was favorable.

Case-patient 2 was a 17-year-old woman who had purchased a domestic rat at the end of December 2008. The rat died within 3 days of purchase and had respiratory symptoms identical to those of the rat that scratched case-patient 1. Six days after the rat died, an inflammatory cutaneous macular lesion appeared at the base of the patient's neck, causing local pain and intense inflammatory reaction. The patient was admitted to the emergency department of Compiègne Hospital. Amoxicillin-clavulanate was prescribed, but the necrotic scab continued to grow, and local pain increased along with fever (39°C) (Figure 1, panel B). Surgery was performed and the outcome was favorable.

Case-patient 3 was a 14-year-old girl. She was admitted to the emergency department of Compiègne Hospital on January 14, 2009. On January 3, she had purchased a rat from the same pet store as case-patient 1. Soon afterward, the rat began to cough and show signs of hemorrhagic lachrymal oozing**;** the rodent died on January 6. On January 13, the patient had maculopapular lesions on the upper right eyelid, on her left shoulder, and at the base of her neck. Due to her deteriorating condition, she was admitted to the emergency department on January 17 with rash characterized by erythema and edema, and painful regional lymphangitis and lymphadenopathy. Surgery was performed and the outcome was favorable.

Case-patient 4 was a 29-year-old woman who reported having been scratched by a rat on January 21, 2009. An inflammatory macule on her clavicle had progressed through papular, vesicular, and pustular stages; she also had fever and malaise. On January 14, she had purchased a domestic rat in the same store as the 3 previous case-patients. The rat had respiratory symptoms similar to the previously infected rats and died on January 21. The patient was admitted to Compiègne Hospital on January 30; examination showed a 20-mm black eschar with a crust, regional lymphangitis, and painful lymphadenopathies (Figure 1, panels C and D). Outcome was spontaneously favorable.

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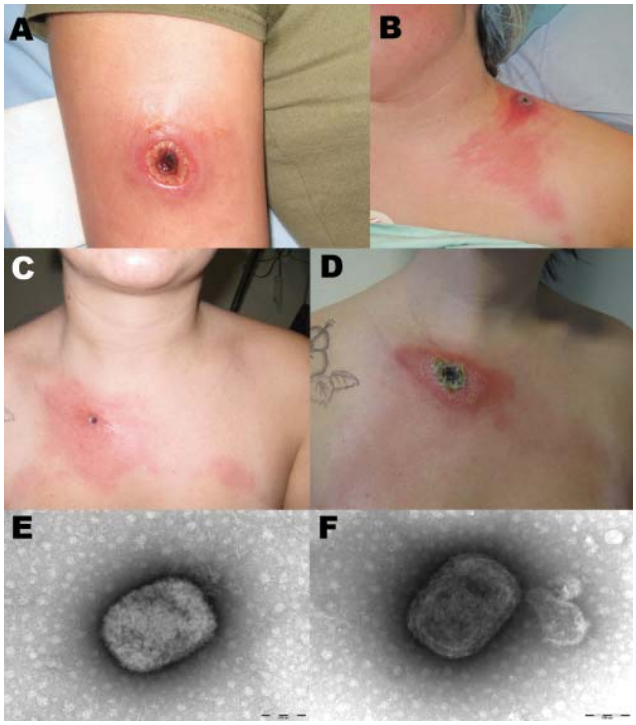


Figure 1. Cowpox virus infection in 3 persons in northern France caused by transmission from infected pet rats. Cutaneous lesions caused by cowpox virus are shown in patient 1 (A), patient 3 (B) and patient 4 (C, D). The 2 latter patients had lymphangitis associated with the local lesion. Panel C was obtained on January 30, 2009, panel D on February 6, 2009. Negative-staining electron microscopy showed mulberry forms with conspicuous but short, randomly arranged surface tubules (E) and capsule forms with deeper stain penetration (F), both highly suggestive of poxvirus. Scale bar for panels E and F = 100 nm.

Biopsy specimens of case-patients 1–3 were sent to the National Reference Center for Rickettsial Diseases (Marseille) on January 21 because anthrax and/or rickettsial disease was suspected. Broad range PCRs were performed for bacteria (16S rRNA) (*7*) and fungi (18S rRNA) (*8*). In the absence of etiology, and based on information provided by the Institut de Veille Sanitaire, negative-stain electron microscopy was performed on January 26. The biopsy samples showed typical poxvirus-like particles (Figure 1, panels E and F). Molecular diagnosis was performed by using PCR targeting a 260-bp fragment in the cowpox hemagglutinin gene (forward primer 5′-TACTTTTGTTACTAATATCATTAG-3′, reverse primer 5′-AGCAGTCAATGATTTAATTGT-3′). Direct sequencing of the PCR product identified cowpox virus by BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the GenBank database. The virus was isolated by using monolayers of Vero cells in 12.5-cm flasks (*9*). When cytopathic effect was obvious, DNA was extracted from the supernatant, and the complete hemagglutinin gene was sequenced from a PCR product (forward primer 5′-CCATTGGAAAAAACACAGTAC-3′, reverse primer 5′-CCAAATATATTCCCATAGTC-3′), amplifying a 1,183-bp region. PCR protocols are available from R.N.C. Electron microscopy and PCR were performed on formalin-fixed scabs from the lesion of case-patient 4. Both test results were positive morphologically for a poxvirus and by PCR for cowpox virus.

The virus was isolated from serum samples of case-patients 1–3 and assessed by cytopathic effect on Vero cells, electron microscopic morphologic identification, positive PCR amplification, and subsequent direct sequencing. The 1,183-bp PCR showed positive results for each of the 4 specimens. Sequences were deposited in GenBank under accession nos. FJ754355–FJ354357. A 1,047-bp sequence was definitive and used for fine comparative genetic analysis with the full-length hemagglutinin gene sequences available in GenBank. The most closely related sequence corresponded to clone cow HA24 of the catpox 5 isolate of cowpox virus, isolated from a cheetah in 1982 in the United Kingdom (AY902254). Genetic heterogeneity with Y902254 comprised 15 mutations; 10 were nonsynonymous, and 3 were insertions. Phylogenetic analyses indicated that sequences corresponding to these cases grouped together and were clearly distinct from other cowpox virus strains previously reported (Figure 2). Sequence analysis indicated that these 4 infections were caused by a virus strain distinct from other cowpox virus sequences retrieved from GenBank.

## Conclusions

Sporadic human cases of cowpox virus infection have occurred in several European countries over the past few years. For most cases the source was domestic cats (*4–6,10,11*). Rat-to-human transmission of cowpox virus was described in the Netherlands, but the source was a wild rat, not a pet rat (*12*). We know of only 1 previous case of human cowpox virus infection that may be linked to a pet rat (*13*). In the 4 cases reported here, the rodent host was clinically sick and rapidly died. All 4 patients reported scratches caused by rat claws, not bites, while handling the rats as pets. In 3 of the 4 cases, fever (39°C) was noticed at the pustular stage, associated with lymphangitis and regional adenopathies. Interviews with the 4 case-patients showed all had purchased or had been in contact with domestic rats originating from the same pet store. Further investigations traced the origin of the cowpox virus-infected rats to a rat breeder in the Czech Republic (*14*).

Recently, similar human cases linked to contact with pet rats have been reported in France, suggesting that the outbreak may involve more cases than were initially realized (*14*). The situations in France and Germany mimic the monkeypox outbreak in the United States, i.e., human

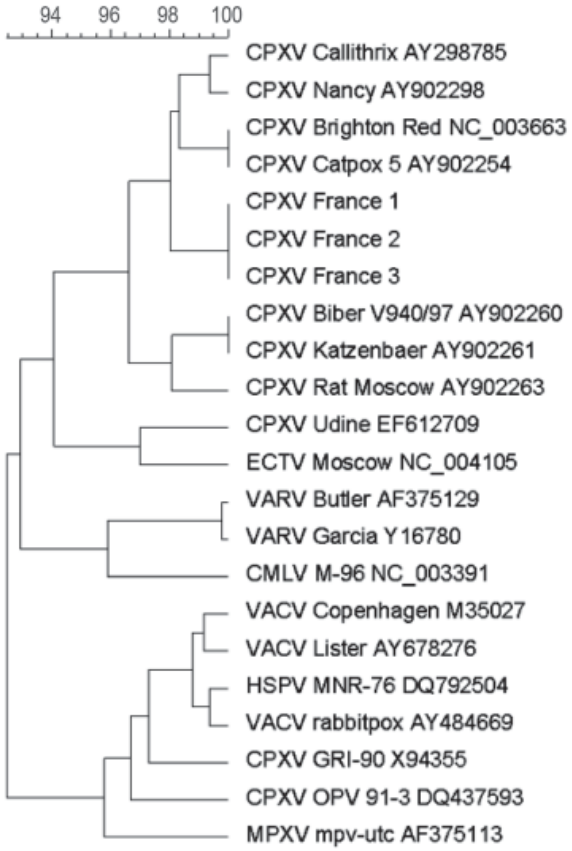


Figure 2. Phylogenetic tree based on nucleotide sequences in the hemagglutinin gene. Sequence information corresponds to virus acronym/strain/GenBank accession number. Phylogenetic study was conducted using MEGA software version 4.0 (www.megasoftware.net). Genetic distances were calculated with the pairwise distance method. Phylogenetic tree were constructed with the neighbor-joining method. CPXV, cowpox virus; ECTV, ectromelia virus; VARV, variola virus; CMLV, camelpox virus; VACV, vaccinia virus; HSPV, horsepox virus; MPXV, monkeypox virus. Scale bar indicates genetic diversity at the nucleotide level.

transmission of the virus by pet prairie dogs contaminated by probable contact with Gambian rats imported from Africa and directly associated with a US pet retailer (*15*). Our study and the US outbreak emphasize the need for extreme caution when humans adopt animals of exotic origin as pets. Our study justifies the establishment of a national diagnostic capability and the corresponding human expertise to enable rapid diagnosis and identification of human pathogens that can cause unimaginable levels of disease in our communities.


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Dr Ninove is a virologist at Marseille University and Hospital who has clinical, research, and teaching activities in the area of common and emerging viruses causing human diseases.

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# Babesiosis Acquired through Blood Transfusion, California, USA

**Van Ngo and Rachel Civen**

Babesiosis was reported in a California resident who received a transfusion of blood products collected in the disease-endemic northeastern region of the United States. Babesiosis should be considered year-round in the diagnosis of febrile and afebrile patients with abnormal blood cell counts who have received blood products from disease-endemic areas.

Babesiosis is an infection of red blood cells (RBCs) caused by various species of the protozoan genus *Babesia*. Most human infections reported in the United States are attributed to *B. microti* and occur most frequently in the Northeast and less commonly in the Midwest (*1*). Infrequently, babesiosis cases have been documented in California and Washington; however, these cases were caused by local *Babesia*-like isolates, including *B. duncani* and a *B. divergens*–like parasite (*1–3*). *B. microti* infection is often asymptomatic but can potentially be severe and even fatal, especially in the elderly, asplenics, and other immunosuppressed persons. Symptoms can be nonspecific, mimicking many systemic infectious diseases, and include fever, chills, myalgias, fatigue, and jaundice caused by hemolytic anemia (*1*).

Babesiosis is transmitted primarily through the bite of an infected tick, typically *Ixodes* spp., although occasionally transmission occurs via transfusion of blood products collected from asymptomatic infected donors (*1*). More than 50 transfusion-related cases have been reported in the United States (*4*). This report describes a transfusion-acquired case of babesiosis caused by *B. microti* in a resident of Los Angeles County, California.

## The Case

On February 12, 2007, a 58-year-old man with metastatic esophageal cancer was admitted to an acute care facility for evaluation of hematemesis and normocytic anemia. The initial examination showed he had hypotension without fever, joint swelling, headaches, or rash. Laboratory evaluation showed a hemoglobin concentration of 8.4 mg/dL, a platelet count of 71,000/mm$^3$, and a leukocyte count of $3.5 \times 10^3$/mm$^3$ with 19% bands. Results of liver function tests showed mild elevations in levels of aspartate transaminase (202 mg/dL), alanine transaminase (33 mg/dL), and total bilirubin (0.7 mg/dL).

An abnormal blood cell count prompted a manual differential count. *Babesia* spp. was identified on a peripheral smear and subsequently confirmed at the Los Angeles County Public Health Laboratory. The result of PCR analysis performed by a commercial laboratory was positive and highly specific for *B. microti* DNA, a result confirmed by the Centers for Disease Control and Prevention (CDC) (Table). The commercial laboratory also performed indirect fluorescent antibody (IFA) testing for *B. microti* and found both acute and convalescent specimens to be negative. Confirmatory testing at CDC corroborated the negative result for the acute specimen but showed the convalescent specimen, collected 8 days after onset, to be positive for *B. microti*, with a total antibody titer of 64. The patient was treated with azithromycin and atovaquone for 7 days, given 2 blood transfusions for anemia, and discharged in stable condition on February 16, 2007.

Before admission, the patient had visited an oncology clinic numerous times for treatment related to his esophageal cancer: radiation therapy in October 2006, 3 chemotherapy courses from October 2006 through February 2007, and blood transfusions in January 2007. The patient received 6 units of packed red blood cells (PRBCs) and 2 units of fresh frozen plasma (FFP) over several clinic visits on January 1 and January 22–24, 2007.

The patient was in Salt Lake City, Utah, from January 13 through January 20, 2007; however, because of poor health, he did not engage in any outdoor activities. At least a year before his admission in 2006, the patient visited an undeveloped property near Klamath Falls, Oregon, where he spent time outdoors. He could not recall ever incurring a tick bite, seeing ticks, or having any animal contact.

The Table summarizes the serologic and PCR results for specimens collected from the patient and 6 PRBC donors. The PRBC units came from 2 blood banks: 1 in Maine (2 units) and 1 in California (4 units). A blood donor from Maine tested positive for *B. microti* by IFA, with a total antibody titer 256, but tested negative *B. microti* by PCR. Testing of specimens from remaining PRBC donors yielded negative results. Specimens from FFP donors were not tested because of the low risk for *Babesia* spp. transmission associated with plasma products.

The implicated donor was a 49-year-old male resident of Maine, where babesiosis is less common than in other states in the northeast. For example, whereas Maine typically reports <12 cases annually, Rhode Island has reported up to 61 cases (*5*,*6*). However, the donor resided in the southern coastal region of the state, where Maine's cases

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Table. Results of serologic testing and PCR analyses of specimens collected from a California resident with babesiosis and donors of packed red blood cells, 2007*

| Specimen source | Residence | Date of transfusion† | Date of specimen collection | *B. microti* test results | |
|---|---|---|---|---|---|
| | | | | PCR | IFA |
| Patient | California | – | Feb 12 | Positive | ≤8‡ |
| | | | Feb 20 | – | 64 |
| Donor 1 | Maine | Jan 1 | Feb 26 | Negative | 256 |
| Donor 2 | Maine | Jan 1 | Feb 26 | – | ≤8 |
| Donor 3 | California | Jan 22 | Feb 21 | – | ≤8 |
| Donor 4 | California | Jan 23 | Feb 22 | – | ≤8 |
| Donor 5 | California | Jan 24 | Feb 21 | – | ≤8 |
| Donor 6 | California | Jan 24 | Feb 21 | – | ≤8 |

*IFA, immunofluorescent antibody.
†Involving blood products from specified donor.
‡≤8 is considered a negative titer for total *B. microti* antibody. IFA tests conducted at the Centers for Disease Control and Prevention Reference Diagnostic Laboratory.

are concentrated (*5*). He frequented tick-infested areas and is likely to have become infected in late August 2006, when he sought treatment for fever, chills, weight loss, and fatigue and was tested for various infections, including Lyme disease and ehrlichiosis. At that time, he was not tested for babesiosis. His health improved without a specific diagnosis or treatment, and he remained asymptomatic, but he evidently was parasitemic when he donated blood on December 20, 2006. Blood products from this donation were included in the transfusion the patient received on January 1, 2007. Between October 2005 and the blood donation on December 20, 2006, the donor made 3 donations.

All other recipients of blood products from this donor were residents of Maine. They were notified of the need for serologic testing; none were reported to be infected (V. Rea, pers. comm.).

## Conclusions

Babesiosis was documented in a man with metastatic cancer who resided in an area nonendemic for *B. microti*. On the basis of laboratory and epidemiologic information, we concluded that the patient acquired the infection via transfusion of infected PRBCs donated in a disease-endemic area thousands of miles away. The 12-day period from donation to transfusion was within the maximum 35 days that *B. microti* has been known to remain viable in refrigerated blood (*7*). The period from time of transfusion exposure until positive smear was ≈6 weeks; incubation periods for transfusion-related cases have ranged from weeks to many months (B. Herwaldt, pers. comm.). We had tentatively hypothesized that the patient might have acquired the infection in Oregon, where he spent substantial time outdoors, but remained asymptomatic until he became ill with cancer; however, we rejected this hypothesis because human cases of babesiosis have never been documented in Oregon (or in Utah where the man also visited) and because infections acquired in western states are more likely to be caused by local *Babesia* agents.

This case demonstrates that, even among transfused patients who show atypical symptoms of babesiosis, the possibility of infection should be considered if they have received blood products from disease-endemic areas and display abnormal blood cell counts, such as low iron and low leukocyte counts. Generalized debilitation associated with cancer and chemotherapy may have masked *Babesia*-related symptoms in this patient and undermined his immune response. This case also underscores the widening seasonal and geographic boundaries of babesiosis. Tick-borne babesiosis usually peaks from July through September (*4*), but because asymptomatic *Babesia* infection can persist for months to years, especially in untreated persons (*7*,*8*), transfusion-associated infection can occur throughout the year. Geographic limitations in babesiosis are virtually erased by the mobility of donors and blood products. The blood bank involved in this case has blood collection centers in California and Maine but provides blood products to hospitals throughout southern California and the East Coast. Medical evaluation for babesiosis in both febrile and afebrile transfusion patients should include a Giemsa-stained thin blood smear, an acute serologic evaluation by IFA testing and a convalescent serologic evaluation by IFA testing taken 4–6 weeks apart (*9*), and PCR evaluation of whole blood.

The varied clinical spectrum of babesiosis makes its detection in blood donors challenging. This case exemplifies the limitations in screening healthy asymptomatic donors for babesiosis. Available screening tests to detect *Babesia* spp. postdonation are not cost-effective and have inadequate sensitivity (*7*,*10*). Nucleic acid testing and inactivation procedures may provide useful options for detecting *Babesia* spp. in the future (*7*,*11*). Until effective screening procedures are available, however, diagnosis of babesiosis in blood donors will continue to be based primarily on clinical observation.

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Ms Ngo is an epidemiologist and Dr Civen is a medical epidemiologist with the vector-borne disease unit of the Acute Communicable Disease Control program of the Los Angeles County Department of Public Health. Their research interests include the surveillance and epidemiology of vector-borne diseases affecting Los Angeles County.

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# etymologia

## *Babesia*

[bə-beʹ ze-ə]

A genus of protozoa of the order Piroplasmida, named for Victor Babès (1854–1926), a Romanian bacteriologist who discovered a parasitic sporozoon in ticks in 1885. The parasites occur within the erythrocytes of various vertebrates and cause babesiosis, a tick-borne infection of domestic animals and humans.

Babès was also a coauthor of the first text on bacteriology (Bacteria and Their Role in the Anatomy and Pathological Histology of Contagious Diseases, with French scientist A.V. Cornil); the first to demonstrate the presence of tuberculous bacilli in the urine of infected patients; a founder of serum therapy; and the first to introduce rabies vaccination to Romania.

**Sources:** Dorland's illustrated medical dictionary, 31st edition. Philadelphia: Saunders; 2007; www.whonamedit.com; www.ici.ro/romania/en/stiinta/babes.html

# Probable Congenital Babesiosis in Infant, New Jersey, USA

**Sonia Sethi, David Alcid, Hemant Kesarwala, and Robert W. Tolan, Jr.**

Only 2 neonates with transplacentally or perinatally acquired (congenital) babesiosis have been reported. We describe a probable third congenital case of babesiosis in a 26-day-old infant; transmission was determined on the basis of a blood smear from the infant (15% parasitemia) and serologic results from the infant and mother.

Victor Babes first described the pathogen of babesiosis in 1888 (*1*). Babesiosis is a tick-borne malaria-like illness transmitted by the same *Ixodes* spp. ticks that transmit *Borrelia burgdorferi* (*2*). It is endemic to the northeastern and northwestern United States and also occurs in Europe and parts of Asia. Babesiosis is an intraerythrocytic parasitic infection that ranges from subclinical to severe (possibly fatal) disease with fever, thrombocytopenia, hemolytic anemia, and hyperbilirubinemia. Appropriate antimicrobial drug therapy, transfusion, and exchange transfusion remain the mainstays of treatment.

Babesiosis occurs rarely among neonates, although it is gaining increasing attention as an emerging tick-borne zoonosis. In 1987, Esernio-Jenssen et al. (*3*) reported an apparent case of transplacentally or perinatally transmitted congenital babesiosis. In 1997, New et al. (*4*) reported another case. We describe a third case of probable congenital babesiosis in a 26-day-old infant with 15% parasitemia. She was treated successfully with atovaquone (Mepron; GlaxoSmithKline, Research Triangle Park, NC, USA) and azithromycin (Zithromax; Pfizer, New York, NY, USA).

## The Case

A 26-day-old, 8-pound, full-term infant girl was transferred to Saint Peter's University Hospital for evaluation of fever and hyperbilirubinemia. For 1 week, she was not feeding well and was gagging and irritable. On the day of admission, her mother noted fever and yellow eyes. The mother (a migrant crop worker) reported having had an uneventful pregnancy, labor, and delivery, except for having been bitten by 2 ticks at 8 months' gestation while picking crops in New Jersey. She did not seek treatment. The mother had not traveled elsewhere in the United States during her pregnancy. Knowledge about earlier travel to *Babesia*-endemic areas would have been helpful in understanding the mother's infection, but this information was unavailable. The infant had no history of tick exposure; she had been outdoors only for visits to the pediatrician.

Physical examination showed an alert but pale infant weighing 4.4 kg; her temperature was 101.8°F (38.7°C), pulse rate 160/min, respiratory rate 36/min, blood pressure 90/40 mm Hg, and oxygen saturation 99% while breathing room air. Her conjunctivae were icteric. Her liver and spleen were palpable 4 cm and 5 cm below their respective costal margins. No hemorrhagic lesions or tick bites were noted. The rest of her physical examination findings were unremarkable except for a diaper rash.

Initial laboratory findings included a hemoglobin level of 8.8 g/dL (indices within normal limits); leukocyte count of 9.0/mm$^3$ with 3% bands, 18% neutrophils, 72% lymphocytes, 7% monocytes; and platelet count of 34,000/mm$^3$. Blood chemistry concentrations included total and indirect bilirubin 5.9 mg/dL (reference range 0.1–1.2 mg/dL); alanine aminotransferase 18 IU/L; aspartate aminotransferase 53 IU/L; alkaline phosphatase 108 IU/L; blood urea nitrogen 6 mg/dL; creatinine 0.3 mg/dL; and C-reactive protein 54 mg/dL (reference range 1.0–10.0 mg/dL). Peripheral blood smear demonstrated evidence of hemolysis and was consistent with *Babesia microti* infection (although *B. duncani* is indistinguishable from *B. microti* on peripheral smear) and ≈15% parasitemia (Figure).

Subsequently, the infant's lactate dehydrogenase concentration was found to be 1,912 IU/L (reference range 313–618 IU/L) and later rose to 2,535 IU/L (Table 1). The infant's *Babesia* immunoglobulin (Ig) G and IgM titers by immunofluorescent antibody (IFA), which are genus specific but not species specific, were 256 (reference <16) and 40 (reference <20), respectively (both tests were performed by Quest Diagnostics–Nichols Institute, Chantilly, VA, USA). Lyme IgG Western blot plus 2 Lyme IgM Western blots, performed early during hospitalization and just before discharge, were negative. The mother's peripheral blood smear did not show any parasites, but her *Babesia* IgG and IgM titers by IFA were ≥1,024 and 80, respectively, and her Lyme serology was positive. The mother refused additional testing. Despite the variability in sensitivity and specificity of commercially available serologic tests (particularly the IFA for *Babesia* IgM), *Babesia* serologic results were not confirmed at a reference laboratory. Species-specific PCR was not performed.

After concluding that this infant had probable congenital babesiosis, we began treating her with oral atova-

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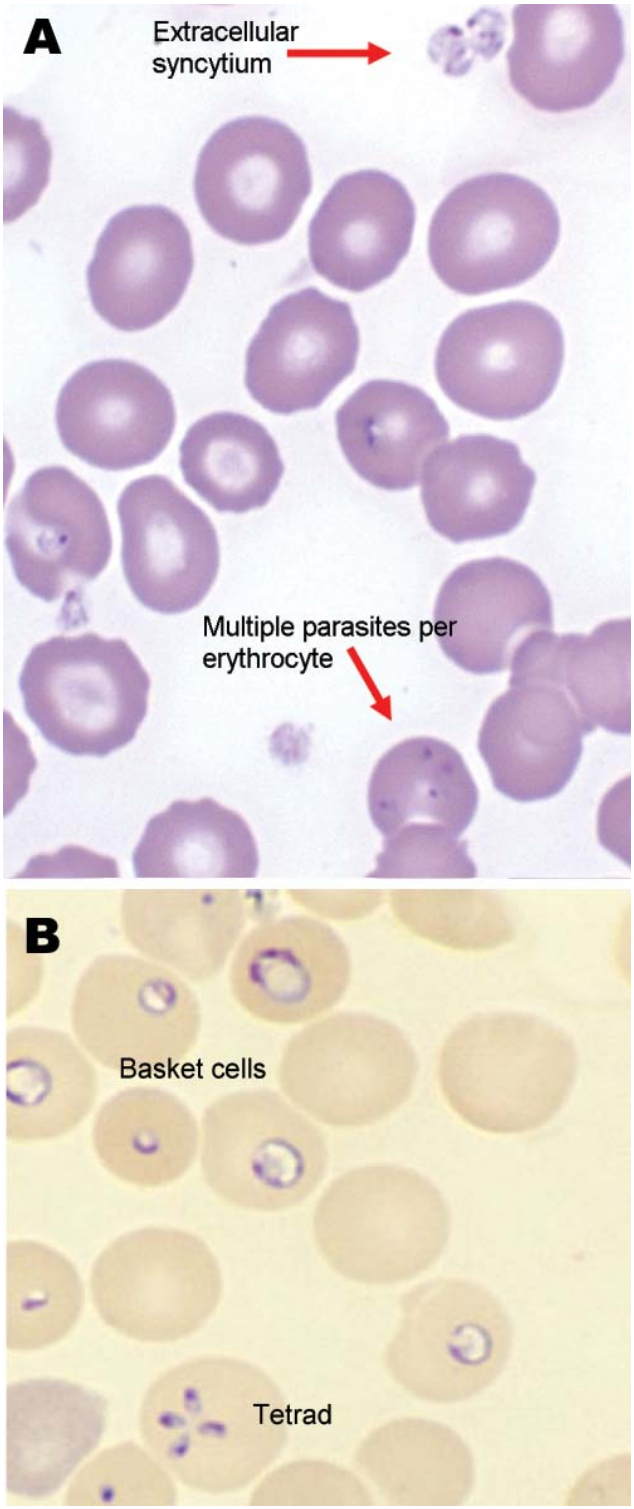


Figure. Giemsa-stained (A) and Wright-stained (B) peripheral blood smear from a newborn with probable *Babesia microti* infection. Parasitemia was estimated in this newborn at ≈15% based on the number of parasites per 200 leukocytes counted. The smear demonstrated thrombocytopenia and parasites of variable size and morphologic appearance and an absence of pigment. Magnification ×1,000

quone (40 mg/kg/d) in 2 divided doses and azithromycin (12 mg/kg/d) once per day. The infant received 1 transfusion with packed red blood cells on hospital day 3 because of continued hemolysis, but she did not require exchange transfusion despite having a high initial parasite count. The infant's parasitemia decreased rapidly, and she responded well to treatment (Table 1). She was discharged after 8 days and was to complete a 10-day course of atovaquone and azithromycin (which were well tolerated); she was subsequently lost to follow-up.

## Conclusions

Of 10 cases of babesiosis in neonates that have been reviewed (*5*), 2 were congenital (*3*,*4*), 2 were transmitted by a tick bite (*6*), and 6 were associated with transfusions (*5*,*7*–*9*). The 2 congenital cases (*3*,*4*) are compared to our probable congenital case (Table 2). All 10 of the affected neonates were reported to have <9% parasitemia (*5*). The illness ranged from no symptoms in 2 infants transfused with contaminated blood (*8*) to symptomatic disease (as in our infant) with fever and hepatosplenomegaly in 5 of 7 (71%), hemolytic anemia in 8 of 10 (80%), indirect hyperbilirubinemia in 4 of 5 (80%), and thrombocytopenia in 7 of 9 (78%) (*5*). Five of 8 (63%) patients required erythrocyte transfusion (*5*). The infant we describe had all of these manifestations as well as a higher parasite count than described previously (*5*). Clearly, the spectrum of neonatal babesiosis is variable and must be more fully elucidated, as must determinants of the illness's clinical course and parasite clearance. In neonates, the degree of parasitemia may not parallel the severity of the babesiosis.

The combination of quinine sulfate and clindamycin hydrochloride for treatment of a newborn with transfusion-associated babesiosis was described in 1982 and subsequently became the first accepted treatment (*7*). A combination of azithromycin with atovaquone for 7 to 10 days has emerged as an alternative regimen (*8*,*10*–*11*), having been used successfully in 2 neonates (*8*,*10*) and several adults (*11*) in whom it appears to be safe and effective. Finally, the addition of azithromycin or atovaquone to the clindamycin hydrochloride plus quinine sulfate regimen has been proposed (*2*,*8*), particularly if parasitemia is slow to resolve.

Recently, our understanding of babesiosis and the methods of testing for it have improved dramatically. Because babesiosis (and congenital babesiosis) is an emerging tick-borne zoonosis, it is worthwhile to review the state-of-the-art approach to its diagnosis in the context of the limitations to diagnosis inherent in this particular case, including its retrospective nature, the mother's lack of insurance and resultant unwillingness to undergo any additional laboratory testing, and the loss to follow-up of the infant and her migrant family.

Table 1. Clinical and laboratory data for infant with probable congenital babesiosis*

| Clinical/laboratory data | Day of hospitalization | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| Maximum daily temperature, ºF | 102.7 | 101.9 | 100.6 | 98.0 | 98.8 | 99.6 | 99.4 | 98.8 |
| Hepatomegaly, cm below costal margin | 4 | 4 | 4 | 5 | Stable | Stable | Smaller | Smaller |
| Splenomegaly, cm below costal margin | 5 | 5 | 5 | 5 | Stable | Stable | Smaller | Smaller |
| Lowest daily hemoglobin level, g/dL | 8.8 | 8.1 | 7.0† | 9.8 | 8.6 | 7.4 | 8.5 | 9.6 |
| Lowest daily platelet count, $10^3$/$mm^3$ | 34 | 30 | 23 | 34 | 45 | 112 | 185 | 340 |
| Parasitemia, % | 15.2 | Present‡ | Present‡ | 2 | <1 | NA | 0 | NA |
| Highest daily total bilirubin level, mg/dL | 5.9§ | NA | 3.6 | 3.9 | 2.7 | 2.1 | 1.7 | 1.3 |
| Highest daily lactate dehydrogenase level, IU/L | NA | NA | 1,912¶ | 2,481 | 2,535 | 2,286 | 2,239 | 1,566 |
| Highest daily C-reactive protein level, mg/dL | 54# | NA | 130 | 82 | 62 | 39 | NA | NA |
| Treatment | CRO, ATO, AZI | CRO, ATO, AZI | CRO, ATO, AZI, PRBC | CRO, ATO, AZI | CRO, ATO, AZI | CRO, ATO, AZI | CRO, ATO, AZI | CRO, ATO, AZI |

*NA, not assessed; CRO, ceftriaxone sodium; ATO, atovaquone; AZI, azithromycin; PRBC, packed red blood cells.
†Before PRBC transfusion.
‡Not quantified.
§Reference range 0.1–1.2 mg/dL.
¶Reference range 313–618 IU/L.
#Reference range 1.0–10.0 mg/dL.

