

EMERGING INFECTIOUS DISEASES

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A Peer-Reviewed Journal Tracking and Analyzing Disease Trends Vol.8, No.9, September 2002



Biofilms: Microbial Life on Surfaces



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Biofilms: Microbial Life on Surfaces

Rodney M. Donlan*

Microorganisms attach to surfaces and develop biofilms. Biofilm-associated cells can be differentiated from their suspended counterparts by generation of an extracellular polymeric substance (EPS) matrix, reduced growth rates, and the up- and down-regulation of specific genes. Attachment is a complex process regulated by diverse characteristics of the growth medium, substratum, and cell surface. An established biofilm structure comprises microbial cells and EPS, has a defined architecture, and provides an optimal environment for the exchange of genetic material between cells. Cells may also communicate via quorum sensing, which may in turn affect biofilm processes such as detachment. Biofilms have great importance for public health because of their role in certain infectious diseases and importance in a variety of device-related infections. A greater understanding of biofilm processes should lead to novel, effective control strategies for biofilm control and a resulting improvement in patient management.

For most of the history of microbiology, microorganisms have primarily been characterized as planktonic, freely suspended cells and described on the basis of their growth characteristics in nutritionally rich culture media. Rediscovery of a microbiologic phenomenon, first described by van Leeuwenhoek, that microorganisms attach to and grow universally on exposed surfaces led to studies that revealed surface-associated microorganisms (biofilms) exhibited a distinct phenotype with respect to gene transcription and growth rate. These biofilm microorganisms have been shown to elicit specific mechanisms for initial attachment to a surface, development of a community structure and ecosystem, and detachment.

A Historical Basis

A biofilm is an assemblage of surface-associated microbial cells that is enclosed in an extracellular polymeric substance matrix. Van Leeuwenhoek, using his simple microscopes, first observed microorganisms on tooth surfaces and can be credited with the discovery of microbial biofilms. Heukelekian and Heller (1) observed the "bottle effect" for marine microorganisms, i.e., bacterial growth and activity were substantially enhanced by the incorporation of a surface to which these organisms could attach. Zobell (2) observed that the number of bacteria on surfaces was dramatically higher than in the surrounding medium (in this case, seawater). However, a detailed examination of biofilms would await the electron microscope, which allowed high-resolution photomicroscopy at much higher magnifications than did the light microscope. Jones et al. (3) used scanning and transmission electron microscopy to examine biofilms on trickling filters in a wastewater treatment plant and showed them to be composed of a variety of organisms (based on cell morphology). By using a specific polysaccharide-stain called Ruthenium red and coupling this with osmium tetroxide fixative, these researchers were also able to show that the matrix material surrounding and enclosing cells

in these biofilms was polysaccharide. As early as 1973, Characklis (4) studied microbial slimes in industrial water systems and showed that they were not only very tenacious but also highly resistant to disinfectants such as chlorine. Based on observations of dental plaque and sessile communities in mountain streams, Costerton et al. (5) in 1978 put forth a theory of biofilms that explained the mechanisms whereby microorganisms adhere to living and nonliving materials and the benefits accrued by this ecologic niche. Since that time, the studies of biofilms in industrial and ecologic settings and in environments more relevant for public health have basically paralleled each other. Much of the work in the last 2 decades has relied on tools such as scanning electron microscopy (SEM) or standard microbiologic culture techniques for biofilm characterization. Two major thrusts in the last decade have dramatically impacted our understanding of biofilms: the utilization of the confocal laser scanning microscope to characterize biofilm ultrastructure, and an investigation of the genes involved in cell adhesion and biofilm formation.

Biofilm Defined

A biofilm is an assemblage of microbial cells that is irreversibly associated (not removed by gentle rinsing) with a surface and enclosed in a matrix of primarily polysaccharide material. Noncellular materials such as mineral crystals, corrosion particles, clay or silt particles, or blood components, depending on the environment in which the biofilm has developed, may also be found in the biofilm matrix. Biofilm-associated organisms also differ from their planktonic (freely suspended) counterparts with respect to the genes that are transcribed. Biofilms may form on a wide variety of surfaces, including living tissues, indwelling medical devices, industrial or potable water system piping, or natural aquatic systems. The variable nature of biofilms can be illustrated from scanning electron micrographs of biofilms from an industrial water system and a medical device, respectively (Figures 1 and 2). The water system biofilm is highly complex, containing

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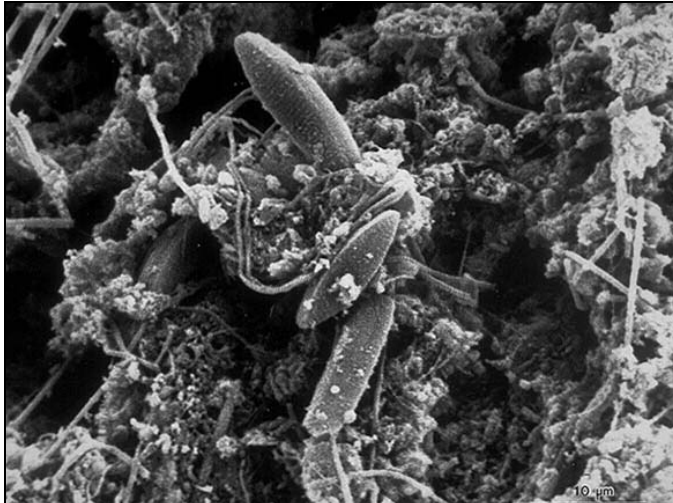


Figure 1. Scanning electron micrograph of a native biofilm that developed on a mild steel surface in an 8-week period in an industrial water system. Rodney Donlan and Donald Gibbon, authors. Licensed for use, American Society for Microbiology Microbe Library. Available from: URL: <http://www.microbelibrary.org/>

corrosion products, clay material, fresh water diatoms, and filamentous bacteria. The biofilm on the medical device, on the other hand, appears to be composed of a single, coccoid organism and the associated extracellular polymeric substance (EPS) matrix.

Attachment

The solid-liquid interface between a surface and an aqueous medium (e.g., water, blood) provides an ideal environment for the attachment and growth of microorganisms. A clear picture of attachment cannot be obtained without considering the effects of the substratum, conditioning films forming on the substratum, hydrodynamics of the aqueous medium, characteristics of the medium, and various properties of the cell surface. Each of these factors will be considered in detail.

Substratum Effects

The solid surface may have several characteristics that are important in the attachment process. Characklis et al. (6) noted that the extent of microbial colonization appears to increase as the surface roughness increases. This is because shear forces are diminished, and surface area is higher on rougher surfaces. The physicochemical properties of the surface may also exert a strong influence on the rate and extent of attachment. Most investigators have found that microorganisms attach more rapidly to hydrophobic, nonpolar surfaces such as Teflon and other plastics than to hydrophilic materials such as glass or metals (7–9). Even though results of these studies have at times been contradictory because no standardized methods exist for determining surface hydrophobicity, some kind of hydrophobic interaction apparently occurs between the cell surface and the substratum that would enable the cell to overcome the repulsive forces active within a certain distance from the substratum surface and irreversibly attach.

Conditioning Films

A material surface exposed in an aqueous medium will inevitably and almost immediately become conditioned or coated by polymers from that medium, and the resulting chemical modification will affect the rate and extent of microbial attachment. Loeb and Neihof (10) were the first to report the formation of these conditioning films on surfaces exposed in seawater. These researchers found that films were organic in nature, formed within minutes of exposure, and continued to grow for several hours. The nature of conditioning films may be quite different for surfaces exposed in the human host. A prime example may be the proteinaceous conditioning film called “acquired pellicle,” which develops on tooth enamel surfaces in the oral cavity. Pellicle comprises albumin, lysozyme, glycoproteins, phosphoproteins, lipids, and gingival crevice fluid (11); bacteria from the oral cavity colonize pellicle-conditioned surfaces within hours of exposure to these surfaces. Mittelman noted that a number of host-produced conditioning films such as blood, tears, urine, saliva, intervascular fluid, and respiratory secretions influence the attachment of bacteria to biomaterials (12). Ofek and Doyle (13) also noted that the surface energy of the suspending medium may affect hydrodynamic interactions of microbial cells with surfaces by altering the substratum characteristics.

Hydrodynamics

In theory, the flow velocity immediately adjacent to the substratum/liquid interface is negligible. This zone of negligible flow is termed the hydrodynamic boundary layer. Its thickness is dependent on linear velocity; the higher the velocity, the thinner the boundary layer. The region outside the boundary layer is characterized by substantial mixing or turbulence. For flow regimes characterized as laminar or minimally turbulent, the hydrodynamic boundary layer may substantially affect cell-substratum interactions. Cells behave as particles in a liquid, and the rate of settling and association with a submerged surface will depend largely on the velocity characteris-

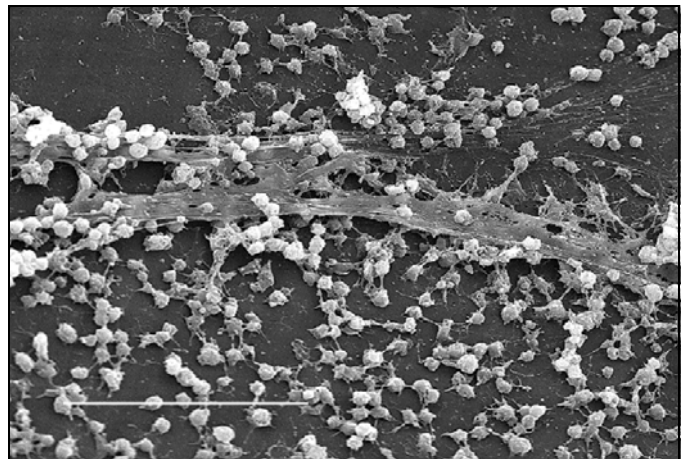


Figure 2. Scanning electron micrograph of a staphylococcal biofilm on the inner surface of an indwelling medical device. Bar, 20 μm. Used with permission of Lippincott Williams & Wilkins.

tics of the liquid. Under very low linear velocities, the cells must traverse the sizeable hydrodynamic boundary layer, and association with the surface will depend in large part on cell size and cell motility. As the velocity increases, the boundary layer decreases, and cells will be subjected to increasingly greater turbulence and mixing. Higher linear velocities would therefore be expected to equate to more rapid association with the surface, at least until velocities become high enough to exert substantial shear forces on the attaching cells, resulting in detachment of these cells (14). This finding has been confirmed in studies by Rijnaarts et al. (15) and Zheng et al. (16).

Characteristics of the Aqueous Medium

Other characteristics of the aqueous medium, such as pH, nutrient levels, ionic strength, and temperature, may play a role in the rate of microbial attachment to a substratum. Several studies have shown a seasonal effect on bacterial attachment and biofilm formation in different aqueous systems (17,18). This effect may be due to water temperature or to other unmeasured, seasonally affected parameters. Fletcher (19,20) found that an increase in the concentration of several cations (sodium, calcium, lanthanum, ferric iron) affected the attachment of *Pseudomonas fluorescens* to glass surfaces, presumably by reducing the repulsive forces between the negatively charged bacterial cells and the glass surfaces. Cowan et al. (21) showed in a laboratory study that an increase in nutrient concentration correlated with an increase in the number of attached bacterial cells.

Properties of the Cell

Cell surface hydrophobicity, presence of fimbriae and flagella, and production of EPS all influence the rate and extent of attachment of microbial cells. The hydrophobicity of the cell surface is important in adhesion because hydrophobic interactions tend to increase with an increasing nonpolar nature of one or both surfaces involved (i.e., the microbial cell surface and the substratum surface). Most bacteria are negatively charged but still contain hydrophobic surface components, as noted by Rosenberg and Kjelleberg (22). Fimbriae, i.e., nonflagellar appendages other than those involved in transfer of viral or bacterial nucleic acids (called pili), contribute to cell surface hydrophobicity. Most fimbriae that have been examined contain a high proportion of hydrophobic amino acid residues (22). Fimbriae play a role in cell surface hydrophobicity and attachment, probably by overcoming the initial electrostatic repulsion barrier that exists between the cell and substratum (23). A number of aquatic bacteria possess fimbriae, which have also been shown to be involved in bacterial attachment to animal cells (23). Rosenburg et al. (24) and Bullitt and Makowski (25) provided evidence for the role of fimbriae in bacterial attachment to surfaces.

Other cell surface properties may also facilitate attachment. Several studies have shown that treatment of adsorbed cells with proteolytic enzymes caused a marked release of attached bacteria (26,27), providing evidence for the role of

proteins in attachment. Bendinger et al. (9) found that mycolic acid-containing organisms (*Corynebacterium*, *Nocardia*, and *Mycobacterium*) were more hydrophobic than were nonmycolic acid-containing bacteria, and increase in mycolic acid chain length generally coincided with increase in hydrophobicity. For most strains tested, adhesion was greater on hydrophobic materials. The O antigen component of lipopolysaccharide (LPS) has also been shown to confer hydrophilic properties to gram-negative bacteria. Williams and Fletcher (28) showed that mutants of *P. fluorescens* lacking the O antigen adhered in greater numbers to hydrophobic materials.

As early as 1971, Marshall et al. (29) provided evidence based on SEM that attached bacteria were associated with the surface via fine extracellular polymeric fibrils. Fletcher et al. (30) found that treatment of attached freshwater bacteria with cations resulted in contraction of the initial adhesives (decrease in the cell distance from the substratum), supporting the idea that this material was an anionic polymer. Cations have been shown to cross-link the anionic groups of polymers (such as polysaccharides), resulting in contraction. Beech and Gaylarde (31) found that lectins inhibited but did not prevent attachment. Glucosidase and N-acetylglucosaminidase reduced attachment for *P. fluorescens*, while NAG reduced attachment for *Desulfovibrio desulfuricans*. Lectins preferentially bind to polysaccharides on the cell surface or to the EPS. Binding of lectins by the cells would minimize the attachment sites and affect cell attachment if polysaccharides were involved in attachment. Zottola (32) confirmed the role of polysaccharides in attachment in studies with *Pseudomonas fragi*.

Korber et al. (33) used motile and nonmotile strains of *P. fluorescens* to show that motile cells attach in greater numbers and attach against the flow (backgrowth) more rapidly than do nonmotile strains. Nonmotile strains also do not recolonize or seed vacant areas on a substratum as evenly as motile strains, resulting in slower biofilm formation by the nonmotile organisms. Flagella apparently play an important role in attachment in the early stages of bacterial attachment by overcoming the repulsive forces associated with the substratum.

In light of these findings, cell surface structures such as fimbriae, other proteins, LPS, EPS, and flagella all clearly play an important role in the attachment process. Cell surface polymers with nonpolar sites such as fimbriae, other proteins, and components of certain gram-positive bacteria (mycolic acids) appear to dominate attachment to hydrophobic substrata, while EPS and lipopolysaccharides are more important in attachment to hydrophilic materials. Flagella are important in attachment also, although their role may be to overcome repulsive forces rather than to act as adsorbents or adhesives.

The attachment of microorganisms to surfaces is a very complex process, with many variables affecting the outcome. In general, attachment will occur most readily on surfaces that are rougher, more hydrophobic, and coated by surface "conditioning" films. An increase in flow velocity, water temperature, or nutrient concentration may also equate to increased

attachment, if these factors do not exceed critical levels. Properties of the cell surface, specifically the presence of fimbriae, flagella, and surface-associated polysaccharides or proteins, also are important and may possibly provide a competitive advantage for one organism where a mixed community is involved. Table 1 summarizes the variables important in cell attachment and biofilm formation.

Gene Regulation by Attached Cells

Evidence is mounting that up- and down-regulation of a number of genes occurs in the attaching cells upon initial interaction with the substratum. Davies and Geesey (34) demonstrated *algC* up-regulation in individual bacterial cells within minutes of attachment to surfaces in a flow cell system. This phenomenon is not limited to *P. aeruginosa*. Prigent-Combaret et al. (35) found that 22% of these genes were up-regulated in the biofilm state, and 16% were down-regulated. Becker et al. (36) showed that biofilms of *Staphylococcus aureus* were up-regulated for genes encoding enzymes involved in glycolysis or fermentation (phosphoglycerate mutase, triosephosphate isomerase, and alcohol dehydrogenase) and surmised that the up-regulation of these genes could be due to oxygen limitation in the developed biofilm, favoring fermentation. A recent study by Pulcini (37) also showed that *algD*, *algU*, *rpoS*, and genes controlling polyphosphokinase (PPK) synthesis were up-regulated in biofilm formation of *P. aeruginosa*. Prigent-Combaret et al. (35) opined that the expression of genes in biofilms is evidently modulated by the dynamic physicochemical factors external to the cell and may involve complex regulatory pathways.

Biofilm Structure

Extracellular Polymeric Substances

Biofilms are composed primarily of microbial cells and EPS. EPS may account for 50% to 90% of the total organic carbon of biofilms (38) and can be considered the primary matrix material of the biofilm. EPS may vary in chemical and physical properties, but it is primarily composed of polysaccharides. Some of these polysaccharides are neutral or polyanionic, as is the case for the EPS of gram-negative bacteria. The presence of uronic acids (such as D-glucuronic, D-galacturonic, and mannuronic acids) or ketal-linked pyruvates confers the anionic property (39). This property is important because it allows association of divalent cations such as calcium and

magnesium, which have been shown to cross-link with the polymer strands and provide greater binding force in a developed biofilm (38). In the case of some gram-positive bacteria, such as the staphylococci, the chemical composition of EPS may be quite different and may be primarily cationic. Hussain et al. (40) found that the slime of coagulase-negative bacteria consists of a teichoic acid mixed with small quantities of proteins.