Diagnosis of congenital babesiosis requires definitive evidence of babesiosis, including evidence from reference laboratory species-specific IFA testing, PCR confirmation, and evidence from reference laboratory evaluation of peripheral blood smears, particularly blood smears with high parasitemia (necessary because of the numerous species of *Babesia* endemic to the United States, including *B. microti*, *B. divergens*–like, *B. duncani*, MO-1, CA-1, and WA-1). Accurate diagnosis also requires collection of extensive epidemiologic information about patients with suspected infections, including their recent and remote travel history, exposure to ticks, transfusion or transplant. Follow-up for recrudescence is important, particularly for the immunocompromised patient. Our report of a probable third case of congenital babesiosis illustrates the variability in the manifestations and clinical course of the illness, suggesting a need for improvement in how the disease is recognized and for evaluation of current treatment modalities.

Dr Sethi is a pediatric resident at The Children's Hospital at Monmouth Medical Center in Long Branch, New Jersey. She is interested in pediatric cardiology.

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Table 2. Selected clinical data from the first 2 reported cases of congenital babesiosis (*3,4*) and the probable case described in this article*

| Clinical data | Reference case 1 | Reference case 2 | Present case |
|---|---|---|---|
| Infant's age at time of illness | 30 d | 5 wk | 26 d |
| Time of maternal tick bite before delivery | 1 wk | 7 wk | 4 wk |
| Serologic test results for *Babesia* spp. | | | |
| Mother | Pos | Pos | Pos |
| Infant | Pos | Pos | Pos |
| Clinical findings | Fever, irritability, pallor, hepatosplenomegaly | Lethargy, poor feeding, pallor | Fever, poor feeding, irritability, pallor, scleral icterus, hepatosplenomegaly |
| Parasitemia, % | 5 | 4.4 | 15 |
| Treatment (duration) | Ampicillin and gentamicin (3 d); clindamycin and quinine sulfate (10 d) | Clindamycin and quinine sulfate (12 d); azithromycin (10 d) | Ceftriaxone (8 d); atovaquone and azithromycin (10 d) |

*All infants' history of tick exposure was negative, and all recovered.

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# Genotype Distribution and Sequence Variation of Hepatitis E Virus, Hong Kong

**Wai-Yip Lam, Rickjason C.W. Chan, Joseph J.Y. Sung, and Paul K.S. Chan**

Most acute cases of infection with hepatitis E virus (HEV) in Hong Kong were autochthonous, sporadic, and occurred in older adults. All except 1 isolate belonged to genotype 4; most were phylogenetically related to swine isolates. The epidemiology is similar to that in industrialized countries, where zoonosis is the major source of HEV infection in humans.

Hepatitis E virus (HEV) is a major cause of enterically transmitted acute hepatitis throughout Asia, the Middle East, and Africa. Large outbreaks resulting from fecal contamination of drinking water are confined mainly to developing countries (*1*), whereas sporadic cases in industrialized countries are thought to be zoonotic, with swine being the most likely reservoir (*2*). Studies in Hong Kong reported a seroprevalence of 16%–19% (*3*). No large outbreak has been recorded in Hong Kong, and the sporadic cases are believed to be imported (*4*). Our study examined the epidemiology and genotype distribution of HEV infections in Hong Kong in an effort to improve control of this disease.

## The Study

We studied patients admitted to the Prince of Wales Hospital, Hong Kong Special Administrative Region, who had laboratory-confirmed acute hepatitis E during 2002–2007. Acute HEV infection was diagnosed on the basis of clinical manifestations of acute hepatitis, elevation of hepatic parenchymal enzyme levels, and presence of anti-HEV immunoglobulin (Ig) M as determined by an HEV IgM kit (Biotec Laboratories Ltd., Suffolk, UK). Clinical data were retrieved from patients' records in the computerized clinical management system of the hospital. HEV RNA was then amplified from patients' stored serum by using a nested reverse transcription–PCR (RT-PCR) specific for open reading frame (ORF) 2 with external primers ConsORF2-s1 (5′-GACAGAATTRATTTCGTCGGCTGG-3′) and ConsORF2-a1 (5′-CTTGTTC RTGYTGGTTRT-CATAATC-3′) and internal primers ConsORF2-s2 (5′-GTYGTCTCRGCCAATGGCGAGC-3′) and ConsORF2-a2 (5′-GTTCRTGYTGGTTRTCATAATCCTG-3′) (*5*). Positive samples were subjected to another nested RT-PCR targeting ORF1 with external primers HE5-1 (5′-TCGATGCCATGGAGGCCC-3′) and HEVORF1-1as (5′-GGCCATTGCCTCCGCAACATC-3′) and internal primers HE5-2 (5′- GCCYTKGCGAATGCTGTGG-3′) and HEVORF1-2as (5′- ACCATCAAAGCAGTAAGTC-CG-3′) (*6*). The ORF2 (145-bp) and ORF1 (364-bp) PCR products were sequenced, and sequence fragments were aligned by CLUSTALX 2.0 (*7*). Phylogenetic trees were constructed by using the neighbor-joining method (*8*), and rooted phylogenetic trees were generated by using PAUP* version 4.0b (*9*). Bootstrapping values obtained from 1,000 resamplings of the data were performed to assess the robustness of trees (*10*). The final tree was obtained with the FigTree program, version1.1.2 (*11*). Nucleotide sequence similarity among isolates was analyzed by using the Bioedit software (*12*). The sequence data were deposited in GenBank under accession nos. FJ438395–FJ438427 and FJ438428–FJ438460, respectively.

We identified 57 patients, of whom 56 were Chinese. All patients were negative for acute hepatitis A and B markers. Nineteen were females (none pregnant) 14–82 years of age (median age 57 years, interquartile range [IQR] 43–74 years); 38 were men 26–76 years of age (median age 52 years, IQR 38–67 years). No significant difference in age was observed ($p = 0.323$ by Mann-Whitney U test). Eleven patients were hepatitis B carriers, 1 had cirrhosis, and 1 was a hepatitis C carrier.

Forty-eight (84%) patients had no history of travel during the prior 6 months. Nine (16%) had traveled outside Hong Kong (6 to People's Republic of China, 1 to Macau, 1 to South Korea, 1 to the United States) 1–4 weeks before illness onset. All cases were sporadic.

The highest liver function levels recorded for the women were serum alanine aminotransferase (ALT) 261–6,500 IU/L (median 1,280 IU/L, IQR 434–8,322 IU/L, reference <58 IU/L). Serum alkaline phosphatase (ALP) levels ranged from 111 to 469 IU/L (median 214 IU/L, IQR 178–268 IU/L, reference 45–145 IU/L), and total serum bilirubin levels ranged from 10 to 565 μmol/L (median 85 μmol/L, IQR 30–146 μmol/L, reference, <15 μmol/L). The highest liver function levels recorded for the men were serum ALT 253–4,525 IU/L (median 1,714 IU/L, IQR 1,043–2,608 IU/L, reference <58 IU/L), serum ALP 76–912 IU/L (median 188 IU/L, IQR 132–261 IU/L, reference 35–100 IU/L), and total serum bilirubin 10–544 μmol/L (median 112 μmol/L, IQR 72–227 μmol/L, reference <15 μmol/L).

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No fulminant hepatitis was recorded, and all patients recovered.

Thirteen patients had other medical conditions including end-stage renal failure, diabetes, ischemic heart disease, colon cancer, system lupus erythematous, thyroitoxicosis, hepatitis B liver cirrhosis, and previous liver transplantation. Patients with a medical condition had significantly higher levels of serum ALP (median 267 IU/L, IQR 86–1,703 IU/L) than did those without medical conditions (median 192 IU/L, IQR 131–164 IU/L; p = 0.03 by Mann-Whitney U test).

Phylogenetic analyses of the ORF2 fragments from 46 patients and ORF1 fragments from 33 patients showed complete agreement (Figure), with most (45 [98%]) belonging to genotype 4. The remaining isolate was genotype 3 (HK14) obtained from a woman who had no history of travel. Most of the Hong Kong isolates clustered closely with a swine isolate reported from Guangxi Province, China (accession no. EU676172). Furthermore, the ORF2 phylogenetic tree showed our isolates were closely related to those reported recently from Beijing, China (accession nos. EU107400–EU107474) (*13*).

We further analyzed the sequence variation of 32 HEV genotype 4 isolates for which ORF1 and ORF2 sequences were generated in the current study. The nucleotide sequence similarity was 79.9% for the ORF1 region (nt 170–448), and 86.4% for ORF2 (nt 6409–6504). For comparison, the sequence similarity for HEV isolates collected elsewhere that had been deposited in GenBank was 73.8% for ORF1 (nt 170–448) and 74.0% for ORF2 (nt 6409–6504). Regions within nt 171–221, 280–310, and 6,461–6,495 were most conserved and represented the best targets for primer or probe design.

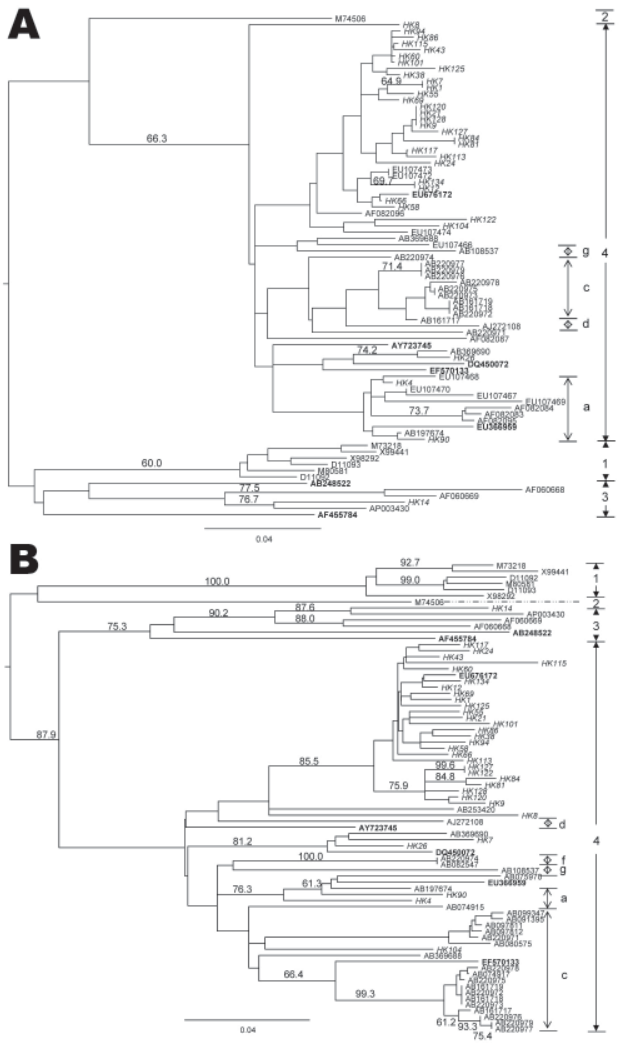


Figure. Phylogenetic tree showing the relationship of hepatitis E virus (HEV) isolates from Hong Kong. Trees were constructed by the neighbor-joining method based on the partial nucleotide sequence of the open reading frame (ORF) 2 (A) and ORF1 (B) regions of HEV samples. Genotypes are indicated by numbers and subtypes by letters on the right. Branch lengths are proportional to genetic distance. Scale bars indicate 0.04 nt substitutions per position. Bootstrap values for the various branches are shown as percentages of trees obtained from 1,000 resamplings of the data. Sequences used for phylogenetic analysis were isolates from genotype 1: Burma (GenBank accession nos. M73218; D10330), Pakistan (M80581), India (X98292; X99441), and China (D11092; D11093); genotype 2: Mexico (M74506); genotype 3: USA (AF060668; AF060669), Japan (AP003430); and genotype 4: China (AB108537; AB197674; AF082083; AF082084; AF082087; AF082095; AF082096; AJ272108; EU107466-74), Japan (AB074915; AB074917; AB080547; AB091395; AB097812; AB099347; AB161717-19; AB220971-79; AB253420; AB369688; AB369690). Branches of swine HEV genotype 3 sequences (AB238522; AF455784 [experimentally infected swine]) and swine HEV genotype 4 sequences (AY723745; DQ450072; EF570133; EU366959; EU676172) are included in the analysis. Accession numbers in **boldface** are swine isolates. All isolates from the current study have a prefix HK followed by a number in italics.

## Conclusions

Our study showed that most HEV cases in Hong Kong were sporadic and autochthonous. Although a substantial proportion (21%) of patients were hepatitis B or C carriers, all diseases had a self-limiting course. This finding could be related to the circulation of relatively mild genotypes 3 and 4 in this locality. The epidemiology of HEV in Hong Kong resembled industrialized countries with a predilection for older adults, rather than older children and young adults as occurs in developing countries. Patients with HEV infections were older than those with hepatitis A, which peaked in persons 20–29 years of age, as reported by Chau et al. (*14*).

The distribution of HEV genotypes is related to geographic location and the mode of spread (*2*). Genotype 1 is epidemic in developing countries in Asia and North Africa. Genotype 2 is found in Mexico and in central African countries. Genotypes 1 and 2 occur only in humans. Genotype 3 is widely distributed and has been isolated from humans

in North and South America, Europe, Japan, and the Pacific region and in domestic pigs in many countries except in Africa. Genotype 4 has been isolated from humans in China, Japan, Taiwan, and Vietnam and from domestic pigs, boar, and deer in many countries (*2*,*5*,*6*). Genotype 4, the predominant genotype in Hong Kong, is less virulent; it is responsible for occasional cases of clinical hepatitis in industrialized countries. A recent study from Guangzhou in southern China showed that most (39/41) HEV isolates found there were similar to Burmese-like isolates (genotype 1) (*15*). Although Hong Kong is near Guangzhou, none of our isolates were genotype 1. This finding could imply that the poor hygienic conditions required for sustaining the circulation of genotypes 1 and 2 do not exist in Hong Kong.

HEV infections in Hong Kong are mainly acquired locally. The sporadic nature, older age of affected patients, and predominance of genotype 4 correspond with the epidemiology in industrialized countries where zoonosis is the major source of infection. Public health control should focus on zoonotic, especially swine, foodborne transmission as a source of human HEV infection in Hong Kong.

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# Canine Leishmaniasis in Southeastern Spain

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To examine prevalence changes and risk factors for canine leishmaniasis, we conducted a cross-sectional seroprevalence study and a survey during April–June 2006. Seroprevalence had increased at the meso-Mediterranean bioclimatic level over 22 years. Risk was highest for dogs that were older, large, lived outside, and lived at the meso-Mediterranean level.

It has been suggested that climate change has the potential to change the transmission intensity of vector-borne diseases such as leishmaniasis, but supporting literature is lacking (*1*,*2*). Because long-term quality data on leishmaniasis caused by *Leishmania infantum* and its vector (*3*–*9*) are available for the Alpujarras region of southeastern Spain (Figure 1), this is an ideal area for studying changes in the prevalence of canine leishmaniasis in a changing environment. Our study objectives were to determine whether any changes had occurred in the prevalence of canine leishmaniasis over 22 years and to identify risk factors for this disease.

## The Study

To achieve the first objective, we conducted a cross-sectional study in the Alpujarras from April through June 2006. We then compared current leishmaniasis seroprevalence data with data from 2 cross-sectional surveys conducted in 1984 and 1991 (*3*–*5*). The villages sampled for all 3 studies were similar and had been selected at random from within each of the 3 bioclimatic levels (thermo-, meso-, and supra-Mediterranean) that comprise the inhabited zone of the Alpujarras (Figure 1) (*10*). For each level, respectively, altitudes are 0–700, 600–900, and 900–1,800 m above sea level; annual mean temperatures are 17–19, 13–17, and 8–15°C; and annual rainfalls are 200–350, 600–1,000, and 1,000–1,600 inches. The dates for sample collection were set to coincide with organized events at which dogs were gathered (e.g., antirabies vaccination campaigns). All 3 surveys used indirect immunofluorescence for diagnosis; protocols and positivity threshold were identical. Dogs with a titer ≥160 were considered positive. To determine and compare the existence or lack of statistically significant differences between present and past prevalence rates, we used the $\chi^2$ or Fisher exact test.

To achieve the second objective, we conducted a survey. The owners of the dogs included in the cross-sectional study conducted in the Alpujarras from April to June 2006 were asked to complete an epidemiologic record for each dog tested; data on the animal and its environment were recorded for subsequent use in univariate and multivariate logistic regression analyses (Tables 1, 2). Density data were included for 2 vectors, *Phlebotomus perniciosus* and *P. ariasi* sandflies, captured with sticky traps in June 2006 in the same villages in which the surveys were conducted.

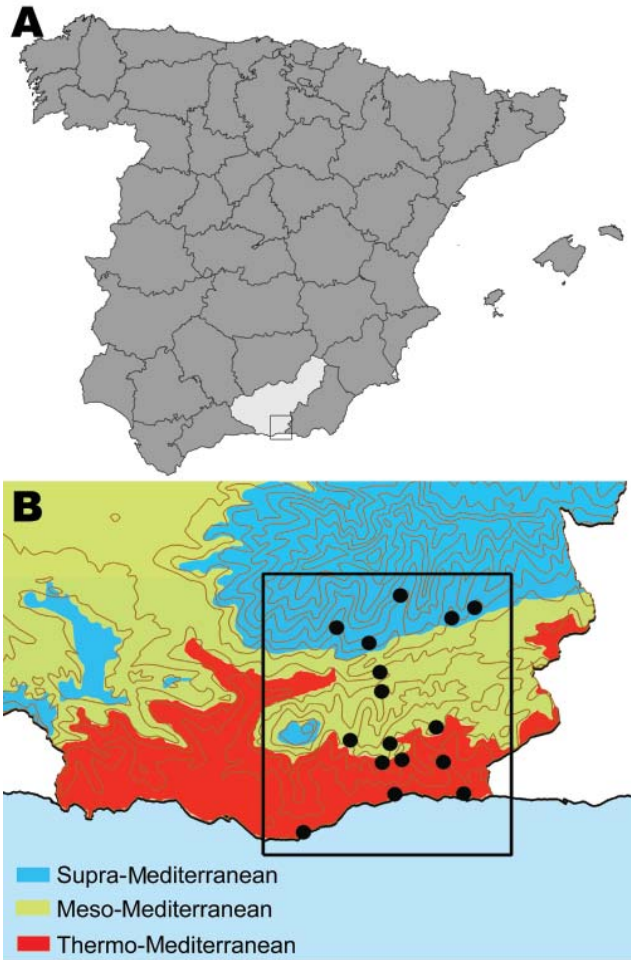


Figure 1. A) Location of the Alpujarras in southeastern Spain (37º00'–37º20'N and 3º00'–3º30'W). B) Bioclimatic levels (shading) and villages (black dots) where serum samples were collected from dogs to examine for leishmaniasis prevalence and sandflies were collected to estimate densities, April–June 2006. Of 1,675 sandflies captured, 269 were identified by morphologic appearance as *Phlebotomus perniciosus* (density 0–165 specimens/m$^2$) and 22 as *P. ariasi* (0 and 11 specimens/m$^2$).

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No retrospective entomologic analysis was performed as had been done for canine leishmaniasis.

In our 2006 survey, 57 (13.0%) of 439 dogs had an antibody titer ≥160 (seroprevalence rate), 268 (61.0%) dogs had titers 20–80, and 114 (26.0%) had no titer. In terms of bioclimatic level, canine leishmaniasis seroprevalence was 20.1% at the meso-Mediterranean, 13.3% at the thermo-Mediterranean, and only 1.1% at the supra-Mediterranean levels. Statistically significant differences ($\chi^2$ test $p<0.001$) indicate that these differences are not random. The evolution-in-time analysis (Figure 2) shows how over 22 years (1984–2006), seroprevalence of canine leishmaniasis has

Table 1. Possible factors associated with canine leishmaniasis, southeastern Spain*

| Variables | No. dogs | % Dogs with canine leishmaniasis | Relative likelihood | p value |
|---|---|---|---|---|
| Bioclimatic level | 439 | 13.0 | – | 0.005 |
| Thermo-Mediterranean | 210 | 13.3 | Ref | – |
| Meso-Mediterranean | 139 | 20.1 | 1.640 | 0.092 |
| Supra-Mediterranean | 90 | 1.1 | 0.073 | 0.011 |
| Habitat | 438 | 13.0 | – | 0.999 |
| Rural | 435 | 13.1 | Ref | – |
| Urban/peri-urban | 3 | 0.0 | <0.001 | – |
| Sex | 435 | 12.4 | – | 0.178 |
| Male | 253 | 14.2 | Ref | |
| Female | 182 | 9.9 | 0.662 | |
| Age, y | 421 | 13.0 | – | <0.001 |
| ≤4 | 232 | 9.5 | Ref | |
| >4 | 189 | 18.0 | 2.094 | |
| Weight, kg | 405 | 13.6 | – | 0.048 |
| ≤25 | 316 | 11.9 | Ref | |
| >25 | 89 | 20.0 | 1.859 | |
| Fur length | 349 | 15.5 | – | 0.069 |
| Short/ medium | 255 | 17.6 | Ref | |
| Long | 94 | 9.6 | 0.494 | |
| Activity | 439 | 13.0 | – | <0.001 |
| Pet | 258 | 8.5 | Ref | – |
| Hunting | 133 | 15.0 | 1.899 | 0.052 |
| Other† | 48 | 31.3 | 4.876 | <0.001 |
| Location during daytime | 373 | 15.3 | – | <0.001 |
| House | 151 | 4.0 | Ref | – |
| Outside | 122 | 28.7 | 9.722 | <0.001 |
| In kennels | 100 | 16.0 | 4.603 | 0.002 |
| Location at night | 435 | 13.1 | – | 0.001 |
| Outdoors | 186 | 19.4 | Ref | |
| Indoors | 249 | 8.4 | 0.384 | |
| Travel away from home | 438 | 13.0 | – | 0.461 |
| No | 396 | 12.6 | Ref | |
| Yes | 42 | 16.7 | 1.384 | |
| Clinical signs of leishmaniasis | 439 | 13.0 | – | 0.122 |
| No | 413 | 12.3 | Ref | |
| Yes | 26 | 23.1 | 2.129 | |
| Fly protection | 375 | 15.2 | – | 0.969 |
| No | 361 | 15.0 | Ref | |
| Yes | 14 | 21.4 | 1.551 | |
| *Phlebotomus perniciosus* density | 439 | 13.0 | – | 0.005 |
| <4 sandflies/$m^2$ | 303 | 9.9 | Ref | |
| ≥4 sandflies/$m^2$ | 136 | 19.9 | 2.254 | |
| *P. ariasi* density | 439 | 13.0 | – | <0.001 |
| <6 sandflies/$m^2$ | 383 | 10.7 | Ref | |
| ≥6 sandflies/$m^2$ | 56 | 28.6 | 3.337 | |

*Univariate analysis by logistic regression. Dogs considered to have canine leishmaniasis were those with antibody titer ≥160. We investigated the existence of interaction and/or confusion between variables by constructing and comparing logistic regression models. The statistical analysis was performed using the software package SPSS 15.0 (www.spss.com). Confusion was noted between the location during daytime and location at night, so location during daytime was excluded from the multivariate analysis. No interaction was detected between any pair of independent variables. Ref, referent.
†Guard dogs (n = 34), sheepdogs (n = 8), stray dogs (n = 4), dogs in kennel (n = 2).

Table 2. Factors associated with canine leishmaniasis, southeastern Spain, final model*

| Variables (no. dogs) | OR (95% CI) | p value |
|---|---|---|
| Bioclimatic level | – | 0.001 |
| Thermo-Mediterranean (189) | – | – |
| Meso-Mediterranean (134) | 0.538 (0.196–1.476) | 0.228 |
| Supra-Mediterranean (82) | 0.013 (0.001–0.126) | <0.001 |
| Age, y | – | 0.001 |
| ≤4 (224) | – | |
| >4 (181) | 3.223 (1.604–6.474) | |
| Weight, kg | – | 0.069 |
| ≤25 (316) | – | |
| >25 (89) | 1.985 (0.948–4.156) | |
| Activity | – | 0.00 |
| Pet (232) | – | – |
| Hunting (129) | 2.401 (1.060–5.442) | 0.036 |
| Other (44)† | 4.831 (1.909–12.226) | 0.001 |
| Location at night | – | <0.001 |
| Indoors (229) | – | |
| Outdoors (176) | 3.304 (1.704–6.406) | |
| *Phlebotomus perniciosus* density | – | <0.001 |
| <4 sandflies/m$^2$ (277) | – | |
| ≥4 sandflies/m$^2$ (128) | 7.029 (2.632–18.769) | |

*Determined by multivariate logistic regression. With age as continuous variable (0.1–25 years, average 4.7 years), odds ratio (OR) = 1.142; with weight as continuous variable (2–60 kg, average = 17.7), OR = 1.047. CI, confidence interval.
†Guard dogs, sheepdogs, stray dogs, and dogs in kennels.

progressively increased at the meso-Mediterranean level, climbing from 9.2% in 1984 to 15.4% in 1991 and finally to 20.1% in 2006 (p = 0.015); in contrast, no significant changes have taken place in global prevalence or in the other 2 bioclimatic levels studied (*3*–*5*; this study).

Dogs at greatest risk for canine leishmaniasis in the disease-endemic region of the Alpujarras were large dogs (>25 kg) and older dogs (>4 years) that worked as guard dogs or sheepdogs, slept outdoors, and lived at the thermo- or meso-Mediterranean level in a village such as Torvizcón, where the *P. perniciosus* density is ≥4 sandflies/m$^2$. Risk for these dogs was 54,571× greater than for dogs that were kept as pets, were small, were <4 years of age, and slept inside a house in a village such as Mecina Bombarón or Pórtugos at the supra-Mediterranean level where *P. perniciosus* density is <4 sandflies/m$^2$.

## Conclusions

Among the drivers of global change that have the potential to influence vector-borne diseases, climatic and nonclimatic (socioeconomic, demographic, and environmental) factors have been cited (*1*,*2*). Although it is not easy to attribute our findings—progressive increase in seroprevalence at the meso-Mediterranean level and drop and subsequent rise at the thermo-Mediterranean level (Figure 2)—to 1 or more drivers, we can attempt to find some form of association in the changes that have occurred in the Alpujarras throughout these 2 decades. Studies on climate change in Spain confirm a warming tendency (reflected at the global level), which provides evidence that temperatures have been increasing for a quarter of a century (*11*,*12*). Thus, we must assume that changes in temperature, rainfall, or humidity will have equally affected the 3 bioclimatic levels researched in the Alpujarras and may have influenced the spatial and temporal distribution and the seasonal dynamics of sandflies. An increase was detected in the period of *P. perniciosus* activity in the region; these effects were probably more notable at the meso-Mediterranean level because this is where the density of this vector species is at its highest (*6*; Martín-Sánchez et al., unpub. data). During the time period researched, the human population remained

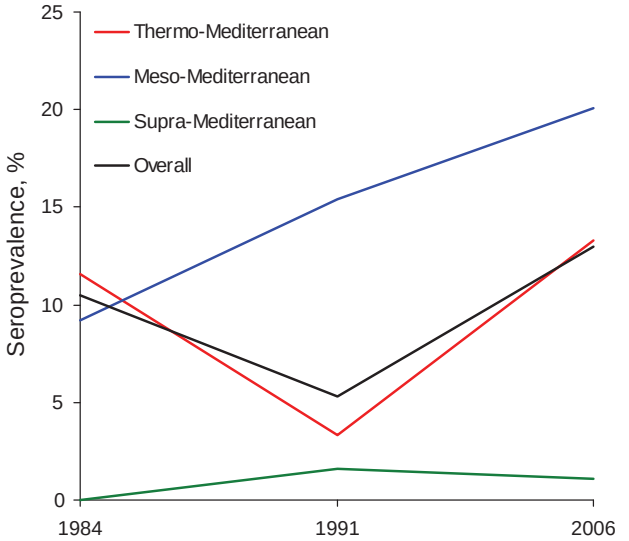


Figure 2. Canine leishmaniasis seroprevalence rates in the Alpujarras, Spain, 1984–2006, by time and bioclimatic level.

constant in the Alpujarras; it has, however, progressively decreased in the disperse populations and increased in population centers. Unfortunately, no official figures for the canine population are available, although we were informed by staff of local town halls that in 1991, the 615 dogs analyzed accounted for ≈100% of the total number of dogs registered in the villages sampled (*3*). The Alpujarras economy is based mainly on agriculture and livestock. One change during the period studied was the gradual disappearance of livestock enclosures in rural population centers. These enclosures appear to create a substantial risk for infection with *Leishmania* spp. (*13*,*14*) and are ideal places for sandfly blood sucking, mating, and oviposition (*15*).

With respect to risk factors, the increase in canine leishmaniasis seroprevalence as animal's age increases seems logical because in a leishmaniasis-endemic area, the greater the age, the longer the animal will have been exposed to sandflies and the greater the probability of having been bitten by an infected female sandfly. Seroprevalence of canine leishmaniasis also increases gradually with weight, which could be attributed to the vector being more attracted to larger animals. Dogs that sleep outdoors are at greater risk than those sleeping indoors because the density of the vector (*P. perniciosus*) is greater outdoors than inside a house. The association between *P. perniciosus* density and canine leishmaniasis seems logical, considering that *P. perniciosus* is the main vector species in the Alpujarras. This is the only species found to have been infected in this region (*8*,*9*), although *P. ariasi* is a proven vector in the nearby region of the Axarquia (*13*).

In the Alpujarras, the percentage of dogs kept as pets has increased from 42% in 1991 (*3*) to 59% in 2006 ($p<0.001$). This increase corresponds to a general increase in standard of living and, along with other factors such as the disappearance of livestock enclosures from within population centers, would have acted to reduce transmission. Only the extension of the vector's activity period, which was detected at some trap sites, would have acted to increase transmission. Such extension of activity may be related to the increase in temperature brought about by climate change (*11*,*12*).

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# Real-Time Surveillance for Respiratory Disease Outbreaks, Ontario, Canada

**Adam van Dijk, Jeff Aramini, Graham Edge, and Kieran M. Moore**

To validate the utility of a chief complaint–based emergency department surveillance system, we compared it with respiratory diagnostic data and calls to Telehealth Ontario about respiratory disease. This local syndromic surveillance system accurately monitored status of respiratory diseases in the community and contributed to early detection of respiratory disease outbreaks.

The threat of emerging infectious diseases, such as severe acute respiratory syndrome and pandemic influenza, makes early detection of health events critical for effective control and intervention of such outbreaks. By using alternative, electronic data sources (*1*), real-time syndromic surveillance systems have the potential to detect outbreaks of respiratory disease before conventional diagnosis- and laboratory-based surveillance identifies them. Many of these alternative data, such as sales of over-the-counter drugs, telephone health hotlines, and emergency department (ED) triage data, already are routinely collected.

ED syndromic surveillance typically uses a patient's chief complaint (CC) as recorded by a triage nurse. Respiratory CC data correlate strongly with discharge data (*2*). CC data from EDs have been successfully integrated into several surveillance systems (*3*,*4*), including the ED system in Kingston, Ontario, Canada (*5*,*6*). CC data are effective for early identification of influenza outbreaks (*7*,*8*).

In September 2004, the Queen's University Emergency Syndromic Surveillance Teama created a real-time emergency department surveillance system (EDSS) to monitor respiratory and gastrointestinal CCs temporally and spatially. Seven hospitals from the southeastern Ontario region fed electronic CC data into the system. Routine patient data collected without identifiers include date and time of visit, demographic information, 5-digit postal code of residence, Canadian Triage Acuity Score, and CC or reason for visit. The National Ambulatory Care Reporting System (NACRS) gathers data for hospital- and community-based ambulatory care, day surgery, outpatient clinics, and EDs. Records contain patient diagnosis according to International Classification of Diseases, 10th Revision–Canadian Enhancement (ICD-10-CA) codes. Every hospital in Ontario submits data to NACRS, and the system includes demographic, clinical, and administrative data for the entire province (*9*). Telehealth Ontario is the teletriage helpline available free to all Ontario residents 24 hours a day, 7 days a week. Callers are connected to skilled nurses who assess symptoms over the phone and assist callers in making the most appropriate healthcare decision (*10*). Each caller's demographics are recorded, and each call is assigned a guideline after the nurse has gone through a list of questions about why the person has called.