EPS is also highly hydrated because it can incorporate large amounts of water into its structure by hydrogen bonding. EPS may be hydrophobic, although most types of EPS are both hydrophilic and hydrophobic (39). EPS may also vary in its solubility. Sutherland (39) noted two important properties of EPS that may have a marked effect on the biofilm. First, the composition and structure of the polysaccharides determine their primary conformation. For example, many bacterial EPS possess backbone structures that contain 1,3- or 1,4- β -linked hexose residues and tend to be more rigid, less deformable, and in certain cases poorly soluble or insoluble. Other EPS molecules may be readily soluble in water. Second, the EPS of biofilms is not generally uniform but may vary spatially and temporally. Leriche et al. (41) used the binding specificity of lectins to simple sugars to evaluate bacterial biofilm development by different organisms. These researchers' results showed that different organisms produce differing amounts of EPS and that the amount of EPS increases with age of the biofilm. EPS may associate with metal ions, divalent cations, other macromolecules (such as proteins, DNA, lipids, and even humic substances) (38). EPS production is known to be affected by nutrient status of the growth medium; excess available carbon and limitation of nitrogen, potassium, or phosphate promote EPS synthesis (39). Slow bacterial growth will also enhance EPS production (39). Because EPS is highly hydrated, it prevents desiccation in some natural biofilms. EPS may also contribute to the antimicrobial resistance properties of biofilms by impeding the mass transport of antibiotics through the biofilm, probably by binding directly to these agents (42).

Biofilm Architecture

Tolker-Nielsen and Molin noted that every microbial biofilm community is unique (43) although some structural attributes can generally be considered universal. The term biofilm is in some ways a misnomer, since biofilms are not a continuous monolayer surface deposit. Rather, biofilms are very

Table 1. Variables important in cell attachment and biofilm formation

Properties of the substratum	Properties of the bulk fluid	Properties of the cell
Texture or roughness	Flow velocity	Cell surface hydrophobicity
Hydrophobicity	pH	Fimbriae
Conditioning film	Temperature	Flagella
	Cations	Extracellular polymeric substances
	Presence of antimicrobial agents	

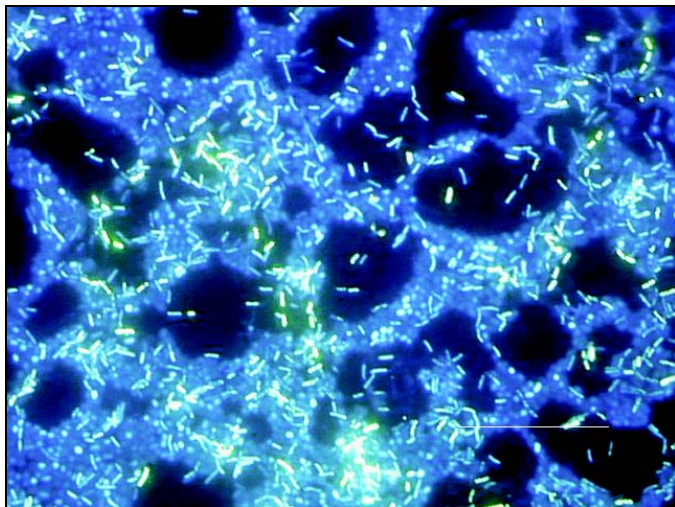


Figure 3. Polymicrobial biofilm grown on a stainless steel surface in a laboratory potable water biofilm reactor for 14 days, then stained with 4,6-diamidino-2-phenylindole (DAPI) and examined by epifluorescence microscopy. Bar, 20 μ .

heterogeneous, containing microcolonies of bacterial cells encased in an EPS matrix and separated from other microcolonies by interstitial voids (water channels) (44). Figure 3 shows a biofilm of *P. aeruginosa*, *Klebsiella pneumoniae*, and *Flavobacterium* spp. that has developed on a steel surface in a laboratory potable water system. This image clearly depicts the water channels and heterogeneity characteristic of a mature biofilm. Liquid flow occurs in these water channels, allowing diffusion of nutrients, oxygen, and even antimicrobial agents. This concept of heterogeneity is descriptive not only for mixed culture biofilms (such as might be found in environmental biofilms) but also for pure culture biofilms common on medical devices and those associated with infectious diseases. Stoodley et al. (45) defined certain criteria or characteristics that could be considered descriptive of biofilms in general, including a thin base film, ranging from a patchy monolayer of cells to a film several layers thick containing water channels. The organisms composing the biofilm may also have a marked effect on the biofilm structure. For example, James et al. (46) showed that biofilm thickness could be affected by the number of component organisms. Pure cultures of either *K. pneumoniae* or *P. aeruginosa* biofilms in a laboratory reactor were thinner (15 μ and 30 μ respectively), whereas a biofilm containing both species was thicker (40 μ). Jones et al. noted that this could be because one species enhanced the stability of the other.

Biofilm architecture is heterogeneous both in space and time, constantly changing because of external and internal processes. Tolker-Nielsen et al. (47) investigated the role of cell motility in biofilm architecture in flow cells by examining the interactions of *P. aeruginosa* and *P. putida* by confocal laser scanning microscopy. When these two organisms were added to the flow cell system, each organism initially formed small microcolonies. With time, the colonies intermixed, showing the migration of cells from one microcolony to the other. The microcolony structure changed from a compact structure to a

looser structure over time, and when this occurred the cells inside the microcolonies were observed to be motile. Motile cells ultimately dispersed from the biofilm, resulting in dissolution of the microcolony.

Interaction of Particles

Structure may also be influenced by the interaction of particles of nonmicrobial components from the host or environment. For example, erythrocytes and fibrin may accumulate as the biofilm forms. Biofilms on native heart valves provide a clear example of this type of interaction in which bacterial microcolonies of the biofilm develop in a matrix of platelets, fibrin, and EPS (48). The fibrin capsule that develops will protect the organisms in these biofilms from the leukocytes of the host, leading to infective endocarditis. Biofilms on urinary catheters may contain organisms that have the ability to hydrolyze urea in the urine to form free ammonia through the action of urease. The ammonia may then raise the pH at the biofilm-liquid interface, resulting in the precipitation of minerals such as calcium phosphate (hydroxyapatite) and magnesium ammonium phosphate (struvite) (49). These minerals can then become entrapped in the biofilm and cause encrustation of the catheter; cases have been described in which the catheter became completely blocked by this mineral build-up. Minerals such as calcium carbonate, corrosion products such as iron oxides, and soil particles may often collect in biofilms of potable and industrial water systems, providing yet another example of particle interactions with biofilms (50).

The Established Community: Biofilm Ecology

The basic structural unit of the biofilm is the microcolony. Proximity of cells within the microcolony (or between microcolonies) (Figure 4A and B) provides an ideal environment for creation of nutrient gradients, exchange of genes, and quorum sensing. Since microcolonies may be composed of multiple species, the cycling of various nutrients (e.g., nitrogen, sulfur, and carbon) through redox reactions can readily occur in aquatic and soil biofilms.

Gene Transfer

Biofilms also provide an ideal niche for the exchange of extrachromosomal DNA (plasmids). Conjugation (the mechanism of plasmid transfer) occurs at a greater rate between cells in biofilms than between planktonic cells (51–53). Ghigo (54) has suggested that medically relevant strains of bacteria that contain conjugative plasmids more readily develop biofilms. He showed that the F conjugative pilus (encoded by the *tra* operon of the F plasmid) acts as an adhesion factor for both cell-surface and cell-cell interactions, resulting in a three-dimensional biofilm of *Escherichia coli*. Plasmid-carrying strains have also been shown to transfer plasmids to recipient organisms, resulting in biofilm formation; without plasmids these same organisms produce only microcolonies without any further development. The probable reason for enhanced conjugation is that the biofilm environment provides minimal shear

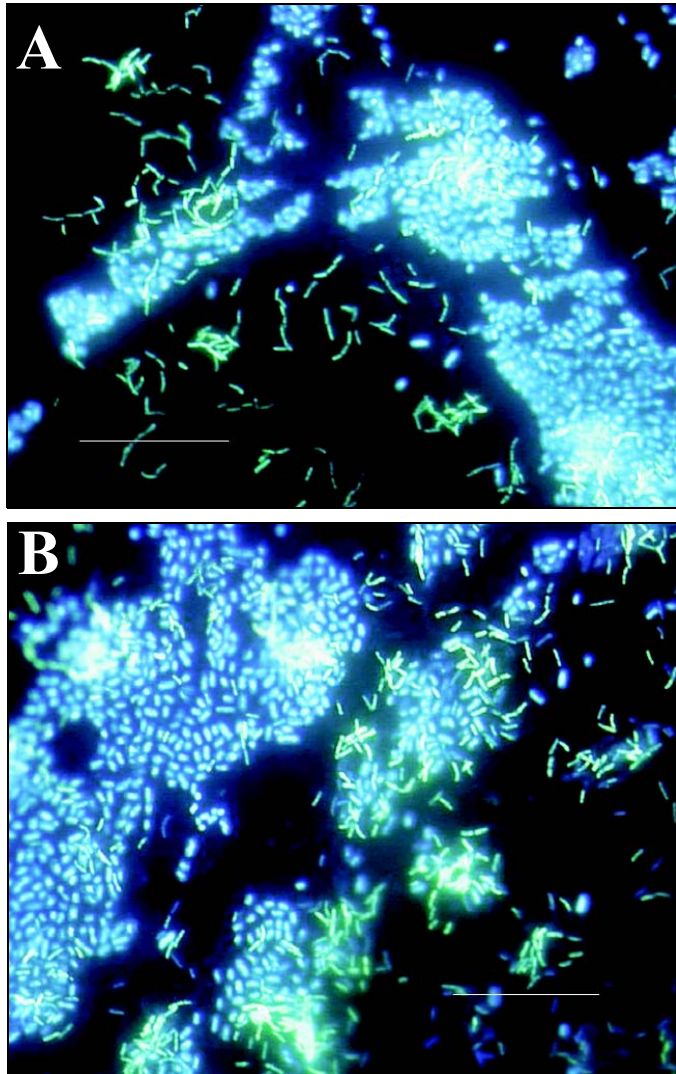


Figure 4A and B. Polymicrobial biofilms grown on stainless steel surfaces in a laboratory potable water biofilm reactor for 7 days, then stained with 4,6-diamidino-2-phenylindole (DAPI) and examined by epifluorescence microscopy. Bar, 20 μ .

and closer cell-to-cell contact. Since plasmids may encode for resistance to multiple antimicrobial agents, biofilm association also provides a mechanism for selecting for, and promoting the spread of, bacterial resistance to antimicrobial agents.

Quorum Sensing

Cell-to-cell signaling has recently been demonstrated to play a role in cell attachment and detachment from biofilms. Xie et al. (55) showed that certain dental plaque bacteria can modulate expression of the genes encoding fimbrial expression (*fimA*) in *Porphyromonas gingivalis*. *P. gingivalis* would not attach to *Streptococcus cristatus* biofilms grown on glass slides. *P. gingivalis*, on the other hand, readily attached to *S. gordonii*. *S. cristatus* cell-free extract substantially affected expression of *fimA* in *P. gingivalis*, as determined by using a reporter system. *S. cristatus* is able to modulate *P. gingivalis* *fimA* expression and prevent its attachment to the biofilm.

Davies et al. (56) showed that two different cell-to-cell sig-

naling systems in *P. aeruginosa*, *lasR-lasI* and *rhlR-rhlI*, were involved in biofilm formation. At sufficient population densities, these signals reach concentrations required for activation of genes involved in biofilm differentiation. Mutants unable to produce both signals (double mutant) were able to produce a biofilm, but unlike the wild type, their biofilms were much thinner, cells were more densely packed, and the typical biofilm architecture was lacking. In addition, these mutant biofilms were much more easily removed from surfaces by a surfactant treatment. Addition of homoserine lactone to the medium containing the mutant biofilms resulted in biofilms similar to the wild type with respect to structure and thickness. Stickler et al. (57) also detected acylated homoserine lactone signals in biofilms of gram-negative bacteria on urethral catheters. Yung-Hua et al. (58) showed that induction of genetic competence (enabling the uptake and incorporation of exogenous DNA by transformation) is also mediated by quorum sensing in *S. mutans*. Transformational frequencies were 10–600 times higher in biofilms than planktonic cells.

Predation and Competition

Bacteria within biofilms may be subject to predation by free-living protozoa, *Bdellovibrio* spp., bacteriophage, and polymorphonuclear leukocytes (PMNs) as a result of localized cell concentration. Murga et al. (59) demonstrated the colonization and subsequent predation of heterotrophic biofilms by *Hartmannella vermiformis*, a free-living protozoan. Predation has also been demonstrated with *Acanthamoeba* spp. in contact lens storage case biofilms (60).

James et al. (46) noted that competition also occurs within biofilms and demonstrated that invasion of a *Hyphomicrobium* sp. biofilm by *P. putida* resulted in dominance by the *P. putida*, even though the biofilm-associated *Hyphomicrobium* numbers remained relatively constant. Stewart et al. (61) investigated biofilms containing *K. pneumoniae* and *P. aeruginosa* and found that both species are able to coexist in a stable community even though *P. aeruginosa* growth rates are much slower in the mixed culture biofilm than when grown as a pure culture biofilm. *P. aeruginosa* grow primarily as a base biofilm, whereas *K. pneumoniae* form localized microcolonies (covering only about 10% of the area) that may have greater access to nutrients and oxygen. Apparently *P. aeruginosa* can compete because it colonizes the surface rapidly and establishes a long-term competitive advantage. *K. pneumoniae* apparently survives because of its ability to attach to the *P. aeruginosa* biofilm, grow more rapidly, and out-compete the *P. aeruginosa* in the surface layers of the biofilm.

Interactions of Pathogenic Organisms

Several frank bacterial pathogens have been shown to associate with, and in some cases, actually grow in biofilms, including *Legionella pneumophila* (59), *S. aureus* (62), *Listeria monocytogenes* (63), *Campylobacter* spp. (64), *E. coli* O157:H7 (65), *Salmonella typhimurium* (66), *Vibrio cholerae*

(67), and *Helicobacter pylori* (68). Although all these organisms have the ability to attach to surfaces and existing biofilms, most if not all appear incapable of extensive growth in the biofilm. This may be because of their fastidious growth requirements or because of their inability to compete with indigenous organisms. The mechanism of interaction and growth apparently varies with the pathogen, and at least for *L. pneumophila*, appears to require the presence of free-living protozoa to grow in the biofilm (59). Survival and growth of pathogenic organisms within biofilms might also be enhanced by the association and metabolic interactions with indigenous organisms. Camper et al. (65) showed that *Salmonella typhimurium* persisted in a model distribution system containing undefined heterotrophic bacteria from an unfiltered reverse osmosis water system for >50 days, which suggests that the normal biofilm flora of this water system provided niche conditions capable of supporting the growth of this organism.

The picture of biofilms increasingly is one in which there is both heterogeneity and a constant flux, as this biological community adapts to changing environmental conditions and the composition of the community.

Dispersal

Biofilm cells may be dispersed either by shedding of daughter cells from actively growing cells, detachment as a result of nutrient levels or quorum sensing, or shearing of biofilm aggregates (continuous removal of small portions of the biofilm) because of flow effects.

The mechanisms underlying the process of shedding by actively growing cells in a biofilm are not well understood. Gilbert et al. (69) showed that surface hydrophobicity characteristics of newly divided daughter cells spontaneously dispersed from either *E. coli* or *P. aeruginosa* biofilms differ substantially from those of either chemostat-intact biofilms or resuspended biofilm cells. These researchers suggested that these differences might explain newly divided daughter cells' detachment. Hydrophobicity was lowest for the newly dispersed cells and steadily increases upon continued incubation and growth.

Alginate is the major component of the EPS of *P. aeruginosa*. Boyd and Chakrabarty (70) studied alginate lyase production in *P. aeruginosa* to determine whether increased expression of this enzyme affected the size of the alginate molecules (and therefore adhesion of the organisms). Inducing alginate lyase expression substantially decreased the amount of alginate produced, which corresponded with a significant increase in the number of detached cells. The authors suggested that the role of *algL* (the gene cassette for alginate lyase production) in wild type *P. aeruginosa* may be to cause a release of cells from solid surfaces or biofilms, aiding in the dispersal of these organisms. Polysaccharidase enzymes specific for the EPS of different organisms may possibly be produced during different phases of biofilm growth of these organisms.

Detachment caused by physical forces has been studied in greater detail. Brading et al. (71) have emphasized the importance of physical forces in detachment, stating that the three main processes for detachment are erosion or shearing (continuous removal of small portions of the biofilm), sloughing (rapid and massive removal), and abrasion (detachment due to collision of particles from the bulk fluid with the biofilm). Characklis (72) noted that the rate of erosion from the biofilm increases with increase in biofilm thickness and fluid shear at the biofilm-bulk liquid interface. With increase in flow velocity, the hydrodynamic boundary layer decreases, resulting in mixing and turbulence closer to the biofilm surface. Sloughing is more random than erosion and is thought to result from nutrient or oxygen depletion within the biofilm structure (71). Sloughing is more commonly observed with thicker biofilms that have developed in nutrient-rich environments (72). Biofilms in fluidized beds, filters, and particle-laden environments (surface waters) may be subject to abrasion.

Detachment is probably also species specific; *P. fluorescens* disperses and recolonizes a surface (in a flow cell) after approximately 5 h, *V. parahaemolyticus* after 4 h, and *V. Harveyi* after only 2 h (73). This process probably provides a mechanism for cells to migrate from heavily colonized areas that have been depleted of surface-adsorbed nutrients to areas more supportive of growth.

The mode of dispersal apparently affects the phenotypic characteristics of the organisms. Eroded or sloughed aggregates from the biofilm are likely to retain certain biofilm characteristics, such as antimicrobial resistance properties, whereas cells that have been shed as a result of growth may revert quickly to the planktonic phenotype.

A Public Health Perspective

Clinical and public health microbiologists' recognition that microbial biofilms are ubiquitous in nature has resulted in the study of a number of infectious disease processes from a biofilm perspective. Cystic fibrosis, native valve endocarditis, otitis media, periodontitis, and chronic prostatitis all appear to be caused by biofilm-associated microorganisms. A spectrum of indwelling medical devices or other devices used in the health-care environment have been shown to harbor biofilms, resulting in measurable rates of device-associated infections (74). Table 2 provides a listing of microorganisms commonly associated with biofilms on indwelling medical devices. Biofilms of potable water distribution systems have the potential to harbor enteric pathogens, *L. pneumophila*, nontuberculous mycobacteria, and possibly *Helicobacter pylori*. What is less clear is an understanding of how interaction and growth of pathogenic organisms in a biofilm result in an infectious disease process. Characteristics of biofilms that can be important in infectious disease processes include a) detachment of cells or biofilm aggregates may result in bloodstream or urinary tract infections or in the production of emboli, b) cells may exchange resistance plasmids within biofilms, c) cells in biofilms have

Table 2. Microorganisms commonly associated with biofilms on indwelling medical devices

Microorganism	Has been isolated from biofilms on
<i>Candida albicans</i>	Artificial voice prosthesis Central venous catheter Intrauterine device
Coagulase-negative staphylococci	Artificial hip prosthesis Artificial voice prosthesis Central venous catheter Intrauterine device Prosthetic heart valve Urinary catheter
<i>Enterococcus</i> spp.	Artificial hip prosthesis Central venous catheter Intrauterine device Prosthetic heart valve Urinary catheter
<i>Klebsiella pneumoniae</i>	Central venous catheter Urinary catheter
<i>Pseudomonas aeruginosa</i>	Artificial hip prosthesis Central venous catheter Urinary catheter
<i>Staphylococcus aureus</i>	Artificial hip prosthesis Central venous catheter Intrauterine device Prosthetic heart valve

dramatically reduced susceptibility to antimicrobial agents, d) biofilm-associated gram-negative bacteria may produce endotoxins, and e) biofilms are resistant to host immune system clearance. Please refer to the online appendix for an expanded discussion of each of these mechanisms (URL: <http://www.cdc.gov/ncid/eid/vol8/no9donlan.htm>).

A Prospectus for Future Research

Research on microbial biofilms is proceeding on many fronts, with particular emphasis on elucidation of the genes specifically expressed by biofilm-associated organisms, evaluation of various control strategies (including medical devices treated with antimicrobial agents and antimicrobial locks) for either preventing or remediating biofilm colonization of medical devices, and development of new methods for assessing the efficacy of these treatments. Research should also focus on the role of biofilms in antimicrobial resistance, biofilms as a reservoir for pathogenic organisms, and the role of biofilms in chronic diseases. The field of microbiology has come to accept the universality of the biofilm phenotype. Researchers in the fields of clinical, food and water, and environmental microbiology have begun to investigate microbiologic processes from a biofilm perspective. As the pharmaceutical and health-care industries embrace this approach, novel strategies for biofilm prevention and control will undoubtedly emerge. The key to success may hinge upon a more complete understanding of what makes the biofilm phenotype so different from the planktonic phenotype.

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biofilms on indwelling medical devices, biofilms and antimicrobial resistance, and interaction of pathogens with potable water biofilms.

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PERSPECTIVE

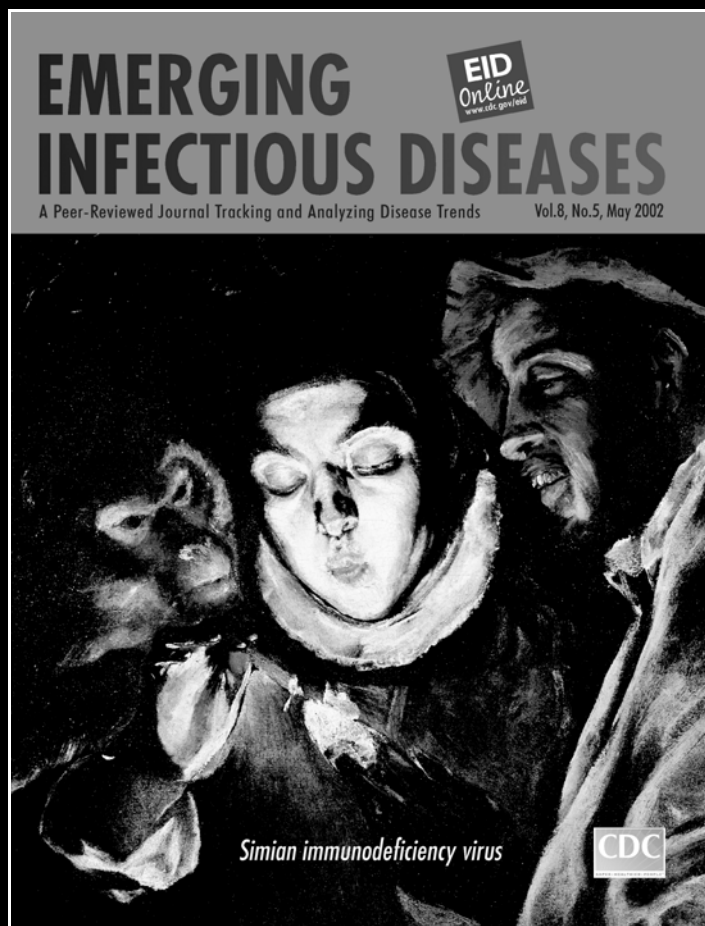
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If he dared, a philosopher dreaming before a water painting by Monet would develop the dialectics of the iris and water lily, the dialectics of the straight leaf and the leaf that is calmly, peacefully, heavily lying on the water's surface. This is the very dialectic of the aquatic plant. Reacting to some kind of spirit of revolt, the one wants to spring up against its native element. The other is loyal to its element. The water lily has understood the lesson of calm taught by still waters. With such a dialectical dream, one might feel the soft, extremely delicate verticality that can be seen in the life of still waters. But the painter feels all that instinctively and knows how to find in the reflections a sure principle that makes up, vertically, the peaceful world of water.

—Gaston Bachelard (1884–1962), French philosopher, about the work of painter Claude Monet



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A New Name (*Pneumocystis jiroveci*) for *Pneumocystis* from Humans

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The disease known as *Pneumocystis carinii* pneumonia (PCP) is a major cause of illness and death in persons with impaired immune systems. While the genus *Pneumocystis* has been known to science for nearly a century, understanding of its members remained rudimentary until DNA analysis showed its extensive diversity. *Pneumocystis* organisms from different host species have very different DNA sequences, indicating multiple species. In recognition of its genetic and functional distinctness, the organism that causes human PCP is now named *Pneumocystis jiroveci* Frenkel 1999. Changing the organism's name does not preclude the use of the acronym PCP because it can be read "*Pneumocystis pneumonia*." DNA sequence variation exists among samples of *P. jiroveci*, a feature that allows reexamination of the relationships between host and pathogen. Instead of lifelong latency, transient colonization may be the rule.

Clinical Importance of *Pneumocystis*

The disease known as *Pneumocystis carinii* pneumonia (PCP) is one of the leading causes of illness and death in persons with impaired immunity. The disease has been described in immunocompromised patients for many years, including outbreaks in malnourished young children in orphanages in Iran in the 1950s (1–6). The AIDS epidemic, however, marked the beginning of the disease's impact on a substantial number of patients. PCP has long been the most common serious AIDS-defining opportunistic infection in the United States. The introduction of highly active antiretroviral therapy (HAART) for the treatment of HIV infection has been accompanied by substantial reductions in mortality and the incidence of opportunistic infections, including PCP (7). Despite these advances, *Pneumocystis* remains a major pathogen in HIV-infected persons who either are not receiving or are not responding to HAART and among those who are unaware of their HIV status. PCP is also of clinical importance in people immunocompromised for reasons other than HIV, such as organ transplantation or chemotherapy for malignant diseases (8). In addition, *Pneumocystis* infection has been documented recently in persons who are mildly immunocompromised, including those with chronic lung disease (9).

Need for a Change in Nomenclature

Pneumocystis organisms were first reported by Chagas in 1909 (10), but he mistook them for a morphologic form of *Trypanosoma cruzi*. Within a few years of this first report, further studies established that the microbe in question was not a trypanosome but a new species altogether, named *Pneumocystis carinii* (11).

From the time of its discovery, until late in the 1980s, *Pneumocystis* was widely thought to be a protozoan. These views were based on several criteria: 1) strong similarities in microbe morphology and host pathology, 2) absence of some phenotypic features typical of fungi, 3) presence of morphologic features typical of protozoa, 4) ineffectiveness of antifungal drugs, and 5) effectiveness of drugs generally used to treat protozoan infections. Some investigators pointed out that *Pneumocystis* organisms exhibit morphologic similarities to fungi (2). Nevertheless, the protozoan hypothesis remained predominant until 1988, when DNA analysis demonstrated that *Pneumocystis* is a fungus, albeit an odd one, lacking in ergosterol and very difficult to grow in culture (12,13).

Soon after the proper classification of *Pneumocystis* had been determined at the kingdom level, additional DNA data showed that *Pneumocystis* organisms in different mammals are quite different. These data led to interim name changes (14), but it was not until 1999 that the first valid new binomial appeared. The organism that causes human PCP is now named *Pneumocystis jiroveci* Frenkel 1999 (pronounced "yee row vet zee"), in honor of the Czech parasitologist Otto Jirovec, who is credited with describing the microbe in humans (15). The primary purpose of this article is to explain what led to the name change and why the new name is necessary, useful, and workable for all concerned. For a more extensive review of the systematics and nomenclature of *Pneumocystis*, see Stringer's review of workshops on the subject (16). The DNA sequence information that led to the renaming of *Pneumocystis* organisms also provided the tools needed to better understand the relationships between these microbes and the hosts they inhabit. Thus, the secondary purpose of this article is to sum-

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marize data on these relationships, focusing on current views on the relationship between *P. jiroveci* and humans.

Complexity of the Genus

One reason that a definitive nomenclature has been slow to develop is that *Pneumocystis* organisms have been difficult to study. Attempts to develop an in vitro culture system have had limited success. Cultivation of *Pneumocystis* organisms in vitro requires a large seed population and supports rather modest increases in organism number for a very limited period of time (17). An exception to the rule was recently reported (18); however, this method has not been established in other laboratories. The fastidiousness of *Pneumocystis* organisms greatly hampered early efforts to understand them. Fortunately, advances in DNA analysis technology allowed progress in the absence of a robust culture system.

Pneumocystis jiroveci as a Distinct Species

Phenotypic differences between *P. jiroveci* and other species of *Pneumocystis* were noted decades ago (19). More recent descriptions echo these reports (20). On the basis of phenotypes, Frenkel first proposed the name *Pneumocystis jiroveci* in 1976. The name was not validly published, however, under the then-prevailing specifications of the International Code of Zoological Nomenclature. Thus, the name did not gain acceptance at that time.

The first indication of a molecular difference between *P. jiroveci* and *Pneumocystis* from laboratory animals came from analyses of protein sizes (21,22). However, the importance of these differences was difficult to judge because the *Pneumocystis* was prepared directly from the lung of the host, leaving open the possibility that differences could have been due to extrinsic factors such as contamination with host proteins, host-mediated modification of *Pneumocystis* proteins, or presence of dead *Pneumocystis* organisms.

DNA analysis provided the information needed to clarify the issue and to establish that the organisms from humans and other animals are quite different (23). The most powerful approach has been to use polymerase chain reaction (PCR). Wakefield developed primers that amplify DNA from all known species of *Pneumocystis* (24,25). When these primers have been used on human-derived samples of *Pneumocystis*, the only DNA found has been that of *P. jiroveci*. Moreover, *P. jiroveci* DNA has not been found in lung samples from any other mammals, including nonhuman primates (26). The PCR data are supported by the results of sequencing cloned genes. Several genes or gene fragments have been cloned from human-derived *Pneumocystis* (27–30). In all cases, the gene sequence is very different from its orthologues in *Pneumocystis* organisms from other host species. Genetic divergence data also argue that *P. jiroveci* is a distinct species. The 18S rRNA sequences from *P. jiroveci* (i.e., human-derived) and *P. carinii* (i.e., rat-derived) differ by 5%. This level of divergence is comparable with that between *Pneumocystis* organisms and *Taphrina deformans* (a plant fungal pathogen), whose 18S

rRNA sequences differ by approximately 6%. In contrast, species in the genus *Saccharomyces* can differ by as little as 1% at the 18S rRNA locus.

The genetic divergence between *P. jiroveci* and other *Pneumocystis* organisms is typical of the genus. When *Pneumocystis* from different host species are compared by DNA sequence analysis, they always differ (23,25,31–33). In addition, experiments with rats, mice, ferrets, and monkeys have demonstrated host-species specificity (34–36). For example, when *Pneumocystis* organisms were taken from a rat and transferred to a mouse, proliferation was not evident, and no disease resulted (34). In contrast, when *Pneumocystis* organisms from a rat were transferred to another rat, they proliferated to a very high number and caused severe disease. Transfer experiments that seem to show lack of specificity have been reported, but these reports did not show that the proliferating organisms were the same species of *Pneumocystis* as those introduced, leaving open the possibility that endogenous organisms were responsible for the infection.

Pneumocystis organisms might be obligate parasites that have evolved to survive in a particular host species. Co-evolution of parasite and host might be expected in such a case. Note, in this regard, that *P. jiroveci* is most similar to organisms isolated from other primates (37). This finding fits with the obligate parasite conjecture. However, the host specificity data also fit with an alternative scenario: there could be many free-living species of *Pneumocystis*, one of which is capable of invading humans, others of which are capable of invading nonhuman primates, and the like. In this scenario, the similarity between *P. jiroveci* and the *Pneumocystis* organisms found in nonhuman primates would reflect the similarities between humans and other primates. If *P. jiroveci* is not an obligate parasite, finding it outside the human body should be possible. *P. jiroveci* DNA has been detected in samples of airborne fungal spores (24) and in a sample of pond water (38). However, the number of *P. jiroveci* in the environment seems to be very low, leaving open the possibility that these “free forms” of the organism may have been deposited by humans. *P. jiroveci* could be an obligate parasite, spores of which can survive in the environment long enough to infect a new host, should one be encountered. Resolving this question awaits the availability of a system capable of detecting infectious *Pneumocystis* organisms in the air, water, or soil.

Soon after DNA sequence data began to appear, name changes were suggested (14,39). However, naming new species seemed premature to many because of concerns about the possibility of creating false species by misinterpreting the importance of a limited amount of DNA sequence data. Consequently, a provisional trinomial nomenclature was adopted. This system referred to the different kinds of *Pneumocystis* organisms as special forms of *P. carinii*. Under this system, *P. jiroveci* was called *P. carinii* formae specialis *hominis* (*P. carinii* f. sp. *hominis*). After these provisional nomenclature changes were instituted, more DNA sequence data were obtained, and by 2001, it became clear that the organism caus-

ing PCP in humans should be recognized as a distinct species. The name *P. jiroveci* had already been published in a valid manner in 1999 (15); however, publication of a name does not necessarily lead to its use. Therefore, at the 2001 International Workshops on Opportunistic Protists held in Cincinnati, Ohio, approximately 50 researchers from around the world, including clinicians, epidemiologists, and laboratory scientists, met to discuss the desirability and appropriateness of retaining the currently used trinomial nomenclature system, as opposed to assigning (or using) new species names. The group unanimously endorsed a proposal to rename the organisms currently known as special forms of *P. carinii* as species in the genus *Pneumocystis* and drew up guidelines for the creation of the new species names (16). Consequently, in keeping with the International Code of Botanical Nomenclature, it is no longer correct, either biologically or taxonomically, to refer to the human *Pneumocystis* organism as *P. carinii*. *P. carinii* now refers exclusively to the organism formerly known as *P. carinii* f. sp. *carinii*, one of the two *Pneumocystis* species found only in rats.

The consensus achieved at the workshop will help to make published reports on *Pneumocystis* more uniform with respect to nomenclature. Such uniformity will clarify communication among all who are interested in this genus and the disease caused by its members. Hopefully, all future reports pertaining to *P. jiroveci* will use its new name.

Acronym "PCP" Retained

Given the compelling evidence that the human form of *Pneumocystis* is a separate species, the most important objection to designating it as such has been the problem that this name change could create in the medical literature, where the disease caused by *P. jiroveci* is widely known as PcP, or PCP. This problem can be avoided by taking the species name out of the disease name. Under this system, PCP would refer to *Pneumocystis pneumonia*. This simple modification in the vernacular accommodates the name change pertaining to the *Pneumocystis* species that infects humans. Furthermore, adopting this change makes the acronym appropriate for describing the disease in every host species, none of which, except rats, is infected by *P. carinii*.

Multiple Strains of *P. Jiroveci*

DNA sequence polymorphisms are often observed in isolates of *P. jiroveci*, suggesting that numerous strains of this species exist. Loci that have been favorite targets for sequence analysis include the mitochondrial large subunit ribosomal RNA gene, the mitochondrial small subunit rRNA gene, the internal transcribed spacer regions of the nuclear rRNA gene (ITS), the *arom* gene, and the dihydropteroate synthase (DHPS) gene. The first three of these loci are considered to be under little if any selective pressure and presumably serve as indicators of genetic changes that are phenotypically neutral. The changes in the *arom* gene may also be considered neutral

because they effect no change in the amino acid sequence of the enzyme. By contrast, the polymorphisms in the DHPS gene may be due to selection (see below). Techniques other than DNA sequencing have been used to detect genotypic variation. These include the use of type-specific oligonucleotide probes to detect variation at the ITS regions (40) and detection of single-strand conformation polymorphism (SSCP) at multiple loci (41).