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## The Study

We conducted an investigation to verify and validate the utility of a triage CC-based EDSS in southeastern Ontario as a tool for monitoring respiratory disease by comparing it retrospectively with data from NACRS and Telehealth Ontario. This study was part of a broader research project approved by the Queen's Research Ethics Board and adheres to the principles and policies for the protection of personal health information charter.

We retrospectively studied data for July 4, 2004–March 31, 2006. Daily counts of discharges of persons with respiratory disease based on ICD-10-CA codes were obtained from the NACRS database, and counts of respiratory CCs were likewise collected from EDSS. Patient location was determined by forward sortation address (i.e., first 3 digits of postal code) and health unit code in the NACRS database and by specific reporting hospital in the EDSS data set. Weekly Telehealth Ontario counts of respiratory disease based on guidelines were also obtained. We categorized Telehealth Ontario calls into episodes of upper or lower respiratory disease on the basis of a priori classification schemes verified by other research (*6*,*11*–*13*). Telehealth Ontario calls were geolocated by forward sortation address. All data were compiled into weekly totals (Sunday–Saturday) from each of the 3 nonidentifiable data sets.

During July 2004–March 2006, EDSS contained 29,668 reports of respiratory diseases in persons seeking care at 1 of the 7 area hospitals. During the same period, Telehealth Ontario received 4,247 calls about upper and lower respiratory disease, and NACRS recorded 19,315 cases of respiratory disease from southeastern Ontario. Analysis comparing the EDSS respiratory CCs with the Telehealth Ontario calls about respiratory disease (Figure) resulted in a Spearman correlation coefficient of 0.91, indicating good correlation. Analysis comparing the EDSS respiratory CC to all NACRS respiratory disease diagnoses resulted in a Spearman correlation coefficient of 0.98, indicating very good correlation. All correlations were highly

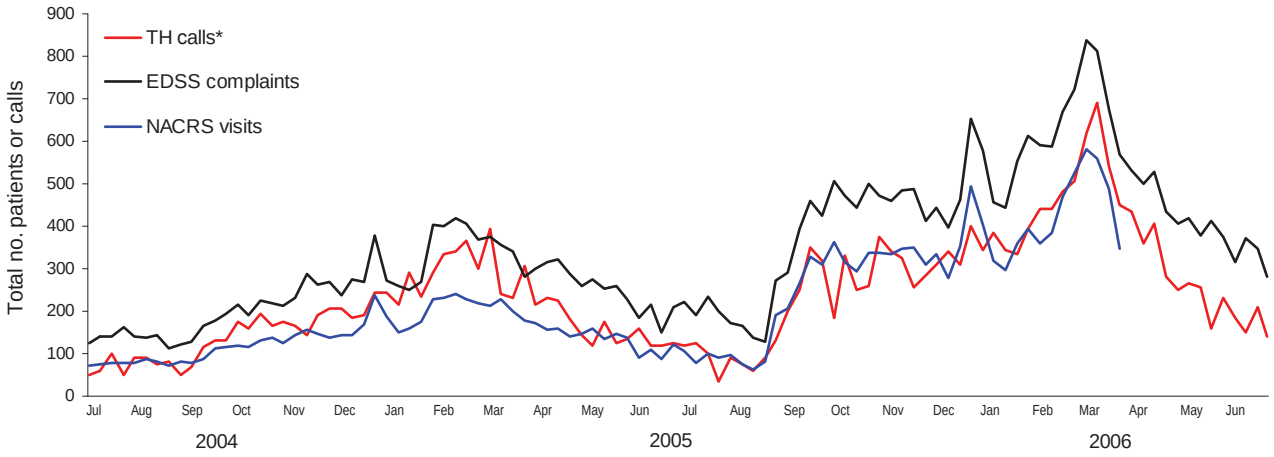


Figure. Weekly totals of emergency department surveillance system (EDSS) respiratory chief complaints, National Ambulatory Care Reporting System (NACRS) respiratory visits, and calls to Telehealth Ontario (TH) about respiratory illness, Ontario, Canada, July 2004–June 2006.

significant ($p<0.0001$). Correlations were highest and most significant when no time lags were included in the models.

## Conclusions

This study verified that ED CC data can be used as a timely source of surveillance for respiratory diseases. ED CCs in southeastern Ontario strongly correlated in time with NACRS respiratory discharge diagnoses and calls to Telehealth Ontario about respiratory disease. NACRS data are unavailable to public health stakeholders in a timely enough fashion to be useful for day-to-day monitoring of respiratory disease trends in the community, whereas ED CCs are available electronically and in real time.

As expected, EDSS CCs about respiratory disease peaked during the influenza seasons (early November 2004–mid-April 2005 and early December 2005–early May 2006) (Figure); however, EDSS CCs continued to fluctuate during the influenza off season, probably because of other respiratory pathogens. The nature of the EDSS data did not allow us to separate respiratory complaints related to influenza from those related to other pathogens. Further analysis is needed to correlate CC subsets with laboratory-positive influenza cases.

One consideration for interpreting EDSS data is that the hospitals in this surveillance system include large referral hospitals that have high volumes of patients visiting from outside our health unit boundaries. Although this large referral area may result in higher counts of visits than reflected in the NACRS database, we believe it does not affect our interpretation of the data. Even though each hospital is required to submit data, it is still possible that not all records from each hospital are sent on time to NACRS.

Although the public health system has accepted syndromic surveillance as a useful tool, doubts remain about its anticipated early warning benefits (*14*). These potential benefits cannot be tested because no large-scale outbreaks have occurred since the inception of our real-time syndromic surveillance system. Protocols of many pandemic-preparedness plans include monitoring of real-time systems. When a pandemic occurs, syndromic surveillance may be able to help healthcare workers recognize a potential outbreak, which theoretically could help them mitigate effects on society earlier. Our study demonstrates that in southeastern Ontario, ED CCs accurately reflect respiratory conditions of patients in the area. The correlations found strongly suggest that EDSS accurately monitors respiratory disease in the community and contributes to early detection of respiratory disease outbreaks. We continue to monitor the system from day to day and have increased the number of reporting hospitals to 9. Future studies will use laboratory data to assess the value of advanced warning on a number of syndromes captured in our system.

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# Possible Seasonality of *Clostridium difficile* in Retail Meat, Canada

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We previously reported *Clostridium difficile* in 20% of retail meat in Canada, which raised concerns about potential foodborne transmissibility. Here, we studied the genetic diversity of *C. difficile* in retail meats, using a broad Canadian sampling infrastructure and 3 culture methods. We found 6.1% prevalence and indications of possible seasonality (highest prevalence in winter).

*Clostridium difficile* infection (CDI) has been associated with increased illness and death in Canada since 2000 (*1*,*2*). Although multiple genotypes with higher levels of virulence and antimicrobial resistance have been recognized (*1*,*3*), little is known about risk factors for CDI acquisition outside healthcare facilities.

In a 2005 study, we found *C. difficile* in 20% of retail meats sampled in Canada (*4*). Limitations to that study included limited geographic representation, nonsystematic sampling, and the use of a nonvalidated culture method. These sampling limitations prevent valid extrapolations. Broader sampling and a better understanding of the culture methods were thus required to reassess the prevalence of retail meat contamination with *C. difficile*. Here, we determined the prevalence of *C. difficile* in retail meat by using a broad-based government sampling infrastructure, compared 3 culture methods, characterized recovered isolates, and evaluated month-to-month variability in *C. difficile* recovery.

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## The Study

Retail meats were obtained from 2 randomly selected census divisions per week from various retailers across Canada as part of the active retail surveillance component of the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) (*5*). We tested random packages of ground beef as well as veal chops from milk-fed calves; the packages were purchased by CIPARS in Ontario, Québec, and Saskatchewan, Canada, from January through August 2006. Purchased packages were sent to the Laboratory of Foodborne Zoonoses, Québec (ground beef), and to the Canadian Research Institute for Food Safety, Ontario (veal chops), where 35-g composite samples were made. Rinsates were prepared by mixing 25 g of meat and 225 mL of buffered peptone water (placed in a stomacher for 15 min). Rinsates (12 mL) and the remains of the composite samples (10 g) were then sent to the University of Guelph for *C. difficile* testing. Sample size estimations indicated that 211 packages were adequate to verify a prevalence of 20% ± 8% ($\alpha = 0.05$, power = 0.8; Stata sampsi command [Stata Corp., College Station, TX, USA]).

A total of 214 meat samples were cultured by using 3 methods. One method, used in an earlier study (*4*), was tested in duplicate to assess reproducibility. All protocols had an enrichment phase of 7 days (Table 1), followed by ethanol treatment of culture sediments (96%, 1:2 [vol/vol], 30 min), and inoculation onto solid agar for colony identification (*4*,*6*).

Suspected colonies (swarming, nonhemolytic) were subcultured onto 5% sheep blood agar. *C. difficile* was preliminarily identified with L-proline aminopeptidase activity (Pro Disc; Remel, Lenexa, KS, USA) but confirmed by PCR detection of the triose phosphate isomerase gene (*7*).

PCR ribotyping and detection of genes for toxins A (*tcdA*), B (*tcdB*), binary toxin (*cdtB*), and toxin regulator (*tcdC*) were performed as previously described (*4*,*8*,*9*). Isolates having either *tcdA*, *tcdB*, or *cdtB* were classified as toxigenic (*10*).

Resulting PCR ribotypes were visually compared to representative PCR ribotypes previously identified in cattle (n = 8, 2004), retail meats (n = 4, 2005), and humans (n = 39, 2004–2006) in Ontario and Québec, Canada (*2*,*4*,*11*). The first meat-derived isolate of each PCR ribotype and 1 matching human isolate were submitted to the Centers for Disease Control and Prevention, Atlanta, Georgia, USA, for *Sma*I pulsed-field gel electrophoresis (PFGE) and toxinotyping (*1*).

We tested selected isolates to determine the MICs of clindamycin, levofloxacin, moxifloxacin, and gatifloxacin by using the Etest (AB Biodisk, Solna, Sweden) and interpreted the results after the isolates were incubated for 48 h on *Brucella* agar (*12*). Controls included *C. difficile* strain ATCC 700057.

Table 1. Proportion of retail meat packages yielding *Clostridium difficile* in 4 culture replicates and estimated method sensitivity, Canada, 2006*†

| Sample | Culture method | | % Samples with *C. difficile* | | | Culture sensitivity, %‡ |
|---|---|---|---|---|---|---|
| | Enrichment | Agar | Ground beef | Veal from milk-fed calves | Both‡ | |
| Rinsate | TCDMNB | CDMNA | 2.7 (4/149)§ | 0 (0/65) | 1.9 (4/214) | 31 |
| Meat¶ | TCDMNB | CDMNA | 2.7 (4/149)§ | 1.5 (1/65) | 2.3 (5/214) | 39 |
| Meat¶ | TCDMNB | CDMNA | 1.3 (2/149)§ | 1.5 (1/65) | 1.4 (3/214) | 23 |
| Meat | TCCFB | Blood | 1.3% (2/149) | 1.5 (1/65) | 1.4 (3/214) | 23 |
| Total of contaminated packages# | | | 6.7 (10/149) | 4.6 (3/65) | 6.1 (13/214)‡ | 100 |

*Rinsate, sediment; TCDMNB, in-house *C. difficile* broth (CM0601; Oxoid, Basingstoke, UK) supplemented with cysteine hydrochloride, moxalactam, norfloxacin (CDMN, SR0173E; Oxoid), and 0.1% sodium taurocholate (Sigma-Aldrich, Inc., St. Louis, MO, USA) (*4*); Meat, 2 g; CDMNA, *C. difficile* agar supplemented with CDMN and 7% laked horse blood (SR0048C; Oxoid); TCCFB, broth supplemented with D-cycloserine and cefoxitin (SR0096E; Oxoid) and 0.1% sodium taurocholate; Blood, 5% defibrinated sheep blood.
†Poor test agreement was found among and between cultures (κ –0.28; p>0.9).
‡Culture sensitivity calculation based on parallel interpretation of all 4 cultures (standard comparator) and 6.1% of overall contamination. Duplicate testing sensitivity ranged from 46.2% (6/13) to 61.5% (8/13).
§Represents 2 packages that simultaneously tested positive in 2 culture replicates.
¶Protocol previously used to test meat; duplicate run (*4*).
#No statistical differences were found between ground beef and veal in any culture replicate (p>0.1).

Culture binary data were analyzed by using a randomized block design approach with a conditional logistic regression analysis (SAS Institute, Cary, NC, USA) and p value estimations with Monte Carlo simulations. Exact tests for pairwise comparisons were based on LogXact 7 and a Fortran program (Cytel Inc, Cambridge, MA, USA). Kappa, $\chi^2$, and Fisher exact tests were also used. Significance was held at p<0.05.

In total, 149 ground beef and 65 veal chop samples, obtained from 210 retailers in Canada, were cultured for *C. difficile* (Figure 1). The numbers of samples tested per month were 12, 49, 34, 5, 73, 31, 0, and 7, from January through August in 2006; 3 samples lacked sampling dates.

Combining the results from 4 cultures, we found the prevalence of *C. difficile* was 6.7% (10/149) in ground beef and 4.6% (3/65) in veal chops from milk-fed calves.

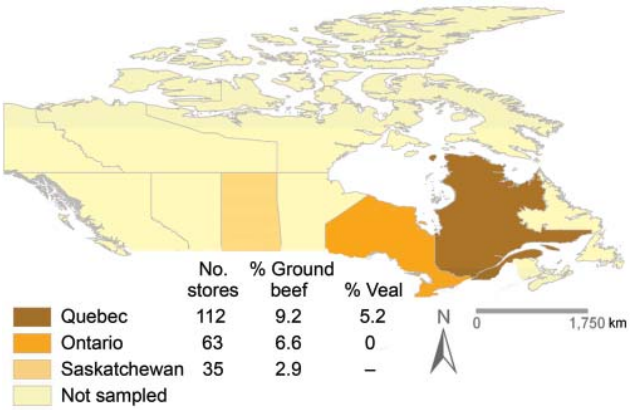


Figure 1. Distribution of retail grocery stores sampled (n = 210) and proportion with contaminated meat. The overall proportion of stores with ≥1 meat package contaminated with *Clostridium difficile* was 5.7%. No statistical differences were observed when comparing the proportions of ground beef contamination in Québec, Ontario, and Saskatchewan, Canada (p>0.2). No comparisons for veal chops were made because Québec was the main source of this commodity; veal from milk-fed calves was not available in Saskatchewan, and only 3 stores had this type of veal during sampling in Ontario.

The combined prevalence was 6.1% (13/214). The prevalence of *C. difficile* recovery determined by using different culture methods varied from 1.4% to 2.3%, but no culture agreement or reproducibility was observed (p>0.1). Overall, the individual diagnostic sensitivity of each method was low (<39%; Table 1).

When month-to-month variability was considered, *C. difficile* was more commonly isolated from meat in January and February (11.5%, 7/61) than during the remaining 5 months of the study (4%; 6/150; p = 0.041). This finding indicates possible seasonality, although further studies are needed.

A total of 28 *C. difficile* isolates were cultured from 13 meat packages (22 from ground beef; 6 from veal). PCR ribotyping showed 8 distinct genotypes, 7 of which were toxigenic and present in 10 (77%) meat packages (Table 2). Genotypes resembling human PCR ribotype 027/NAP1 were found in 30.8% (n = 4) of positive samples, and PCR ribotypes 077/NAP2 and 014/NAP4, formerly reported in cattle and retail meats (*3*,*4*), were identified in 23.1% (n = 3) and 15.4% (n = 2) of samples, respectively. Multiple genotype contamination was also documented (2 PCR ribotypes/sample, n = 2).

PFGE confirmed that selected meat and human PCR ribotypes were identical (Figure 2). Fluoroquinolone and clindamycin resistance was common (41.6%–58.3%) among isolates tested (Figure 2).

## Conclusions

In contrast to our first study (*4*), this study evaluated the genetic diversity of *C. difficile* in retail meats in a large area of Canada and tested 1–2 samples per store to prevent clustering. Thus, the overall prevalence observed (6.1%) was lower than that of previous studies in Canada (20%) (*5*) and the United States (42%) (*13*). Although different sampling and culture methods may account for the different prevalences, taken altogether, these studies support recent concerns regarding food safety.

Table 2. Molecular characteristics of 15 representative *Clostridium difficile* strains isolated from 13 of 214 retail meat packages tested in Canada, 2006*

| Type† | % (no.) | Toxin genes‡ | *tcdC* deletion | Toxinotype | PFGE§ | Product–culture | Month | Province |
|---|---|---|---|---|---|---|---|---|
| M26 | 23.1 (3) | $A^-B^-$, *cdtB*$^-$ | NA | Nontypeable | Unnamed | VC–C3 | Feb | QC |
| | | | | | – | GB–C2 | Jan | ON |
| | | | | | – | GB–C2 | Jun | SK |
| 077¶ | 23.1 (3) | $A^+B^+$, *cdtB*$^-$ | No | 0 | NAP2 | GB–C3 | Jan | QC |
| | | | | | – | GB–C1 | Jan | ON |
| | | | | | – | GB–C3 | Jan | QC |
| J¶ | 23.1 (3) | $A^+B^+$, *cdtB*$^+$ | 18 bp | III | NAP1 | GB–C4 | May | ON |
| | | | | | NAP1a | GB–C4 | Jun | ON |
| | | | | | – | VC–C4 | Feb | QC |
| 014¶ | 15.4 (2) | $A^+B^+$, *cdtB*$^-$ | No | 0 | NAP4 | GB–C1 | May | QC |
| | | | | | – | GB–C2 | Jan | QC |
| C | 7.7 (1) | $A^+B^+$, *cdtB*$^-$ | No | – | – | GB–C1 | Jan | ON |
| F | 7.7 (1) | $A^-B^+$, *cdtB*$^-$ | No | VIII | NAP9 | GB–C2 | Jan | QC |
| H | 7.7(1) | $A^+B^+$, *cdtB*$^-$ | No | 0 | Unnamed | GB – C1 | Jun | QC |
| K | 7.7(1) | $A^+B^+$, *cdtB*$^+$ | 18 bp | III | NAP1-r | VC–C2 | Aug | QC |

*PFGE, pulsed-field gel electrophoresis; NA, not amplified because it lacks pathogenicity locus; VC, veal chops; GB, ground beef; C1, rinsate/TCDMNB; C2, meat/TCDMNB; C3, meat/TCDMNB duplicate; C4, meat/TCFFB; QC, Québec; ON, Ontario; SK, Saskatchewan; –, not performed.
†Bidet's PCR ribotyping method (*9*); 077 and 014; representative ribotypes with international nomenclatures assigned by Dr Jon Brazier, University of Wales, Wales, in a previous study (*11*). M26, non-toxigenic Canadian meat ribotype lacking pathogenicity locus (pers. com., M. Rupnik, University of Maribor, Slovenia) (*5*).
‡A, B; *tcdA* and *tcdB* genes. *cdtB,* binding segment of binary toxin; – and + superscripts indicate absence or presence of the gene. *tcdC* gene: no deletions (≈345 bp); 18 bp, deletion type B/C (*8*).
§Nomenclature at the Centers for Disease and Control and Prevention, Atlanta, GA, USA. NAP1, North America PFGE type 1.
¶Meat PCR ribotypes matching concurrent local and international human ribotypes (*2,3*). Note that 28 *C. difficile* isolates initially identified were grouped into 15 strains based on molecular characteristics and source of origin; 2 meat samples simultaneously harbored 2 strains.

Duplicate cultures, irrespective of method, could yield higher rates of *C. difficile* recovery from meat. However, the sensitivity of duplicate testing of meat is still suboptimal (46.2%–61.5%) compared with the sensitivity reached by one of our methods (*4*) in human stool samples (>95%) (*6*). Suboptimal performance might be due to reduced culture selectivity and nonhomogeneous distribution or a low number of spores.

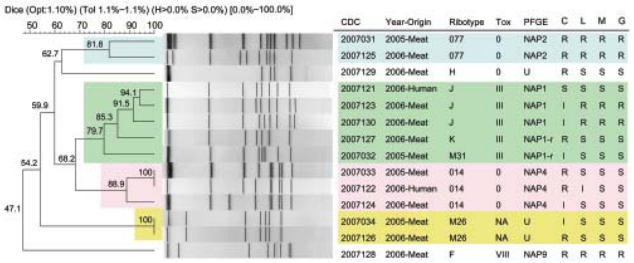


Figure 2. Pulsed-field gel electrophoresis (PFGE)–*SmaI* dendogram of *Clostridium difficile* isolates of meat and human origin in Canada. Representative PCR ribotypes 077, 014, M31, and M26 are of meat origin from 2005 (*4,11*). PCR ribotype designations are described in Table 2. Note the genetic similarity (94.1%–100%) and antimicrobial resistance profiles between human and meat isolates, especially PCR ribotypes 014 and J. Also note the genetic similarity (81.8%–100%) between meat isolates from 2005 and 2006 for multidrug-resistant epidemic PCR ribotype 077, clindamycin-variable, PCR ribotype 014, and nontoxigenic PCR ribotype M26. Resistance to all 4 antimicrobial drugs was observed in meat isolates of ribotypes 077 and F, which also yielded the highest level of clindamycin resistance (>256 μL/mL; breakpoint: ≥6 μL/mL). The breakpoints for moxifloxacin (*12*) were also used for levofloxacin and gatifloxacin. R (resistant), S (susceptible), and I (intermediate) represent antimicrobial profiles. CDC, Centers for Disease Control and Prevention; NAP, North America PFGE type; NAP1-r, NAP-related strain; Tox, toxinotyping nomenclature (M. Rupnik, Maribor, Slovenia); U, unnamed.

In addition to cross-contamination at slaughter and during processing, it is possible that contamination of muscle tissue with *C. difficile* spores occurs preharvest. In horses, *Clostridium* spores have been recovered from muscle tissue in healthy horses (*14*), and a recent muscle sample yielded *C. difficile* in a healthy cow (unpub. data). Translocation from the intestines and deposition of dormant spores in muscle are reasonable assumptions that need investigation.

The increased recovery of *C. difficile* from meat in winter suggests that a seasonal component might exist. This component is currently uncertain, but a possible epidemiologic link between this observation and the seasonality observed in human disease (*15*) and the high rate of *C. difficile* toxins in calves in winter (*11*) requires further elucidation.

The *C. difficile* genotypes identified in this and other studies (especially the NAP1 clone and PCR ribotypes 077 and 014) (*3*,*4*,*11*) provide further molecular evidence that spore dissemination through foods should be considered. Although ingestion of spores does not necessarily imply infection, this study supports the potential for foodborne transmissibility and raises questions about possible seasonality.

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# Novel Respiratory Virus Infections in Children, Brazil

**Maria Carolina M. Albuquerque, Gisele P.A. Pena, Rafael B. Varella, George Gallucci, Dean Erdman, and Norma Santos**

Recently discovered respiratory viruses were detected in 19 (9.2%) of 205 nasal swab specimens from children in Brazil with respiratory illnesses. Five each were positive for human metapneumovirus (HMPV) alone and human bocavirus (HBoV) alone, 3 for human coronaviruses (HCoV-HKU1 or -NL63) alone, and 6 for more than 1 recently discovered virus.

Viral infections are among the leading causes of respiratory disease in children. Most of these infections are caused by respiratory syncytial virus (RSV), influenza virus A or B (FluV), parainfluenza virus (PIV), rhinovirus (RV), or adenovirus (AdV). Several recently discovered viruses, such as human metapneumovirus (HMPV), human bocavirus (HBoV), and the human coronaviruses (HCoVs) NL63 and HKU1, have been identified as potential respiratory pathogens (*1*). In addition, 2 new human polyomaviruses (HPyVs), KIPyV and WUPyV, have been detected in patients with respiratory infections (*1*). In Brazil, epidemiologic studies have demonstrated the extent to which viruses cause respiratory illness in children (*2–4*). However, because such studies have focused on the most common viral pathogens, the extent to which the novel respiratory viruses are etiologic agents of respiratory disease in Brazilian children remains unknown. In this study, we sought to investigate the occurrence of respiratory infections associated with HMPV, HBoV, HCoV-HKU1, HCoV-NL63, KIPyV, and WUPyV among children in Brazil.

## The Study

The study protocol was reviewed and approved by the research ethics committees of the Institute of Puericulture and Pediatrics Martagão Gesteira of the Federal University of Rio de Janeiro and the Educational Foundation of Serra dos Órgãos of Teresópolis. The parents of all children involved in the study gave informed consent for their children's participation in accordance with Resolution 196/96 of the Brazilian Ministry of Health.

Nasal swabs from 205 children (median age 3.3 years; range 1 month to 15 years) with acute upper or lower respiratory illnesses were collected from March 2006 through October 2007 and tested for viral pathogens. Acute respiratory illness was defined by the presence of rhinorrhea, cough, respiratory distress, or sore throat, associated or not with fever, for a maximum duration of 7 days. The specimens were collected from hospitalized patients, emergency departments, and walk-in clinics at 2 university hospitals in the cities of Rio de Janeiro and Teresópolis. Relevant clinical information, including patients' hospitalization status, age, sex, and clinical symptoms, was collected during the first medical visit by means of a standard questionnaire.

The nasal swabs were immersed in 1 mL of virus transport media and kept at –70°C until processing. Nucleic acid was extracted from 200 μL of the sample by using the Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA) or RNAgents kit (Promega) according to the manufacturer's instructions. Specimens were tested for presence of FluV A and B (*5*), PIV 1- 4 (*6*), AdV (*7*), RSV (*8*), RV (*8*), HMPV (*9*), HBoV (*10*), WUPyV (*11*), and KIPyV (*12*) by conventional PCR assays as previously described. A real-time PCR protocol was used for detection of HCoVs (229E, OC43, NL63, and HKU1) (*13*).

Of the 205 samples tested, 63 (30.7%) were positive for at least 1 of the viral pathogens specified above. Nineteen (9.2%) were positive for at least 1 of the newly described viruses: 5 for HMPV only, 5 for HBoV only, 3 for HCoV-HKU1 or HCoV-NL63 only, and 6 for co-infections with these viruses, including 2 samples positive for KIPyV or WUPyV. Of the samples positive for common respiratory viruses, 33 were positive for rhinovirus only, 5 for FluV A only, 3 for RSV only, and 1 each for HCoV-OC43 and AdV only. Two samples were positive for >1 common respiratory viruses, and PIV was not detected (online Appendix Table, available from www.cdc.gov/EID/content/15/5/806-appT.htm). The age of the patients infected with the newly described viruses ranged from 4 months to 11 years (median 2.7 years). The most frequent clinical symptoms were fever, rhinorrhea, cough, sore throat, wheezing, bronchiolitis, and pneumonia (Table). Although the specimens were collected from symptomatic children and a wide range of viruses were screened for by highly sensitive methods, less than one third of the samples were positive. Perhaps if we had collected nasopharyngeal swabs or aspirates, the percentage of samples that tested positive would have been higher.

The diagnosis for most patients infected with HCoV-NL63 or HCoV-HKU1 was pneumonia. Of the 3 patients with single infections, 2 were hospitalized and 1 was treated at the emergency department; the 2 patients with mixed

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Table. Clinical symptoms observed among patients with a single infection, by virus, Brazil, 2006–2007*

| Clinical symptom | % Patients (no. positive/no. tested) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | HCoV-HKU1 or NL63 | HMPV | HBoV | RV | FluV A | RSV | HCoV-OC43 | AdV |
| Fever | 100 (3/3) | 80 (4/5) | 80 (4/5) | 52 (17/33) | 40 (2/5) | 100 (3/3) | 0 (0/1) | 100 (1/1) |
| Rhinorrhea | 33 (1/3) | 60 (3/5) | 60 (3/5) | 67 (22/33) | 80 (4/5) | 100 (3/3) | 0 (0/1) | 0 (0/1) |
| Cough | 33 (1/3) | 100 (5/5) | 80 (4/5) | 67 (22/33) | 40 (2/5) | 33 (1/3) | 0 (0/1) | 100 (1/1) |
| Sore throat | 0 (0/3) | 20 (1/5) | 20 (1/5) | 6 (2/33) | 60 (3/5) | 0 (0/3) | 0 (0/1) | 0 (0/1) |
| Wheezing | 33 (1/3) | 40 (2/5) | 80 (4/5) | 27 (9/33) | 20 (1/5) | 0 (0/3) | 0 (0/1) | 100 1/1) |
| Bronchiolitis | 33 (1/3) | 20 (1/5) | 0 (0/5) | 12 (4/33) | 0 (0/5) | 0 (0/3) | 100 (1/1) | 0 (0/1) |
| Pneumonia | 100 (3/3) | 0 (0/5) | 40 (2/5) | 27 (9/33) | 20 (1/5) | 0 (0/3) | 0 (0/1) | 0 (0/1) |

*HCoV, human coronavirus; HMPV, human metapneumovirus; HBoV, human bocavirus; RV, rhinovirus; FluV A, influenza virus A; RSV, respiratory syncytial virus; AdV, adenovirus.

infection of HCoV-NL63 and HMPV or RV were treated at walk-in clinics.

Patients with HMPV infections had a myriad of symptoms, including fever, cough, rhinorrhea, wheezing, and sore throat. Of the 5 patients with single infections, 4 were treated at walk-in clinics and 1 was treated at an emergency department. A patient co-infected with HMPV and HCoV-NL63 was treated at a walk-in clinic, and a patient co-infected with HMPV and KIPyV and 1 co-infected with HMPV, HCoV-OC43, AdV, and RV were treated at an emergency department. The patient co-infected with KIPyV was a 4-year-old boy with cough, fever, rhinorrhea, and wheezing.

HBoV was detected in samples from 5 patients as a single infection and in samples from 2 patients as a co-infection with RV or WUPyV. Three patients had pneumonia (2 single infections and 1 co-infection with RV). Two of the 3 were treated at walk-in clinics; 1 of the patients with only HBoV infection was hospitalized. The patient co-infected with WUPyV was a 10-month-old boy who had been treated at an emergency department after exhibiting cough, rhinorrhea, and laryngomalacia.

## Conclusions

Previous studies have documented the importance of respiratory virus infections among pediatric patients in Brazil (*2*–*4*). However, the effect of the so-called emerging respiratory viruses on the children of Brazil is yet to be clarified. Few studies have demonstrated the circulation of HMPV among Brazilian children (*4*,*14*), and to our knowledge, none have described the circulation of HBoV, HCoV, or HPyVs as respiratory pathogens in Brazil, although 1 study did report the presence of HBoV in the stools of Brazilian children with gastroenteritis (*15*). Our finding that HMPV, HBoV, HCoV-HKU1 or HCoVB-NL63, or the newly described KIPyV or WUPyV was present in 9.2% of the tested samples suggests that these viruses could be important respiratory pathogens in the country. However, further investigative studies that include appropriately matched control groups will be necessary to demonstrate that these novel viruses act as etiologic agents of respiratory disease in Brazil.

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# Reduced Fluoroquinolone Susceptibility in *Salmonella enterica* Isolates from Travelers, Finland

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We tested the fluoroquinolone susceptibility of 499 *Salmonella enterica* isolates collected from travelers returning to Finland during 2003–2007. Among isolates from travelers to Thailand and Malaysia, reduced fluoroquinolone susceptibility decreased from 65% to 22% (p = 0.002). All isolates showing nonclassical quinolone resistance were from travelers to these 2 countries.

Fluoroquinolones are the most commonly used antimicrobial agents for the treatment of salmonellosis in adult patients (*1*). The proportion of nontyphoidal strains of *Salmonella enterica* with reduced fluoroquinolone susceptibility has increased during recent years in many countries (*1*–*3*). In Finland, fluoroquinolone susceptibility of salmonella has been surveyed since 1995 by analyzing isolates from patients who acquired the disease either at home or abroad. From 1995 through 2004, reduced fluoroquinolone susceptibility (MIC ≥0.125 μg/mL) increased significantly (4.0% to 39%) among all foreign *Salmonella* isolates (*3*,*4*). The increase was most prominent among isolates from Southeast Asia, especially Thailand (*4*).

Until 2002, all *Salmonella* isolates worldwide with reduced ciprofloxacin susceptibility were uniformly resistant to nalidixic acid (*3*–*5*); i.e., they exhibited the conventional quinolone resistance phenotype. In 2003, we identified *Salmonella* isolates that showed reduced susceptibility to ciprofloxacin but were either susceptible (MIC <32 μg/mL) or only low-level resistant (MIC = 32 μg/mL) to nalidixic acid. All *Salmonella* isolates with this nonclassical quinolone resistance phenotype were from travelers returning from Thailand or Malaysia (*6*). We undertook this study to survey the recent incidence of reduced fluoroquinolone susceptibility among nontyphoidal strains of *S. enterica* acquired by Finnish travelers abroad and to define the epidemiology of the nonclassical quinolone-resistant *Salmonella* population.