Genotyping has produced data from hundreds of *P. jiroveci* samples. Most studies have targeted one locus for analysis, but several multilocus studies have been reported (41–44). The allelic sequence polymorphism common in *P. jiroveci* is not seen in *P. carinii* (rat-derived *Pneumocystis*). However, *P. carinii* populations differ with respect to chromosome size, and several different strains have been identified by analysis of chromosome sizes (45,46). The possibility of chromosome size variation in *P. jiroveci* has not been adequately addressed because this analysis requires more organisms than are typically available from patients.

New Perspectives on Infection

Genotyping samples of *P. jiroveci* provides a method for exploring epidemiologic issues. For example, one study examined the possibility that the low incidence of PCP in African HIV-infected persons might be due to the presence or absence of certain strains of *P. jiroveci*. However, samples of *P. jiroveci* from Zimbabwe, Brazil, the United States, and the United Kingdom have exhibited no major differences in genotypes (47). Another example is a study in which genotyping at four different genetic loci was used to compare isolates of *P. jiroveci* collected before (1968–1981) and after (1982 to present) the beginning of the AIDS pandemic (48). Pre- and postpandemic samples were the same except for a single base polymorphism (in the mitochondrial large subunit rRNA gene) found in the pre-pandemic samples only. These data show that the large increase in incidence of PCP was not accompanied by a shift in the kinds or frequencies of strains of *P. jiroveci*.

Strain analysis has also led to observations that are difficult to reconcile with the traditional view of the relationship between *P. jiroveci* and humans. The traditional theory holds that clinically important infection results from reactivation of a latent infection that was acquired during childhood. While infection of young children appears to be common, latent *P. jiroveci* has not been directly observed in healthy adults. In addition, indirect evidence is difficult to reconcile with life-long latency.

The latency issue is important for several reasons. Under the reactivation of latent infection theory, little rationale exists for instituting measures to minimize the risk of infection during adulthood because this infection has already occurred. On the other hand, person-to-person transmission of the disease would have important public health implications for medical centers that treat HIV-infected patients or other immunocompromised persons (42–44,49–52). Furthermore, transmission from

patients who are undergoing treatment for PCP might enhance the opportunity for drug resistance to arise. By contrast, the generation of drug resistance would be less of a concern if most or all infections were due to transmission from an immunocompetent person, such as a young child's mother, or another child (i.e., someone who is not being treated for PCP). Under these conditions, drug-resistant strains, if they arose, would not spread very effectively.

PCP develops in infants infected with HIV perinatally, suggesting that *P. jiroveci* was present in these infants' environments early in their lives (53). Evidence of *P. jiroveci* has also been found in some victims of sudden infant death syndrome (SIDS) (54). In normal, healthy children, serologic data have long indicated that infection of young children is common. Most children develop anti-*Pneumocystis* antibodies early in life, and the prevalence of these antibodies appears to increase with age (48,55). Recently, *P. jiroveci* has been linked to clinical illness in normal, healthy infants (51). *P. jiroveci* DNA was identified in nasopharyngeal aspirates obtained during episodes of mild respiratory infection in 24 (32%) of 74 infants. Seroconversion developed by 20 months of age in 67 (85%) of 79 infants who remained in the study and occurred in the absence of any symptoms of disease in 14 (18%). These reports confirm previous ones showing infection of children (1,3,4). Young children may be a reservoir of infectious *P. jiroveci* in the community.

Although infection of children seems common, little evidence exists for lifelong latency. Using PCR, Wakefield found no evidence of *P. jiroveci* in bronchoalveolar lavage fluid from 10 healthy persons (56). Peters replicated this result in post-mortem lung tissue from 15 immunocompetent adults (56,57). (The techniques used to detect *P. jiroveci* have found it in HIV-negative adults but only those with other health problems [58].) Studies on recurrent PCP have shown that different *P. jiroveci* genotypes are present during different PCP episodes in patients with repeat episodes of PCP, a result suggestive of infection proximal to the time of disease (42–44). Recent infections of adults are also suggested by the high frequency of mutations that cause changes in the sequence of the DHPS gene, the enzyme associated with sulfonamide resistance in other pathogens (59–61). These mutations have not been detected in patients in whom PCP occurred at a time before the widespread use of sulfonamides to treat and prevent it (62) but are common in today's patients, even in those with no known exposure to sulfonamides (61,63). Mutant DHPS genes have been found in a variety of *P. jiroveci* genetic backgrounds, suggesting that selection for DHPS mutations is an ongoing process (64).

An alternative approach to exploring the importance of latency is employing population genetics and epidemiology to test the following hypothesis. If lifelong latency is important, adult patients who reside far from their birthplace should have the strain of *P. jiroveci* common in their place of birth, not in their place of residence. Data pertaining to this hypothesis are

now available (64). The strains infecting adult patients were more similar to those common in their place of residence than their place of birth, suggesting that infections had been recently acquired, rather than carried since early childhood.

Latent *P. jiroveci* have not been found in healthy adults, but proving that they do not exist is practically impossible. A single organism anywhere in the body could be sufficient to maintain a latent infection. Therefore, the possibility of latency remains. However, latent infections may be transitory, and humans who have eliminated the microbe may be subject to reinfection. The observations described above seem more consistent with this "transient colonization" scenario than with lifelong latency.

Summary

The microbe that causes PCP in humans is a distinct phylogenetic fungal species called *Pneumocystis jiroveci*. This species has been difficult to find in the environment, has not been found in nonhuman hosts, and is either absent in healthy adults or present at very low levels. In contrast, *P. jiroveci* is fairly common in humans who have depressed immune function. The number of *P. jiroveci* in a person appears to be dependent on the degree of immune dysfunction, suggesting that the species is adapted to exploit this dysfunction, growing to very high numbers in the severely immunodeficient and to lesser extents when immune function is less impaired. *P. jiroveci* may be eliminated when immune function is optimal. Genetic variants of the organism are common, providing markers for epidemiologic studies. Studies using these markers have raised questions about the role of latency in PCP. Recurrent PCP can be accompanied by shifts in genotype. Some patients are infected by genotypes more common in their place of residence than in their birthplace. Variable loci include the gene encoding an enzyme targeted by sulfonamides, suggesting transmission from treated patients to others at risk. While these observations, combined with the scarcity of *P. jiroveci* in healthy adults, do not exclude latency as a cause of PCP, they suggest that long-term latency is not the only source of this disease.

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Human *Metapneumovirus* as a Cause of Community-Acquired Respiratory Illness

Joanne Stockton,* Iain Stephenson,† Douglas Fleming,‡ and Maria Zambon*¹

Human *metapneumovirus* (HMPV) is a recently identified *Paramyxovirus* first isolated from hospitalized children with acute respiratory tract infections (ARTI). We sought evidence of HMPV infection in patients who had visited general practitioners, had influenzalike illnesses (ILI), and had negative tests for influenza and *Human respiratory syncytial virus* (HRSV). As part of national virologic surveillance, sentinel general practices in England and Wales collected samples from patients of all ages with ILI during winter 2000–01. Reverse transcriptase-polymerase chain reaction (PCR) for HMPV, influenza A (H1 and H3), influenza B, and HRSV (A and B) was used to screen combined nose and throat swabs. PCR products from the HMPV-positive samples were sequenced to confirm identity and construct phylogenetic trees. Of 711 swabs submitted, 408 (57.3%) were negative for influenza and HRSV; HMPV was identified in 9 (2.2%) patients. HMPV appears to be associated with community-acquired ARTI. The extent of illness and possible complications related to this new human virus need to be clarified.

Despite control of many infectious diseases in the industrialized world, acute viral respiratory tract infections (ARTI) remain a leading cause of illness. Although usually self-limiting in healthy adults, these infections are responsible for a substantial loss of productive time and are important factors in the illness and death of the elderly population. Various genetically diverse viruses, often with multiple types, may cause respiratory illness; of these, influenza receives the greatest attention (1). *Human respiratory syncytial virus* (HRSV) is also increasingly implicated as an important pathogen (2).

The association between the incidence of ARTI and excess winter deaths in the United Kingdom is well recognized (1). Regression modeling associates excess winter deaths with influenza and HRSV but also suggests that other pathogens may be involved (3).

Studies of the impact of respiratory virus infections are limited by difficulty in distinguishing respiratory pathogens clinically and in the laboratory (4,5). Despite improved sensitivity with diagnostic techniques such as reverse transcriptase-polymerase chain reaction (RT-PCR), approximately 40% of specimens from patients with community-acquired respiratory illnesses during peak winter months contain no identified viral pathogen (2,5,6).

A new pneumovirus, Human *Metapneumovirus* (HMPV), has recently been isolated in the Netherlands (7). The *Pneumovirinae* subfamily is classified into *Pneumovirus*, containing HRSV, and *Metapneumovirus* genera. In 2001, Van den Hoogen et al. (7) reported the detection of HMPV in nasopharyngeal aspirates taken in a 10-year period from 28 hospital-

ized children and infants with respiratory tract infections who had signs and symptoms similar to those of HRSV infection.

Establishing sensitive methods for virus detection helps to clarify the relative contribution of different pathogens to the extent of illness in the community. This information is important for future development of specific antiviral therapies and vaccines. We examined specimens submitted from patients seen in general practice with influenzalike illnesses (ILI) during winter 2000–01 to detect HMPV as a possible cause of influenza- and HRSV-negative ILI.

Materials and Methods

Sentinel General Practice Networks

Clinical episodes of ILI are recorded by continuous monitoring in approximately 75 sentinel practices in England and Wales, covering a population of 700,000. New episodes of illness are noted and weekly returns submitted to the Royal College of General Practitioners research unit. ILI was defined as symptoms of fever, cough, and muscle pains with duration of ≤ 5 days (8,2). Virologic surveillance of ILI is performed by a subset of the sentinel practices. Combined nose and throat swabs are taken from persons diagnosed with ILI at the time they see a clinician, which is often several days after onset of illness. Swabs are mailed in virus transport medium to the Central Public Health Laboratory for analysis. Samples are divided into aliquots, labeled, and then frozen at -80°C on receipt.

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¹MZ and JS designed the study; DF and MZ organized the sampling. JS performed the polymerase chain reaction analysis and designed the sequencing strategy. IS collected clinical data. All authors contributed to the writing of the manuscript.

HRSV and Influenza Detection

Detection of HRSV and influenza was performed prospectively on receipt of samples by using multiplex PCR as previously described (2,8).

HMPV Detection

Stored nucleic acid from 348 samples negative for influenza and HRSV was analyzed by using a PCR for HMPV (7). No optimization of the PCR detection was undertaken. Detection of HPMV was based on primers located in the L gene. For a further 60 (14.8%) samples from which the nucleic acid stored was insufficient, an aliquot of the original clinical material was re-extracted by using a Magnapure (Roche Diagnostics GmbH, Mannheim, Germany) automated extraction machine according to the manufacturer's instruction; RT was performed as described previously (8). Twenty microliters of cDNA was added to 80 μ L of reaction mix, which yielded a final concentration of 20 mM Tris-HCl pH8.4, 10 mM MgCl₂, 50 mM KCl, and 3 U *Taq* polymerase and 50 pmol of each primer. Amplification, using a DNA Engine thermocycler (MJ Research, Essex, England), consisted of 1 cycle at 94°C for 2 min, followed by 35 cycles of 94°C 1 min, 58°C 1 min, and 72°C for 1 min. Amplicons of the expected size (171 bp) were visualized by agarose gel electrophoresis. PCR sensitivity was determined by cloning of the PCR product into TOPO PCR4 vector (Invitrogen Corp., San Diego, CA) according to the manufacturer's instructions. Plasmid preparations were performed by using a Qiagen minprep kit (QIAGEN, Inc., Valencia, CA), according to the manufacturer's instructions. DNA quantitation followed by PCR, using the original primers and conditions (as above) with the addition of 0.1 mM of each deoxynucleoside triphosphate per reaction, was undertaken using the plasmid plus insert as template. Total RNA at the time of extraction was estimated by spectrophotometry at A₂₆₀.

The resulting amplicons were sequenced by using a Ceq Dye terminator sequencing kit (Beckman Coulter, Inc., Somerset, NJ) according to the manufacturer's instructions. Sequenced reactions were run on a CEQ 2000 capillary sequencer (Beckman Coulter). Resulting sequence was BLAST searched; 98% identity to deposited sequences of HMPV was seen.

Phylogenetic Analysis

Sequences were aligned by using the program Megalign (Lasergene, DNA STAR, Inc., Madison, WI) and exported into PAUP* (URL: <http://paup.csit.fsu.edu/about.html>). Maximum likelihood trees were created by using the K81 model of evolution and bootstrapped x1000.

Results

ILI Clinical Diagnosis

A total of 711 swabs were submitted from cases of ILI seen at the subgroup of 16 sentinel practices supplying virologic

specimens. These cases constituted approximately 65% of the total consultations of ILI in some of these practices from October 1, 2000, through March 30, 2001, covering a population of 241,000. Swabs were obtained from all age groups: 79 (11%) from those <5 years; 115 (16%) from those 5–14.9 years; 300 (42%) from those 15–39.9 years; 179 (25%) from those 40–64.9 years; and 37 (5%) from those >65 years; for 1 sample the age was unknown.

HMPV PCR Sensitivity

The sensitivity of the PCR was determined to be at least 1 femtogram of DNA template and 0.32 μ g of a total RNA preparation.

HMPV

A total of 408 specimens were negative for influenza and HRSV; of these, 405 (99.3%) samples were available for analysis and examined for HMPV by RT-PCR. Nine (2.2%) of these were positive for HMPV by PCR. Samples were unrelated geographically or epidemiologically. Clinical history and findings are summarized in the Table. HMPV was detected in samples from a child <1 year old, from four persons ages 18–64 years, and four persons ages >65 years. Six (67%) of the nine had clinical evidence of lower respiratory tract involvement. Four (44%) received antibiotic therapy at the time they were seen by a clinician. All made a complete recovery. Figure 1 shows timing of HMPV-positive sample collection relative to the circulation of HRSV and influenza, and the rate of clinical ILI consultations throughout the study period.

HMPV Phylogeny

The phylogenetic analysis of the L gene sequences from our patients and nine previously reported (7) showed at least two possible clusters of sequences. The sequences obtained from the adult patients cluster with those obtained from children from our study and those previously reported (7) (Figure 2).

Discussion

The extent of illness in the community caused by circulating respiratory viruses is currently underestimated. Problems in diagnosing these viruses include capture of suitable specimens for virologic diagnosis, limitations caused by transport of labile viruses, and sampling from adult subjects, who may shed fewer viruses than children (9). However, PCR is more sensitive for detecting a range of respiratory viruses than are culture or serologic methods and has been shown to be a robust diagnostic tool (10).

We have found evidence of HMPV in approximately 2.2% of cases of ILI that were negative for HRSV and influenza. If no other HMPV were detected in the rest of the samples (i.e., as a dual infection with either HRSV or influenza), the overall detection rate in this population would be 1.3% with the use of the PCR method. We cannot exclude the possibility that HMPV is carried asymptotically in the human respiratory

Table. Clinical information on patients with influenzalike illness and positive *Metapneumovirus* polymerase chain reaction results, seen by general practitioners^a

Sex	Age (yr)	Past medical history	Influenza vaccine	Clinical signs and symptoms (days symptoms persist/total days ill)
F	46	None	No	(7/7) febrile respiratory symptoms, sore throat, malaise, and lethargy. Chest clear
F	20	None	No	(2/7) sore throat, unproductive cough, sternal pain, wheeze. Signs: 37.4°C; chest clear
M	1	None	No	(4/7) coughing, vomiting. OE: 37.2°C, chest and abdomen normal
F	75	Mild hypertension	Yes	(4/7) febrile respiratory illness, cough. Signs: 37.4°C, bilateral basal crackles
F	57	COPD ^b	Yes	(5/7) coryza, sore throat, thick green sputum. Signs: poor air entry and bilateral crackles
M	65	Mild hypertension	No	(3/7) cough, upper respiratory symptoms. Chest clear
F	73	None	Yes	(6/7) cough, green sputum, and dyspnea
M	74	COPD, IHD	Yes	(5/7) cough, malaise, sputum, breathlessness
M	46	None	No	(6/7) days sore throat, sputum, wheeze, breathlessness Signs: PEF ^b 260 mL/min, wheeze

^aAll made a full recovery.

^bCOPD, chronic obstructive pulmonary disease; IHD, ischemic heart disease; OE, on examination; PEF, peak expiratory flow rate.

tract and is of no clinical importance, nor the possibility of dual infection. Our estimate, therefore, is likely to be a minimum one. Respiratory virus coinfections appear to occur at a rate of 1% to 3% in various sample sets (2,11). Nonetheless, as the specimens are taken from symptomatic patients seeking medical consultation at the time of respiratory illness, the assumption that the pathogen detected is responsible for the illness is reasonable.

When HMPV was first isolated, it was associated with symptoms of ARTI in infants and children similar to those seen in HRSV infection, which ranged in severity from self-limiting mild respiratory illness to respiratory failure requiring ventilation. Therefore, diagnosing HMPV infection and differentiating it from other respiratory viruses may be impossible on clinical grounds alone. All the specimens were negative for other respiratory viruses, and it was concluded that HMPV was likely to be the responsible pathogen.

Our results are consistent with the association of HMPV with acute respiratory infections in winter (7) and demonstrate that this virus is associated with at least a proportion of mild, community-acquired, self-limiting respiratory illnesses in all age groups. Initial seroprevalence data documented that all children were seropositive by 5 years of age (7), and our results imply that, like HRSV, HMPV is capable of causing clinically important reinfection in late childhood or adult life.