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## The Study

Our study included 499 *S. enterica* isolates collected during 2003–2007 from Finnish travelers returning from abroad; due to a technical error, in 2004 only 99 foreign *Salmonella* isolates were sent to us from the National Salmonella Reference Center. The first 100 foreign *Salmonella* isolates identified during January of each year were collected. An isolate was designated to be of foreign origin if the patient had reported travel abroad during the month before the specimen was obtained. Epidemiologic information regarding travel destination was collected from the forms accompanying each isolate.

MICs of ciprofloxacin and nalidixic acid for the isolates were determined by using plate agar dilution (*7*). The MIC breakpoint value for reduced ciprofloxacin susceptibility was chosen as ≥0.125 μg/mL on the basis of earlier publications (*4*,*5*,*8*). The breakpoints for nalidixic acid were 16 μg/mL for susceptibility and 32 μg/mL for resistance, according to Clinical and Laboratory Standards Institute guidelines (*7*).

Pulsed-field gel electrophoresis (PFGE) was performed using PulseNet standardized protocol with few modifications (*9*). We considered any difference between 2 profiles to be sufficient to distinguish 2 different PFGE profiles.

Data concerning the number of travelers from Finland to countries of interest (i.e., countries with *Salmonella* isolates showing reduced fluoroquinolone susceptibility) during the study months were received from Statistics Finland (www.stat.fi). Susceptibility data were analyzed by using WHONET5.4. For statistical analyses, we summarized data on the basis of the number and proportion of *Salmonella* isolates with reduced fluoroquinolone susceptibiity. The trend over years was analyzed using a logistic regression model with year as a covariate; $p<0.05$ was considered significant. Statistical analyses were performed using SAS for Windows version 9.1.3 (SAS Institute Inc., Cary, NC, USA).

Of the 499 *S. enterica* isolates collected, 227 came from travelers returning to Finland from Thailand or Malaysia (Table). Among all *Salmonella* isolates, reduced fluoroquinolone susceptibility decreased from 48% in 2003 to 34% in 2007 (p = 0.029). Among the isolates from Thailand and Malaysia, the decrease was 65% to 32% (p = 0.002) (Figure 1, panel A). However, when excluding isolates from

Table. Quinolone susceptibility of 499 *Salmonella* isolates collected from travelers returning to Finland, by country visited, 2003–2007*

| Country visited | No. (%) isolates | No. isolates with CIP MIC ≥0.125 μg/mL | No. isolates with NAL MIC >32 μg/mL | No. isolates with NAL MIC ≤32 μg/mL | No. trips from Finland during study months† |
|---|---|---|---|---|---|
| Thailand‡ | 212 (42.5) | 98 | 67 | 31 | 122,472 |
| Spain§ | 66 (13.2) | 25 | 25 | 0 | 426,822 |
| Brazil | 41 (8.2) | 0 | 0 | 0 | 25,009 |
| Egypt | 40 (8.0) | 17 | 17 | 0 | 47,291 |
| India | 32 (6.4) | 7 | 7 | 0 | 26,233 |
| Malaysia | 15 (3.0) | 6 | 1 | 5 | 2,597 |
| Vietnam | 9 (1.8) | 2 | 2 | 0 | 7,555 |
| Tanzania | 9 (1.8) | 2 | 2 | 0 | 1,259 |
| Portugal | 8 (1.6) | 7 | 7 | 0 | 35,899 |
| Morocco | 5 (1.0) | 3 | 3 | 0 | 8,708 |
| Other areas | 62 (12.4) | 14 | 14 | 0 | |
| Total | 499 (100) | 181 | 145 | 36 | |

*CIP, ciprofloxacin; NAL, nalidixic acid.
†Data collected from statistics reports, Finland, based on the no. of Finnish travelers to these countries.
‡No. Finnish travelers to Thailand from 2003 to 2007: 21,873; 22,882; 17,661; 21,941; and 38,115, respectively.
§Includes Canary Islands.

Thailand and Malaysia, the reduced fluoroquinolone susceptibility remained fairly stable from 2003 to 2007 (31% vs. 37%; p = 0.787).

Among all *Salmonella* isolates, conventional quinolone resistance decreased significantly during the study, from 39% in 2003 to 22% in 2007 (p = 0.012). This decrease was even more conspicuous among isolates from Thailand and Malaysia (47% vs. 12%; p = 0.0014) (Figure 1, panel B).

The nonclassical quinolone resistance phenotype first appeared in 2003. Subsequently, 36 *Salmonella* isolates showing this resistance pattern have been identified. From 2003 through 2007, the yearly proportions of *Salmonella* isolates showing the nonclassical quinolone resistance phenotype were 9%, 8%, 2%, 5%, and 12%, respectively; there was no significant difference from year to year (p = 0.720). Among the isolates from Thailand and Malaysia, the nonclassical quinolone resistance varied; however, the difference from year to year was not significant (p = 0.878) (Figure 1, panel B).

The 499 *Salmonella* isolates in this study were collected from travelers returning from 43 different countries; isolates with reduced ciprofloxacin susceptibility were collected from travelers returning from 18 countries (Table). All 36 isolates showing the nonclassical resistance phenotype were from Thailand or Malaysia; 47% of the isolates showing the conventional quinolone resistance phenotype were from these 2 countries.

The nonclassical quinolone resistance phenotype was found in 7 different serovars, of which *S. enterica* serovar Corvallis and *S. enterica* serovar Stanley were the most prevalent; among the conventional quinolone resistance phenotype, *S. enteritidis* and *S. virchow* were the most prevalent. The 36 isolates belonging to the nonclassical phenotype were distinguished by PFGE to 16 different PFGE patterns (Figure 2).

## Conclusions

Reduced fluoroquinolone susceptibility among nontyphoidal strains of *S. enterica* among travelers from Finland, as a whole, decreased significantly during the study period, 2003–2007 after having constantly increased for several

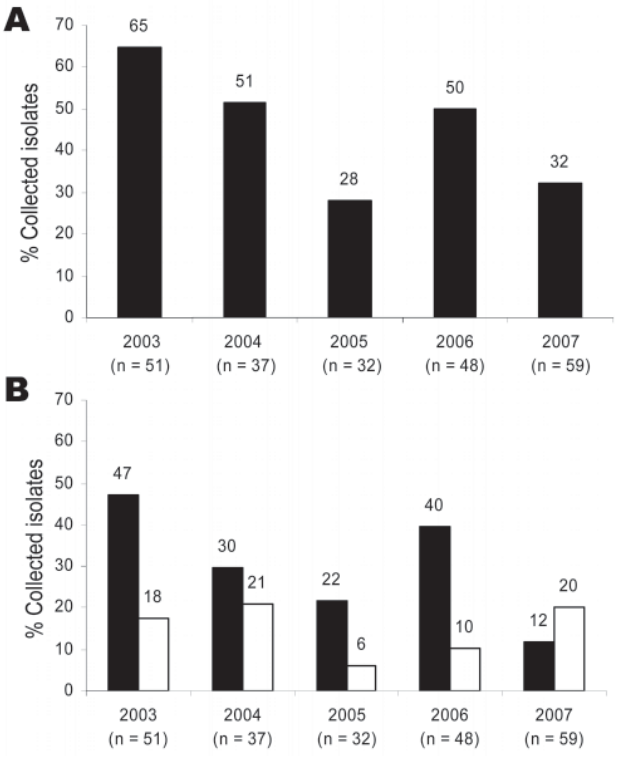


Figure 1. Ciprofloxacin susceptibility and quinolone resistance in 227 *Salmonella enterica* isolates collected from travelers returning to Finland from Thailand or Malaysia, 2003–2007. A) Percentage of isolates showing reduced ciprofloxacin susceptibility (black bars, MIC ≥0.125 μg/mL, p = 0.002). B) Percentage of isolates showing conventional (black bars, MIC of nalidixic acid >32 μg/mL, p = 0.0014) or nonclassical (white bars, MIC of nalidixic acid ≤32 μg/mL, p = 0.878) quinolone resistance phenotype.

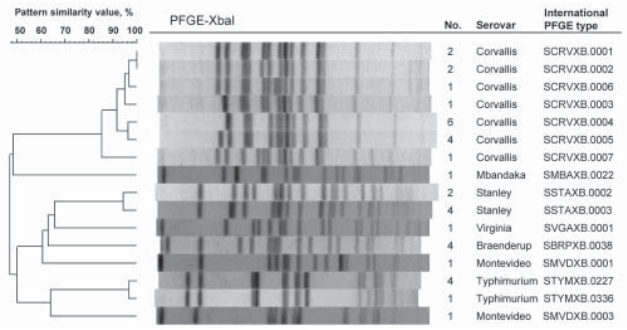


Figure 2. Dendrogram showing the clonal relationships among 36 isolates of *Salmonella enterica* collected from travelers returning to Finland from Thailand or Malaysia, 2003–2007. These isolates showed the nonclassical quinolone resistance phenotype (i.e., reduced susceptibility to ciprofloxacin [MIC ≥0.125 μg/mL] and susceptibility or low-level resistance to nalidixic acid [MIC ≤32 μg/mL]). No., number of *Salmonella* isolates belonging to a certain pulsed-field gel electrophoresis (PFGE) pattern.

years (*3*,*4*). The significant decrease (p = 0.029) from 2003 to 2007 was driven by isolates from Southeast Asia; the proportion of resistant strains from the other travel destinations remained fairly stable. Notably, this recent decreasing trend in resistance was not linked with reduced travel to Thailand, as the number of tourists from Finland going to Thailand more than doubled from 2005 to 2007 (Table).

In addition, after the emergence of the nonclassical quinolone resistance phenotype among *S. enterica* in Thailand and Malaysia, these isolates have persisted in that area. Our study shows a minor variation in the proportion of this resistance phenotype among the whole quinolone-resistant population. For example, in 2005 the proportion of the nonclassical phenotype isolates was only 6%, but in that year, the proportion of all isolates with reduced ciprofloxacin susceptibility was small. This result is most likely due to the collection of the foreign isolates soon after the tsunami catastrophe in December 2004; travelers from Finland took >20% fewer trips to Thailand in 2005 than in 2004 (Table).

All of the *Salmonella* isolates showing the nonclassical quinolone resistance phenotype were from Thailand or Malaysia. Despite this geographic stability, isolates of the nonclassical phenotype were nonclonal, as shown by PFGE. These findings provoke the question of whether the emergence, persistence, and confinement of those isolates in this area might have something to do with the living conditions of the residing population. In 2007, the proportion of the nonclassical phenotype surpassed that of the conventional phenotype for the first time (20% vs. 12%; Figure 1, panel B). This increase in nonclassical phenotypes may be an emerging trend that needs to be under close surveillance.

The nonclassical quinolone-resistant population may prove hard to identify in those microbiological laboratories that use only nalidixic acid to screen for reduced fluoroquinolone susceptibility in salmonella isolates. It is to be expected that this screening approach may fail due to susceptibility or only low-level resistance to nalidixic acid in these isolates (*10*–*12*). Isolates collected from travelers returning from Thailand or Malaysia should especially be examined for fluoroquinolone susceptibility because nalidixic acid screening test results may no longer be predictive of fluoroquinolone resistance. At the present time, the nonclassical phenotype appears to be mainly confined to Thailand and Malaysia (*10*,*11*), but given the continuous increase in global travel, these isolates may emerge in other parts of the world.

## Acknowledgments

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# Molecular Epidemiology of Feline and Human *Bartonella henselae* Isolates

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Multiple locus variable number tandem repeat analysis was performed on 178 *Bartonella henselae* isolates from 9 countries; 99 profiles were distributed into 2 groups. Human isolates/strains were placed into the second group. Genotype I and II isolates shared no common profile. All genotype I isolates clustered within group B. The evolutive implications are discussed.

*Bartonella henselae* is the zoonotic agent of cat-scratch disease and has been associated with bacillary angiomatosis, bacillary peliosis, endocarditis, osteomyelitis, and neuroretinitis (*1*). It is usually present in low numbers in infected human tissues, whereas cats, the natural reservoir for the bacterium, are prone to persistent bacteremia. Therefore, most *B. henselae* isolates are of feline origin. Two genotypes, based on 16S rDNA differences, have been described (*1*). Genotype I was more frequently observed in humans in some countries where most cats are infected with genotype II (*2*,*3*). Genotype II is more prevalent in cats in Europe, Australia, and the United States (*2*–*6*), and most feline isolates from Asia belong to genotype I (*7*–*9*). In a recent study, 3 (1.6%) of 191 *B. henselae* isolates harbored 2 different 16S rDNA copies and could not be assigned to a distinct genotype (*10*). However, most isolates harbored 2 identical 16S rDNA copies and were assigned to either type I or II, confirming that delineation of *B. henselae* isolates in two 16S rDNA types is generally reasonable.

Multiple-locus variable number tandem repeat analysis (MLVA) was recently developed for *B. henselae* typing (*6*). The results are produced in an intrinsically quantitative form, called a profile, corresponding to the number of basic units in an isolate for each variable number tandem repeat (VNTR). MLVA was more discriminatory (*11*) than the other widely used typing techniques, such as pulsed-field gel electrophoresis (*2*), multilocus sequence typing (MLST) (*12*) and multispacer typing (MST) (*13*). In our protocol, MLVA involves the amplification of 5 main VNTR loci, BHV-A to -E, for *B. henselae* VNTRs (*6*).

## The Study

We analyzed 178 *B. henselae* isolates/strains from various sources (Table 1): 156 (88%) feline isolates/strains, 21 (11%) from diseased humans, and 1 isolate from a sick dog. The number of alleles varied from 7 (BHV-E) to 22 (BHV-B). Most of the European isolates (all but 1 of feline origin) (*2*,*4*,*6*) and of the American isolates/strains (North Carolina and California) (*5*,*14*), of which 85% were of feline origin, belonged to genotype II (89% and 64.6%, respectively). The Asian isolates (all but 1 of feline origin) (*7*–*9*) and the Australasian isolates (60% of human origin) (*12*), mainly belonged to genotype I (89.6% and 65%, respectively).

Ninety-nine different MLVA profiles were observed (Table 1), corresponding to an average number of isolates per profile of 1.8 (Table 2). Sixty-nine of these profiles were found in only 1 isolate or strain (67%), and 30 were observed in >1 isolate. Among these, none was shared by genotype I and genotype II isolates. Diversity index (DI) was 0.98 (Table 1). Diversity was observed in both genotypes because genotype-specific DIs were almost identical (Table 1).

MLVA profiles appeared location-specific because only 4 (13%) of the 30 profiles observed in >1 isolate/strain were present in >1 continent (Table 2). Within continents, no marked dominance of a given profile was observed, and continent-specific DIs were similar (Table 1).

Of the 99 *B. henselae* profiles, 12 were obtained from the 21 human isolates/strains and 1 from the dog, whereas 92 profiles were obtained from the 156 feline isolates. Five profiles were common to 5 human and 11 feline isolates. Among the 30 profiles observed in ≥2 isolates, 23 were observed only in feline isolates (Table 2). The proportion of genotype I profiles was significantly higher in human-specific profiles than in cat-specific profiles ($p = 0.01$, by Fisher test).

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Table 1. Description of *Bartonella henselae* isolates and strains tested, global diversity of the typing system, and diversity variations according to 16S rDNA genotype, continent, and host*

| Characteristics | No. isolates/ strains | No. profiles | Average no. isolates/profiles | DI | No. alleles (minimum–maximum no. repeats) A | B | C | D | E |
|---|---|---|---|---|---|---|---|---|---|
| All *B. henselae* | 178 | 99 | 1.8 | 0.98 | 8 (9–16) | 22 (5–37) | 14 (1–25) | 8 (1–9) | 7 (1–7) |
| 16S rDNA genotype I | 64 | 44 | 1.5 | 0.98 | 6 | 12 | 11 | 6 | 6 |
| 16S rDNA genotype II | 114 | 55 | 2.0 | 0.97 | 6 | 15 | 7 | 7 | 6 |
| Location | | | | | | | | | |
| Europe | 80† | 42 | 1.9 | 0.95 | 6 | 12 | 6 | 6 | 5 |
| Asia | 29‡ | 22 | 1.3 | 0.98 | 7 | 10 | 8 | 4 | 3 |
| USA | 49§ | 28 | 1.7 | 0.95 | 4 | 12 | 7 | 6 | 6 |
| Australia–New Zealand | 20¶ | 11 | 1.8 | 0.87 | 4 | 5 | 5 | 4 | 6 |
| Host | | | | | | | | | |
| Human + dog | 22# | 12 | 1.8 | 0.87 | 2 | 6 | 6 | 3 | 6 |
| Healthy cat | 156 | 92 | 1.7 | 0.98 | 8 | 20 | 11 | 8 | 7 |

*DI, diversity index; A, BHV-A; B, BHV-B; C, BHV-C; D, BHV-D; E, BHV-E.
†Denmark, 18 (*7*); France, 23 (*7*); Germany, 27 (*2*); UK, 12 (*5*).
‡Japan, 12 (*8*); Philippines, 7 (*9*); Thailand, 10 (*10*).
§California: 36, including 5 owners and their 11 cats (*6,14*); North Carolina, 12 (provided by Ed Breitschwerdt). Reference strain Houston 1.
¶Australia, 18 and New Zealand, 2 (*3,12*).
#21 human isolates from 1 German patient with bacillary angiomatosis (BA) (*2*), 1 Japanese patient with cat-scratch disease (provided by S. Maruyama), 12 Australian human patients with cat-scratch disease (*3*), 5 California human patients with BA (*14*), 1 North Carolina patient with a wide range of symptoms, including fatigue, joint pain, insomnia, headache, blurred vision, irritability (provided by Ed Breitschwerdt), plus the reference strain Houston 1 (ATCC 49882, initially isolated from a patient with BA) (*3*), and 1 isolate from a dog with endocarditis (provided by Ed Breitschwerdt).

For BHV-A, only 2 alleles (14 and 15 copies) were found in isolates from humans, whereas all 8 identified alleles were observed in cat isolates. The number of repeats differed significantly between sick humans and healthy cats ($p = 0.02$, by Fisher test).

Relationships between the 99 MLVA profiles were analyzed by unweighted pair group method with arithmatic mean (UPGMA), using a categorical distance, with a *B. koehlerae* isolate used as an outgroup. To take into account that UPGMA is sensitive to taxa entry order, we computed the majority-rule consensus tree of 500 dendrograms built with random taxa entry order. MLVA profiles were grouped into 2 main groups named A and B (online Appendix Figure, available from www.cdc.gov/EID/content/15/5/813.htm). Group A (26 profiles), was exclusively constituted by genotype II feline isolates. Group B (73 profiles), to which all human isolates belonged, further divided in 2 subgroups, Ba and Bb. Subgroup Ba (38 profiles) was exclusively composed of genotype I isolates, including the reference strain Houston I and a homogenous subgroup, Ba1, containing 84% of the Asian isolates. Finally, 83% of subgroup Bb isolates belonged to genotype II (29/35 profiles).

The utility of MLVA for molecular epidemiologic analysis of clusters was tested using isolates from California cats and their owners (*14*). Five human–cat groups of *B. henselae* isolates were analyzed. For 1 cat-human pair of isolates, which belonged, respectively, to genotype II and genotype I, major profile differences were observed, as expected. The 4 other cat-human groups, which possessed the same genotype, also had the same MLVA profile with the 5 tested BHV, as well as with the 6 additional BHV (F–K) and variant alleles for BHV-A and/or B (*6*). Sequencing confirmed these results.

## Conclusions

Our results confirm that VNTRs are excellent molecular markers for confirming or excluding the responsibility of a given cat in the transmission of *B. henselae* to a human. In California, the profile identity observed within 4 clusters further supports the hypothesis that all these humans acquired infection from their respective domestic cat contacts.

MLVA enabled a clear separation between genotypes I and II, because no profile was shared between both genotypes. The dendrogram showed a high level of discrimination between 16S rDNA genotypes in the *B. henselae* population tested. Interestingly, the groups and subgroups delineated by MLVA were the same as those defined by MLST, a standard method for phylogenetic analysis (*12*). The same was observed with MST (*13*). The isolates of the subgroup Bb appeared divergent and distant from each other and from subgroup Ba that contains almost all genotype I profiles (98%). Moreover and despite possible clustering for some of the isolates, none of the 21 human isolates was present in group A. Interestingly, as for most of the human patients, the isolate obtained from the ill dog also belonged to genotype I.

These observations suggest that all genotype I isolates could be phylogenetically derived from genotype II isolates located in group B but not in group A, as already suggested using MLST (*15*). This observation could mean that genotype II isolates belonging to group B are closer to genotype I isolates than to genotype II isolates belonging to group

Table 2. Distribution of *Bartonella henselae* isolates/strains by 16S rDNA genotype, host, and location for profiles with ≥2 isolates*

| VNTR profile | | | | | No. isolates | 16S rDNA genotype | | Host | | Location | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | B | C | D | E | | I | II | Human | Healthy cat | Europe | Asia | USA | Aus-NZ |
| 10 | 14 | 2 | 2 | 1 | 14 | | 14 | | 14 | 14 | | | |
| 9 | 15 | 2 | 1 | 1 | 8 | | 8 | | 8 | 5 | | 3 | |
| 10 | 15 | 2 | 2 | 1 | 8 | | 8 | | 8 | 8 | | | |
| 14 | 34 | 2 | 7 | 4 | 8 | | 8 | 1 | 7 | | | 8 | |
| 14 | 22 | 10 | 5 | 3 | 7 | 7 | | 7 | | | | | 7 |
| 14 | 32 | 8 | 7 | 4 | 6 | | 6 | 2 | 4 | | | 6 | |
| 14 | 20 | 10 | 7 | 5 | 4 | 4 | | 2 | 2 | 3 | | 1 | |
| 9 | 14 | 2 | 2 | 1 | 4 | | 4 | | 4 | 4 | | | |
| 10 | 15 | 2 | 1 | 1 | 4 | | 4 | | 4 | 4 | | | |
| 15 | 20 | 10 | 8 | 2 | 4 | 4 | | | 4 | | 4 | | |
| 13 | 14 | 6 | 5 | 4 | 3 | | 3 | | 3 | 3 | | | |
| 15 | 20 | 10 | 8 | 4 | 3 | 3 | | 1 | 2 | | | 3 | |
| 13 | 31 | 6 | 5 | 5 | 2 | 2 | | | 2 | 2 | | | |
| 9 | 14 | 2 | 1 | 1 | 2 | | 2 | | 2 | 1 | | 1 | |
| 9 | 15 | 2 | 2 | 1 | 2 | | 2 | | 2 | 2 | | | |
| 13 | 34 | 10 | 8 | 3 | 2 | 2 | | | 2 | | 2 | | |
| 14 | 36 | 8 | 7 | 4 | 2 | | 2 | | 2 | | | 2 | |
| 13 | 32 | 8 | 7 | 4 | 2 | | 2 | | 2 | | | 2 | |
| 9 | 15 | 2 | 1 | 3 | 2 | | 2 | | 2 | | 1 | 1 | |
| 14 | 32 | 8 | 7 | 1 | 2 | | 2 | 2 | | | | | 2 |
| 14 | 26 | 6 | 8 | 4 | 2 | | 2 | | 2 | | | | 2 |
| 15 | 32 | 10 | 8 | 5 | 2 | 2 | | | 2 | | | | 2 |
| 14 | 11 | 6 | 7 | 4 | 2 | | 2 | | 2 | 2 | | | |
| 13 | 20 | 7 | 8 | 2 | 2 | 2 | | | 2 | | | 2 | |
| 14 | 20 | 6 | 1 | 2 | 2 | 2 | | 1 | 1 | | | 2 | |
| 10 | 15 | 3 | 1 | 1 | 2 | | 2 | | 2 | 2 | | | |
| 10 | 15 | 3 | 2 | 1 | 2 | | 2 | | 2 | 2 | | | |
| 14 | 20 | 10 | 8 | 2 | 2 | 2 | | | 2 | | 2 | | |
| 14 | 18 | 10 | 1 | 3 | 2 | 2 | | | 2 | | 2 | | |
| 15 | 20 | 10 | 1 | 2 | 2 | 2 | | | 2 | | 2 | | |

*VNTR, variable number tandem repeat; Aus-NZ, Australia and New Zealand; A, BHV-A; B, BHV-B; C, BHV-C; D, BHV-D; E, BHV-E.

A; it also raises an important clinical question: Are feline genotype II isolates belonging to group A nonpathogenic for humans? Genotype I isolates could represent the most pathogenic isolates for humans within a group of potentially zoonotic isolates, all belonging to group B and could represent an ultimate evolutionary step toward human infection. Additionally, within group B, the differences in the number of BHV-A repeat units observed between isolates from patients (humans, dog) versus cat isolates suggest that this specific VNTR could constitute a marker for the ability to cross the species barrier from reservoir cats to susceptible species, independent of the 16S rDNA genotype.

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# *Clostridium difficile* in Ready-to-Eat Salads, Scotland

**Marwah M. Bakri, Derek J. Brown, John P. Butcher, and Alistair D. Sutherland**

Of 40 ready-to-eat salads, 3 (7.5%) were positive for *Clostridium difficile* by PCR. Two isolates were PCR ribotype 017 (toxin A–, B+), and 1 was PCR ribotype 001. Isolates were susceptible to vancomycin and metronidazole but variably resistant to other antimicrobial drugs. Ready-to-eat salads may be potential sources for virulent *C. difficile*.

Over the past decade, *Clostridium difficile* infection has become a prominent cause of healthcare-associated infection. Although *C. difficile* has been thought of traditionally as a predominantly nosocomial infection, the incidence of community-acquired cases has increased recently, as has the incidence of cases from other healthcare settings such as nursing homes (*1*). Notably, some evidence has shown that *C. difficile* may be brought into the healthcare environment by asymptomatic carriers (*2*). The reported carriage rates of *C. difficile* in healthy adults have varied from 0% to 3% in Europe to up to 15% in Japan (*3*). Little is known, however, about the prevalence of *C. difficile* in the environment and how it may be transmitted to humans.

*C. difficile* has been found in a variety of environments, including water, soil, animal feces, and foods (*4*,*5*); these findings suggest that *C. difficile* may be transmitted to humans through food, although no foodborne cases have been reported. Because ready-to-eat foods have been implicated in foodborne disease outbreaks associated with *Salmonella* species (*6*) and *Escherichia coli* O157 (*7*), we examined ready-to-eat salads for the presence of *C. difficile*.

## The Study

We tested 50-g samples from each of 40 packaged ready-to-eat salads purchased from 7 Glasgow supermarkets from May 1 through June 30, 2008, for the presence of *C. difficile* spores. We essentially used the CDMN (*C. difficile*, moxalactam, norfloxacin) agar method of Rodriguez-Palacios et al. (*4*) but also used direct plating and enrichment broth culture. The contents of the 40 salads generally differed, and any salads with the same contents carried different supermarket brands, which eliminated replicate sampling.

Isolates were identified as toxigenic (having genes for toxins A and B) by PCR as previously described (*8*,*9*), and ribotypes were identified by PCR (*10*). The MICs of 6 antimicrobial drugs for these isolates were determined by using E-test strips (AB Biodisk, Solna, Sweden). The following MIC breakpoints were used to define resistance to these drugs: metronidazole, ≥32 μg/mL; vancomycin, ≥16 μg/mL; cefotaxime, 64 μg/mL; erythromycin, ≥8 μg/mL; moxifloxacin, ≥8 μg/mL; and clindamycin, ≥8 μg/mL (Clinical and Laboratory Standards Institute, Wayne, PA, USA).

*C. difficile* spores were detected in 3 (7.5%) of the 40 salad samples after culturing in enrichment broth (Table 1). Thirty-five (87.5%) of the salads were marked as imported from European Union countries; the remaining 5 were from UK suppliers. The 3 contaminated salads were not of UK origin. The 3 isolates were found to be toxinogenic by PCR; 2 were PCR ribotype 017, and 1 was PCR ribotype 001 (Table 1).

The MICs of 6 antimicrobial drugs for each isolate and the resistance profile of each isolate are shown in Table 2. None of the isolates was resistant to vancomycin or metronidazole, and only the 001 isolate was resistant to moxifloxacin and erythromycin. All 3 isolates were resistant or intermediately resistant to clindamycin and cefotaxime; breakpoints for these drugs were highest for the 001 isolate.

Isolates were obtained after being cultured in enrichment broth and not by direct plating, which suggests that spore counts were low (<3.0 CFU/g). The infectious dose required to colonize the healthy human gut is, however, unknown. Isolates were of PCR ribotypes 001 (a common clinical isolate in Scotland [*11*]) and 017 (a common European PCR ribotype containing isolates that are negative for toxin A and positive for toxin B [*12*]). No isolate was resistant to vancomycin or metronidazole, which is in accord with findings for other *C. difficile* isolates found in Scotland (*11*), but recent studies have highlighted the emergence of increased resistance to metronidazole among *C. difficile* isolates in England (*13*).

In general, the PCR ribotype 001 isolate was more drug resistant than the 017 isolates; it was the only isolate resistant to moxifloxacin and erythromycin and had the highest breakpoints to clindamycin and cefotaxime. In a 2005 study in which 271 *C. difficile* isolates from the UK were examined, all were found to be resistant to cefotaxime (*14*).

Table 1. PCR profile for toxins A and B and PCR ribotype of *Clostridium difficile* isolates, Scotland, 2008

| Sample no./description | Toxin A | Toxin B | PCR ribotype |
|---|---|---|---|
| 13/baby leaf spinach | – | + | 017 |
| 24/organic mixed leaf salad | – | + | 017 |
| 35/organic lettuce | + | + | 001 |

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Table 2. Susceptibility of 3 *Clostridium difficile* isolates to 6 antimicrobial drugs, by source of isolate, Scotland, UK, 2008*

| Antimicrobial drug | Source of isolate | | |
|---|---|---|---|
| | Baby leaf spinach (MIC, µg/mL) | Organic mixed leaf salad (MIC, µg/mL) | Organic lettuce (MIC, µg/mL) |
| Metronidazole | S (0.125) | S (0.094) | S (0.75) |
| Vancomycin | S (0.50) | S (0.38) | S (1.0) |
| Moxifloxacin | S (0.50) | S (0.75) | R (256) |
| Clindamycin | I (4.0) | I (6.0) | R (8.0) |
| Erythromycin | S (1.5) | S (0.75) | R (192) |
| Cefotaxime | I (48) | R (64) | R (256) |

*S, sensitive; R, resistant; I, intermediate.

## Conclusions

The isolation of these PCR ribotypes from ready-to-eat salads is of concern and highlights the potential risk associated with consuming these salads, particularly since they are not cooked before being consumed. The consumption of these foods by vulnerable groups could possibly lead to *C. difficile* colonization and an increase in the asymptomatic *C. difficile* carriage rate among humans, thus increasing the risk for *C. difficile* transference within the healthcare environment (*2*). The presence of *C. difficile* in ready-to-eat salads could result from environmental contamination or transmission by food handlers. Further work is needed to investigate foods as a source of this pathogen and also to assess the role of soil and animals as its reservoirs.

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# *Clostridium difficile* in Retail Meat Products, USA, 2007

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To determine the presence of *Clostridium difficile*, we sampled cooked and uncooked meat products sold in Tucson, Arizona. Forty-two percent contained toxigenic *C. difficile* strains (either ribotype 078/toxinotype V [73%] or 027/toxinotype III [NAP1 or NAP1-related; 27%]). These findings indicate that food products may play a role in interspecies *C. difficile* transmission.

The incidence and severity of *Clostridium difficile* infections (CDIs) are increasing in North America (*1*), probably because of emergence of an epidemic strain (NAP1/BI/027, toxinotype [TT] III) (*2*,*3*). *C. difficile* transmission occurs primarily in healthcare facilities, but community-associated CDI (CA-CDI) appears to be increasing and may now account for 20%–45% of positive diagnostic assay results (*4*,*5*). Up to 35% of patients with CA-CDI report no antimicrobial agent use within 3 months before disease onset (*4*,*5*), although nonantimicrobial drugs (e.g., proton pump inhibitors, nonsteroidal antiinflammatory agents) are also implicated as risk factors (*4*). Sources of *C. difficile* acquisition in community settings are unknown.

CDI is increasingly important in food animals (*6*). Infection rates of >95% have been documented among neonatal pigs in farrowing facilities, resulting in diarrhea and typhlocolitis (*6*). Toxigenic *C. difficile* is also implicated as a cause of diarrhea in calves (*7*). *C. difficile* was identified in raw meat intended for pet consumption (*8*) and in ≈20% of retail ground beef in Canada (*9*). We report the isolation of *C. difficile* from uncooked and ready-to-eat meats in retail markets in a US metropolitan area.

## The Study

Packaged meats were purchased from 3 national-chain grocery stores in the Tucson, Arizona, area on 3 occasions at 1-month intervals from January to April 2007. Products sampled were both uncooked (ground beef, ground pork, ground turkey, pork sausage, and pork chorizo) and ready to eat (beef summer sausage, pork braunschweiger) (Table). Pork chorizo was produced and distributed locally; all other samples were national brands. Products with different sell-by dates (a surrogate for production date) were sampled for each meat type. Samples were not representative of all meat products in each grocery store.

For each sample, 1 g of meat was added to two 10-mL tubes of prereduced brain heart infusion (BD, Franklin Lakes, NJ, USA), which had been supplemented with 0.5% yeast extract (BD), 0.05% DL-cysteine (Sigma-Aldrich, St. Louis, MO, USA), and 0.1% taurocholate (MP Biomedicals, Solon, OH, USA). One tube was heat shocked (80°C, 10 min), and both were then incubated anaerobically at 37°C for 72 h. Aliquots were subcultured onto taurocholate cycloserine cefoxitin fructose agar (TCCFA) (*10*) and incubated anaerobically for 24–72 h at 37°C. Colonies were subcultured onto anaerobic blood agar, TCCFA (with or without antimicrobial agents), and confirmed as *C. difficile* by *p*-cresol odor, yellow-green fluorescence under UV illumination, a positive L-proline aminopeptidase reaction, and negative indole reaction.

Isolates were characterized by PCR ribotyping (*11*), toxinotyping (*3*), and pulsed-field gel electrophoresis (PFGE) (*12*). Presence of *tcdA, tcdB, cdtB* (binary toxin), and deletions in *tcdC* was determined by PCR (*2*).

MICs were determined by Etest (AB Biodisk, Solna, Sweden) on *Brucella* blood agar with vitamin K and hemin (Remel, Lenexa, KS, USA) that was incubated anaerobically at 35°C. Reference interpretive criteria for *C. difficile* susceptibility to clindamycin and moxifloxacin were used; MICs for levofloxacin and gatifloxacin were interpreted by using criteria for moxifloxacin (*13*). *Bacteroides fragilis* ATCC 25285, *B. thetaiotaomicron* ATCC 29741, *C. difficile* ATCC 700057, and *Enterococcus faecalis* ATCC 29212 were included as controls.

Proportions were compared by $\chi^2$ or Fisher exact test. Thirty-seven (42.0%) of 88 retail meats yielded *C. difficile*, including 42.4% of beef, 41.3% of pork, and 44.4% of turkey products (Table). Ready-to-eat products were more commonly culture positive (11/23; 47.8%) than were uncooked meats (26/65; 40.0%), although the difference was not significant ($p = 0.34$). The highest percentages of *C. difficile* isolates were recovered from pork braunschweiger (62.5%) and ground beef (50.0%). Culture-positive results came from both heat-shocked and non–heat-shocked cultures, whereas culture-negative specimens were negative in both types of culture, and no specimen was positive by both methods (not shown). No association was found with the meat processor, the sell-by date, the store, or the month sampled (not shown). Multiple independent cultures from 2 braunschweiger samples yielded indistinguishable isolates

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Table. Source and characteristics of *Clostridium difficile* isolates obtained from retail meats sold in Tuscon, Arizona, USA, 2007*

| Meat product | No. samples cultured | Total no. (%) positive | Ribotype | Toxinotype | Δ*tcdC*, bp† | PFGE type | No. (%) pos |
|---|---|---|---|---|---|---|---|
| Ground beef (uncooked) | 26 | 13 (50) | 027 | III | 18 | NAP1 | 1 (3.8) |
| | | | | | | NAP1-related | 2 (7.7) |
| | | | 078 | V | 39 | NAP7 | 8 (30.8) |
| | | | | | | NAP8 | 2 (7.7) |
| Summer sausage (ready to eat) | 7 | 1 (14.3) | 027 | III | 18 | NAP1 | 1 (14.3) |
| Ground pork (uncooked) | 7 | 3 (42.9) | 027 | III | 18 | NAP1-related | 1 (14.3) |
| | | | 078 | V | 39 | NAP7 | 2 (28.6) |
| Braunschweiger (ready to eat) | 16 | 10 (62.5) | 027 | III | 18 | NAP1 | 2 (12.5) |
| | | | | | | NAP1-related | 1 (6.2) |
| | | | 078 | V | 39 | NAP7 | 7 (43.8) |
| Chorizo (uncooked) | 10 | 3 (30.0) | 027 | III | 18 | NAP1-related | 1 (10.0) |
| | | | 078 | V | 39 | NAP7 | 2 (20.0) |
| Pork sausage (uncooked) | 13 | 3 (23.1) | 027 | III | 18 | NAP1-related | 1 (7.7) |
| | | | 078 | V | 39 | NAP7 | 2 (15.4) |
| Ground turkey (uncooked) | 9 | 4 (44.4) | 078 | V | 39 | NAP7 | 4 (44.4) |
| Totals | 88 | 37 (42.0) | 027 | III | 18 | NAP1 | 4 (4.4) |
| | | | | | | NAP1-related | 6 (6.7) |
| | | | 078 | V | 39 | NAP7 | 25 (27.8) |
| | | | | | | NAP8 | 2 (2.2) |

*All samples were positive for *cdtB*, which encodes the binding component of binary toxin. PFGE, pulsed-field gel electrophoresis.
†Deletions in *tcdC* regulatory gene.

in the same meat sample (10/10 from 1 package and 12/12 from another; not shown), which suggests that a single strain may predominate when *C. difficile* is present. Our percentage of recovery of *C. difficile* from retail meat products is higher than that reported (20%) in a similar study of Canadian ground beef (*9*), possibly because of differences in culture methods, the meats sampled, or national or geographic variation.

Isolates were grouped into ribotype 078/TT V (27/37, 73.0%) and ribotype 027/TT III (10/37, 27.0%). Strain types were not specific to meat type, store, or sampling month (Table). All isolates were PCR positive for binary toxin (*cdtB*), *tcdA,* and *tcdB*. Characteristic 18-bp and 39-bp deletions in *tcdC* were present in 027/TT III and 078/TT V isolates, respectively (*2*,*12*). PFGE divided 027/ TT III isolates into NAP1 (≥80% related to human NAP1) and NAP1-related (78% related to human NAP1) groups and 078/TT V isolates into NAP7 and NAP8 groups (Figure).

Ribotype 027 isolates are described almost exclusively in context of the current human epidemic strain, NAP1/027/TT III (*2*). In this study, we also found 027/TT III isolates that were only 78% similar to NAP1 (i.e., NAP1-related). Ribotype 078 strains were previously uncommon causes of healthcare-associated CDI in humans (*12*), but now they are emerging in pigs and calves with diarrhea (*7;* J.S. Weese, pers. comm.) and in persons with CDI (*12*). Two epidemiologically unrelated 078/TT V isolates from human CDI patients are indistinguishable by PFGE from pig isolates (*12*).

The 078/TT V isolates were uniformly susceptible to levofloxacin, moxifloxacin, and gatifloxacin. Like human TT V isolates (*12*), most 078/TT V meat isolates were nonsusceptible to clindamycin (56% resistant, 41% intermediate). This may not be surprising given the widespread use of tylosin, erythromycin, virginiamycin, and lincomycin in food animals and the potential for selection of macrolide-lincosamide-streptogramin resistance (*14*).

NAP1 isolates have demonstrated high-level resistance to levofloxacin, moxifloxacin, gatifloxacin (>32 μg/mL), and clindamycin (>256 μg/mL), consistent with current human strains (*2*). NAP1-related isolates were susceptible to levofloxacin, moxifloxacin, and gatifloxacin but resistant to clindamycin, similar to the pattern of historic NAP1 strains (*2*).

## Conclusions

Fluoroquinolones are widely used in human therapy, and the current epidemic strain may have emerged because of its resistance to these agents. Fluoroquinolone use is limited in food animal production (*14*), with the exception of enrofloxacin for treatment of bovine respiratory disease (now approved for use in swine).

The source of *C. difficile* in retail meats may involve antemortem deposition of spores in the animal's muscle or other tissues, fecal or environmental contamination of carcasses, or contamination during processing. Spores could persist in packing plants, resulting in contamination of carcasses or food products during processing. Contamination may also occur in retail meat markets.

Direct or indirect human-to-human transmission is responsible for most healthcare-related CDIs (*15*) and most likely contributes to CA-CDI. Therefore, stopping such

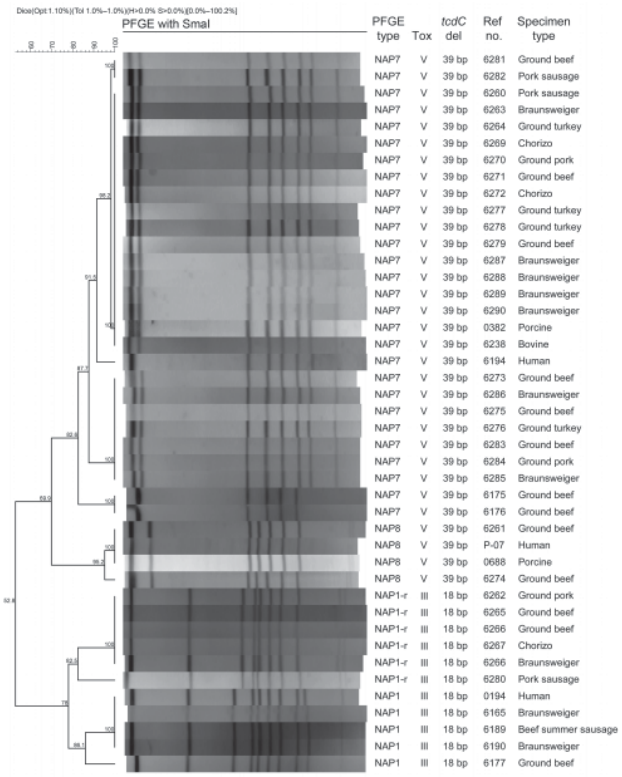


Figure. Origin, NAP types, and relatedness of strains from foods and humans, Arizona, USA, 2007. All strains were positive by PCR for binary toxin. Scale bar indicates genetic relatedness. Tox, toxinotype; Ref, reference; NAP1-r, NAP1-related.

transmission remains the critical control point for preventing most human CDIs. Nonetheless, our findings highlight the potential both for selection of virulent or resistant strains in animals and interspecies transmission through the food supply. Our data do not prove transmission of *C. difficile* from foods to humans but highlight the need for studies to characterize risks posed by this organism in the human food supply.

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# Bovine Kobuvirus in Europe

**To the Editor**: Picornaviruses (family *Picornaviridae*) are small, nonenveloped viruses with single-stranded, positive-sense genomic RNA. Picornaviruses are currently divided into 8 genera: *Enterovirus, Aphthovirus, Cardiovirus, Hepatovirus, Parechovirus, Erbovirus, Teschovirus,* and *Kobuvirus* (*1*). To date, the genus *Kobuvirus* consists of 2 officially recognized species, *Aichi virus* and *Bovine kobuvirus,* and 1 porcine kobuvirus as a candidate species (*2*–*4*). Aichi virus (strain A846/88) was first isolated in 1991 from feces of a person with acute gastroenteritis (*2*). Bovine kobuvirus (strain U-1) was detected in 2003 in bovine serum and fecal samples from clinically healthy cattle (*3*); in 2008, it was isolated from cattle with diarrhea (*5*). Aichi virus and bovine kobuvirus were first isolated in Japan. Porcine kobuvirus (strain S-1-HUN) was recently identified from domestic pigs in Hungary (*4*). Aichi viruses have been also detected in other countries in Asia (*6*), Europe (*7*,*8*), South America (*7*), and northern Africa (*9*). Bovine kobuvirus, however, has not been detected outside Asia (Japan and Thailand) (*3*,*5*).

Kobuvirus genomes are ≈8.2–8.4 kb and have a typical picornavirus genome organization, including leader (L) protein following structural (VP0, VP3, and VP1) and nonstructural (2A–2C and 3A–3D) regions (*1*,*3*,*4*). The genetic identity on coding regions of Aichi virus, bovine kobuvirus strain U-1, and porcine kobuvirus strain S-1-HUN is between 35% (L protein) and 74% (3D region) (*3*,*4*). We report the detection of bovine kobuvirus in Europe.

In February 2002, a total of 32 fecal samples were collected from cattle (*Bos taurus*) in a closed herd of 870 animals in central Hungary; age groups were 1–9 days (n = 6), 14–17 days (n = 4), 6–7 months (n = 5), and 1–7.6 years (n = 17). In February 2008, 26 more samples were collected from animals <20 days of age on this farm. On the sampling days, no diarrhea was reported.

Reverse transcription–PCR was performed by using a new generic kobuvirus primer (UNIV-kobu-F, forward, 5′-TGGAYTACAAG(/R)ATGTTTTGATGC-3′, corresponding to nucleotides 7491–7512 of strain U-1 and UNIV-kobu-R, reverse, 5′-TGTTGTTRATGATGGTGTTGA-3′, corresponding to nucleotides 7686–7707 of strain U-1). The primer design was based on the viral sequences of the Aichi virus (AB040749), bovine kobuvirus strain U-1 (bovine, AB084788), and porcine kobuvirus strain S-1-HUN (porcine, EU787450), which amplify a 216-nt region of 3D (RNA-dependent RNA polymerase region) of all species. The continuous 862-nt 3D and 3′ untranslated region (UTR) of the genome was determined by using 5′/3′RACE Kit (2nd Generation; Roche Diagnostics GmbH, Mannheim, Germany) and primers UNIV-kobu-F and Z20-F-7729 (5′-CCAACATCCTGACTTCTCTCCT-3′, corresponding to nucleotides 7729–7750 of strain U-1). PCR products were sequenced directly in both directions by using the BigDye Reaction Kit (Applied Biosystems, Warrington, UK), the PCR primers, and an automated sequencer (ABI PRISM 310 Genetic Analyzer; Applied Biosystems, Stafford, TX, USA). Phylogenetic analysis was conducted by using MEGA version 4.1 (*10*). The sequence of this bovine kobuvirus strain (kobuvirus/bovine/Aba-Z20/2002/Hungary) was submitted to GenBank under accession no. FJ225406.

Of the 32 samples collected in 2002, two (6.25%), from 1-year-old animals, were positive for bovine kobuvirus; however, no kobuvirus was found in the samples from 2008. The 2 partial 3D regions (216 nt) were genetically identical. Strain kobuvirus/bovine/Aba-Z20/2002/Hungary (FJ225406) had 89%–94% nucleotide and 96%–100% amino acid identities to the 19 known Asian bovine kobuvirus strains in GenBank. Strain Z20 had 93% and 95% nucleotide identities to U-1 in 3D/3′-UTR (862 nt) and 3′-UTR (174 nt) regions, respectively. Phylogenetic analysis of the overlapping partial 3D nucleotide sequences of bovine kobuvirus strain Z20 from Hungary, together with all published bovine kobuvirus strains available in the GenBank database, are shown in the Figure. Aichi virus and porcine kobuvirus were included in the tree as outlier viruses. The phylogenetic tree confirmed that strain Z20 belonged to bovine kobuviruses (Figure).

Our detection of bovine kobuviruses in Europe confirms a wider geographic presence of this type of picornavirus in cattle and suggests that bovine kobuvirus is common and potentially distributed worldwide. Genetic diversity was seen, based on the 3D regions of bovine kobuviruses; however, this region shows the highest genetic identity among the kobuvirus genetic regions (*3*,*4*). Strain Z20 also confirms the 174-nt 3′-UTR region of bovine kobuvirus. At this time it is not clear what diseases (including gastroenteritis) are associated with bovine kobuvirus (*3*,*5*). In addition to the bovine kobuvirus, 2 other RNA viruses that are transmitted by the fecal–oral route (genotypes GIII/1 and GIII/2 of bovine noroviruses and rotavirus) were detected at the same time from these apparently healthy animals. More epidemiologic and molecular studies are required to determine the relevance, distribution, and diversity of bovine kobuvirus in cattle.

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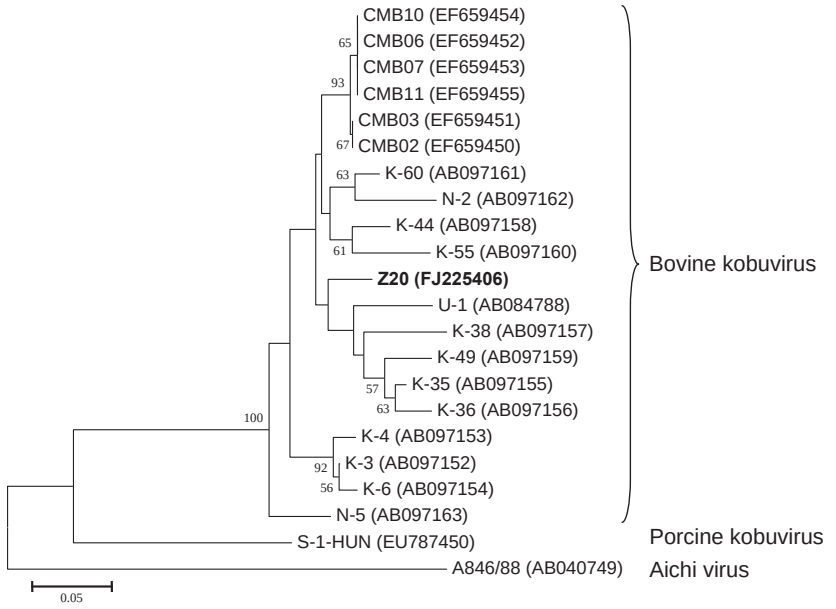


Figure. Phylogenetic tree of bovine kobuvirus (kobuvirus/bovine/Aba-Z20/2002/Hungary, in **boldface**) based on the 455-nt fragment of the kobuvirus 3D regions. The phylogenetic tree was constructed by using the neighbor-joining clustering method with distance calculation and the maximum composite likelihood correction for evolutionary rate with help of MEGA version 4.1 software (*10*). Bootstrap values (based on 1,000 replicates) are given for each node if >50%. Reference strains were obtained from GenBank. Scale bar indicates nucleotide substitutions per site.

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## Candidate Porcine *Kobuvirus*, China

**To the Editor**: The picornaviruses constitute a large, diverse family of positive-sense RNA viruses, which comprise 8 genera: *Enterovirus*, *Aphthovirus*, *Cardiovirus*, *Hepatovirus*, *Parechovirus*, *Erbovirus*, *Kobuvirus*, and *Teschovirus* (*1*). The genus *Kobuvirus* contains 2 known species: *Aichi virus,* which was identified in humans in 1989 and was found to be associated with human acute gastroenteritis (*2*), and *Bovine kobuvirus,* which was identified in 2003 in apparently healthy cattle (*3*). In our study, we identified a candidate novel strain of kobuvirus from porcine fecal specimens; this strain is markedly different from Aichi virus and bovine kobuvirus.

Using reverse transcription–PCR (RT-PCR) to characterize calicivirus in porcine fecal specimens with a primer pair of p289/VN3T20 designed for a 3-kb fragment of the virus, we observed an unexpected band on aga-

rose gel electrophoresis (*4*). After purification and sequencing, the 1,185-bp fragment was found to share 73% similarity with the 3D region of bovine kobuvirus. A pair of primers was then designed from this sequence and synthesized (forward: 5′-TGGAC GACCAGCTCTTCCTTAAACAC-3′ and reverse: 5′-AGTGCAAGTGCAAGT CTGGGTTGCAGCCAACA-3′; 495 bp) to screen other porcine samples for the virus by PCR. Our samples were 322 fecal specimens collected during 2006–2007 from healthy piglets <15 days of age from 3 different farms and several sporadically distributed families that raised pigs in Lulong County, China. Of the 322 samples, 97 were positive. All products were sequenced, and 18 were chosen randomly for deposit in GenBank under accession nos. FJ459895–FJ459912.

To further characterize the virus, we designed primers corresponding to the viral protein (VP) 0 region of kobuvirus on the basis of conserved sequences deduced by comparing the sequences of bovine kobuvirus (GenBank accession no. NC_004421) and Aichi virus (GenBank accession no. NC_001918). An 823-bp fragment was examined and then submitted, together with the 1185-bp sequence, to GenBank under accession no. FJ493623. The obtained sequences were analyzed by using the DNASTAR software package (www.dnastar.com) and were compared with other sequences in GenBank by using BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi).

The results demonstrate that the 3D partial region of the novel strain has nucleotide homology of 73% and 70% to that of bovine kobuvirus and Aichi virus, respectively. The sequence in the VP0 region was less conserved; nucleotide and amino acid homologies were 69% and 71%, respectively, to those of bovine kobuvirus and 66% and 69%, respectively, to those of Aichi virus.

Partial sequences of the 3D region have been used to deduce phylogenetic and taxonomic relationships among picornaviruses; these sequences have been particularly useful for placing viruses within species or genera or for comparing viruses of different genera or families (*5*). We constructed phylogenetic trees by using MEGA software version 3.1 (www.megasoftware.net). The phylogenetic analysis showed a single genetic lineage for the novel virus, close to *Kobuvirus* but phylogenetically distinct from both bovine kobuvirus and Aichi virus, which suggests that the porcine fecal specimen contained a candidate novel species of *Kobuvirus* (Figure).

The filtered fecal samples positive for the virus were inoculated onto RD cells. After 3 serial passages, no obvious cytopathic effect in RD cells was noted. The cells and supernatants in every passage were collected separately for RNA extraction. We then used semiquantitative RT-PCR with glyceraldehyde-3-phosphate dehydrogenase as an internal reference

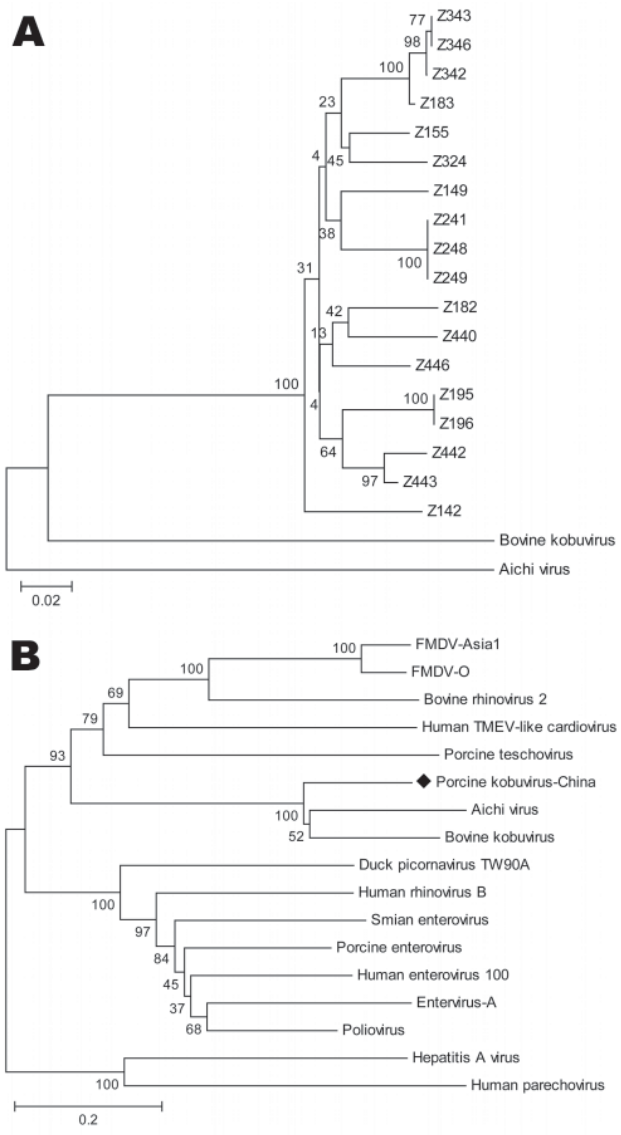


Figure. A) Phylogenetic tree of the partial sequences in the 3D region of the candidate novel virus, Aichi virus, and bovine kobuvirus. B) Relationships between the candidate novel virus and other picornaviruses based on nucleotide differences in the 3D region. FMDV, foot and mouth disease virus; TMEV, Theiler's murine encephalomyelitis virus. Scale bars indicate nucleotide substitutions per site.

to detect the virus and determine the amount of the viral RNA in the medium. Cells and supernatants of the 3 passages were virus positive, and the control inoculated with phosphate-buffered saline was virus negative. However, with each passage, the amount of viral RNA in the medium decreased. Whether the positive result was caused by residual viruses of the initial inoculation or by the decreased propagation of the virus in the cells is not clear. Further studies, such as continuous serial passages and neutralization assay, are needed to determine the final activity of the virus in RD cells, as well as in other cells such as Vero and HeLa, because several species of picornaviruses have been identified as causing persistent infections in these cells in vitro (*6–10*).

In conclusion, we report the genetic characterization and biological properties of a new agent in China. Of note, while we were preparing this article, a similar article from Hungary was published (*5*). After comparing our 1,185-bp sequence with the sequence from Hungary, we found that our sequence was 171 bp longer at the 3′ end and 50 bp shorter at the 5′ end and that the truncated sequence in the middle (same length) had a nucleotide homology of 92.1%. Phylogenetic analysis indicated that the 2 sequences may share the same origin (Figure, panel B). In addition, prevalence of our virus (30.12%) was higher than that of the virus from Hungary (13.3%). Further studies are needed to determine the complete genome and the relevance of the candidate porcine *Kobuvirus* as a causative agent of disease in pigs and a potential zoonotic agent.

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## Postoperative Panophthalmitis Caused by Whipple Disease

**To the Editor:** The clinical spectrum of Whipple disease has widely expanded since its etiologic agent, *Tropheryma whipplei*, was isolated in 2000 (*1*). Systematic 16S rDNA sequencing unexpectedly identified *T. whipplei* in patients for whom blood cultures were negative for endocarditis, spondylitis, and uveitis (*2*). Features common to these conditions and to Whipple disease include long-standing, unexplained arthralgia and deterioration of the patient's condition after treatment with immunosuppressive drugs (*2*). We report an unexpected case of postoperative panendophthalmitis identified by systematic 16S rDNA sequencing of a vitreous sample in a patient who had unexplained arthralgia and had been given topical corticosteroids after cataract surgery.

A 78-year-old woman in France underwent left eye phacoemulsification with intraocular lens implantation in May 2005 and retinal surgery followed by local corticoid application in April 2006. She had experienced cortisone-resistant polyarthralgia for 2 years before the first surgery. In July 2006, she showed decreased vi-

sual acuity (20/1,000) and a painful, red eye. Chronic postoperative endophthalmitis was suspected, and the patient underwent anterior chamber paracentesis (ACP). Parameters included $0.614 \times 10^9$ eosinophils/L in the blood and an erythrocyte sedimentation rate of 70 mm in the first hour.

Sequencing of 16S rDNA of the ACP specimen showed 99.9% similarity with that of *T. whipplei* (GenBank accession no. AJ551273). A specific PCR confirmed this result in the ocular sample and detected *T. whipplei* in saliva and stool samples, whereas blood and cerebrospinal fluid were negative for the organism by PCR. Duodenal biopsy specimens were negative by periodic acid–Schiff staining, specific immunohistochemical analysis, and PCR.

The patient was treated with topical corticosteroids, cycloplegic drugs, doxycycline (200 mg/d), hydroxychloroquine (200 mg 3×/d), and sulfamethoxazole/trimethoprim (1,600 mg and 320 mg 3×/d) (*2*). She was hospitalized for 7 days in the ophthalmology department and for 4 days in the infectious disease department. At 8-month follow-up, visual acuity had improved (20/50) despite intraocular inflammation with a Tyndall effect, moderate capsular opacification, decreased vitreitis, macular edema, and retinal macular abnormalities shown by optical coherence tomography. *T. whipplei* DNA was again not detected by PCR in saliva and stool samples at 8-month follow-up, and the patient remained free of symptoms at 16-month follow-up when treatment was stopped.

Diagnosis of Whipple disease uveitis was confirmed by detection of *T. whipplei* DNA in the ocular sample by 2 laboratories that used 2 molecular targets and negative controls. *T. whipplei* was identified by 16S rDNA sequencing and by detection of *T. whipplei*–specific repeat sequences. Further investigations detected *T. whipplei* in saliva and stool samples. Uveitis was the initial manifestation of Whipple disease, although patient evaluation showed a 2-year history of idiopathic, corticoresistant polyarthralgia described as a hallmark of Whipple disease (*2*). Initial unexplained eosinophilia in blood was observed, as in several confirmed cases of Whipple disease (*2*).

Uveitis has been reported in Whipple disease (*2*), but <20 patients had *T. whipplei* in a diseased eye (Table). *T. whipplei* has been found by periodic acid–Schiff staining of foamy macrophages, electron microscopy, and immunocytochemical detection in ocular monocytes (*3–10*). Detection of *T. whipplei* DNA (*6–10*) has been confirmed by sequencing in only 2 patients, including the case reported herein.

Diagnosis of *T. whipplei* uveitis in our patient was made 3 months after ocular surgery. The patient's condition was diagnosed as chronic postoperative panendophthalmitis, which raised the issue of nosocomial transmission of *T. whipplei*. We have reported a correlation between diagnosis of *T. whipplei* uveitis and a history of ocular surgery (*7*). By reanalyzing detailed published reports, we found that 11 of 19 patients with intraocular demonstration of *T. whipplei* had a history of ocular surgery before documentation of Whipple disease uveitis (Table). *T. whipplei* has not been reported as be-

Table. Characteristics of 19 patients with Whipple disease uveitis documented by presence of *Tropheryma whipplei* in a diseased eye*

| Patient. no. | Age, y/sex | Class | Location | Postoperative uveitis | Use of local or systemic steroids | Microscopy, PAS stain | EM | PCR | Reference |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 52/M | I | B | – | No | + | + | ND | (*3*) |
| 2 | 60/M | A | B | – | No | + | + | ND | (*5*) |
| 3 | 56/M | A | B | + | Yes | + | ND | ND | (*4*) |
| 4 | 47/M | A | B | – | Yes | + | ND | ND | (*4*) |
| 5 | 65/M | A | B | + | Yes | + | + | + | (*6*) |
| 6 | 59/F | A | B | + | Yes | + | + | + | (*7*)† |
| 7 | 53/F | Pa | U | – | Yes | + | ND | + | (*7*)† |
| 8 | 65/M | I | U | + | Yes | + | ND | + | (*7*)† |
| 9 | NR/NR | NR | NR | NR | NR | ND | ND | + | (*8*) |
| 10 | 65/M | I | U | + | Yes | ND | ND | +‡ | (*7*) |
| 11 | 81/M | P | U | + | Yes | ND | ND | +‡ | (*7*) |
| 12 | 35/M | P | U | NR | NR | ND | ND | +‡ | (*7*) |
| 13 | 46/M | P | U | – | No | ND | ND | +‡ | (*7*) |
| 14 | 3/F | A | U | – | No | ND | ND | +‡ | (*7*) |
| 15 | 90/F | A | U | + | Yes | ND | ND | +‡ | (*7*) |
| 16 | 69/M | P | U | + | Yes | ND | ND | +‡ | (*7*) |
| 17 | 20/F | A | U | + | Yes | ND | ND | +‡ | (*7*) |
| 18 | 74/F | Pa | U | + | Yes | ND | ND | +‡ | (*7*) |
| 19 | 78/F | P | U | + | Yes | ND | ND | +‡ | (*7*) |

*PAS, periodic acid–Schiff; EM, electron microscopy; I, intermediate; B, bilateral; ND, not done; A, anterior; Pa, panuveitis; U, unilateral; NR, not reported; P, posterior.
†Reviewed by Drancourt et al. (*7*).
‡These patients were considered to have suspected cases.

ing responsible for nosocomial infection. Items used during the patient's ocular surgery were confirmed to be disposable and nonreused.