We consider that our data on the prevalence of HMPV in a sentinel physician surveillance scheme, used for combined clinical-virologic monitoring, are likely to be a minimum estimate of this virus' contribution to acute respiratory infections, for several reasons. Our detection strategy depends on the use of PCR targets in the L gene of a paramyxovirus and has been determined by sequence availability. A limitation of the current study may be the sensitivity of the PCR used. Although the sensitivity was determined for a DNA target and total RNA preparation, no information is currently available, for comparison, on the sensitivity of the PCRs that other studies have used (12,13). Nevertheless, we consider these estimates may

be taken as a robust minimum value. A differential gene transcription present in the *Paramyxoviridae* as a whole suggests that targeting diagnostics to genes that are transcribed more proximally may be a more sensitive approach. As virus quantitation methods are developed and in vitro transcription methods are established, determination of copy number sensitivity will become possible. Currently, no methods for virus quantitation in cell culture are established, and the full genome sequence has only very recently been published (14). Furthermore, no information exists on the efficiency of HMPV detection directly from nose and throat swab specimens, without culture amplification as was done in the Dutch study (7). As only patients presenting with ILI symptoms were sampled in this surveillance cohort, most acute respiratory infections that occur outside this surveillance definition are unsampled, thereby increasing the likelihood of underestimation of HMPV as a cause of all respiratory infections. Since sampling only occurs during the winter season (October–March), we cannot

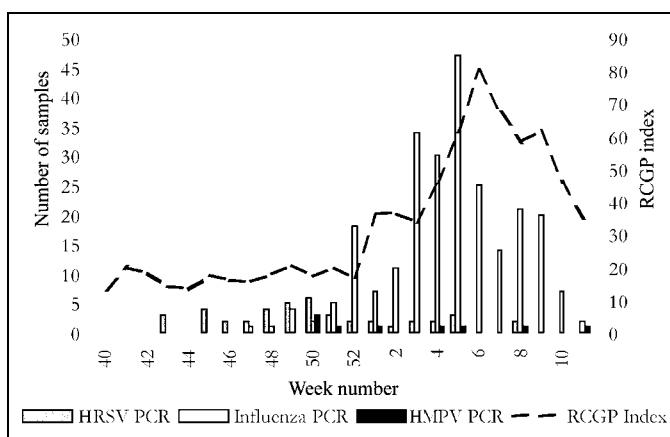


Figure 1. Incidence of influenzalike illness consultations in England during winter 2000–01 and timing of collection of positive samples for human *Metapneumovirus* (HMPV). HRSV, *Human respiratory syncytial virus*; PCR, polymerase chain reaction; RCGP, Royal College of General Practitioners. RCGP index is the consultation rate per 100,000 of the population for ILI.

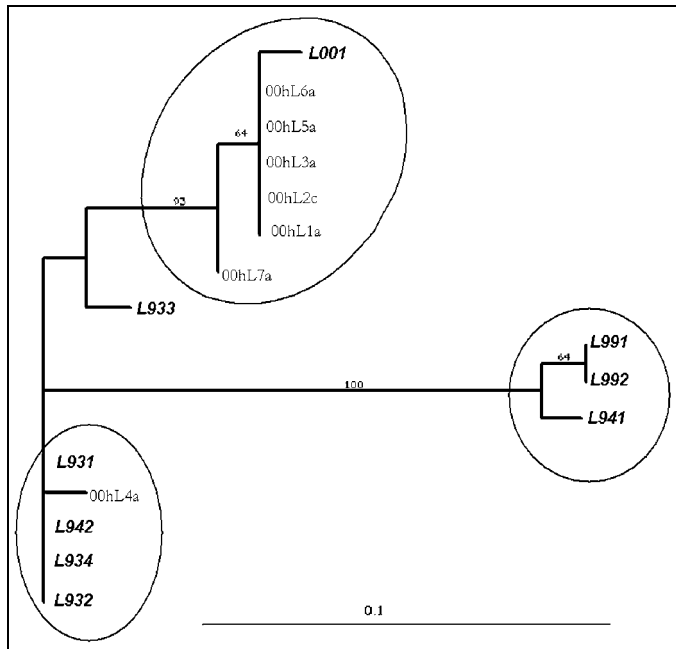


Figure 2. Phylogenetic tree showing sequence analysis of human *Metapneumovirus* (HMPV). Isolates prefixed with L were obtained from GenBank and represent isolates from the Netherlands. Isolates prefixed with 00hL are from this study; the following number indicates strain designations throughout the season: a, sample from an adult; c, sample from a child (<15 years). Scale shown is proportional to number of nucleotide substitutions per site.

determine whether the virus circulates year-round or whether it has seasonal peaks during the summer or winter months. Clearly, HMPV cocirculates with influenza and HRSV (Figure 1, data from this national surveillance program published on the PHLS web [URL: http://www.phls.co.uk/topics_az/influenza/Activity0102/graph12.pdf]), which adds to the potential for clinical diagnostic confusion.

Four out of five ILI cases in adults <65 years of age or with cardiorespiratory conditions in which HMPV was detected occurred in persons who had received influenza vaccination in accordance with national vaccination policy. This finding indicates that HMPV infection may cause an illness difficult to distinguish from influenza in the elderly and may be one of the reasons for underestimating the clinical efficacy of influenza vaccine in the elderly when clinical endpoints are used.

Sequencing analysis was performed on seven out of nine of our isolates by using primers that amplify a portion of the L gene. By analogy with other paramyxoviruses, the L gene codes for the viral polymerase. This gene typically shows less variability than genes coding for surface proteins. These results are consistent with the limited published evidence for genetic variation between HMPV isolates, suggesting the possibility of more than one type (7). Analysis of the structural membrane proteins that are more commonly associated with genetic or antigenic drift may identify further variability. Accurately determining relationships between strain clusters may also require sequencing of larger portions of the genome.

This study has identified, for the first time, HMPV in adults and children in the community. Phylogenetic analysis

confirmed that similar strains are circulating in adults and children at the same time, a feature also seen with influenza and HRSV infection (2).

More sensitive diagnostic tools need to be developed to ascertain a true estimate of the extent of illness caused by HMPV in the general community.

Although ILI and ARTI are common illnesses and usually self-limiting in healthy persons, these infections have a major impact on the overall health of the population with substantial loss of productive time. Continuous surveillance of respiratory pathogens is important for public health. Assessing the role of individual pathogens is important for the potential development of vaccines and for considering specific antiviral therapy. In the future, we will consider including HMPV detection in our routine surveillance programs of respiratory illness. Whether this interesting new virus, HMPV, plays a major clinical role in winter hospital admissions and excess deaths in different age groups of the general population remains to be seen.

Acknowledgments

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The sequence information generated from this study is deposited in EMBL under accession numbers AJ420267–AJ420273.

Dr. Stockton has a doctorate in respiratory syncytial virus and is currently employed as a postdoctoral virologist in the Public Health Laboratory Service. Current scientific interests are the application of molecular tools to the diagnosis of respiratory viral infections, the importance of strain variation in *Human respiratory syncytial virus*, and the detection of new respiratory viruses.

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Molecular Epidemiology of Measles Viruses in the United States, 1997–2001

Paul A. Rota,* Stephanie L. Liffick,* Jennifer S. Rota,* Russell S. Katz,* Susan Redd,* Mark Papania,* and William J. Bellini*

From 1997 to 2001, sequence data from 55 clinical specimens were obtained from confirmed measles cases in the United States, representing 21 outbreaks and 34 sporadic cases. Sequence analysis indicated the presence of 11 of the recognized genotypes. The most common genotypes detected were genotype D6, usually identified from imported cases from Europe, and genotype D5, associated with importations from Japan. A number of viruses belonging to genotype D4 were imported from India and Pakistan. Overall, viral genotypes were determined for 13 chains of transmission with an unknown source of virus, and seven different genotypes were identified. Therefore, the diversity of *Measles virus* genotypes observed in the United States from 1997 to 2001 reflected multiple imported sources of virus and indicated that no strain of measles is endemic in the United States.

An important component of laboratory surveillance for measles is the genetic characterization of wild-type viruses (1). This genetic information provides a powerful adjunct to standard epidemiologic data for describing the transmission pathways of *Measles virus* (MeV). Molecular epidemiology supports classical epidemiology in cases for which the source of imported MeV is known by confirming that the viral genotype obtained is consistent with the genotype known to be circulating in the country or region from which the case was imported. Molecular epidemiology fills in the gaps of information when classical epidemiology fails to discover the source of MeV, by providing a likely source on the basis of the genotypic information.

Monitoring the pattern of measles genotypes in an area can help document the effectiveness of control measures. For example, in areas that have endemic transmission of measles, virologic surveillance of cases detects a limited number of genotypes. On the other hand, in areas where endemic transmission of virus has been interrupted, a variety of genotypes are detected, reflecting the multiple sources of imported viruses. Virologic surveillance has already been used to help document the interruption of transmission of measles in the United States (2–4) and Australia (5). In addition, genetic analysis of viruses provides a means to differentiate vaccine-associated cases of measles from cases caused by infection with wild-type virus. Current surveillance protocols call for the collection of appropriate specimens for virologic surveillance during all phases of measles control. For countries such as the United States that are in the elimination phase of measles control, the goal is to collect a specimen for viral isolation along with a serum sample at first contact with each suspected case.

Genetic characterization of wild-type MeV is based on sequence analysis of two variable regions on the viral genome.

The targets for molecular epidemiologic studies are the 450 nucleotides coding for the 150 amino acids comprising the COOH-terminus of the nucleoprotein and the entire protein-coding region of the hemagglutinin gene. Based on these sequences, a number of genotypes have been identified (2,3,5–18). The World Health Organization (WHO) recognizes 20 genotypes and one proposed genotype (19–21), including several new genotypes that have been identified in the last 3 years (14,15,21–23). The prototype (Edmonston) strain of measles as well as all the currently used measles vaccines are in genotype A (21).

The purpose of this report is to describe the genetic characteristics of wild-type measles viruses isolated in the United States during 1997–2001. Overall, the results show a pattern consistent with the continued interruption of endemic transmission. Viruses representing several of the recently described genotypes were associated with imported cases in the United States, and this information has increased our understanding of the degree of genetic diversity in wild-type measles viruses.

Methods and Materials

Throat swabs and urine sediments were obtained from patients with serologically confirmed cases of measles. Clinical specimens were inoculated onto B95a cells (24), and the cells were observed for cytopathic effect (CPE). Inoculated cells were blind-passaged up to three times before being discarded. Cells were harvested when the CPE was maximal. Total cellular RNA was extracted from infected B95a cells or directly from clinical specimens, if virus isolation was not successful, by the guanidinium acid-phenol method (25). cDNA corresponding to the 565 nucleotides coding for the COOH-terminus of the nucleoprotein (N) and the full-length open reading frame for the hemagglutinin (H) gene were synthesized by using *Avian myeloblastosis virus* reverse transcriptase and amplified by polymerase chain reaction (PCR), as

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described previously (2,3). Sequences of the PCR products were derived by automated sequencing with the BigDye terminator chemistry according to the manufacturer's protocol (Perkin Elmer-Applied Biosystems, Foster City, CA), and reaction products were analyzed on an automatic sequencer (ABI 373, ABI 3100, Perkin Elmer-Applied Biosystems). Sequence data were analyzed by using version 10.1 of the Genetics Computer Group Sequence Analysis Software Package (Genetic Computer Group, Madison, WI) (26). Genotypes were assigned on the basis of phylogenetic analyses performed by using PAUP version 4.0 (27). All phenograms were drawn as unrooted trees. Sequences described were deposited in GenBank under accession numbers AY037009–AY037048.

MeV genotype information was combined with epidemiologic information for each confirmed measles case. The epidemiologic information was gathered through case investigation by state and local health departments and reported to the Centers for Disease Control and Prevention through the National Notifiable Diseases Surveillance System.

Results

For the years 1997–2001, the number of reported measles cases in the United States has been at record low levels. A total of 138 cases were reported in 1997, 100 cases/year in both 1998 and 1999, 86 cases in 2000 (4), and 108 cases for 2001 (provisional data). Most cases were imported from other countries or spread from imported cases.

During this 5-year period, 47 outbreaks consisting of ≥ 3 epidemiologically linked, confirmed cases occurred. These outbreaks were very small: only three outbreaks had >10 cases. Specimens for viral isolation were obtained from 31 (64%) of 48 outbreaks, and at least one virus was isolated in tissue culture from 19 of the 31 outbreaks from which specimens were submitted (Table 1). In addition, reverse transcriptase (RT)-PCR was successfully used to detect measles RNA in clinical specimens from two outbreaks when attempts to isolate virus failed (Table 1). Overall, genetic information was obtained from 21 (44%) measles outbreaks, while specimens from 10 outbreaks failed to yield a viral isolate or positive PCR signal. The inability to isolate MeV or detect measles RNA was due to failure to collect specimens within 5 days after the onset of rash or to improper storage. In addition to the outbreaks, 176 sporadic cases and 28 chains of transmission with 2 cases were reported during this period. Twenty-eight measles viruses were isolated from sporadic cases and two-case chains (Table 1); six additional specimens from sporadic cases were positive for measles RNA by RT-PCR.

Viruses representing 11 of the 20 genotypes recognized by WHO in 2001 (20) were isolated in the United States in 1997–2001 (Tables 1 and 2). Among the 13 chains of transmission for which an imported source of MeV was not detected by classical epidemiology, seven different genotypes were identified (Table 2).

Thirteen (24%) of the measles sequences detected from chains of transmission in the United States in 1997–2001

belonged to genotype D6, which has previously been associated with importation from European countries (2,3). Within the last 6 years, viruses in genotype D6 have been isolated in Brazil, Uruguay, Argentina, United Kingdom, Spain, Germany, Russia, Poland, and Luxembourg (9,10,16,28). During 1997–2001, genotype D6 viruses were imported into the United States from Italy, Greece, Ukraine, Croatia, Cyprus, and the United Kingdom. During 1997, viruses from genotype D6 were imported into the United States from the large measles outbreak that occurred in São Paulo, Brazil, and spread to other South American countries (29–33). The viruses that were isolated from cases imported from Brazil to Minnesota (MN33-97) and Pennsylvania (PA28-97) during 1997 had sequences identical to those obtained from measles viruses isolated during the outbreak in Brazil. Measles virus WA31-97 was isolated at the same time as MN33-97 and PA28-97 from a case in Washington with an unknown source of infection. The sequence of WA31-97 was identical to those of the two viruses imported from Brazil, suggesting that this virus may also have been imported from Brazil (Figure).

Twelve (22%) of the 55 sequences obtained from viral isolates or directly from clinical specimens were placed in genotype D5 (Tables 1 and 2). Genotype D5, along with D3, is one of the genotypes known to have endemic circulation in Japan (11). Epidemiologic investigations indicated that Japan was the source of infection for 6 of the 10 sporadic cases and two of the outbreaks. The largest outbreak in the United States during 1997–2001 occurred in Anchorage, Alaska, during 1998. The outbreak started 4 weeks after a case was imported from Japan to Anchorage. No direct epidemiologic link between this imported case and the outbreak was discovered. However, since the imported case and the outbreak occurred in the same place and within two generations of transmission, they were likely epidemiologically related. Although no viral specimens were received for the imported case, the sequence of the virus isolated during the outbreak was closely related to the sequences of viruses known to be circulating in Japan. Genotype D5 was also detected in specimens from a small outbreak that occurred in New York in July 2001 among a group of Japanese students who were visiting a university in New York City.