Topical drops of corticosteroids commonly applied during cataract surgery for intraocular lens implantation penetrate ocular structures. An alternative hypothesis is that corticosteroids applied during ocular surgery reactivate a latent ocular infection. Our review indicated that 13 of 19 patients with documented *T. whipplei* uveitis had received topical or systemic corticosteroids before the diagnosis (Table) (*7*). Worsening of Whipple disease has been reported in patients receiving corticoid therapy for arthralgia (*10*). We speculate that our patient had an asymptomatic ocular infection before surgery.

This case shows that ocular surgery and use of topical corticosteroids that penetrate ocular structures could reactivate a latent *T. whipplei* ocular infection. We suggest that patients with postoperative panendophthalmitis be tested for *T. whipplei* by PCR.

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## *Klebsiella pneumoniae* Carbapenemase, Canada

**To the Editor:** Carbapenems are used to treat life-threatening infections caused by extremely drug-resistant gram-negative pathogens; these drugs represent the last line of defense in the antimicrobial drug armamentarium against serious or invasive infection (*1*). The rapid global spread of *Klebsiella pneumoniae* that produces *K. pneumoniae* carbapenemase (KPC), especially in the northeastern United States (e.g., New York state), is of major concern (*2*,*3*). KPC β-lactamases belong to the family of serine carbapenemases and are usually found in *K. pneumoniae* and *Escherichia coli*. KPC hydrolyzes β-lactam agents, thereby reducing their action. KPC activity has been reported, albeit less frequently, in other family *Enterobacteriaceae* (*K. oxytoca*, *Enterobacter* spp., *Salmonella* spp., *Citrobacter freundii*, and *Serratia* spp.) as well as in *Pseudomonas aeruginosa* (*1*).

The $bla_{KPC}$ genes have been identified on conjugative plasmids and pose an infection control problem because plasmids could theoretically be transmitted from one species to another (*4*). The few therapeutic options for treating infections caused by organisms containing these β-lactamases are aminoglycosides, glycylcyclines, polymyxins, or combinations (*1*). A major concern is that routine susceptibility testing methods based on existing breakpoints can falsely identify KPC producers as susceptible to carbapenems. Such results pose the potential risk for increased illness and death, longer hospital stays, and nosocomial spread of infection.

In 2008, the Public Health Laboratory in Toronto received clinical isolates of *K. pneumoniae* from urine and sputum of 1 patient. The hospital laboratory had forwarded the isolates to the

Public Health Laboratory because they were possible KPC producers. The patient was a 73-year-old man with a history of emphysema and hypertension, seen at a tertiary care hospital in the Toronto area, 80 miles from the New York state border, for a laparoscopic right radical nephrectomy because of hypernephroma. He had no risk factors for acquisition of KPC producers, e.g., travel to the United States or prior carbapenem exposure.

Susceptibility testing of *K. pneumoniae* was performed by the agar dilution method, using breakpoints set by the Clinical and Laboratory Standards Institute (*5*,*6*). The sputum isolate (7315) was susceptible to meropenem (MIC 4 μg/mL), and the urine isolate (7184) was intermediately susceptible (MIC 8 μg/mL). The *K. pneumoniae* isolates were screened for extended-spectrum β-lactamases (ESBLs) and AmpC production according to Ontario guidelines (*7*).

Briefly, to screen for ESBL enzymatic activity, a double-disk diffusion method was used: a clavulanic acid–containing disk was placed adjacent to a disk containing one of several cephalosporins such as ceftazidime and cefotaxime. Enhanced killing of the organism in the area between the drug with and without clavulanate indicates ESBL. Cefoxitin resistance (zone ≤17 mm) indicates AmpC-like β-lactamase activity. In addition, testing for ESBL/AmpC was performed according to Clinical and Laboratory Standards Institute guidelines (*6*). When the screening result for ESBL or AmpC is positive, the clinical laboratory issues a warning that no β-lactam except carbapenems can effectively treat this infection. The Table summarizes results of initial susceptibility testing and supplementary laboratory testing for KPC.

The initial result was consistent with a possible AmpC/ESBL producer for the sputum and urine isolates (*6*,*7*). However, because the patient responded poorly to empiric vancomycin and imipenem therapy and because of the elevated MIC to meropenem for isolate 7184, further laboratory testing was conducted to rule out the possibility of carbapenemase activity.

The modified Hodge test is a phenotypic test proposed to confirm the presence of carbapenemase activity such as KPC in *K. pneumoniae* and *E. coli* (*8*). Universal primers for $bla_{KPC}$ family, Uni-KPC-F (5′-ATGTCACTGTATCGCCGTCT-3′) and -R (5′-TTACTGCCCGTTGACGCCC-3′), were used for the entire 882-bp coding sequence. Amplicons were bidirectionally sequenced by using the BigDye Terminators method and a 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and primers Uni-KPC-F and -R. Multiple nucleotide and protein sequence alignments were performed with the ClustalW2 software (www.ebi.ac.uk/Tools/clustalw2/index.html). To aid the clinician, an Etest method was used to measure the MIC of this KPC-producing *K. pneumoniae* isolate to colistin (0.5 μg/mL) and tigecycline (2.0 μg/mL). However, before this information could be used, the patient had died of respiratory failure, presumably caused by *K. pneumoniae*. Infection control measures and laboratory screening were undertaken in the hospital to limit transmission to other patients.

This report shows that KPC-producing organisms such as *K. pneumoniae* may pose a major risk for clinical disease and a challenge for infection control if they were to spread to other hospitals in Canada. Current testing algorithms focus on ESBL- and AmpC-producing gram-negative bacteria, which may not detect KPC-producer strains. We suggest that reference laboratories validate a screening method coupled with confirmatory phenotypic assay for carbapenemase activity for suspected organisms, especially *K. pneumoniae* and *E. coli*. Our in-house validation studies confirm that use of the ertapenem disk followed by the modified Hodge test to confirm carbapenemase activity may be effective (D.R. Pillai et al., unpub. data). Public health officials should be aware that this report further expands the international distribution of KPC-producing *K. pneumoniae*.

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Table. Results of initial susceptibility and supplementary testing for *Klebsiella pneumoniae* carbapenemase in urine and sputum samples from 73-year-old man, Canada*

| Isolate | MIC, μg/mL† | | | | | | Disk diffusion results, mm | | | | | Initial report‡ | Final report§ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | AMP | FOX | CIP | GEN | CTRX | MEM | FOX | CAZ | CAC | CTX | CTC | | |
| 7184 | >16 | >16 | >2 | 8 | >32 | 8 | 16 | 0 | 14 | 13 | 15 | AmpC/ESBL | KPC |
| 7315 | >16 | >16 | >2 | 8 | >32 | 4 | 0 | 0 | 8 | 13 | 15 | AmpC/ESBL | KPC |

*AMP, ampicillin; FOX, cefoxitin; CIP, ciprofloxacin; GEN, gentamicin; CTRX, ceftriaxone; MEM, meropenem; CAZ, ceftazidime; CAC, ceftazidime-clavulanic acid; CTX, cefotaxime; CTC, cefotaxime-clavulanic acid; ESBL, extended-spectrum β-lactamase; KPC, *Klebsiella pneumoniae* carbapenemase.
†MIC values for clinical isolates 7184 (urine) and 7315 (sputum) were obtained by using agar macrodilution.
‡Initial screening for ESBL or AmpC β-lactamase activity, performed by Kirby Bauer disk diffusion according to Clinical Laboratory Standards Institute guidelines (*6*,*7*), suggested ESBL or AmpC β-lactamase activity.
§Supplementary modified Hodge test; PCR (specific for $bla_{KPC}$ family), and DNA sequencing confirmed the presence of KPC activity due to $bla_{KPC-2}$.

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## *Cryptosporidium* sp. Rabbit Genotype, a Newly Identified Human Pathogen

**To the Editor:** Most human cases of cryptosporidiosis are caused by *Cryptosporidium parvum* or *C. hominis*, but pathogenicity of some unusual *Cryptosporidium* species/genotypes is uncertain (*1*). In July 2008, an outbreak caused by *Cryptosporidium* sp. rabbit genotype was linked to consumption of tap water in Northamptonshire, England (*2*). On June 23 and 24, *Cryptosporidium* oocysts were detected by operational monitoring of treated water at a surface water treatment works. A precautionary boil-water notice was implemented on June 25.

Enhanced surveillance for cases was established by the health protection team on June 25 in the affected area. Eight single-well immunofluorescent microscopy slides, on which oocysts were detected by water company sampling of the distribution system, were sent to the UK Cryptosporidium Reference Unit, Swansea, for typing. Slides contained 49–259 oocysts. Coverslips were removed after softening the seal with nail polish remover. Fixed material was resuspended from the slides by thorough scraping of the entire well with a pipette tip twice with 50 μL lysis buffer AL (QIAGEN, Crawley, UK) and twice with 50 μL reverse osmosis water to a final volume of 200 μL. Oocysts were disrupted in 3 dry ice/methanol freeze-thaw cycles, and DNA was extracted by using the QIAamp DNA Mini Kit (QIAGEN), which involved digestion with proteinase K in lysis buffer AL at 56°C for 30 min, purification in a spin column, elution in 50 μL buffer AE, and storage at –20°C (*3*).

*Cryptosporidium* oocysts were also detected by direct immunofluorescent antibody test (IFAT) (CryptoCel; TCS Biosciences, Buckingham, UK) in large bowel contents from a rabbit carcass removed by the water company from a tank at the water treatment works. Oocysts were separated from fecal debris by flotation, resuspended in reverse osmosis water (*4*), and processed as above.

*Cryptosporidium* species were identified by bidirectional sequencing of PCR products generated by nested PCR for the small subunit (SSU) rRNA gene (*5*) from 4 DNA aliquots of each sample. SSU rDNA sequences from 7 water samples, containing 49–197 oocysts, and the rabbit isolate were homologous with isolates from rabbits in the People's Republic of China (*6*) and the Czech Republic (*7*) (GenBank accession nos. AY120901 and AY273771, respectively) (online Appendix Table, available from www.cdc.gov/EID/content/15/5/829-appT.htm). One sample from 1,391 L of water contained 259 oocysts but was not amplified. Other cryptosporidia were not identified.

Human stool samples from 34 local laboratory-identified cases of cryptosporidiosis in the affected area were sent to the UK Cryptosporidium Reference Unit for typing. To differentiate rabbit genotype from *C. hominis* (*1*), enhanced typing by SSU rRNA nested PCR–restriction fragment length polymorphism analysis with *Ssp*I and *Vsp*I (*1*,*5*) was used for all isolates submitted to the UK Cryptosporidium Reference Unit during July and August. Samples from 23 cases (22 primary and 1 secondary) with rabbit genotype profiles were identified by visualization of 472-, 267-, and 109-bp bands generated by digestion with *Ssp*I (*1*). All case-patients lived in the area affected by the water supply incident and had onset dates consistent with exposure by drinking water consumption or by person-to-person spread. All 23 samples were homologous to AY120901 and AY273771 (online Appendix Table). Of the other 11 samples, 6 were not confirmed by IFAT or PCR, 2 were *C. hominis*, 1 was *C. parvum*, and 2 were not typeable.

Sequences of the heat shock protein (HSP) 70 gene (*8*) and, to identify subtype family, the 60-kDa glycoprotein (gp60) gene (*9*) were determined for 7 water isolates and the rabbit and 9 outbreak case isolates. All HSP70 sequences were homologous with AY273775 from a rabbit in the Czech Republic (*7*) (online Appendix Table). One water sample, the rabbit sample, and 8 human samples amplified the gp60 gene. These sequences were homologous with each other, but distinct from those published for *C. hominis* (subtype family I), *C. parvum* (subtype family II), *C. meleagridis* (subtype family III), and *C. fayeri* (subtype family IV) (*10*). Each rabbit genotype isolate had 18 TCA (serine) tandem repeats in the gp60 microsatellite region. We propose subtype family Va, subtype A18 for these isolates. This subtype differs from the rabbit genotype previously identified in a human in the United Kingdom (*1*) (subtype VaA22) (GenBank accession no. EU437420) and from rabbits in the Czech Republic (subtype VbA19) and China (subtype VbA29). Sequences generated during this study have been deposited in GenBank under accession nos. FJ262724–FJ262734.

Six additional persons infected with *Cryptosporidium* sp. rabbit genotype were identified by testing 394 stool samples that were routinely submitted for typing from diarrheic patients in July and August from throughout the UK. All persons had onset dates inconsistent with the affected period and were from other regions of the UK. This finding may indicate a low background level of rabbit genotype cases; however, prevalence is currently unknown.

The *Cryptosporidium* rabbit genotype has been identified as the etiologic agent in an outbreak of diarrheal disease and should be considered a human pathogen. Further studies commissioned by the Drinking Water Inspectorate (England and Wales) and funded by the Department of Environment, Food and Rural Affairs UK are underway.

This study was funded by the Welsh Assembly Government to support *Cryptosporidium* outbreak investigations in England and Wales.

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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.

# Acceptance of Public Health Measures by Air Travelers, Switzerland

**To the Editor:** Infectious diseases can spread rapidly by air travel, as did severe acute respiratory syndrome (SARS) in 2003. Public health measures at airports might protect passengers and employees from such diseases and delay spread into the general population. The SARS epidemic was contained largely through traditional public health interventions (*1*,*2*). These interventions included recommendations to postpone nonessential travel, provide public health information and face masks, screen passengers at entry or exit by questionnaire, measure ear temperatures, provide medical examinations, isolate case-patients, and quarantine contacts.

For a future influenza pandemic, the World Health Organization does not encourage entry screening for any pandemic phase but leaves this decision for screening to each country (*3*). Switzerland currently considers entry screening, albeit not by infrared thermal scanning (*4*,*5*). Many travelers to developing countries do not obtain health information or use preventive measures (*6*). However, to be effective, public health measures must be communicated to and accepted by travelers. Current knowledge on acceptance of public health measures by air passengers is limited. Compliance with quarantine measures seems to depend on consistency of policies and credibility of public health messages (*7*).

To investigate passenger knowledge, communication preferences, and acceptance of public health measures for a hypothetical respiratory disease pandemic, we conducted a cross-sectional survey among passengers departing from EuroAirport Basel-Mulhouse-Freiburg (Haut-Rhin, France) to European destinations, and from Zurich Airport (Kloten, Switzerland) to Asian or North American destinations during the summer of 2007. Data were collected by a pretested, self-administered, 23-item questionnaire (in English, French, and German), which was distributed to all adult passengers in the departure waiting areas. Information was analyzed by basic statistical methods, $\chi^2$ and *t* tests, and logistic regression adjusting for sex, age group, airport, residence region, solitary traveling, and business travel.

A total of 2,633 passengers were approached and asked to participate. The response rate was 71%. Most passengers who refused to participate did so because of language difficulties. After we excluded passengers <18 years of age, data for 1,880 participants (1,081 at EuroAirport and 799 at Zurich Airport) were analyzed. Mean (SD) age was 39.8 (14.7) years; 54% were female, 58% had a university degree, 97% were currently feeling healthy, 30% were traveling alone, 37% were traveling for business reasons, 30% were residents of Switzerland, 42% were residents of other European countries, and 15% were residents of the Americas.

Passengers were asked about acceptance of public health measures in a hypothetical severe respiratory disease pandemic. Results are shown in the online Appendix Figure (available from www.cdc.gov/EID/content/15/5/831-appF.htm). A total of 71.6% would cancel their trip if postponement of nonessential travel was recommended, 93.7% would wear face masks, 93.2% would fill out a health questionnaire, and 89.1% would accept having their ear temperature measured on arrival. If fever were detected, 88.1% would undergo a short physical examination. If persons were diagnosed with a disease and were receiving treatment, 92.3% would accept isolation for 7 days. If feeling healthy but were seated next to someone with a cough on the airplane, 69.2% would accept 7-day quarantine at home (residents of Switzerland) or hotel (travelers to Switzerland) and would monitor their health. Fewer persons from the Asia-Pacific region would accept these requirements. Male passengers and all passengers >30 years of age indicated they would be more compliant than other passengers with nearly all measures. However, many female travelers explained that they would not consider traveling during a pandemic.

There were no differences in questionnaire responses between the 2 airports. Other questions concerned information status and seeking. In a pandemic, 93.5% of passengers would acquire information before departure about the situation and preventive measures; 67% would consult the Internet, 59% their family doctor, 49% the media, 37% health authorities, 25% their travel agent, 23% travel medicine centers, 20% the airport, and 17% friends and relatives. For their current trip, 22.4% sought pretravel advice on infectious diseases. This seeking of advice was more frequent in those departing to overseas destinations from Zurich Airport. Information sources were the family doctor (38%), the Internet (34%), the media (26%), friends and relatives (22%), a travel agency (19%), health authorities (11%), travel medicine centers (10%), and the airport (7%). A total of 17.4% noticed the official posters regarding avian influenza.

Because the study was conducted when no major international disease outbreaks were occurring, passengers answered hypothetical questions about an imaginary future pandemic. Therefore, attitudes and behavior might be difficult to predict from these results and would depend on the perceived severity of the pandemic disease. Similar surveys among the general population showed comparable results. During an influenza pandemic, 75% of Europeans would avoid public transportation (*8*). In the United States, 86% would stay at home in quarantine and 94% would stay in isolation (*9*). A survey in Hong

Kong Special Administrative Region, People's Republic of China reported 74% would wear face masks in public, 87% would make declarations at border crossings, and 88% would comply with quarantine policies (*10*).

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## Near-Fatal Multiple Organ Dysfunction Syndrome Induced by *Plasmodium malariae*

**To the Editor:** We report a case of *Plasmodium malariae*–related multiple organ dysfunction syndrome (MODS) in a healthy immunocompetent patient. Despite extensive investigation, *P. malariae* was the only pathogen identified. The patient's isolates had a combination of mutant alleles that could possibly explain the severity of the infection.

Five weeks after returning to France in November 2006 from Côte d'Ivoire, a 28-year-old French soldier was admitted to our surgical intensive care unit (University Hospital, Rennes, France) because of fever and MODS of suspected infectious origin. The patient had stopped taking his doxycycline for antimalarial chemoprophylaxis 3 days before his admission. During those 3 days he began to experience myalgia, fatigue, nausea, and vomiting but no fever. He took no medication. He then became unable to move his lower legs and experienced paresthesia just before his condition rapidly deteriorated. He was found at home by the local Emergency Medical Service (EMS) in respiratory distress and shock and required immediate orotracheal intubation for mechanical ventilation. When admitted to the intensive care unit, he had severe acute respiratory distress syndrome ($PO_2$/$FiO_2$, 65 mm Hg; $PCO_2$, 90 mm Hg; with positive end expiratory pressure of 12 cm $H_2O$). Transthoracic echocardiography and pulmonary artery catheterization showed severe global hypokinesia with a left ventricular ejection fraction of <10%, right ventricular dilatation, and low pulmonary artery occlusion pressure. Blood tests showed disseminated intravascular coagulation with $30 \times 10^9$/L platelets, an international normalized ratio of 3.54, an activated partial thromboplastin time >180 s, and D-dimers at 25.6 μg/mL. He had severe mixed acidosis (pH 6.9 and arterial lactate 4.2 mmol/L) and acute renal failure. Blood cultures were performed. A thin-blood film showed *Plasmodium* spp. within the red blood cells (parasitemia 0.4%). Rapid fluid resuscitation was carried out and epinephrine was given, along with intravenous quinine (1,000 mg over 4 h, then 1,500 mg/d) and broad-spectrum antimicrobial drugs (cefotaxime and ofloxacine).

Massive acidosis developed (pH 6.61; lactate 8.8 mmol/L). A brief cardiac arrest required chest compressions and extracorporeal membrane oxygenation (ECMO) after venoarterial femoral cannulation at the bedside. Continuous venovenous hemofiltration was started. APACHE II and SAPS 2 scores were 38 and 93, respectively. Drotrecogin-alpha (activated) was given as a 96-h infusion.

Extensive microbiologic investigations included tests for common bacteria at usual sampling sites and tests for specific arboviruses, *Lep-*

*tospira* spp., *Rickettsia* spp., and parasites other than *Plasmodium* spp. Results were negative. *P. malariae* was found in the thick-blood film.

The patient's cardiac and pulmonary functions stabilized over the next week. Epinephrine and ECMO were stopped. Surgical exploration of extensive lower-limb necrosis showed arterial thrombosis and *Serratia marcescens* infection. Amputation was necessary of the right leg at the thigh and of the left lower leg after 1 month of hospitalization. The patient was discharged from intensive care 90 days after hospital admission. One year later he was fully recovered and was using prostheses.

Almost all deaths from malaria are related to *P. falciparum* infection. We are unaware of previous reports of near-fatal imported *P. malariae* infection (*1*). Although we cannot definitively exclude bacterial co-infection, results were negative from blood cultures drawn before the first antimicrobial drug dose and from all other microbiologic tests. To look for factors that might explain the unusual disease severity, we conducted additional investigations. The thick blood film showed *P. malariae* trophozoites, schizonts, and gametocytes (285/μL). Rapid diagnostic tests were positive for pan–*Plasmodium* lactate dehydrogenase (pLDH) and aldolase but negative for histidine-rich protein 2, which is specific for *P. falciparum*. Nested PCR with specific primers for *P. falciparum* and *P. malariae*, followed by sequence analysis of the SSUrRNA gene, and nested PCR, followed by sequence analysis of the pLDH gene, were negative for *P. falciparum*, positive for *P. malariae*, and negative for *P. knowlesi* (*2*).

The patient gave written informed consent to tests for genetic polymorphisms associated with severe sepsis, coagulation disorders, and MODS (*3*). Several of these polymorphisms were found (Table). He had a mannose-binding lectin (MBL) haplotype associated with low MBL levels (2 variants in the promoter region [homozygous at –550 and heterozygous at –221] and 1 in the first exon [heterozygous for codon 54 mutation]) (*5*). He was homozygous for 4 gene polymorphisms associated with higher susceptibility or severity of severe sepsis or both and ARDS: *CD14*, lymphotoxin alpha, *TNF-alpha*, *IRAK-1*, and *IL-6* (*6*–*9*). Furthermore, he was homozygous for a *PAI-1* variant associated with decreased fibrinolysis and a higher risk for amputation, skin grafting, and death in meningococcal disease and trauma (*10*). Of 11 patients with uncomplicated *P. malariae* whom we screened for these polymorphisms, none had such a combination of high-risk polymorphisms as did our patient. Thus, the genetic background of our patient may have contributed to the severity of *P. malariae* infection.

Table. Results of genetic screening for single nucleotide polymorphisms known to be associated with sepsis severity in patient with *Plasmodium malariae* infection, France*

| Gene | rs | Wild type | Heterozygous | Homozygous |
|---|---|---|---|---|
| Pathogen detection | | | | |
| TLR2 | 5743708 | X | | |
| TLR2 | 5743704 | X | | |
| TLR4 | 4986790 | X | | |
| TLR5 | 5744168 | X | | |
| CD14 | 2569190 | | | X |
| MD-2 | –1625C/G | X | | |
| FcγRIIa | 1801274 | | X | |
| MBL2 | 52 A/D | X | | |
| MBL2 | 54 A/B | | X | |
| MBL2 | 57 A/C | X | | |
| MBL2 | –550 H/L | | | X |
| MBL2 | –221 X/Y | | X | |
| MBL2 | +4 P/Q | X | | |
| TLR signaling | | | | |
| IRAK1 | 1059703 | | | X |
| TIRAP | 8177374 | | X | |
| IκB | 3138053 | | X | |
| IκB | 2233406 | | X | |
| Inflammation | | | | |
| Lymphotoxin α | 909253 | | | X |
| TNF α | 1800629 | | | X |
| ACE | 17236674 | | | X |
| MIF | 755622 | | X | |
| IL-6 | 1800795 | | | X |
| IL-10 | 1800896 | X | | |
| Coagulation | | | | |
| PAI-1 | 1799768 | | | X |
| Factor V | 6025 | X | | |

*Except for MD-2 and MBL2, rs is the nomenclature used to describe the variants (initially described by den Dunnen and Antonarakis [*4*]). TLR, toll-like receptor; MBL2, mannose-binding lectin 2; IRAK-1, interleulin-1 receptor-associated kinase; TIRAP, toll interleukin-1 receptor-associated protein; IκB:, inhibitor of Nf-κB; TNF, tumor necrosis factor; ACE, angiotensin-converting enzyme; MIF, macrophage migration inhibitory factor; IL, interleukin; PAI-1, plaminogen activator inhibitor-1.

Despite extensive testing, we found no cause for this near-fatal case of MODS except *P. malariae* infection. An unusual combination of genetic polymorphisms may explain the extreme severity of this classically mild infection.

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## Pulmonary Involvement and Leptospirosis, Greece

**To the Editor:** Since the leptospirosis outbreak associated with pulmonary hemorrhage in Nicaragua in 1995 (*1*), pulmonary manifestations of leptospirosis are often recognized in many countries; reported incidence has ranged from 20% to 70% (*2–4*). The severe pulmonary form of leptospirosis is accompanied by pulmonary hemorrhage, which directly results in high death rates (*2*,*5*). In Greece (population 11 million), leptospirosis cases in humans occur every year, usually from June to November (summer and autumn), with a peak in August. The annual incidence rate of the disease is 3 cases per 1 million population (*6*). Clinical presentation varies from a flu-like syndrome to Weil disease, which includes jaundice, renal failure, and hemorrhagic complications. Studies on leptospirosis in Greece have been limited, and no reports have focused on pulmonary involvement.

During 1998–2007, we tested samples from 650 patients with suspected leptospirosis or hemorrhagic fever with renal syndrome (i.e., hantavirus infection). Various hospitals of northern Greece sent these samples to our laboratory (a World Health Organization Collaborating Center for Reference and Research on Arboviruses and Hemorrhagic Fever) for analysis. Because both diseases are endemic to Greece and have similar clinical, epidemiologic, and seasonal characteristics (*7*), all samples sent to our laboratory for testing either for leptospirosis or for hantavirus infection are always tested for both (*8*).

Leptospirosis was confirmed for 123 patients, 10 (8.1%) of whom died (Table). For 72 case-patients, paired samples were available. A commercial ELISA (Leptospira IgG/IgM, Institute Virion/Serion GmbH, Würzburg, Germany) was used to detect immunoglobulin (Ig) G and IgM against *Leptospira* spp.. A nested PCR, which amplifies a 289-bp fragment of the 16S rDNA gene, was used to detect bacterial DNA (*9*). IgM concentrations >20 U/mL indicated acute infection. Samples with borderline results were tested in parallel with a second sample taken from the patient 1 week later. IgG concentrations were considered only for paired samples, and a case was considered as acute leptospirosis when a ≥4-fold titer rise of IgG, or IgG seroconversion, was detected. When samples were taken before the sixth day of illness, initial diagnosis was achieved by PCR. In 6 of 10 fatal cases, leptospirosis was diagnosed only by PCR because antibodies were not detectable. Epidemiologic and clinical information for patients was collected from chart review, following a protocol approved by the medical school review board.

All 123 patients resided in northern Greece. Most (82.1%) were male; patients were 6–83 years of age (me-

Table. Leptospirosis cases and pulmonary involvement, Greece, 1998–2007

| Year | No. cases (no. fatal cases) | No. cases with pulmonary involvement (no. fatal cases) |
|---|---|---|
| 1998 | 12 (0) | 0 (0) |
| 1999 | 9 (2) | 3 (0) |
| 2000 | 7 (1) | 1 (0) |
| 2001 | 11 (0) | 1 (0) |
| 2002 | 13 (2) | 4 (2) |
| 2003 | 13 (1) | 6 (1) |
| 2004 | 20 (3) | 10 (3) |
| 2005 | 14 (1) | 3 (1) |
| 2006 | 17 (0) | 5 (0) |
| 2007 | 7 (0) | 2 (0) |
| Total | 123 (10) | 35 (7) |

dian 51 years). Fifty-two patients were farmers; 9 were sewer workers; and 6 and 2 patients, respectively, reported gardening and other recreational exposures before becoming ill. Cases occurred in all months; 27% occurred in August. Fever, as well as elevated levels of serum urea and creatinine, occurred in all patients; 55 (44.7%) had jaundice, and 46 (37.4%) had thrombocytopenia. Weil disease was present in 27 (22%). In our case-series, jaundice appeared to be a common sign, in contrast to recent studies in other countries in which the icteric form of the disease was observed in only 10% of cases (*10*). Median age of the 10 patients who died was 50 years (range 39–78 years). Half of the patients who died had the icteric form of the disease, 4 had the typical signs and symptoms of Weil disease, and 1 had central nervous system involvement.

Thirty-five (28.5%) patients had pulmonary signs and symptoms, either when admitted to the hospital or during hospitalization. Eight of these had acute respiratory distress syndrome, 6 had multiple organ dysfunction syndrome, and 6 had acute respiratory insufficiency; the remainder had hemoptysis and/or dyspnea, according clinician notes in the medical charts. More than half of the patients had abnormal radiographic findings, mainly bilateral bronchoalveolar infiltrations. Seven (20%) of the 35 case-patients with pulmonary involvement died, a significantly higher death rate than that for case-patients without pulmonary involvement (3.4%, $p<0.01$). Respiratory symptoms were recognized during the first phase of the disease, as other studies have reported (*10*). We found no significant difference in death rates between males and females ($p = 0.629$).

Pulmonary involvement seems to be common and associated with a high death rate for patients with severe leptospirosis cases in our setting. Clinicians in Greece should include leptospirosis in the differential diagnosis of syndromes with associated pulmonary manifestations.

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# Chikungunya Outbreak, Singapore, 2008

**To the Editor:** Chikungunya virus, an arbovirus belonging to the family *Togaviridae*, genus *Alphavirus*, was first isolated in Tanzania in 1953 (*1*). The first outbreak in Asia was documented in Bangkok, Thailand, in 1958. Since then, outbreaks have been reported in Cambodia, Vietnam, Laos, Myanmar, Malaysia, the Philippines, and Indonesia (*2*). In Indonesia, a 1972 serosurvey suggested widespread distribution of chikungunya infection, and numerous outbreaks have reemerged since 2001 (*3*). Malaysia reported its first outbreak between December1998 and February 1999 and a reemergence in an isolated northwestern coastal town in 2006 (*4*).

In Singapore, although dengue fever has been endemic since the 1960s, the first chikungunya case was not reported until 2006. In 2007, 10 imported cases were reported to Singapore's Ministry of Health (*5*). Notably, Taiwan reported a case involving a returning student from Singapore in November 2006, suggesting the possibility of autochthonous transmission in Singapore (*6*).

Located in tropical Southeast Asia, Singapore has remained vigilant in the surveillance of chikungunya. A 2002/2003 serosurvey on 531 healthy young adults showed only 2 (0.3%) persons with chikungunya antibodies (*7*). We describe an outbreak of autochthonous chikungunya transmission in Singapore and discuss removal of infectious human reservoirs from transmission areas as an outbreak control strategy.

On January 14, 2008, a local case of chikungunya infection was detected through the general practitioners' laboratory-based surveillance system established by Singapore's Environmental Health Institute in 2006. The Ministry of Health responded with a massive active surveillance exercise. A total of 2,626 people who resided or worked within a 150-m radius of the index case-patient's address were screened for chikungunya infection by reverse transcription–PCR (RT-PCR), using primers adapted from Hasebe et al. (*5*,*8*). Persons with an acute febrile illness, signs or symptoms compatible with chikungunya fever (fever, joint pain, or rash), or those with positive RT-PCR results were referred to the Communicable Disease Centre at Tan Tock Seng Hospital (CDC/TTSH), the national infectious disease referral center in Singapore.

During the outbreak period from January 14 to February 21, 2008, chikungunya infection was confirmed for 13 patients (*5*). Of these, 10 acutely symptomatic patients (all men; median age 35 years, range 22–69 years) were isolated at CDC/TTSH until fever resolved and a negative chikungunya RT-PCR test result was obtained. During hospitalization, patients' temperatures were monitored every 4 hours and daily chikungunya RT-PCR tests were performed. Viral load profiles were derived from an external standard curve generated by 10-fold serially diluted virus from a concentration of $10^8$ pfu/mL, using crossing-point values.