Viruses from genotype D4 were isolated from eight chains of transmission during 1997–2001; in seven of these chains, a foreign source of infection was identified (Tables 1 and 2). Importations from Kenya were associated with a 15-case outbreak in Virginia in 1999 and a sporadic case in Minnesota in 2001. At this time, no information is available about the genotypes of wild-type measles viruses circulating in Kenya, but a genotype D4 virus was imported into the United States from Kenya in 1996 (1). A genotype D4 virus imported from Ethiopia was responsible for a six-case outbreak in Vermont during 2000. In 2001, a genotype D4 virus was isolated from a small outbreak in Massachusetts, which was traced to a student from Pakistan. During 1999, two genotype D4 viruses were isolated from unlinked, imported cases from India. Genotype D4 is known to be circulating in Pakistan, Southern Africa, India,

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Table 1. Summary of virologic surveillance for measles, United States, 1997–2001

Virus/specimen ^a	Abbreviation	Date	Activity ^b	Genotype ^a	Source ^c
MVi/Michigan.USA/3.97	MI3-97	1/97	Sporadic case	D5	Japan
MVi/Minnesota.USA/13.97	MN13-97	3/97	Sporadic case	H2	Vietnam
MVi/Pennsylvania.USA/17.97	PA17-97	4/97	Outbreak, 4 cases	D4	Unknown
MVi/Florida.USA/15.97/2	FL15-97	4/97	Sporadic case	D5	Japan
MVi/Texas.USA/18.97	TX18-97	4/97	Outbreak, 3 cases	D6	Europe
MVi/Florida.USA/19.97	FL19-97	5/97	Sporadic case	D6	Italy
MVi/California.USA/22.97	CA22-97	5/97	Sporadic case	C2	Germany
MVi/Nevada.USA/20.97	NV20-97	5/97	Sporadic case	H1	China
MVi/Massachusetts.USA/27.97	MA27-97	7/97	Outbreak, 4 cases	D6	Greece/Italy
MVi/Minnesota.USA/33.97	MN33-97	8/97	Outbreak, 5 cases	D6	Brazil
MVi/Massachusetts.MA.USA/30.97	MA30-97	7/97	Sporadic case	D6	Ukraine
MVi/Pennsylvania.USA/28.97	PA28-97	7/97	Sporadic case	D6	Brazil
MVi/Washington.USA/31.97	WA31-97	7/97	Sporadic case	D6	Unknown
MVi/Massachusetts.USA/2.98	MA2-98	1/98	Sporadic case (1)	H1	China
MVi/Washington.USA/17.98	WA17-98	4/98	Sporadic case (1)	D6	Croatia
MVi/Indiana.USA/16.98	IN16-98	4/98	Outbreak: 3 cases	C2	Zimbabwe
MVi/New York.USA/16.98	NY16-98	4/98	Sporadic case (1)	D6	Germany
MVi/California.USA/23.98	CA23-98	6/98	Sporadic case (1)	D5	Japan
MVi/Vermont.USA/28.98	VT28-98	7/98	Sporadic case (1)	D6	Cyprus
MVi/Alaska.USA/32.98	AK32-98	9/98	Outbreak: 33 cases	D5	Japan
MVi/California.USA/7.99	CA7-99	2/99	Outbreak: 4 cases	D4	India
MVi/Washington.USA/12.99	WA12-99	3/99	Outbreak: 3 cases	D8	Italy
MVi/Connecticut.USA/16.99	CT16-99	4/99	Sporadic case (1)	D4	India
MVi/Texas.USA/28.99	TX28-99	7/99	Outbreak: 3 cases	D8	UK
MVi/Virginia.USA/37.99	VA37-99	9/99	Outbreak: 15 cases	D4	Kenya
MVi/Illinois.USA/50.99	IL50-99	12/99	Sporadic case	D7	Sweden
MVi/Michigan.USA/52.99	MI52-99	12/99	Outbreak: 6 cases	D6	UK
MVi/California.USA/1.00	CA1-00	1/00	Sporadic case	D4	Japan
MVi/New York.USA/7.00	NY7-00	2/00	Outbreak: 9 cases	D6	UK
MVi/Washington.USA/6.00	WA6-00	2/00	Sporadic case (1)	D5	Japan
MVi/California.USA/8.00	CA8-00	2/00	Sporadic case (1)	D6	Turkey
MVi/New York.USA/11.00	NY11-00	3/00	Sporadic case	D2	Ireland
MVi/Alaska.USA/16.00	AK16-00	4/00	Sporadic case	H2	Unknown
MVs/Hawaii.USA/20.00	HI20-00	5/00	Sporadic case	D5	Japan
MVs/California.USA/24.00	CA24-00	5/00	Outbreak (5)	G2	Unknown
MVs/Florida.USA/25.00	FL25-00	6/00	Sporadic case	H1	Unknown
MVi/Vermont.USA/24.00	VT24-00	6/00	Outbreak: 6 cases	D4	Ethiopia
MVi/Michigan.USA/35.00	MI35-00	9/00	Sporadic case	D5	Unknown
MVi/Kansas.USA/43.00	KS43-00	11/00	Sporadic case	D5	Unknown
MVi/California.USA/49.00	CA49-00	12/00	Outbreak: 3 cases	D3	Philippines
MVs/Washington.USA/2.01	WA2-01	1/01	Outbreak: 11 cases	H1	Korea
MVi/Maryland.USA/5.01	MD5-01	1/01	Outbreak: 4 cases	D3	Philippines

Table 1. (continued) Summary of virologic surveillance for measles, United States, 1997–2001

Virus/specimen ^a	Abbreviation	Date	Activity ^b	Genotype ^a	Source ^c
MVi/Massachusetts.USA/6.01	MA6-01	2/01	Outbreak: 3 cases	D4	Pakistan
MVs/Illinois.USA/5.01	IL5-01	2/01	Sporadic case	H1	Korea
MVi/Minnesota.USA/9.01	MN9-01	2/01	Sporadic case	H1	Unknown
MVi/Washington.USA/9.01	WA9-01	2/01	Sporadic case	H1	China
MVi/California.USA/13.01	CA13-01	3/01	Sporadic (1)	D5	Unknown
MVs/Hawaii.USA/22.01	HI22-01	5/01	Sporadic case	D5	Unknown
MVs/California.USA/31.01	CA31-01	7/01	Sporadic case	D5	Japan
MVi/NewYork.USA/28.01	NY28-01	7/01	Outbreak: 4 cases	D5	Japan
MVi/Minnesota.USA/35.01	MN35-01	8/01	Sporadic case	D4	Kenya
MVi/Minnesota.USA/36.01	MN36-01	9/01	Sporadic case	H2	Unknown
MVs/Arizona.USA/35.01	AZ35-01	9/01	Sporadic case	D7	Unknown
MVi/California.USA/38.01/1	CA38-01/1	9/01	Outbreak: 3 cases	D7	Europe
MVi/California.USA/38.01/2	CA38-01/2	9/01	Outbreak: 6 cases	D7	Unknown

^aStrain name and genotypes as recognized by the World Health Organization (20). MVi indicates that sequence was obtained from a viral isolate; MVs indicate sequence was obtained directly from the specimen.

^bFor sporadic cases, the number of spread cases is indicated in parentheses.

^cSource identified by standard epidemiologic techniques.

and Ethiopia (17,21,34). A genotype D4 virus (CA1-00) was isolated from a single imported case in California that was traced to Japan. This finding was unusual because genotype D4 viruses have never been detected in Japan despite extensive virologic surveillance.

Previous studies had shown that wild-type measles viruses isolated in the People's Republic of China were members of a distinct genotype designated H1 (14). More recent information indicated that measles viruses circulating in both China and Vietnam were members of clade H but were sufficiently divergent from each other to be considered two separate genotypes, H1 and H2, respectively (35). Analysis of viruses imported from both China and Vietnam showed that these new genotype designations will be useful in epidemiologic surveillance. In 1997 and 1998, two viruses from genotype H1 were isolated from cases imported from China, and one virus in genotype H2 was isolated from a case imported from Vietnam. Two other viruses from genotype H2, AK16-00 and MN36-01, were isolated from sporadic cases that occurred in Alaska during 2000 and Minnesota during 2001, respectively. The sources could not be identified by standard epidemiologic investigation.

During 2001, genotype H1 viruses were isolated from an outbreak in Washington (WA2-01) and four sporadic cases in Florida (FL26-00), Washington (WA9-01), Illinois (IL5-01), and Minnesota (MN9-01). Concurrently, the Republic of Korea was experiencing a measles epidemic that began in 2000. Korea was identified as the source of the Washington outbreak and the sporadic case in Illinois. The sequences from the Washington outbreak and the sporadic case in Illinois were identical to those of genotype H1 viruses isolated in Korea (36). The person diagnosed with measles in Florida in 2000

had traveled to Los Angeles and Las Vegas shortly before onset of illness and probably was infected by a genotype H1 virus (FL26-00) while in transit. The sequence of FL26-00 was identical to that of the two viruses imported from Korea, suggesting that the source of this case may have also been the Korean outbreak (Figure). The sequence of the virus (WA9-01) from the sporadic case in Washington, which was imported from China, was very closely related to the sequences of the Korean viruses. This finding suggests that the genotype H1 viruses circulating in both China and Korea are closely related (Figure). The sequence of the virus identified in the Minnesota

Table 2. Frequency of detection of measles genotypes, United States, 1997–2001

Genotype	No. ^a	Source ^b
D6	13	European countries, Brazil, unknown
D5	12	Japan, unknown
D4	8	India, Kenya, Ethiopia, Pakistan, Japan, unknown
H1	7	China, Korea, unknown
C2	2	Germany, Zimbabwe
D8	2	Italy, United Kingdom
H2	3	Vietnam, unknown
D3	2	Philippines
D2	1	Ireland
D7	4	Sweden, Europe, unknown
G2	1	Unknown

^aNumber indicates the number of times a genotype was associated with either an outbreak or a case.

^bSource of virus, if known, based on standard epidemiologic investigations.

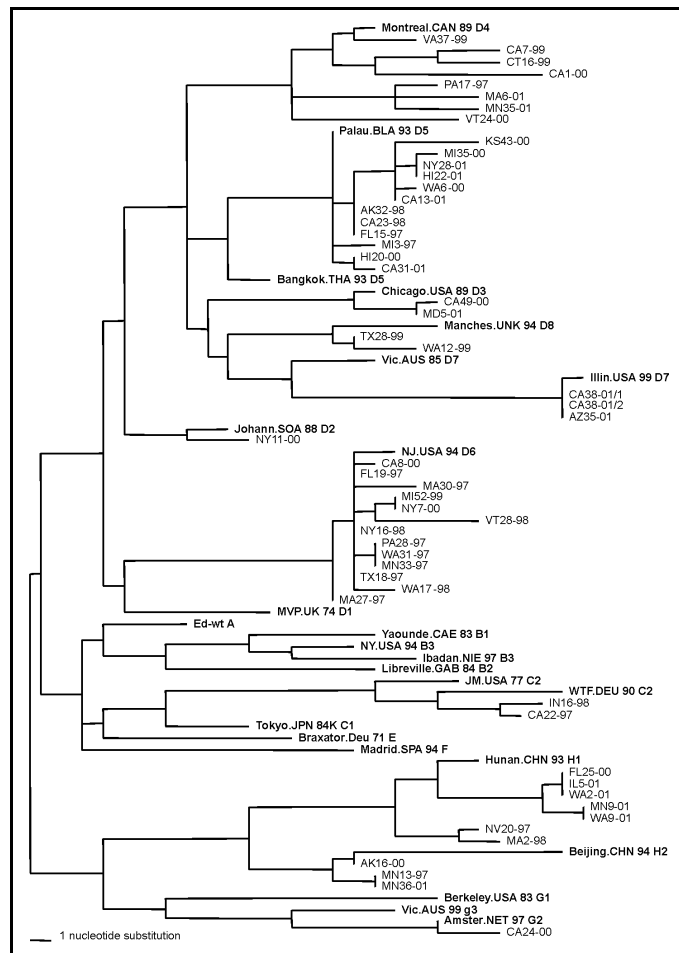


Figure. Genetic relationship between measles viruses isolated in the United States in 1997–2001 and the reference strains established by the World Health Organization (WHO) (20). Phylogenetic tree was based on the nucleotide sequences coding for the COOH-terminus of the nucleoprotein. Strain abbreviations are given in Table 1. Reference strains as established by WHO are shown in bold and designated by their genotype name. The length of the horizontal scale bar represents one nucleotide change.

case (MN9-01) with an unknown source was identical to that of the Washington case imported from China (WA9-01).

Viruses from genotype C2 were isolated from two imported cases in the United States. Detecting a genotype C2 virus (CA2-97) in association with an importation from Germany was not unusual since genotype C2 viruses have frequently been detected in Europe (21), but the isolation of a C2 virus (IN16-98) from a patient returning from Zimbabwe was unexpected. No viral isolates from Zimbabwe have been characterized, and genotype C2 viruses have not been detected in any countries in southern Africa. In imported cases, the source of infection is usually assumed to be the country in which the person was traveling during the incubation period. However, this patient may have been infected while in transit from Africa to the United States via Europe. In fact, in this case, rash onset was 14 days after completion of travel, suggesting that the infection occurred near the end of the trip.

Measles viruses in genotype D2 are known to circulate in both South Africa and Zambia (13,17). A genotype D2 virus was obtained from a single case imported into the United States from Dublin, Ireland, in 2000 (NY11-00). Although genotype D2 viruses are probably not endemic in Ireland, genotype D2 viruses were isolated during an outbreak that occurred in an immigrant community in Dublin that had low vaccination coverage. The sequence of NY11-00 was identical to the sequence of a virus isolated during the Irish outbreak (37).

Viruses from genotype D3, the genotype associated with the resurgence of measles in the United States in 1989–1991, were detected from small outbreaks in California and Maryland in December 2000 and January 2001, respectively. In both outbreaks, the source of infection was the Philippines. The only other genotype D3 virus detected in the United States after 1993 was also imported from the Philippines to California in 1996 (3).

Six of the sequences obtained from cases in 1999 and 2001 (TX28-99, WA12-99, IL50-99, CA38-01/1, AZ35-01, CA38-01/2) were closely related to the recently recognized genotypes D7 and D8 (Figure). A retrospective study showed that viruses from genotype D7 were isolated in Australia as early as 1985 (5). The sequences of IL50-99, CA38-01/1, AZ35-01, and CA38-01/2 were most closely related to the sequences from the Australian genotype D7 viruses. Interestingly, a virus isolated in Canada in 2000 from a case with a travel history to Mexico had a sequence nearly identical to that of IL50-99 (a WHO reference strain for genotype D7), which was imported into the United States from Sweden (38). In 2001, genotype D7 viruses (CA38-01/1, CA38-01/2, AZ35-01) were associated with two small outbreaks in California and a sporadic case in Arizona. One of the outbreaks in California had a European source (Table 1). Genotype D7 viruses were isolated from cases that were imported into El Salvador from Europe (39) as well as from outbreaks in Germany (40), suggesting that D7 may be another endemic European genotype. The two viral isolates from genotype D8, TX28-99 and WA12-99, had identical H gene sequences, although the sources of importation were different. The sequences of these viruses were most closely related to that of a virus isolated in the United Kingdom in 1994 (10), which has been designated the reference strain for genotype D8. Viruses in genotype D8 have been detected in Ethiopia and Nepal in 1998 and 1999, respectively (34,41).

The source of a small outbreak in Los Angeles, California, during May–June 2000 was never identified. While no viral isolate was obtained, measles RNA was detected in some of the clinical samples. Sequences of the PCR product showed that a virus in genotype G2 was responsible for the outbreak. Viruses in genotype G2 are known to be circulating in Indonesia and Malaysia and were associated with importation of virus from Indonesia to the Netherlands (22,23). However, virologic surveillance has not been established in most areas of Asia, and genotype G2 viruses may be circulating in countries other than Malaysia and Indonesia.

Discussion

This study demonstrates the utility of virologic surveillance, especially for countries in the elimination phase of measles control. Sequence data obtained from 55 viral isolates or clinical specimens from measles cases in the United States during 1997–2001 indicated that 11 genotypes of virus were represented. No genotype was detected in a consistent pattern that would indicate endemic transmission. Rather, the diversity of genotypes reflects multiple, imported sources of measles virus. When the source of virus was identified by standard epidemiologic investigation, virologic surveillance helped to confirm the source of the virus and to build a genetic database of viral sequences associated with imported cases from different areas. Virologic surveillance was especially useful for characterizing 13 chains of infection in which the source of infection could not be identified by standard epidemiologic methods. Seven different genotypes were detected in these 13 chains, indicating multiple sources of infection. This finding suggests that these unknown source cases were the result of imported virus and not caused by circulation of an endemic genotype. These results underscore the need to improve the mechanism for obtaining appropriate specimens for viral isolation from all suspected cases, especially outbreak-associated cases. In countries that are in the elimination phase of measles control, obtaining specimens for viral isolation at first contact with all suspected measles cases is important.

Viruses isolated during the resurgence of measles in the United States in 1989–1991 were all in genotype D3, suggesting that D3 viruses had spread throughout the entire country (2,3). Following the resurgence, both standard epidemiologic and virologic surveillance indicated that endemic transmission was interrupted in the United States in 1993 (2,3,42,43). After 1993, only three viruses from genotype D3 were isolated in the United States, and all three were the result of importations from the Philippines (3). The pattern of MeV genotypes observed in the United States in 1993–2001 suggests an absence of indigenous transmission of virus since no genotype was consistently isolated. The pattern of viral genotypes reported for the United States after 1993 has been observed in other areas of the world that have good virologic surveillance and have achieved a high level of measles control. In Canada, the pattern of viral genotypes detected over the last 10 years has been very similar to the pattern in the United States (38). Likewise, virologic surveillance in Victoria, Australia, over a 25-year period suggested that repeated importation of multiple genotypes had occurred (5), and a similar pattern has been reported for the United Kingdom (10). In contrast, in countries that still have indigenous transmission of measles, only a limited number of genotypes are circulating (14,15).

The tremendous reduction of measles cases in the United States after 1991 was due in part to the successful measles control and elimination program initiated by the Pan American Health Organization (PAHO) in the early 1990s. However, during 1997, Brazil had a resurgence of measles, with nearly 50,000 cases reported (44,45). The outbreaks in Brazil eventu-

ally spread to several other South American countries. Genetically homogeneous viruses in genotype D6 viruses were associated with all the recent measles activity in South America except for two cases imported into El Salvador in 2001. In the last 2 years, PAHO has reported a record low number of measles cases in the Americas and <2,000 cases were reported for the year 2000 (46); most cases occurred in the Dominican Republic and Haiti. Virologic surveillance will play a key role in documenting the elimination of endemic transmission of the genotype D6 viruses in South and Central America in the same manner that was used to document the elimination of the genotype D3 viruses in the United States. Efforts are under way to improve laboratory capacity and expand virologic surveillance in the Americas.

Strengthening virologic surveillance activities will not only contribute to our understanding of the transmission pathways of MeV but also increase the sensitivity of measles diagnosis. As the prevalence of disease decreases, the positive predictive value of serologic testing also decreases. Having the laboratory capacity to detect MeV or viral RNA will be especially helpful for measles surveillance in areas where indigenous transmission has been interrupted and many of the suspected cases are sporadic. The identification of new genotypes indicates that our current understanding of the extent of genetic diversity in measles strains throughout the world is incomplete. Virologic surveillance has not been initiated in many countries, and others are just beginning to collect appropriate samples. Virologic surveillance activities need to be initiated or expanded in countries that are in the outbreak control phase of measles control to obtain an accurate record of the pattern of endemic viral genotypes present in all areas of the world.

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Public Health Impact of Reemergence of Rabies, New York

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This report summarizes the spread of a raccoon rabies epizootic into New York in the 1990s, the species of animals affected, and human postexposure treatments (PET). A total of 57,008 specimens were submitted to the state laboratory from 1993 to 1998; 8,858 (16%) animals were confirmed rabid, with raccoons the most common species (75%). After exposure to 11,769 animals, 18,238 (45%) persons received PET, mostly because of contact with saliva or nervous tissue. We analyzed expenditure reports to estimate the cost of rabies prevention activities. An estimated \$13.9 million was spent in New York State to prevent rabies from 1993 to 1998. Traditional prevention methods such as vaccinating pets, avoiding wildlife, and verifying an animal's rabies status must be continued to reduce costly PET. To reduce rabid animals, exposures, and costs, oral vaccination of wildlife should also be considered.

The incidence of human rabies is high in developing countries, and most cases of the illness occur in humans with untreated dog bites (1,2). In developing countries, rabies control in domestic canids has shifted the source of rabies exposures for most humans and domestic animals to wild terrestrial animals. Reported animal rabies cases in the United States have increased dramatically since 1990 in association with the raccoon rabies epizootic in the mid-Atlantic and northeastern states. Before 1990, rabies infections in New York were attributed to red fox and bat variants of the virus. After 1993, rabies testing indicated that the red fox variant no longer existed in the state (3); instead, a raccoon rabies variant had moved into New York State from Pennsylvania in 1990.

Nationwide, the number of reported rabies cases in animals increased from 6,972 in 1991 to 9,495 in 1993, but decreased to 8,224 in 1994, 8,509 in 1997, and 7,961 in 1998 (4–11). Wild animals accounted for 92% of animal rabies cases in the United States, with raccoons reported most frequently, followed by skunks, bats, and foxes. The number of human cases remained low in the same time period, ranging from one case in 1998 to six cases in 1994 (4–11). In 1991, New York State accounted for 14% of reported rabid animals in the United States; this proportion increased to 28% in 1993 (12,13).

The exposure of humans and domestic animals to rabid animals has resulted in an estimated 16,000–39,000 persons per year receiving postexposure prophylaxis treatment (PET) in the United States (14). The estimated cost for human postexposure treatment ranges from \$1,039 to \$4,447 per person (15). Including pet animal vaccinations, the total cost of treatment was recently estimated at \$300 million per year in the United States (16).

New York State has passed a legislative appropriation for rabies prevention and PET. Reimbursement of PET costs not covered by third-party payers was first established more than 50 years ago in response to concerns about potential human deaths from fox rabies in those who could not afford treatment. Since the New York State Department of Health (NYSDOH) disburses these funds, this agency can provide accurate estimates of the cost of postexposure rabies treatments in the state. In addition, NYSDOH's active rabies laboratory conducts all diagnostic work in the state, excluding New York City, which has its own laboratory (although test result data from New York City are also reported).

Initial analyses of rabies treatments for four New York counties in 1993 and 1994 have been previously published (13). In this study, we examine the reemergence of rabies in New York and summarize information on the spread of rabid animals, the type of animals involved, trends in human exposures to rabid animals, and the intervention strategies to reduce human exposures from 1993 to 1998.

Materials and Methods

In New York State, public health law requires health-care providers with knowledge of a person exposed to an animal suspected of having rabies infection to report the incident to the local health unit (LHU). LHUs are required to have comprehensive rabies control protocols that provide 24-hour availability of county staff to manage possible exposures, including 10-day confinement and observation of apparently healthy dogs and cats responsible for exposures; collection, preparation, and submission of animal specimens to the rabies laboratory for prompt rabies examination; authorization of human PET; and provision of pet vaccination clinics. Annually, LHUs must submit to NYSDOH a detailed expenditure report for state-reimbursed costs including PET, laboratory specimen preparation, and pet vaccination clinics. We used fiscal year

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data (April–March) from 1993 to 1998 to estimate the overall cost of human PETs in New York.

A rabies specimen history form accompanies each animal specimen submitted to the New York State Wadsworth Center rabies laboratory for testing. Using this form, we gathered information specific to the specimen regarding species, location of capture, nature of human and animal contacts, and rabies testing results.

A rabies surveillance report form is completed by the LHU for each animal exposure that resulted in human postexposure treatment and for each rabid animal. These surveillance forms are forwarded to the NYSDOH Bureau of Communicable Disease Control for data entry and analysis. Data collected on these reports include animal species, location, type of exposure, and number of humans exposed to the suspected animal.

We matched data from the surveillance reports with data from rabies laboratory specimen history reports. Positive test results with missing surveillance information were actively followed up with LHUs to assure the completeness of exposure and treatment data. The data from laboratory and human exposure reports have been computerized for the years 1993–1998 and are analyzed in this report. To map New York’s counties and the year raccoon rabies was first confirmed in each county, we included data from 1991 to 1997.

Results

From 1993 to 1998, a total of 56,947 animal and 61 human specimens were submitted for rabies testing, with the highest number of tested animals in 1993 (11,896) and the lowest in 1995 (8,032) (Figure 1). The overall proportion of tested animal specimens with laboratory-confirmed rabies virus was 16%, and the number of rabid animals declined from a high of 2,688 (23%) in 1993 to 1,097 (11%) in 1998. However, we did not observe a similar decline in the 18,238 humans who received PET during this period, with the highest number in 1997 (3,373) and the lowest in 1995 (2,422).

The geographic movement of raccoon variant of rabies is shown in Figure 2, which indicates when the variant was first confirmed in each county from 1991 to 1997. By 1998, only three counties reported no rabid raccoons or other animals infected with the raccoon variant of rabies. Although the raccoon rabies variant continued to spread throughout the state in the 1990s, the annual number of raccoons testing positive for rabies decreased from 2,318 in 1993 to 691 in 1998.

From 1993 to 1998, a total of 18,071 animal rabies surveillance reports were received from local health departments (Figure 3). Of these, 8,437 (47%) were for exposures to animals not submitted for rabies testing. The annual number of surveillance reports without an animal submitted for rabies testing increased from 1,194 in 1993 to 1,714 in 1998.

A total of 8,858 rabies surveillance reports were received on animal specimens with laboratory-confirmed rabies (Figure 3), with 6,302 representing rabid animals in which no human exposure was reported. Of the number of rabid animals associated with human exposures, the species most frequently

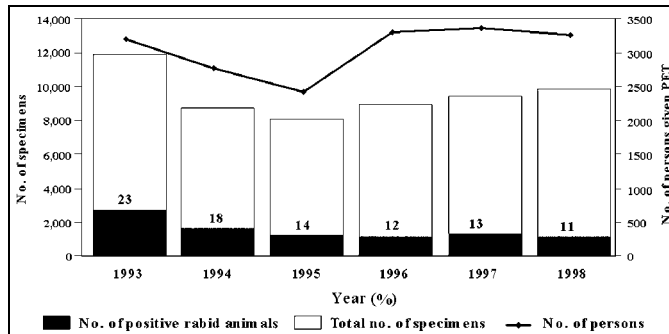


Figure 1. Number of animal specimens tested for rabies, rabid animals, and humans receiving postexposure treatments, New York, 1993–1998.

involved were raccoons (65.2%), skunks (10.4%), bats (7.2%), cats (6.5%), and foxes (5%) (Table 1). For some domestic species, a large proportion of the rabid animals were reported to have exposures resulting in human PET, such as goats (100%), horses (96%), cats (90%), dogs (87%), and cows (82%). High numbers of PETs (>10) for single incidents of rabid animals were documented for many animal species, including raccoons, bats, foxes, cats, cows, deer, dogs, horses, and ferrets. In 1996, 465 persons who attended a county fair received PET because of contact with one rabid goat.

A total of 11,552 persons received PET for exposure to 8,762 animals with specimens unavailable for testing or not

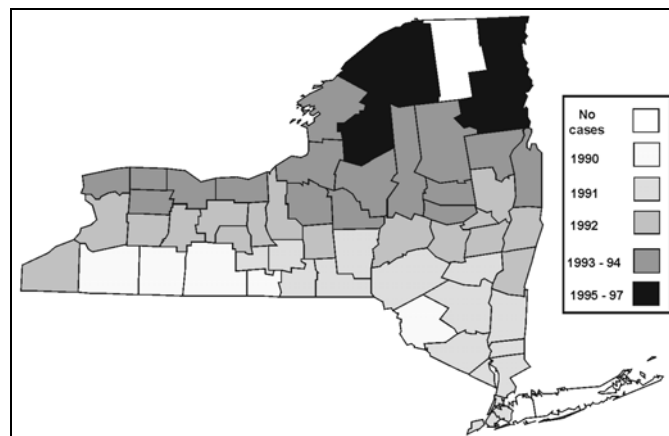


Figure 2. Annual distribution of raccoon-variant rabies when first confirmed within each county, New York, 1991–1997.

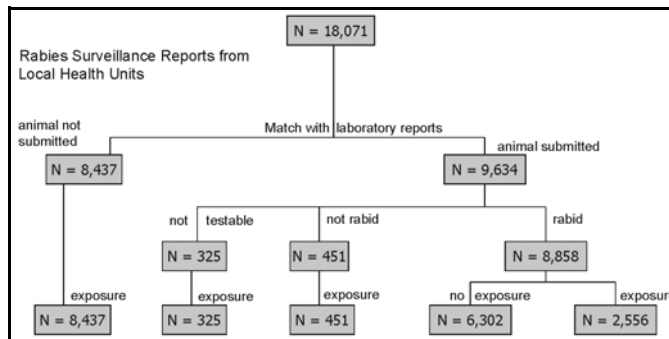


Figure 3. Matching of rabies surveillance reports from local health departments and laboratory reports for submitted animals by test result and human exposure, New York, 1993–1998.

Table 1. Rabid animals by species, human exposure, and postexposure treatment, New York, 1993–1998^a

Animal species	No. (%) of rabid animals		No. (range) ^b of humans receiving PET
	without human exposure	with human exposure	
Raccoon	4,983 (79.1)	1,666 (65.2)	2,944 (1–25)
Skunk	895 (14.2)	266 (10.4)	470 (1–8)
Bat	221 (3.5)	184 (7.2)	377 (1–12)
Fox	101 (1.6)	127 (5.0)	229 (1–10)
Cat	18 (0.3)	166 (6.5)	844 (1–36)
Cow	12 (0.8)	54 (2.1)	246 (1–30)
Woodchuck	44 (0.7)	21 (0.8)	32 (1–5)
Deer	14 (0.2)	10 (0.4)	42 (1–13)
Dog	3 (<0.1)	20 (0.8)	286 (1–37)
Horse	1 (<0.1)	22 (0.9)	139 (1–14)
Beaver	2 (<0.1)	4 (0.2)	9 (1–3)
Goat	0	4 (0.2)	476 (1–465)
Bobcat	0	3 (<0.1)	7 (1–4)
Coyote	1 (<0.1)	2 (<0.1)	2 (1)
Rabbit	1 (<0.1)	2 (<0.1)	12 (5–7)
Sheep	0	2 (<0.1)	7 (2–5)
Ferret	0	2 (<0.1)	16 (3–13)
Other ^c	6 (<0.1)	1 (<0.1)	1 (1)
Total	6,302 (100)	2,556 (100)	6,139 (1–465)

^aPET, postexposure treatment.^bRange of number of PETs for a single exposure incident for a rabid animal.^cOther species included one rabid opossum resulting in human PET, three rabid opossums, one fisher, one pig, and one otter without consequent human PET.

testable because of specimen condition (Table 2). In addition, 547 persons received PET for exposure to 451 animals that had negative rabies virus tests (Table 2). Cats, bats, and dogs each accounted for approximately 25% of the exposures requiring treatment when the suspect animal was unavailable for testing, with raccoons accounting for more than 10% of the exposures. Similarly, these species also accounted for most of the PETs when laboratory testing confirmed that the animal was not rabid, although more than 40% of the unnecessary treatments resulted from cat exposures.

Across all categories of rabies status for the animal, most postexposure treatments were provided because of possible contact with saliva or nervous tissue (44.5%), followed by bite (34.9%) and scratch (5.8%) exposures (Table 3). When the animal tested positive for rabies, a larger proportion of the PETs (82.9%) were for saliva or nervous tissue contact, particularly from raccoons. In contrast, for the suspect or nonrabid animals, most of the PETs were for bite exposures (47.6% and 60%, respectively).

Two fatal human rabies cases related to bat exposure occurred in New York in 1993 and 1995 (the 1995 case was in a Connecticut resident hospitalized in New York) resulting in treatment of 55 and 48 persons, respectively, who had contact with the cases either at home or in the hospital. Although bats represented only 4.6% of the rabid animals in New York, exposure to bats accounted for 25.8% of the PETs, with a total of 4,706 persons receiving PET after exposure to bats in the state. Fifty-one percent of the bat-related PETs were classified as “unknown” in regard to exposure, and 28% were provided because of reported contact with saliva or nervous tissue.

The total expenditure for PETs, laboratory specimen preparation, and pet vaccination clinics increased in New York from \$1.8 million in the 1993–1994 fiscal year to \$2.9 million

in the 1998–1999 fiscal year (Table 4). The estimated average annual statewide cost for the biologics and administration of the PETs was \$1.8 million; the average cost per person for PET was \$927, increasing from \$769 in the 1993 fiscal year to \$1,136 in the 1998 fiscal year.

Discussion

The public health impact of the reemergence of rabies in New York resulting from the spread of raccoon variant in the 1990s was profound in terms of the number of rabid animals diagnosed, humans exposed and treated, and PET costs. Despite the decreasing number of rabid animals during the study period, the increasing number of humans receiving treatment for rabies from 1993 to 1998 appeared to be a result of the high number of suspected rabid animals (untested) and the high number of reported bat exposures following publicity surrounding two bat rabies-related human deaths.

The high proportion of PETs associated with exposures other than bites (9,165/18,238 [50%]) in our review indicates the degree of human fear about possible rabies and the difficulties in interpreting definitions of exposure (17,18). This concern is also indicated by the PET administered to 465 persons exposed to a rabid goat in 1996 and 547 persons exposed to animals that tested negative for rabies from 1993 to 1998. With 41% of 11,552 treated persons exposed to dogs and cats without testable specimens (because of specimen condition), efforts to find these pets to verify their rabies status may be helpful in reducing unnecessary treatments (19). The annual reviews and recommendations on animal rabies control from the National Association of State Public Health Veterinarians (20) should be applied to reduce human exposures to rabid animals and unnecessary rabies PETs.

Table 2. Nonrabid or suspected rabid animals and the number of humans receiving postexposure treatment, by animal species, New York, 1993–1998^a

Animal species	No. (%) of suspected rabid animals ^b	No. (range) ^c of humans receiving PET	No. (%) of nonrabid animals	No. (range) ^c of humans receiving PET
Cat	2,373 (27.1)	2,620 (1–11)	183 (40.6)	220 (1–5)
Bat	2,289 (26.1)	4,181 (1–40)	116 (25.7)	148 (1–4)
Dog	2,000 (22.8)	2,067 (1–15)	49 (10.9)	53 (1–3)
Raccoon	952 (10.9)	1,247 (1–21)	51 (11.3)	67 (1–5)
Skunk	160 (1.8)	211 (1–6)	10 (2.2)	12 (1–3)
Fox	104 (1.2)	125 (1–3)	6 (1.3)	6 (1)
Woodchuck	92 (1.1)	99 (1–3)	9 (2.0)	9 (1)
Squirrel	73 (0.8)	75 (1–2)	6 (1.3)	6 (1)
Opossum	48 (0.5)	51 (1–2)	2 (0.4)	1 (1)
Deer	26 (0.3)	32 (1–4)	3 (0.7)	5 (1–2)
Muskrat	15 (0.2)	15 (1)	5 (1.1)	6 (1–2)
Cow	18 (0.2)	37 (1–2)	1 (0.2)	1 (1)
Rabbit	17 (0.2)	18 (1–2)	1 (0.2)	1 (1)
Rat	16 (0.2)	16 (1)	2 (0.4)	2 (1)
Ferret	12 (0.1)	16 (1–3)	3 (0.7)	3 (1)
Chipmunk	12 (0.2)	13 (1–2)	1 (0.2)	1 (1)
Other	104 (1.2)	133 (1–7)	3 (1.2)	5 (1)
Unknown	450 (5.1)	596 (1–7)	0	0
Total	8762 (100)	11552 (1–40)	451 (100)	547 (1–5)

^aPET, postexposure treatment.^bRabies status of animals could not be determined by testing (animal not submitted for rabies testing or specimen not testable because of specimen condition).^cRange of number of PETs for a single exposure incident to a potentially rabid animal.

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Table 3. Number of humans receiving postexposure treatment, by animal status and type of exposure, New York, 1993–1998

Type of exposure	No. (%) of humans receiving PET ^a			
	Rabid animal	Suspect rabid animal ^b	Nonrabid animal ^c	Total
Bite	538 (0.8)	5,503 (47.6)	328 (60.0)	6,369 (34.9)
Scratch	224 (3.6)	773 (6.7)	56 (10.2)	1,053 (5.8)
Contact with saliva	5,090 (82.9)	2,891 (25.0)	131 (23.9)	8,112 (44.5)
Unknown exposure	287 (4.8)	2,385 (20.6)	32 (5.9)	2,704 (14.8)
Total	6,139 (10)	11,552 (100)	547 (100)	18,238 (100)

^aPET, postexposure treatment.

^bPETs due to exposure to animals not submitted for rabies testing or specimen was not testable because of specimen condition.

^cPETs due to exposure to animals that tested negative for rabies.

A few studies suggest that >\$1 billion per year has been spent recently to prevent rabies in the United States (14), with the vaccination of pet animals accounting for 82% of the expenditures (the cost associated with pet vaccination given by private providers was not available for our study). Our study estimated that \$13.9 million was spent to prevent rabies in New York, where \$10.7 million (77%) was used on PET from 1993 to 1998. The use of PET for 547 persons exposed to nonrabid animals supports the need for better education of health-care providers to determine whether PET is really necessary pending laboratory test results and the need for public education to reduce exposure to rabid animals and minimize contact to exposed pets.