The Table summarizes the presence of viremia and patients' febrile status in relation to the day of illness. High levels of viremia were observed during the first 5 days of illness (median 119,126 pfu/mL, range 360–14,605,314 pfu/mL). Fever lasted a median of 5 days (range 3–10 days); viremia persisted up to day 9 of illness. Our findings concur with those of a European study, suggesting extremely high levels of viremia at the initial stage of chikungunya disease (*9*). Notably, 1 patient (patient 4), who was screened by the Ministry of Health, was observed to have a positive chikungunya RT-PCR test result 1 day before symptom onset. Fever resolution did not predict viral clearance. Of note, 30% of our patients had detectable viremia (376–8,523 pfu/mL), after fever had resolved. We are uncertain of the role of level of viremia in the transmission of chikungunya; more research is needed to address this pertinent public health question.

*Aedes aegypti* mosquitoes were the vectors involved in this outbreak. Viral sequences from our patients showed a close association to the circulating strains in the 2006 Indian Ocean outbreak (GenBank accession nos. EU441882 and EU441883), without the E1-A226V mutation, which can increase transmission of the virus in the alternate vector *Ae. albopictus* (*5*). Virus phylogenetic studies supported the notion that the East African genotype, which emerged in Kenya in 2004 and the Indian Ocean islands in 2005, closely resembling the 2006 outbreak strain in India, arrived in Singapore in January 2008.

Singapore's outbreak containment strategy focused primarily on intensive vector control and rapid removal of infectious human reservoirs through active case finding and isolation. The proportion of asymptomatic infections in this outbreak was not determined. Asymptomatic infections could possibly reduce the effectiveness of control efforts. However, there have been no data thus far supporting chikungunya transmissibility in asymptomatic persons. Detectable viremia before clinical signs and symptoms and high levels of viremia during early illness, as demonstrated in our study and others (*9*,*10*), pose logistical challenges in the timeliness of case detection for isolation.

Singapore remains at risk for chikungunya outbreaks. It has a highly susceptible population, a porous border with large travel volumes from epidemic areas, and effective vectors (both *Ae. aegypti* and *Ae. albopictus*). In the absence of a vaccine, high vigilance for autochthonous transmission and stringent vector control should be maintained along with a swift public health response.

Table. Daily trend of fever and viremia in 10 hospitalized chikungunya patients, Singapore

| Patient no. | Signs and symptoms* | Fever† and chikungunya test results (viral load‡), by day of fever | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | –1 | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 1 | F, A, B, D | | | | | NA<br>+<br>(442.7) | 37.7§<br>+<br>(3.5) | 37.0<br>– | 37.0<br>– | | | | |
| 2 | F, A, H, RE | | | NA<br>+<br>(5,191.4) | 39.6§<br>+<br>(3,655.2) | 38.4<br>+<br>(119.1) | 38.0<br>+<br>(1.3) | 37.6<br>– | 38.5 | 38.0 | 37.4 | | |
| 3 | F, A, R, H | | | | NA<br>+<br>(155.5) | 39.3§<br>+<br>(385.2) | 37.0<br>+<br>(3.7) | 37.2<br>+<br>(0.4) | 37.3<br>– | 37.2<br>– | | | |
| 4 | F, A | NA<br>+<br>(0.8) | | 40.8§<br>+<br>(14,605.3) | 39.9<br>+<br>(14,170.2) | 38.2<br>+<br>(1,007.8) | 37.6<br>+<br>(4.0) | 37.6<br>+<br>(0.2) | 37.4<br>– | 37.0 | | | |
| 5 | F, A | | NA<br>+<br>(371.4) | | 37.0§<br>+<br>(25.9) | 37.8<br>+<br>(0.4) | 36.8<br>– | 36.6<br>– | | | | | |
| 6 | F, D, R, M | | | NA<br>+<br>(524.4) | 37.6§<br>+<br>(0.7) | 38.6<br>+<br>(0.4) | 37.2<br>– | 36.8<br>– | | | | | |
| 7 | F, A, H, EP | | | NA<br>+<br>(18.8) | 38.3§<br>+<br>(7.2) | 37.0<br>+<br>(0.4) | 37.0<br>– | | | | | | |
| 8 | F, A | | | | | | | | NA<br>+<br>(36.6) | 37.5§ | 37.6<br>+<br>(0.3) | 37.7<br>– | 36.4 |
| 9 | F, A, N | | | NA<br>+<br>(406.7) | 38.4§<br>– | 37.8 | 37.1 | 37.1 | | | | | |
| 10 | F, A, M | | | | | | 39.2§ | 36.7<br>+<br>(8.5) | 36.8<br>+<br>(3.6) | 37.1<br>– | 37.4<br>– | 36.8 | |

*At hospitalization. F, fever; A, arthralgia; B, backache; D, diarrhea; H, headache; RE, red eyes; R, rash; M, myalgia; EP, eye pain; N, nausea.
†Day 0, day of fever onset. Maximum temperature expressed in ºC. Light shading indicates self-reported fever; dark shading indicates documented fever (maximum temperature >37.5 ºC). NA, not available.
‡Viral load expressed as × $10^3$ pfu/mL. Reverse transcription–PCR test results for chikungunya: +, positive; –, negative.
§Indicates day patient was hospitalized.

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# Vancomycin-Resistant Enterococci, Point Barrow, Alaska, USA

**To the Editor:** An increasing number of bacterial infections are now difficult or impossible to treat (*1*) because of the misuse of antimicrobial drugs and the epidemic spread of bacterial resistance to these drugs (*2*). The most alarming reports are of methicillin-resistant *Staphylococcus aureus*, extended-spectrum β-lactamase producers, and vancomycin-resistant enterococci (VRE). Although knowledge about dissemination mechanisms is poor, the spread of resistance clearly is not restricted to hospitals but occurs also in the community and in the natural environment (*3*,*4*). Since the 1990s, the epidemiology in the United States has shifted so that most VRE are *Enterococcus faecium*. Recent studies indicate clonal spread of the *E. faecium* CC17 lineage in clinical isolates, exhibiting high-level ampicillin and fluoroquinolone resistance and harboring an enterococcal surface protein–coding *esp* gene (*5*,*6*).

During a polar research expedition to the Beringia region in 2005, we collected fecal samples from birds at sites with no or low human population. The aim was to investigate the current status of resistance dissemination into remote areas of the world. The study site in Alaska was located on the tundra halfway between the city of Barrow and Point Barrow, the northernmost point of the United States (71°23′20″N, 156°28′45″W). Fecal samples from glaucous gulls (*Larus hyperboreus*) were enriched (18 h at 37°C) in brain–heart infusion broth (Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with aztreonam and vancomycin (10 mg/L and 4 mg/L, respectively; ICN Biomedicals Inc., Aurora, OH, USA), followed by spreading on chromID VRE plates (bioMérieux, Marcy l'Etoile, France) and incubated for 48 h at 37°C. Typical colonies were isolated and species identified by biochemical testing, including the Phoenix Automated Microbiology System (Becton Dickinson). MIC was determined for vancomycin, teicoplanin, ampicillin, and ciprofloxacin by using Etest strips (AB Biodisk, Solna, Sweden), and the presence of *vanA*, *vanB*, and *esp* genes was established by PCR with previously described primers (*7*,*8*) (esp primers esp11 and esp12).

Cultures showed 2 isolates of *E. faecium*; MICs for vancomycin and teicoplanin were >256 and 96 μg/mL, respectively, for both isolates. Genotyping determined that they harbored *vanA*. Isolates exhibited high-level ampicillin and ciprofloxacin resistance; MICs were >256 and >32 μg mL, respectively for both isolates. They also harbored the *esp* gene. Isolates came from 2 of 33 sampled glaucous gulls, a species confined to the Arctic regions, that have limited southbound migration during the nonbreeding season.

Clinical isolates of VRE were first found in the late 1980s. In the United States, vancomycin was widely used in human medicine, and outbreaks occurred in hospitals rather than in the community; the opposite was, and is, true in Europe. Because of massive use of glycopeptide antimicrobial drugs, i.e., avoparcin, as growth promoters in domestic animal production until the mid-1990s, VRE can be found in hospitals and the community (*9*).

Our findings show that bacteria resistant to antimicrobial drugs, or resistance genes, already have spread to one of the most remote areas of North America, Point Barrow, Alaska. This spread suggests that few (if any) places on earth may be protected against the spread of such resistance, and the dispersal mechanisms are far more efficient than previously thought. Our data also place the isolates as part of the clinically spread clonal *E. faecium* CC17 lineage, characterized by high-level ampicillin and quinolone resistance and harboring the *esp* gene, thus strongly supporting a human origin. Possible dispersal mechanisms to remote areas include stepwise horizontal transfer between migratory and nonmigratory bird species and anthropogenic transport.

The increasing evolution and spread of antimicrobial drug–resistant bacteria and resistance genes seriously threaten public health and could escalate to catastrophic proportions (*1*). Bacteria and drug resistance are easily transferred between humans and animals and consequently between the environment and clinical settings. Much remains to be learned about the effect of human-associated changes of natural ecosystems on the total effect of resistance. Therefore, our finding of VRE at Point Barrow is important to recognize. Decisive action is needed to establish efficient monitoring programs that include not only surveillance and control of clinical bacterial resistance but also environmental levels of resistance.

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## Use of Templates to Identify Source of Norovirus Outbreak

**To the Editor**: On November 22, 2006, an infection control nurse notified the Marion County (Oregon) Health Department about acute gastroenteritis among persons who had attended a reception at a medical facility on November 16, 2006. With a holiday weekend only hours away, the county health department asked the state health department to join the outbreak investigation.

After interviewing the caterer, organizers, and several attendees, we modified a questionnaire template to reflect potential exposures. Using this questionnaire, we conducted a retrospective cohort study by telephone among reception attendees identified from a ticket list. We defined a case of acute gastroenteritis as reported vomiting or diarrhea (≥3 loose stools within a 24-hour period) within 18–72 hours of the event.

Sanitarians inspected the facility and the caterer's kitchen. We traced implicated oysters (the source of the outbreak) through distribution records; screened stool specimens for norovirus by RT-PCR; tested oysters from the implicated lot for norovirus by qRT-PCR; entered data into a custom outbreak database template; calculated relative risks (RRs) and 95% confidence intervals (CIs) using Epi Info (www.cdc.gov/epiinfo); and assessed the significance of the association between acute gastroenteritis and consumption of implicated oysters by the $\chi^2$ or Fisher exact test.

Approximately 200 persons attended the reception. We called all households on the reception ticket list with identifiable phone numbers and reached a convenience sample of 66 attendees from 50 households. We determined that 10 had cases of acute gastroenteritis, 53 had no symptoms, and 3 (who were excluded from the analysis) had minor symptoms. The median incubation period was 36 hours (range 31–63 hours). None of the 10 attendees with acute gastroenteritis sought medical attention; stool specimens from 2 of them tested positive for norovirus (1 positive for genogroup II and 1 positive for both I and II).

Illness was associated with consumption of raw oysters on the half shell (RR 11.8; 95% CI 2.8–50; p = 0.0001), which was reported by 8 of the 10 attendees with acute gastroenteritis. No other foods were associated with illness. No significant breaches in food-handling procedures were identified. The only food handler who reported illness had eaten several oysters at the event and became ill 36 hours later.

The oysters had been individually quick frozen on the half shell and packed loosely in cartons after being harvested in South Korea by growers approved by the US Food and Drug Administration. For the reception, a single 6-kg box of oysters was thawed and served raw. The box was from a shipment of 2,200 boxes legally imported in October 2006. Boxes from the same shipment had been distributed to 5 states. Oysters from 4 other cartons were consumed (some cooked) at 2 other Oregon locations. Public health officials in other states were notified and asked to report any related illnesses; none were identified.

Noroviruses (genogroups I and II) were detected in oysters from an intact carton of the implicated lot. Sequencing was not attempted. The implicated lot was voluntarily recalled by the national distributor; most of the lot was embargoed or recalled before the oysters were consumed.

Oysters are a recurrent source of outbreaks and sporadic cases of norovirus infection, vibriosis, and other infections (*1*) because they are frequently eaten raw or undercooked (*2*). Microbial monitoring of oyster harvest

areas reduces but does not eliminate the risk for disease associated with consumption of raw or undercooked oysters (*3*).

Oysters from many parts of the world have been implicated in previous norovirus outbreaks (*3*–*6*), including similar norovirus outbreaks in New Zealand in 2004 and 2006 caused by consumption of frozen oysters from South Korea (*6*). In addition, norovirus was detected in 10% of imported oysters in Hong Kong (*7*), and adenoviruses or enteroviruses were identified in 80% of oyster samples from popular harvest areas in South Korea (*8*).

Although widely distributed commercial foods are rarely implicated as a source of norovirus infections, oysters (*3*) and raspberries (*9*) are notable exceptions. Without timely subtyping of virus specimens and a PulseNet-like data-sharing system, cluster linkage is unlikely. Norovirus infections are rarely confirmed by laboratory tests, and sporadic cases are rarely considered notifiable. The outbreak we described was recognized and reported because illnesses clustered in 1 workplace. However, even when outbreaks are reported, they are not always investigated thoroughly. The conventional wisdom that many, if not most, foodborne norovirus outbreaks are caused by contamination at the point of service (*10*) may discourage thorough epidemiologic investigations of these outbreaks.

Because thorough outbreak investigations are time-consuming and gastroenteritis outbreaks are common, resource issues often affect decisions about how intensively to pursue investigations. Our use of integrated questionnaire, data entry, and analysis templates (www.oregon.gov/DHS/ph/acd/keene.shtml) facilitated a quick and efficient response to the outbreak described here. Questionnaire design, interviews, data entry, and analysis were completed within 6 hours of the initial report, and distributors and regulatory agencies worked quickly to recall other oysters from the same source, thus probably preventing additional illnesses. We believe that widespread use of such templates would increase the number of outbreaks that could be investigated thoroughly.

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## Epidemiologic Questions from Anthrax Outbreak, Hunter Valley, Australia

**To the Editor:** Anthrax was introduced into Australia in 1847 near Sydney, New South Wales, and spread along stock routes throughout New South Wales and southern Queensland (*1*). Anthrax was considered endemic to the Hunter Valley, New South Wales, during the 1890s. The last recorded anthrax-related stock losses there occurred on 3 properties in the Upper Hunter Valley in 1939 (*1*).

During the past 4 decades, anthrax has become uncommon in Australia. Clinical cases are seen only spo-

radically in sheep, cattle, and (rarely) horses. Annually, 6–12 properties are affected in unrelated incidents; where cattle are involved, generally only 1–3 animals per property are affected (*2*). Anthrax is confined almost exclusively to a belt running through the center of New South Wales (*3*).

From December 14, 2007, through January 3, 2008, a total of 53 cattle (*Bos taurus*) with peracute anthrax and 1 horse died on 11 properties in the Rouchel area, 20 km east of Aberdeen in the Hunter Valley and 350 km from the anthrax belt (Figure). The area is hilly, rising to ≈550 m, with alluvial soils alongside a stream and rocky basaltic and sandy soils on the slopes. Most properties feature gullies that flow intermittently after rain. The affected properties covered ≈60 km$^2$.

The animals that died in this area were of all ages. Anthrax was not suspected because of a long history of no local activity, but *Bacillus anthracis* was initially confirmed by microscopy of blood smears and subsequently by PCR of blood or other carcass fluid smear scrapings taken when animals were decomposed and microscopy was unreliable (*4*). Dates of discovery of the index case on each property ranged from December 14 to December 29; 1–26 deaths (median 2) occurred per property. Property attack rates varied from 0.9% (1/110 cattle) to 10.7% (3/28 cattle). All stock on infected and 24 neighboring properties were vaccinated in late December; carcasses were burned to ash; movement control, including quarantine, was implemented; and all subsequent stock deaths in the area were investigated to rule out anthrax. One subsequent case occurred when an unvaccinated bull was introduced onto an infected property in late May 2008.

Detailed record review excluded importation of infected feed from known anthrax-endemic areas before the outbreak, and no deaths occurred in stock introduced from these areas during the previous month. Many of the animals died near streams, and waterborne spore dispersal with infection was initially hypothesized. However, the temporal pattern of properties affected, with downstream properties affected before upstream properties; the fact that properties without contiguous streams were affected; and the dilution effect of rapidly flowing streams argued against this transmission route. Because they are septicemic, terminally ill animals with anthrax often seek water (*5*).

The mysterious contemporaneous reemergence of anthrax in this area is unlikely to be explained by mechanical vector-borne transmission because only 1 animal had eye damage, suggesting a crow attack. There was no additional evidence of scavenger attack. No tabanid species (biting) flies were seen on any carcass, and the small number of carcasses and relatively large distances between some properties made mechanical transmission with ocular inoculation by non-biting flies unlikely (*4*).

Both the remarkable survival capability of anthrax spores and a 1-in-100-year rain event probably contributed to the near-simultaneous reemergence of anthrax on multiple properties in this area. Anthrax spores are resistant to biological extremes of heat, cold, pH, desiccation, chemicals, and irradiation, persisting in this state for decades awaiting conditions that favor germination and multiplication (*6*). In June 2007, drought-ravaged Hunter Valley experienced intense flooding; most rain fell in just 3 days (259 mm in the Aberdeen area, compared with the previous 3-year June average of 43 mm), and massive amounts of topsoil washed into gullies and streams. During late 2007, rainfall also was excessive: 132 mm and 129 mm in November and December, respectively, compared with the 3-year average of 87 mm and 65 mm.

The June floods are likely to have unearthed anthrax spores in the area. The question remains whether these spores had been present for >6 decades, concentrating in depressions that collected water and dead vegetation, potentially providing a milieu for germination and multiplication (i.e., incubator areas), a mechanism that has been implicated in wildlife epidemics

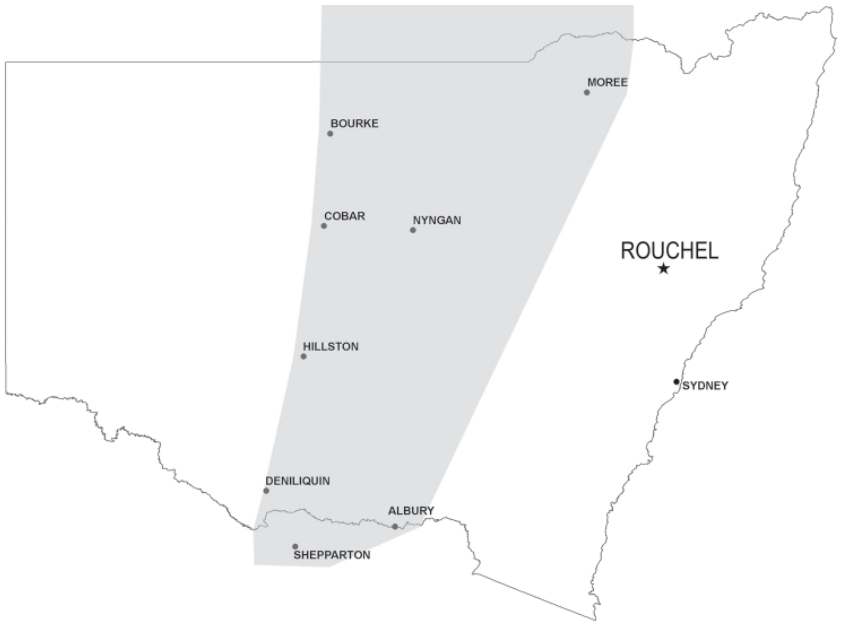


Figure. Map of New South Wales, Australia, showing the anthrax belt (gray shading) and the Rouchel location where anthrax reemerged during December 14, 2007–January 3, 2008.

of anthrax (*7*,*8*). Alternatively, low-grade sporadic infection may have been ongoing since the 1940s and infrequent stock mortality may not have been investigated for anthrax because of a low local index of suspicion, resulting in environmental contamination The extreme weather conditions in the area may have unearthed spores from undiagnosed carcasses, providing simultaneous exposures on multiple properties.

We are currently unable to resolve this epidemiologic conundrum. However, our experience is a timely reminder that veterinary public health authorities should be on high alert for possible anthrax when unexpected livestock deaths follow flooding in areas where anthrax has historically occurred.

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## Distinct Ecologically Relevant Strains of *Anaplasma phagocytophilum*

**To the Editor:** *Anaplasma phagocytophilum* was defined to include *Ehrlichia phagocytophila*, *E. equi*, and the agent of human granulocytic ehrlichiosis. Nevertheless, we and others have found phenotypic and genetic differences from diverse regions and hosts and conclude preliminarily that ecologically separate strains might exist that should be distinguished. Two precedents include ruminant strains of *A. phagocytophilum* in Europe and the Ap-Variant 1 from ruminants and ticks of North America and Europe.

In Europe, *A. phagocytophilum* infects livestock, rodents, and humans, with some species such as European cattle showing severe disease and high antibody prevalence. In contrast, cattle infection is rare in the United States, despite being common in other species. Experimental infection of cattle with California equine–origin strain MRK failed to induce disease or marked rickettsemia (*1*). Thus, even though European strains have ruminant tropism, an equine strain does not.

Ap-Variant 1 is found in ticks and deer in North America. This strain is distinctive in the 16S rRNA, major surface protein 4 (*msp4*), *msp2*, and *ankA* genes (*2*). Deer, goats, and tick-derived cell lines can be infected with Ap-Variant 1, but rodents cannot (*3*). Our recent data examining *A. phagocytophilum* in western North America show at least 2 phenotypes: strains originating from sciurids (chipmunks and tree squirrels) and strains from woodrats (the previously postulated reservoir). In a survey of 2,121 small mammals in areas of California with enzootic Ap-Variant 1, seroprevalence was highest in tree squirrels (71%), woodrats (50%), and chipmunks (up to 28%), and PCR prevalence was highest in tree squirrels (16%) and chipmunks (34%) (*4*). We showed that chipmunks were competent reservoirs for *A. phagocytophilum* through exposure in the field, successful inoculation with strain MRK, and transmission through *Ixodes pacificus* to mice. However, discrepancy in the phenotype of strains originating from woodrats and chipmunks is substantial when these strains are inoculated into horses. One chipmunk strain can infect both rodents and horses (important laboratory animal models for human infection), whereas woodrat strains show restricted rodent-only tropism.

A naturally infected redwood chipmunk was trapped in Mendocino County, California, exsanguinated, and documented to be positive for *A. phagocytophilum* by using real-time

PCR, with a cycle threshold ($C_t$) of 31.31. An adult horse was negative for infection and exposure by PCR and immunofluorescence assay, premedicated with flunixin meglumine and diphenhydramine, and inoculated with 1.5 mL of infected chipmunk whole blood in EDTA. The mare was monitored daily for 16 days, including blood smear, serologic testing, and PCR, and assessment of behavior and attitude, rectal temperature, and legs for edema, swelling, or pain. She became ill 12 days postinoculation, with a body temperature of 40.0ºC, lethargy, depression, and inappetance. Blood smears showed *A. phagocytophilum* morulae in neutrophils, and she was PCR positive on day 13 ($C_t$ 37). Thus, infection from this chipmunk strain was indistinguishable from that induced when human-origin *A. phagocytophilum* was inoculated into this horse. The mare recovered after treatment.

In contrast, woodrat strains show rodent-host tropism but are not infectious to horses. We attempted to infect 3 horses with *A. phagocytophilum* from naturally infected, PCR-positive woodrats from Hoopa Valley, Humboldt County (1 pool of 4, 1 single) and Henry Cowell State Park, Santa Cruz County (N = 1); both sites are enzootic for *A. phagocytophilum*. Woodrats were bled into tubes containing EDTA, and blood was kept cool and screened that day by real-time PCR and serologic testing. The PCR-positive samples were divided into rodent and horse inocula. The horses were negative for infection and exposure using PCR and immunofluorescence assay, premedicated as described above, and then inoculated with 6 mL of *A. phagocytophilum*–infected woodrat blood. These horses never became infected on the basis of clinical signs, serology, blood smears, and PCR. Each horse was reinoculated 1–2 months later with 1.5 mL of an equine-tropic strain (MRK or chipmunk) to verify susceptibility to infection. All 3 became ill within 12–13 days postinoculation, with substantial increase in body temperature (>39.4º C), lethargy, depression, and inappetance. Blood smears showed *A. phagocytophilum* morulae in neutrophils, and the animals were PCR positive and seroconverted. We considered the woodrat inocula unlikely to be noninfectious because aliquots of the same samples produced rickettsemia according to PCR in C3H/HeJ mice and uninfected woodrats. All 3 horses recovered after treatment.

Although some woodrat *A. phagocytophilum* strains are genetically similar to human and equine strains, others differ from sciurid, human, horse, and dog strains, with conserved blocks within the *msp2* gene (*5*). A phylogenetic tree based on the *omp1n* gene clusters a sciurid strain with local horses, distinct from northern California woodrats (online Appendix Figure, available from www.cdc.gov/EID/content/15/5/842.htm). Some woodrat strains have rodent-only tropism; Ap-Variant 1 does not infect rodents. Strains from sciurids and white-footed mice infect multiple laboratory animals and perhaps humans as well. Thus, epidemiologic studies evaluating human risk need to incorporate these distinctions and further ecologic and molecular genetic studies are necessary. With increasing reports of dissimilar genotypes of *A. phagocytophilum* from multiple regions of the world, defining distinct phenotypes and using nomenclature that appropriately clarifies the distinctions are important.

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# Ovine Herpesvirus 2 Infection in Foal, Brazil

**To the Editor:** Malignant catarrhal fever (MCF) is an acute, generalized, and usually fatal disease previously thought to be restricted to mammals of the order Artiodactyla, often members of the subfamilies Bovinae, Cervidae, and Suidae (*1*). Although animals of the order Perissodactyla, family Equidae, have previously been considered not susceptible to ovine herpesvirus 2 (OvHV-2), we show that horses may be infected by this virus.

In July 2006, in the state of Minas Gerais, Brazil, neurologic signs developed acutely in a 6-month-old foal; signs included muscle spasms, rigidity of the neck and limbs, difficulty standing, and hind-limb paralysis. The foal also had severe dyspnea and profuse sweating and died 1 day after the onset of clinical signs.

Histopathologic findings included a marked lympho-histiocytic fibrinoid necrotizing vasculitis affecting small blood vessels and arterioles in the kidney and liver (Figure) and associated with lymphocytic interstitial nephritis and mild multifocal granulomatous hepatitis. The vasculitis lesions were strikingly similar to those observed in cattle with MCF caused by OvHV-2 or alcelaphine herpesvirus 1. Both forms of the disease have a wide spectrum of clinical manifestations, but histopathologic findings for the 2 forms are similar (*2*,*3*). In addition, the foal had severe and diffuse interstitial pneumonia characterized by thickening of the alveolar walls and interstitial accumulation of macrophages, proliferation of type II pneumocytes, and accumulation of cell debri in the alveolar lumen. Granulomatous inflammation, characterized by mild multifocal to coalescent accumulation of epithelioid macrophages, was also observed in the spleen and lymph nodes. Surprisingly, the brain showed only moderate congestion and mild, multifocal, perivascular hemorrhage.

Although horses are not considered susceptible to OvHV-2, histopathologic findings in this case were consistent with MCF-like lesions. Thus, we looked for any history of direct or indirect contact between the affected foal and goats or sheep. Indeed, on this particular farm, horses shared food with 65 goats. On the basis of indirect contact with potential reservoirs of infection, the disease in the foal was suspected of being associated with a member of the MCF virus (MCFV) group (*4*).

To determine whether the disease in the foal was associated with an MCFV, we obtained tissue samples from the foal at necropsy and collected blood from all 3 adult horses, including the dam, and 10 randomly selected goats on the farm. DNA was extracted from these samples, and PCR was performed to detect members of the MCFV group, including OvHV-2 (*5*), caprine herpesvirus 2 (*6*), and alcelaphine herpesvirus 1 (*7*). The tissues from the foal as well as peripheral blood mononuclear cells from all 3 adult horses and 8 tested goats were positive for OvHV-2 only. The adult horses had no clinical signs of infection for at least 8 months after the outbreak.

To confirm the PCR detection of OvHV-2, we purified amplicons obtained from the dam of the affected foal, the affected foal, and 1 goat and processed them for automated sequencing. These nucleotide and deduced amino acid sequences were identical, and we deposited them in GenBank under accession nos. EU244694, EU718486, EU718487. PCR was conducted on tissue samples of the foal to test for differential diagnosis agents equine herpesvirus 1 (*8*), equine herpesvirus 4 (*8*), and equine arteritis virus (*9*). No amplification was observed. Considering that sheep and goats are the most important natural reservoirs of OvHV-2 (*6*), these results support the notion that infected goats were the most likely source of infection for horses in this outbreak.

To further support this diagnosis of OvHV-2 infection, we attempted to detect viral DNA in the vascular wall of the foal's hepatic arterioles that contained fibrinoid necrotizing lympho-histiocytic vasculitis by using laser capture microdissection on sections stained with hematoxylin and

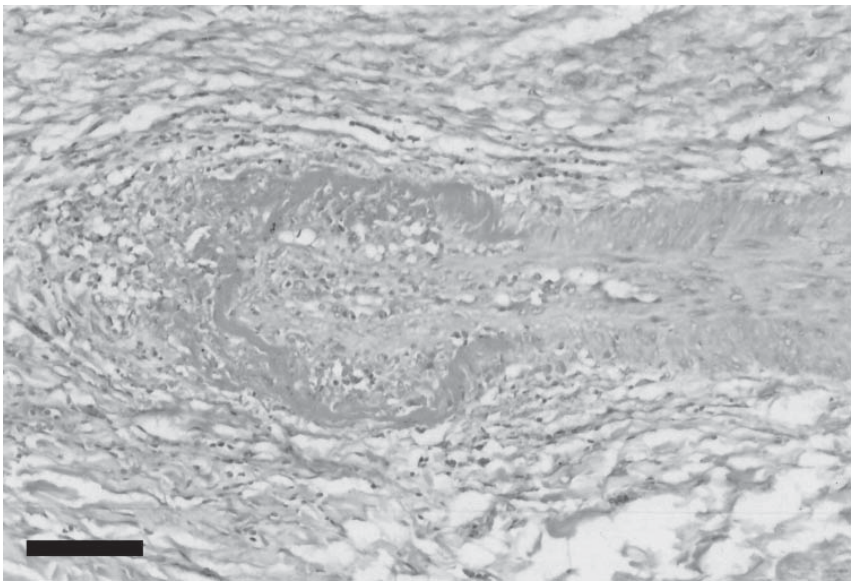

Figure. Liver of foal with vasculitis associated with intralesional ovine herpesvirus 2 DNA, showing segmental, severe, fibrinoid, necrotizing arteriolitis. Hematoxylin and eosin stain. Scale bar = 100 μm.

eosin (*10*). DNA extracted from these areas was PCR positive for OvHV-2, which confirmed the co-localization of OvHV-2 DNA sequences in the site of MCF-like lesions.

Taken together, these findings confirm an emergent infectious disease associated with OvHV-2 infection in a horse, a species previously considered not susceptible to OvHV-2. The finding of vasculitis associated with intralesional OvHV-2 DNA sequences unequivocally demonstrates the pathogenic potential of this virus in foals. However, a cause-and-effect relationship between OvHV-2 infection and interstitial pneumonia as well as the granulomatous inflammation in the liver and spleen could not be established in this case. This report supports the notion that either equine infection is extremely rare or that this strain of OvHV-2 underwent recent modifications that expanded the host range.

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## Community-acquired Methicillin-Resistant *Staphylococcus aureus* ST398 Infection, Italy

**To the Editor:** Community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) has been identified in livestock animals (particularly pigs), veterinarians, and animal farm workers (*1*,*2*). CA-MRSA strains from pigs have been classified most frequently within the multilocus sequence type (ST) 398 (*1*) and have been rarely identified as a cause of invasive infection in humans (*1*,*3*,*4*). We report a case of invasive infection in a pig-farm worker in Cremona, Italy, an intensive animal farming area; the infection was caused by MRSA of swine origin, ST398.

The case-patient was a 58-year-old man admitted to a surgical department in Cremona, Italy, on July 30, 2007, because of a 1-week history of fever and intense pain in his right buttock. He worked on a pig farm, was obese, consumed high volumes of wine (1.5 L/day), was taking medication for hypertension, and had not had recent (<5 years) contact with the healthcare system. At the time of hospital admission, he was moderately ill, oriented, and cooperative. His right buttock was extremely painful. He reported neither recent trauma nor anything that would

explain infection. Laboratory examination showed increased C-reactive protein (298 mg/L) and leukocytosis (28,000 cells/mm$^3$) with neutrophilia (80%). Empiric treatment with intravenous ampicillin-sulbactam was started.

Based on clinical and magnetic resonance imaging data, the diagnosis was cellulitis, pyomyositis, and pelvic multiloculated abscess of the buttock. A needle aspiration of the abscess, guided by computed tomography, was performed. Because of persistent fever (38.5°C), oral ciprofloxacin was added to the patient's treatment regimen on day 3. Blood and abscess cultures yielded MRSA that was sensitive to glycopeptides, rifampin, linezolid, gentamicin, and mupirocin and resistant to co-trimoxazole, macrolides, clindamycin, and fluoroquinolones. After treatment was switched to vancomycin plus rifampin, the patient's general condition improved; he was discharged from the hospital after 24 days.

An epidemiologic investigation of the patient's family and 3 fellow workers and their families was performed; nasal and inguinal swabs were obtained from these 11 persons. Two fellow workers were colonized with *S. aureus*, 1 with methicillin-sensitive *S. aureus* (MSSA) and the other with MRSA. The pig farm, a farrow-to-finish production farm with 3,500 pigs, was screened for MRSA according to guidelines of the European Food Safety Authority (*5*). Dust swabs were taken from 5 areas of the farm; 7 MRSA isolates were detected.

*S. aureus* species identification was confirmed by PCR (*6*). Staphylococcal chromosomal cassette *mec* type (SCC*mec*) was identified by multiplex PCR testing (*7*,*8*). Panton-Valentine leukocidin (PVL) gene detection and *spa* and ST typing were performed as previously described (*9*).

The isolate from the patient belonged to *spa* type t899, was ST398, carried an SCC*mec* type IVa cassette, and was PVL negative. The isolate from the MRSA-colonized worker was a t108 strain carrying SCC*mec* type V. The isolate from the MSSA-colonized worker was identified as t899. The dust swabs yielded 7 isolates: 2 belonged to t899 and carryied SCC*mec* IVa; 5 belonged to t108 and carryied *SCCmec* V. The isolates obtained from the patient, farrowing area 7, and gestation area 1 were indistinguishable (i.e., same *spa* type, SCC*mec* type, and ST profile; Table), thus confirming the animal origin of transmission.

This case highlights other considerations. First, although the isolate, as expected, was PVL negative, its aggressiveness resembled that of PVL-positive strains. Second, all *S. aureus* isolates identified, MRSA and MSSA, belonged to t899 or t108, within the ST398 group, in agreement with the observation of van Dujkeren et al. (*10*) that ST398 MSSA, a possibly virulent strain, may acquire different SCC*mec* cassettes relatively easily. Third, ST398 carriage was high (75%) among workers; 2 of 4 were carriers of MRSA ST398 and 1 was a carrier of MSSA ST398. This strain may be a hazard to the health of pig farmers and a possible cause of zoonotic infection. When treating pig farmers for possible staphylococcal infection, healthcare workers should consider using antimicrobial drugs effective against MRSA and should consider the aggressive resistance pattern observed in this case, which was more similar to hospital-acquired strains than to classic CA-MRSA.

The identification of a case of ST398 endocarditis (*4*) and of a nosocomial outbreak of ST398 in the Netherlands (*3*) may support the hypothesis that the scarce number of infections reported so far may be due to the still-limited spread of ST398 among critically ill patients; emergence among pigs is thought to be recent. As observed by Wulf and Voss, the pathogenicity, aggressiveness, or potential spread of ST398 among humans remains to be ascertained (*1*).

In conclusion, attention should be given to the emergence of MRSA strains among animals, and continuous surveillance in humans should monitor the extent of disease from MRSA ST398, especially in areas of intensive animal farming. Collaboration between infectious disease specialists, microbiologists, and epidemiologists, on both the human and the veterinary sides, should be strengthened and readied for appropriate action whenever complex, zoonotic, public health issues occur.

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Table. Main characteristics of *Staphylococcus aureus* isolates identified from persons and pig-farm environment, Cremona, Italy, 2007*

| Origin of isolate | Sample type | *nuc/mec* | PVL | *spa* type | *mec* type |
|---|---|---|---|---|---|
| Patient | Blood | +/+ | – | t899 | IVa |
| Pig worker 1 | Nasal swab | +/+ | – | t108 | V |
| Pig worker 2 | Nasal swab | +/– | – | t899 | NA |
| Farrowing area 5 | Dust swab | +/+ | – | t108 | V |
| Farrowing area 5 | Dust swab | +/+ | – | t108 | V |
| Farrowing area 6 | Dust swab | +/+ | – | t108 | V |
| Farrowing area 7 | Dust swab | +/+ | – | t108 | V |
| Farrowing area 7 | Dust swab | +/+ | – | t899 | IVa |
| Farrowing area 8 | Dust swab | +/+ | – | t108 | V |
| Gestation area 1 | Dust swab | +/+ | – | t899 | IVa |

*PVL, Panton-Valentine leukocidin; NA, not applicable.

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# *Campylobacter jejuni* in Penguins, Antarctica

**To the Editor:** The wildlife of Antarctica is highly specialized. Although large animal species are limited primarily to penguins and seals, each species is often abundant. The high degree of isolation potentially protects Antarctic wildlife from diseases distributed in other areas of the world (*1*,*2*). Despite Antarctica's isolation, however, human- or animal-related pathogens have been found there, or in the sub-Antarctic islands. For instance, serologic evidence of influenza virus A infections in penguins has been found (*3*), and both *Salmonella* spp. and *Mycobacterium tuberculosis* have been isolated from sub-Antarctic and Antarctic animals (*4*,*5*).

*Campylobacter jejuni* is a leading cause of bacterial gastroenteritis in humans worldwide; it is usually found in the intestinal tract of various farm and wild animals, particularly birds (*6*,*7*). We previously reported finding 3 *C. jejuni* subsp. *jejuni* isolates in macaroni penguins (*Eudyptes chrysolophus*; Figure) from Bird Island (54°00′S, 38°02′W), South Georgia (*1*). Phenotypic tests and 16S rRNA gene sequencing showed that the penguin isolates were identical to each other, and macrorestriction profiling of pulsed-field gel electrophoresis fragments showed that they were very similar to fragments isolated from poultry in Washington in 1984 (*1*). Because the isolates were retrieved from macaroni penguin chicks, we concluded that the animals had acquired the infection locally and that this was likely an instance of introduction of a pathogen to the Antarctic region.

However, restriction fragment pattern resemblance is not identical to genetic relatedness and, given the relevance of the question of origin, this resemblance led us to use a new method for genetic characterization. We reanalyzed the macaroni penguin isolates with multilocus sequence typing (MLST), a method that uses sequence data from 7 unlinked loci for genetic identification (*8*), complemented with *flaA* gene sequencing. A benefit of this method is the increasing availability of epidemiologic databases in which isolates can be compared (e.g., http://pubmlst.org/campylobacter). The isolates were thawed and cultured on conventional blood agar (Columbia agar II containing 8% [vol/vol] whole horse blood) at 42°C in a microaerobic gas environment, with the CampyGen gas-generating system (CN0025A; Oxoid Ltd, Basingstoke, UK) and the BBL GasPak system (BD, Franklin Lakes, NJ, USA). Bacterial DNA was prepared by making a suspension of freshly grown bacterial cells in 200 μL of phosphate-buffered saline (Sigma, St. Louis, MO, USA). Genomic DNA was extracted by use of a Bio Robot M48 (QIAGEN, Hilden, Germany) with a MagAttract DNA mini M48 kit, according to the instructions of the manufacturer. The PCR amplification and nucleotide sequencing followed

the original protocol in principle (*8*). The amplification products were purified and sequenced by using internal separated nested primer pairs.

The 3 isolates from macaroni penguins were all of the same genotype (sequence type [ST]–45) and thus have a common origin. The ST-45 sequence type is the central genotype of the ST-45 clonal complex, a complex often associated with human disease and asymptomatic infection in poultry (*9*,*10*). Indeed, nearly 42% of the ST-45 samples available in the MLST database have been isolated from humans (31% from poultry), and similar percentages have been observed for the ST-45 clonal complex as a whole. The ST-45 clonal complex is large (composed of 195 individual STs) and has been isolated to date from a variety of environmental sources and different geographic regions, with the exception of the Arctic. The isolates were identical at the *flaA* locus, all having the allele 21/peptide 2 designation (http://pubmlst.org/campylobacter/flaA). This particular peptide is found in 31 records in the database and is thus not unique to the penguin isolates.

Our MLST analysis confirms that the *C. jejuni* isolates from the penguins were of a genotype common among humans with disease and among our food animals. *C. jejuni* is not normally distributed among Antarctic animals (*1*,*2*), which indicates that this strain may have been imported through human activities. On Bird Island, such activities were carried out by scientists at the British Antarctic Survey base. At the time of the study, toilet wastes from the station were emptied into the surrounding waters, providing a possible transmission route for human-associated *C. jejuni* to reach wildlife, including penguins. Other possible sources of the *C. jejuni* infections include wastes from passing ships or seabirds that pick up the bacteria during offshore feeding excursions (for albatrosses, these can be ≈1,000 km). Once established in a penguin colony, a gastrointestinal pathogen may be transmitted rapidly among individual birds as they are breeding densely and producing a large amount of feces (guano) in the colony. *C. jejuni* infection in birds is normally not associated with overt disease, but other and possibly more devastating pathogens introduced to Antarctic animals could potentially cause outbreaks.

Figure. Macaroni penguins (*Eudyptes chrysolophus*). Photo by Jonas Bonnedahl

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## Acute Diarrhea in Children after 2004 Tsunami, Andaman Islands

**To the Editor**: The Andaman Islands, population ≈350,000, are a territory of India located in the Bay of Bengal, northwest of Indonesia. On December 26, 2004, these islands were struck by an earthquake measuring 9.1 on the Richter scale (*1*) and by the ensuing Great Asian Tsunami (*2*). The fault slip, which caused permanent land subsidence of several meters (*3*) and ingression of sea water, resulted in the displacement of most survivors, many of whom were forced to live in temporary camps on higher ground for periods of more than a year. About 80% of the water supply lines were broken (*4*) and so were most sewage lines, making the situation ideal for transmission of water-borne diseases.

Because an outbreak of cholera had occurred in the Andaman and Nicobar Islands in 2002 (*5*), we were apprehensive about outbreaks of infectious diseases after the tsunami, particularly among children, who are less immune to most infections; therefore, we increased our efforts to identify and contain these possible outbreaks as quickly as possible. However, except for a cluster of cases of rotaviral diarrhea (*6*), no major infectious disease outbreak occurred among residents of the Andaman Islands in the year that followed the tsunami.

Although the incidence of severe cases of diarrhea among children admitted to G.B. Pant Hospital in Port Blair, the only referral hospital in the Andaman Islands, varied greatly from month to month during 2001–2007, the incidence began decreasing after 2005, as indicated by the 12-month moving average (Figure). The mean number of cases per year fell from 361.4 during 2001–2005 to only 255.0 during 2006 and 2007 ($p = 0.00025$).

The estimated annual incidence of acute diarrhea per 100,000 children in the Andaman and Nicobar Islands was 609 in 2001, 580 in 2002, 595 in 2003, 601 in 2004, 571 in 2005, 370 in 2006, and 420 in 2007. For these incidence estimates, the population at risk during the years 2002–2007 was calculated by extrapolating from the 2001 census population on the basis of an annual population growth rate of 1.53% (the average for 1991–2001) and assuming that children ≤15 years old constituted 36.2% of the total population each year (as they did in 2001). The reduction in the number of acute cases of childhood diarrhea began several months after the tsunami, when the water and sewage systems of the islands had been repaired and renovated in many areas.

According to official reports, the cost of the restoration and renovation of the water and sewage systems after the tsunami was 389.9 million rupees, >2× the projected cost of work on the water and sanitation systems (172.9 million rupees) prior to the tsunami (*4*). In the aftermath of the tsunami, 52 km of new pipelines were laid and 12.5 km of old pipelines were replaced. Water supplies were augmented in 49 areas (*4*). The revamped water and sewage systems eliminated many sources of fecal contamination.

Moreover, by the middle of 2005, post-disaster assistance had been provided by voluntary organizations, missionaries, nongovernmental organizations, and government agencies from mainland India and abroad. This assistance resulted in further improvements in the area's public sanitation infrastructure and hygiene, particularly in the temporary shelters that displaced residents were living in; it also raised awareness among island residents about the threat of water-borne diseases. All of these factors were likely con-

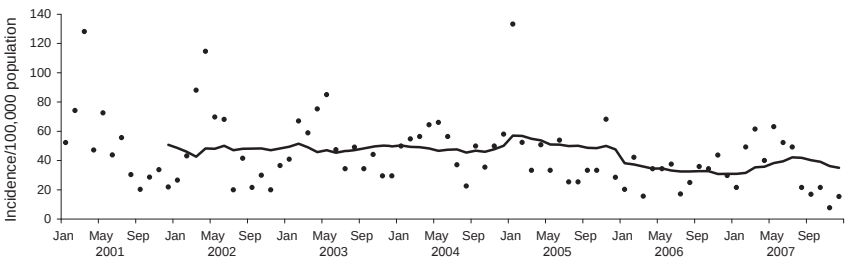


Figure. Estimated monthly incidence (black dots) of acute diarrhea among children ≤15 years of age in the Andaman Islands and 12-month moving average of the monthly incidence (black line), 2001–2007. Data based on cases of disease among children admitted to G.B. Pant Hospital, Port Blair, Andaman and Nicobar Islands, India.

tributors to the decline in the number of cases of acute diarrhea in children after the tsunami. Although out-migration of island residents or a reduction in case detection after the tsunami also could have contributed to the observed decline in cases of diarrhea, no large-scale migration was reported during the period, and disease surveillance systems were in fact strengthened after the tsunami and further strengthened with the introduction of the Integrated Disease Surveillance Program.

In summary, we found that the incidence of acute diarrhea among children of the Andaman Islands decreased within months after the 2004 tsunami. This result highlights the importance of public health and sanitation measures after a natural disaster.

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## Population-Attributable Risk Estimates for *Campylobacter* Infection, Australia

**To the Editor:** Many industrialized countries have a high incidence of *Campylobacter* infections. An estimated 250,000 cases of *Campylobacter* infection occur annually in the United States (*1*), and several sequelae compound the impact of these infections. The incidence of *Campylobacter* infections is also important to policymakers—in the United Kingdom it is used to assess foodborne disease–reduction strategies (*2*)—and governments worldwide rely on the findings of epidemiologic and microbiological studies on *Campylobacter* infection to shape their food-safety policies.

Population-attributable fractions provide added value in case–control studies by helping researchers identify the most important risk factors for a condition on the basis of risk association and frequency of exposure. In an analysis of data from a previous case–control study of *Campylobacter* infection (*3*), Stafford et al. (*4*) used population-attributable fractions to estimate the annual number of *Campylobacter* infection cases among Australians ≥5 years of age that were attributable to each risk factor from that study. Using this technique, they estimated that 50,500 cases annually can be attributed directly to eating chicken.

Population-attributable fractions have been defined as "the proportion of disease cases over a specified time that would be prevented following elimination of … exposure [to the specified risk factors]" (*5*). Therefore, removing exposure to factors not associated with disease risk will not affect disease incidence. Stafford and colleagues implicitly acknowledge this in their methods: "We calculated PARs [population-attributable risks] … for each variable that was significantly associated with an increased risk for infection." It is surprising, therefore, that they subsequently included consumption of cooked chicken in their extrapolation, even though this exposure was not significantly associated with illness (adjusted odds ratio 1.4, 95% confidence interval 1.0–1.9, p = 0.06). Because they attributed 35,500 of the 50,500 cases of *Campylobacter* infection to the consumption of cooked chicken, I believe that Stafford et al. overestimated the role of chicken consumption in cases of *Campylobacter* infection by a factor of 3.4.

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**In Response:** Gillespie (*1*) questions whether we should have treated consumption of cooked chicken as a contributing factor in estimating population-attributable risk (PAR) for *Campylobacter* infection because it was not a significant risk factor (adjusted odds ratio [aOR] 1.4, 95% confidence interval [95% CI] 1.0–1.9, p = 0.06). Although on strict statistical grounds Gillespie is correct, we believe that consumption of cooked chicken warrants consideration as a risk factor on the basis of biological plausibility, possible misclassification of the "cooked" status of chicken eaten by study participants, and previous empirical evidence.

Many case–control studies have demonstrated that consumption of any chicken is a risk factor for *Campylobacter* infection (*2*,*3*), although others have shown that only the consumption of undercooked chicken or chicken eaten outside of the home is a risk factor (*4*,*5*). We acknowledged in our study that some misclassification of exposure is likely in any study of reported food-consumption habits and that such misclassification is a major limitation of case–control studies. Because of the difficulty of determining whether previously eaten chicken had been thoroughly cooked or recontaminated after having been cooked, particularly if it was purchased outside of the home, at least some study participants who reported eating cooked chicken could have acquired their infection from the chicken meat. Moreover, as noted above, results from other case–control studies showing an association between disease risk and consumption of chicken or poultry have been based on study participants' reported consumption of both undercooked and cooked chicken.

In our study, we included chicken consumption in our multivariable models as either "cooked chicken" or "undercooked chicken" but not as "chicken." However, in our univariate analysis, consumption of any chicken (i.e., cooked or undercooked) was significantly associated with illness (OR 1.6, 95% CI 1.2–2.1), and it was also significantly associated with illness when included in a multivariable model as a single variable (aOR 1.7, 95% CI 1.2–2.3). Results of a univariate assessment of various types of cooked chicken meat showed that consumption of chicken fillet (OR 1.2), chicken kebabs (OR 1.7), and bought barbecued chicken (OR 1.2) was each associated with increased risk for illness. Other studies have similarly shown consumption of cooked, fried, or barbecued chicken to be significantly associated with risk for *Campylobacter* infection (*3*,*4*).

The strength of our approach is that we were able to estimate the number of cases of campylobacteriosis attributable to chicken consumption each year and to assess the uncertainty of these estimates. Because we were unable to calculate CIs for the estimated number of cases not detected by surveillance, we used computer simulation to generate an overall distribution of the number of cases that could plausibly be attributed to chicken consumption; the estimated range was 10,000–105,000 cases annually. This represents a conservative approach to assessing the contribution of chicken consumption to *Campylobacter* infections in Australia. Thus, although we concur that the inclusion of cooked chicken as a risk factor for *Campylobacter* infections was debatable on strictly statistical grounds, we believe that including cooked chicken in our estimates of the PAR for and community incidence of these infections was reasonable.

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DOI: 10.3201/eid1505.090080

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# The Travel and Tropical Medicine Manual, 4th Edition

**Elaine C. Jong and Christopher A. Sanford, editors**

**Saunders/Elsevier, Philadelphia, PA, USA, 2008**

**ISBN: 978-1-4160-2613-6**
**Pages: 682; Price: US $55.95**

Like the previous editions of The Travel and Tropical Medicine Manual, the 4th edition continues to successfully teach the fundamentals of travel and tropical medicine and serve as a useful reference. The manual is well-organized and literally (measuring 12 × 20 cm) and figuratively handy. It has many appealing tables and figures, a colorful cover, and a reasonable price. It remains appropriately titled, with some chapters written from a travel perspective and others from a tropical medicine perspective.

The manual contains 45 chapters divided into 7 sections (Pre-Travel Advice, Advice for Special Travelers, Fever, Diarrhea, Skin Lesions, Sexually Transmitted Diseases, and Worms). The current edition has a new coeditor (Christopher A. Sanford) and new chapters on urban medicine and health advice for long-term expatriates. Its audience should continue to be primary care providers who routinely counsel patients on travel-related issues, infectious disease physicians with an interest in travel medicine, other travel medicine practitioners, and trainees doing rotations in travel medicine. However, its technical terminology and concepts prevent the manual from achieving its stated goal of being a "perfect source" for travelers.

The greatest strengths of the manual continue to be its practical suggestions for counseling travelers prior to their departure and evaluating patients with post-travel illnesses. Notable chapters discuss general approaches to travel medicine, immunizations, managing jet lag and motion sickness, counseling HIV-infected travelers, malaria prevention, avoiding and self-treating travelers' diarrhea, evaluating diarrhea in returned travelers, tropical dermatology and sexually transmitted infections. The manual also has outstanding tables in its approach to travel medicine chapter. Other informative tables describe the safe selection of food and water, drugs for preventing and treating traveler's diarrhea, the use of melatonin to prevent jet lag, potential interactions between antiretroviral and travel-related medications, and the differential diagnoses of travel-related skin lesions. The manual wisely refers readers to internet sites such as www.cdc.gov/travel for up-to-date travel advice.

Although the manual has shortcomings, it has no serious deficiencies. Incorporating contributions from 49 authors results in chapters of variable quality, some redundancies, and occasional omissions. A 20-page chapter on water disinfection appears disproportionately lengthy when compared with the immediately preceding 12-page chapter on the prevention and self-treatment of traveler's diarrhea. Although the chapter on women travelers discusses male condoms superficially, this important topic is well addressed in the chapter on sexually transmitted infections. In future editions of the manual, the editors should consider including more than 2 photographs in the 74-page section on skin lesions, using a larger font, and enlarging the figures.

Clinicians who routinely evaluate patients before or after traveling should definitely consider purchasing this manual, reading select chapters in the pretravel and special travelers sections, and keeping it in their travel clinic as a useful reference.

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**Erratum—Vol. 15, No. 3**

A reference was missing from the article Methicillin-Resistant *Staphylococcus aureus* in Poultry (D. Persoons et al.). In the Table accompanying the article, the data on *spa* types isolated from pigs were originally described in de Neeling AJ, van den Broek MJM, Spalburg EC, van Santen-Verheuvel MG, Dam-Deisz WDC, Boshuizen HC, et al. High prevalence of methicillin-resistant *Staphylococcus aureus* in pigs. Vet Microbiol. 2007;122:366–72. The article has been corrected online (www.cdc.gov/EID/content/15/3/452.htm).

DOI: 10.3201/eid1505.999000

# Nothing But Tears

**Damian J. Krysan**

What do I do
My little one,
Lying oh so low?

Is all that I do
For me or for you,
Hoping for a cure?

Do I take your hand,
Do I let you lie,

Do I leave you alone in the snow?

To do no harm,
To close my bag,

To treat you with nothing but tears.

Dr Krysan is assistant professor of pediatrics in the Division of Pediatric Infectious Diseases, University of Rochester Pediatrics, Rochester, New York. His research interests are yeast cell wall biology, *Candida albicans* genetics, and high-throughput screening for antifungal drugs.

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Alexis Rockman (b. 1962) The Farm (2000) Oil and acrylic on wood (243.8 cm × 304.8 cm) Collection of JGS, Inc. Courtesy of the artist.

# Nature Isn't What It Used To Be

**Polyxeni Potter**

"There are also cocks, which are extraordinary size, and have their crests not red as elsewhere, or at least in our country, but have the flower-like coronals of which the crest is formed variously colored," wrote traveler and geographer Megasthenes 17 hundred years ago. "Their rump feathers are neither curved nor wreathed but are of great breadth and they trail them in the way peacocks trail their tails, when they neither straighten nor erect them: the feathers of these Indian cocks are in color golden and also dark-blue like the smaragdus." These impressive cocks, and other fantastic creatures, populated *Ta Indica*, the author's account of India. Megasthenes' flamboyant beasts, some of them sporting nonstandard digits and extra heads, may have come from the stories of people he met in his travels and not his own observations. Nonetheless, they could be describing the creatures of Alexis Rockman, artist, naturalist, author, educator, activist.

"I try to use all of the ways we depict nature and natural history as content," Rockman says, explaining the natural sciences bias of his subjects. "I'm interested in credibility." His love for science and art flourished during childhood in his native New York. "I grew up in the American Museum of Natural History. My mother was an assistant to Margaret Meade … it shaped my perspective. ... Charles R. Knight and Chesley Bonestell were my heroes." His education at the School of Visual Arts complemented long studies of nature in the United States and abroad. "I started out thinking that I would be a scientist. Eventually, over the years, I ended up becoming interested in other types of practices, like certain genres of filmmaking, animation. I think what I ended up doing was really a combination of all those different interests. I've always been interested in the history of the representation of nature."

Rockman's style, now realistic now abstract, has eluded traditional descriptions, "I try to make it as credible as possible without making it boring." Some have seen surrealism in his unusual depictions of plants, animals, and humans, "What I am after … is the disturbing part or the transformative part." Embracing popular culture, he flaunts it with great precision in a manner called hyperrealism in the tradition of Grant Wood, who captured the rural Midwest of the 20th century, especially in his American Gothic. Like pop art icon Andy Warhol, he is comfortable with modern technology and skillful with its agility and interactivity, "computer-manipulated, archetypal images that we've all seen, or if we haven't seen them we feel that we know them." The Farm, on this month's cover, was commissioned by Creative Time, a public arts organization, as a New York City billboard. "I like to put people off-kilter by breaking up expected visual patterns."

The constant struggles between nature and its creatures engage all of Rockman's interests from evolution, climate

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change, and genetic engineering to the failures of technology. Like his idol H.G. Wells, the artist portrays humans as, "the unnatural animal, the rebel child of nature," that, "more and more … turns himself against the harsh and fitful hand that reared him."

The Farm portrays this favorite theme. Developing it follows the artist's usual path of discovery, which involves learning from an expert, in this case a molecular biologist, about genetics and artificial selection. The subject drives the medium. The story unfolds with the clarity of a high-definition screen. A field of soybeans extends as far as the eye can see; against it, an allegorical tableau, in Rockman's words, "the way humans have altered their landscape."

"The way I constructed it is that, as in a lot of Western culture, we read things from left to right," he explained in an interview. "On the left side of the image are the ancestral species of the chicken, the pig, the cow, and the mouse"; on the right, their contemporary versions. Farther to the right are "permutations of what things might look like in the future." The transition from wild cow and boar to familiar barnyard beasts to grotesque technologically engineered models is precise and tactical, and the animals still maintain some original species characteristics. A fruit fly, a strand of DNA, an overmanipulated dog inside a prize-winning blue rosette compete for attention with the cocks placed conspicuously on the fence, straddling the horizon, challenging Megasthenes. Tomatoes created to fit the shipping crate, loaf-shaped watermelons, and multicolor corn complete the picture, occupying several layers of time and genetic activity in the permissive context the artist calls "democratic space."

Rockman's vision of biotechnology is riddled with clues and inside jokes rooted in economic, social, ethical, and other concerns. It's a vision he wants to popularize, "It has to be decipherable to a six-year-old child. I try to construct it as an onion with different layers of meaning and iconography." The Farm succeeds in this regard, perhaps even beyond the artist's intentions. This icon of biotechnology is also the stage for foodborne disease emergence. The soybean farm is much too close to the farm animals, whose fertile waste deposits seep into the nearby water used to irrigate the plants. The fence is useless for keeping out rodents or birds and their microbial deposits. And human manipulation, intended to make larger more efficient food animals, may have unintended consequences. If the DNA strands were animated, they would be turning wildly.

A 2006 multistate outbreak of *E. coli* O157:H7 infection was associated with salad. Samples taken from a stream, cattle manure, and feces from wild pigs on ranches in Salinas Valley, California, implicated spinach, but after this outbreak, leafy greens were seen as subject to this type of contamination. Rockman's iconic overlap of urban, agricultural, and cattle-raising elements in ecosystems containing sigmodontine rodents, which are reservoirs of hantaviruses, is a recipe for hantavirus pulmonary syndrome. The syndrome, now found throughout the United States, is rare but deadly. Mad cow disease emerged in Britain, possibly as an interspecies transfer of scrapie from sheep to cattle and moved around the globe through trade. Spread from human to human is now a threat through contaminated hospital equipment and blood transfusions. People who consume antler velvet as a nutritional supplement may also be at risk for exposure to prions.

"Nature isn't what it used to be," Rockman wrote in one of his books. And of course it never was or ever will be. But the irony of that statement awaits future developments. Because, as H.G. Wells put it, "The only true measure of success is the ratio between what we might have done and what we might have been on the one hand, and the thing we have made and the things we have made of ourselves on the other."

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# EMERGING INFECTIOUS DISEASES

## Upcoming Issue

Past, Present, and Possible Future Human Infection with Influenza Virus A Subtype H7

Diphyllobothriasis Associated with Eating Raw Pacific Salmon

Fluoroquinolone-Resistant *Streptococcus pneumoniae* after 7-Valent Conjugate Vaccination

Geographic Clustering of Leishmaniasis in Northeastern Brazil

Influenza Hospitalization Surveillance, Colorado, USA, 2004–2008

Lineage 2 West Nile Virus as Cause of Fatal Neurologic Disease in Horses, South Africa

Racial Disparity in Tuberculosis Rates, Houston, Texas, USA

*Bartonella quintana* in Lice from Homeless Persons, San Francisco, California, USA

Hantaviruses in Rodents and Humans, Inner Mongolia Autonomous Region, China

Drought, Smallpox, and Emergence of *Leishmania braziliensis,* Northeastern Brazil

Murine Typhus in Children, Yucatan, Mexico

Murine Typhus and Leptospirosis as Cause of Acute Undifferentiated Fever, Indonesia

Recovery from Botulinum Toxin Type F in Adult

Rabies in Ferret Badgers, Southeastern China

Nipah Virus Infection in Dogs, Southwestern Malaysia, 1999

Merkel Cell Polyomavirus Strains in Patients with Merkel Cell Carcinoma

Phocine Distemper Virus in Northern Sea Otters in the Pacific Ocean, Alaska, USA

Vancomycin-Resistant *Staphylococcus aureus,* Michigan, USA, 2007

Diversity of *Anaplasma phagocytophilum* Strains, USA

Increasing Incidence of Zoonotic Visceral Leishmaniasis on Crete, Greece

Complete list of articles in the June issue at
http://www.cdc.gov/eid/upcoming.htm

## Upcoming Infectious Disease Activities

**June 4–5, 2009**
Drug-resistant and Vaccine-escape Hepatitis B Virus Mutants: Emergence and Surveillance
Atlanta, GA, USA
http://www.cdc.gov/hepatitis/hbvsymposium2009

**June 18–21, 2009**
26th International Congress of Chemotherapy and Infection
Sheraton Centre Toronto Hotel
Toronto, Canada
http://www.icc-09.com

**June 21–23, 2009**
6th International Congress on Bartonella as Medical and Veterinary Pathogens
Queen Hotel
Chester, United Kingdom
http://www.liv.ac.uk/university/vets/cpd/documents/All_information_bartonella.pdf

**June 28–July 1, 2009**
18th ISSTDR: International Society for STD Research
QEII Conference Centre
London, United Kingdom
http://www.isstdrlondon2009.com

**August 3–4, 2009**
UC Berkeley CIDER SUMMIT 2009: "Advances in the Control and Epidemiology of Emerging Infectious Diseases"
Sheraton Gateway Hotel
Burlingame, CA, USA
http://www.idready.org/CIDERSummit2009.html

**August 10–21, 2009**
11th International Dengue Course
http://www.ipk.sld.cu/cursos/dengue2009/indexen.htm

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## Article Title:

## Increased Risk for Severe Malaria in HIV-1–infected Adults, Zambia

## CME Questions

**1. Which of the following best explains the large scale of the HIV-1–malaria interaction that has emerged in the last decade?**

A. Longer survival of patients infected with HIV
B. Effects of increased antiretroviral drug use
C. Poor immune function and higher susceptibility
D. Poor control of malaria worldwide

**2. A 28-year-old Zambian patient presents with a fever of 38.5°C, *Plasmodium falciparum* on thick smear with 120 parasites per 200 white blood cells, and jaundice. Which of the following best describes the likely diagnosis?**

A. Uncomplicated malaria
B. Moderately severe malaria
C. Severe malaria
D. HIV and malaria

**3. The study noted the importance of fever as an indicator of severe malaria in patients infected with HIV-1. Which of the following features were most commonly encountered in addition to fever?**

A. Impaired consciousness and jaundice
B. Impaired consciousness and hypoglycemia
C. Multiple convulsions and jaundice
D. Hypoglycemia and jaundice

**4. Which of the following best describes the association between HIV-1 infection and risk for severe malaria in the population studied?**

A. HIV-1 infection is a risk factor for uncomplicated and severe malaria
B. Risk for severe malaria is only increased in patients with HIV-1 with a CD4 count <250 cells/μL
C. HIV-1 infection increases the risk for severe malaria
D. Risk for severe malaria is increased only in patients with AIDS

## 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 |

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**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.

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**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.