Seventy-five percent (24/32) of the human rabies cases in the United States since 1990 have been attributed to bat variants (21–23). The two bat-variant deaths in New York exemplify the new realization that human rabies may result from encounters with bats when bites are unreported or unrecognized. In July 1993, a child without a history of a known bite or other exposure to a suspected rabid animal died from rabies

that was identified as a bat variant (24). In October 1995, a Connecticut resident without history of animal bites but possible exposure to a bat died from rabies in a N. Y. hospital and resulted in 48 state residents receiving PET (25). Bat exposures accounted for 25% of New York's PETs from 1993 to 1998, underscoring the importance of avoiding contact with bats and the need to test bats for rabies when human contact may have occurred (26).

The persistence and spread of rabies in raccoons and domestic animal exposure to this variant continue to be an important issue for public health officials. The reemergence of wildlife rabies in areas like New York (after the fox variant had moved out of the state) as a result of the unimpeded northward spread of the raccoon variant into the state and increased recognition of the importance of bat variants has led to a large number of rabies cases both in domestic and wildlife species and a corresponding number of human rabies PETs. Traditional public health methods of surveillance, public and provider education to avoid exposure to potentially rabid animals, appropriate postexposure prophylaxis, and emphasis on

Table 4. Rabies expenditures for postexposure treatments, laboratory specimen preparation, and pet vaccination clinics, by fiscal year,^a New York, 1993–1998

Type of expenditure	1993–1994	1994–1995	1995–1996	1996–1997	1997–1998	1998–1999
PET ^b	\$1,222,125	\$1,919,606	\$1,257,621	\$1,835,058	\$2,092,572	\$2,347,555
State	\$669,564	\$1,006,471	\$679,902	\$311,356	\$974,079	\$959,362
Local	\$138,415	\$170,284	\$116,368	\$787,500	\$84,630	\$188,723
Other	\$414,146	\$742,851	\$461,351	\$736,202	\$1,033,863	\$1,199,470
Average per person ^c	\$769	\$822	\$824	\$944	\$1,020	\$1,136
Specimens ^d	\$265,037	\$256,518	\$251,796	\$246,794	\$276,219	\$270,184
State	\$200,702	\$234,097	\$231,917	\$226,224	\$254,888	\$250,762
Local	\$64,335	\$22,421	\$19,879	\$20,570	\$21,331	\$19,422
Clinics ^e	\$271,062	\$328,532	\$294,251	\$289,729	\$244,254	\$262,351
State	\$84,671	\$167,763	\$139,456	\$117,840	\$110,145	\$118,002
Local	\$186,391	\$160,769	\$154,795	\$171,889	\$134,109	\$144,349

^aFiscal year is April – March.

^bPET, postexposure treatment; PET costs incurred by the New York State Department of Health (≤\$1,000 per PET), the local health departments, and others (third-party payers).

^cCalculated by dividing total treatment costs by number of persons treated.

^dLaboratory specimen preparation costs incurred by the New York State Department of Health (limits of \$60 per small animal specimen and \$75 per livestock specimen) and the local health departments, for animal euthanasia, head removal, and specimen shipment.

^ePet vaccination clinic costs incurred by the New York State Department of Health (limits of five clinics per year and \$1,000 per clinic) and local health departments.

verifying the negative rabies status of suspect animals to avoid unnecessary treatments will remain important methods for rabies control. However, the major impact of raccoon rabies in human exposure and treatments may also need to be addressed with new wildlife rabies control methods such as oral rabies vaccine (27–29).

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Impact of the El Niño/Southern Oscillation on Visceral Leishmaniasis, Brazil

Carlos Roberto Franke,* Mario Ziller,† Christoph Staubach,† and Mojib Latiff‡

We used time-series analysis and linear regression to investigate the relationship between the annual Niño-3 index from 1980 to 1998 and the annual incidence of visceral leishmaniasis (VL) in the State of Bahia, Brazil, during 1985–1999. An increase in VL incidence was observed in the post-El Niño years 1989 (+38.7%) and 1995 (+33.5%). The regression model demonstrates that the previous year's mean Niño-3 index and the temporal trend account for approximately 50% of the variance in the annual incidence of VL in Bahia. The model shows a robust agreement with the real data, as only the influence of El Niño on the cycle of VL was analyzed. The results suggest that this relationship could be used to predict high-risk years for VL and thus help reduce health impact in susceptible regions in Brazil.

Visceral leishmaniasis (VL) is a widespread parasitic disease in the Old and New Worlds, with a global incidence of 500,000 new human cases each year. VL is the most severe clinical form within the leishmaniasis complex, which is endemic in 88 countries with an at-risk population of approximately 350 million (1). In Brazil, VL affects both humans and animals and is caused by *Leishmania chagasi*, a flagellate protozoan transmitted by the sand fly *Lutzomyia longipalpis* (2). The disease occurs mainly in malnourished young children and is frequently fatal if untreated (3,4). Periodic epidemic waves of VL, observed mainly in northeastern Brazil, have been associated with human migrations to urban areas after long periods of drought (5–8). In this region, El Niño events are related to unusually dry conditions, widespread food scarcity, and migration (9–11). El Niño periods in 1982–1983, 1986–1987, 1991–1993, and 1997–1998 coincided with long droughts recorded by the Superintendence for the Development of the Northeast Brazil (SUDENE). Data from the State of Bahia, analyzed in our study, show that 247 municipalities were affected during the strong El Niño of 1997–98, mainly in the semi-arid inland region, where approximately 200,000 people were included in SUDENE's emergency program, at a cost of an estimated US \$62 million.

El Niño is the strongest interannual climate fluctuation worldwide, characterized by a large-scale warming of the eastern and central equatorial Pacific Ocean. El Niño (also known as El Niño/Southern Oscillation) can be understood as the warm phase of an irregular cycle with an average frequency of 3–4 years. Each event typically lasts for approximately a year, with the peak warming in boreal winter (December–February) and the following spring (March–May) (12). Some studies provide strong evidence of the relationship between El Niño and increased epidemic risk of vector-borne diseases in dis-

tinct regions throughout the world (13,14). This observation is especially true for malaria (15–18). We report the early results of our analysis of the relationship between the El Niño cycle and VL in Brazil.

Methods

The State of Bahia (pop. 13,093,243, in the 2000 census) is situated on the northeast Atlantic coast of Brazil. Its area is 567,295 km², divided into 415 municipalities. The annual number of VL cases from 1985 to 1999 was obtained from the Public Health Secretary of the State of Bahia. In this period in Bahia, 12,413 cases of VL were reported by using a passive case-detection procedure. Diagnosis was based on clinical and epidemiologic features, confirmed by immunofluorescence assay or detection of parasites by examination of smears of bone marrow, lymph node, or splenic aspirate.

For our analysis, we used the mean monthly Niño-3 index for the years 1980 to 1998. This index is the tropical Pacific sea surface temperature (SST) anomaly averaged over 150°W–90°W, 5°N–5°S, obtained from the Hadley Centre dataset (19). To evaluate the relationship between El Niño and VL and to quantify the delay of its possible impact, we calculated the cross-correlation function between the annual incidences of VL per 10,000 inhabitants from 1985 to 1999 and the 12-month moving average of the mean monthly Niño-3 index. The linear temporal trend was removed from both variables before analysis. Additionally, to model the observed dependence, we calculated a linear regression between the annual incidence of VL, the year (to consider the linear temporal trend), and the mean annual Niño-3 index 12 months previously.

Results

We observed that, despite the wide territorial area of Bahia and the complex epidemiologic nature of VL, the cycle of the annual incidence appears to be narrowly associated with both the frequency and duration of El Niño episodes. Low

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incidence levels coincide with the occurrence of El Niño, and an increase in incidence occurs after such climatic events (Figure 1). We found extreme increases of the incidence in relation to the 5-year moving average for the years 1989 (+38.7%) and 1995 (+33.5%).

The cross-correlation function between the annual incidences of VL and the 12-month moving average of the mean monthly Niño-3 index has its strongest negative correlation at a lag of 12 months and its strongest positive correlation at a lag of 36 months (Figure 2). The positive correlation may, in part, result from a broad minimum of the autocorrelation function of the mean monthly Niño-3 index at lags from 16 to 28 months, with autocorrelation values of $-0.54 < \text{correlation coefficient } (r) < -0.45$.

The results of the regression model (root square error [RSE] = 0.209, adjusted coefficient of determination [adj. R²] = 0.465, F test with 2 and 12 degrees of freedom [F_{2,12}] = 7.081, $p = 0.009$) demonstrate that the mean Niño-3 index 12 months previously and the temporal trend can account for approximately 50% of the variance in the annual incidence of VL in Bahia (Figure 3). The model shows a robust agreement with the real data, considering that only the influence of El Niño on the variance of VL incidence was analyzed.

Discussion

Our results show that El Niño episodes are related to variation in the annual incidence of VL in the State of Bahia and suggest that an El Niño-based early warning system for VL may help reduce the health impact of the disease in susceptible regions in Brazil. According to our results, the annual incidence of VL in Bahia tends to reach its lowest level in the first year after El Niño episodes and begins to increase in the second year after El Niño. The delay of this correlation pattern is unusual in light of the well-studied association between malaria and El Niño reported in different regions of the world. Increases in malaria incidence have been reported to accompany El Niño episodes or occur in the year immediately following such episodes (15–18).

Because of the multifactorial interactions involved in this complex system, a simple deterministic explanation for the interannual correlation between El Niño and VL is not possible. However, some climatic and epidemiologic behaviors related to VL could be helpful in drafting a preliminary assumption about pathways by which El Niño can affect the temporal distribution pattern of the disease. In the New World, >90% of VL cases have been reported in Brazil, mainly in the semi-arid northeast region (2,20), where dry and rainy seasons are clearly defined. Furthermore, in this region, evidence indicates that the population density of the sand fly vector is low during the dry season and increases after the end of the rainy season (December–April), reaching its highest density level around May (5,21). The resulting increase in transmission intensity during this seasonal peak of vector density leads to an increase in the reported incidence after this period (4,5,21). In the semi-arid region of Bahia, El Niño episodes coincide with



Figure 1. The Niño-3 index and the incidence of visceral leishmaniasis (VL) in the State of Bahia, Brazil, on a yearly basis. The broken line is the normalized mean annual Niño-3 index, 1980–1998. The solid line shows the annual number of cases of VL per 10,000 inhabitants during 1985–1999.

the rainy season and negate its effect, causing a prolonged drought by connecting the previous dry season, the period of El Niño, and the subsequent dry season. We hypothesize that, in view of the negative influence of dry conditions on the vector density, the long droughts triggered by El Niño events could be expected to be accompanied by prolonged low vector density and low transmission intensity. These exceptional circumstances most likely contribute to a gradual increase in the potential risk of some related epidemiologic factors, such as waning herd immunity, increase in the susceptible population in the endemic areas (as a result of new births and nonimmune immigrants), and displacement of human and animal carriers to regions with populations that lack a protective immunity (4,20). Drought-related food scarcity and growing malnutrition also increase the susceptibility to VL within these risk populations (4,22).

The rainy season at the beginning of the first year after El Niño is followed by an increase in vector density (around

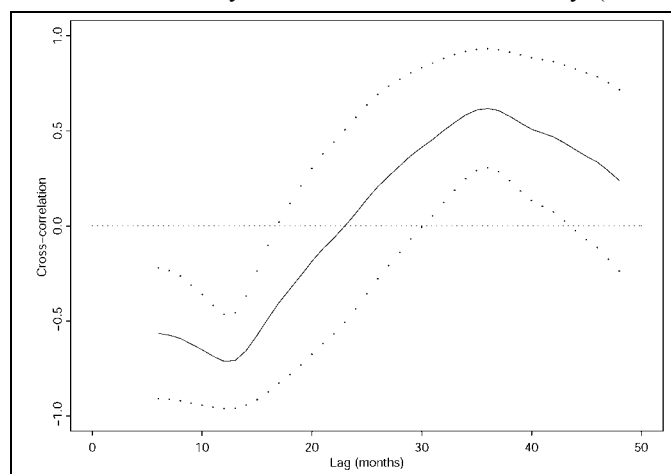


Figure 2. Cross-correlation function between the annual incidences of visceral leishmaniasis from 1985 to 1999 and the 12-month moving average of the mean monthly Niño-3 index (solid line). Broken lines are the corresponding 95% pointwise confidence intervals.

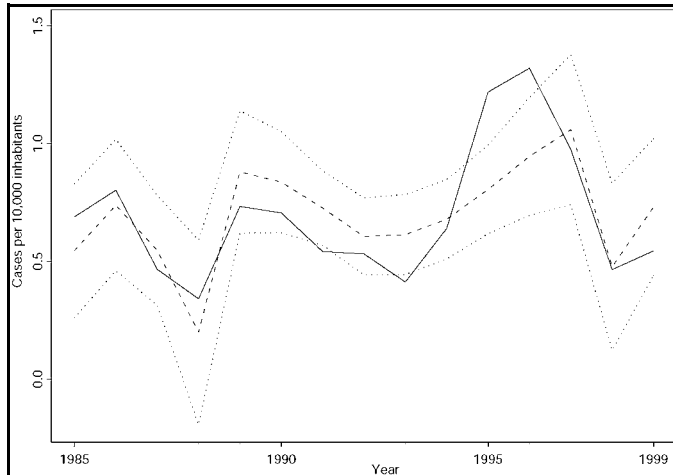


Figure 3. Result of the regression model. The figure represents the annual number of cases of visceral leishmaniasis per 10,000 inhabitants during 1985 to 1999 (solid line), the fitted regression model (broken line), and the corresponding 95% confidence limits (dotted lines).

May), which triggers a severe increase in infection rate in high-risk populations. However, the incubation period (2–6 months) (23), added to the time from the onset of VL symptoms until diagnosis of the disease (approximately 3 months, according to data from the Federal Health Foundation of Bahia [data not shown]), shifts the reporting of most new cases into the second year after El Niño. Although this preliminary assumption provides a plausible explanation of the lag time and intensity of the teleconnection pattern between El Niño and VL cycle in Bahia, the occurrence of distinct climate and vector seasonality patterns on the regional level could lead to differences in the lag time previously discussed.

In accordance with our assumption, the divergent regression fits of the model observed for the years 1991, 1993, 1995, and 1996 most likely reflect the influence of the exceptionally long El Niño period from 1991 to 1993, which led to a lasting reduction in transmission intensity. Still in accordance with our assumption, the annual incidence of VL increased from 1994 to 1996. The divergent regression fits observed for the years 1995 and 1996 might have been caused by the sum of two factors: the critical increase in the related risk factors following a longer El Niño episode and improvement of the surveillance system. The latter occurred in 1994, when new endemic areas were included. This expanded surveillance probably facilitated better reporting of this epidemic phase of the VL cycle in Bahia. However, the low incidence reported for 1997 and 1998 (El Niño period) shows a reduced impact of this new system on the detection of new VL cases. Statistically based forecast models also failed to predict the evolution of this climatic anomaly during 1990–1993 in northeast Brazil (11). Within our limited time-series of VL data, only one longer El Niño period is included. Indeed, our model underestimates the intensity of the impact of this atypical El Niño event on the occurrence of VL. The real impact of this modified surveillance system can only be evaluated through a comparative analysis with future data.

The extreme changes in the annual incidence pattern of VL associated with the atypical occurrence of El Niño during 1991–1993 do, however, provide a possible example of the potential impact of future variations of the El Niño cycle on public health. This observation is especially interesting given the expected rising frequency of El Niño following the continuous increase of greenhouse-gas concentrations in the atmosphere (24).

The results presented here provide the first evidence of the relationship between the El Niño cycle and VL. Greater global understanding of this complex relationship, particularly of the impact of El Niño on the population dynamics of humans, animal hosts, and sand fly vectors, could provide additional tools to predict epidemic risk. The ability to forecast VL on the basis of El Niño activity about 12 months before outbreaks could permit preventive improvements on public health infrastructure, including access to financial resources, technical knowledge, active disease surveillance, and targeted vector control to reduce the risk of the increased transmission. Such forecasts would reduce disease and death from visceral leishmaniasis in susceptible regions.

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Characterization of Flagella Produced by Clinical Strains of *Stenotrophomonas maltophilia*

Doroti de Oliveira-Garcia,*† Monique Dall'Agnol,‡ Mónica Rosales,‡
Ana C.G.S. Azzuz,§ Marina B. Martinez,†§ and Jorge A. Girón‡

Stenotrophomonas maltophilia is an emerging nosocomial pathogen associated with opportunistic infections in patients with cystic fibrosis, cancer, and HIV. Adherence of this organism to abiotic surfaces such as medical implants and catheters represents a major risk for hospitalized patients. The adhesive surface factors involved in adherence of these bacteria are largely unknown, and their flagella have not yet been characterized biochemically and antigenically. We purified and characterized the flagella produced by *S. maltophilia* clinical strains. The flagella filaments are composed of a 38-kDa subunit, SM_{FILC}, and analysis of its N-terminal amino acid sequence showed considerable sequence identity to the flagellins of *Serratia marcescens* (78.6%), *Escherichia coli*, *Proteus mirabilis*, *Shigella sonnei* (71.4%), and *Pseudomonas aeruginosa* (57.2%). Ultrastructural analysis by scanning electron microscopy of bacteria adhering to plastic showed flagellalike structures within the bacterial clusters, suggesting that flagella are produced as the bacteria spread on the abiotic surface.

S *tenotrophomonas* (formerly *Pseudomonas* and *Xanthomonas*) *maltophilia* is a widespread environmental microorganism that has become an important opportunistic pathogen associated with nosocomial colonization and infection (1–7). These organisms have been recovered from water faucets, water traps, respirometers, sinks, suction catheters, and occasionally, from cultures of the hands of hospital personnel (5,8). Infection and colonization of implantable medical devices such as catheters and intravenous cannulae represent a major risk for hospitalized patients. *S. maltophilia* can cause septicemia, endocarditis, conjunctivitis, mastoiditis, meningitis, postoperative wounds, abscesses, urinary tract infections, and pneumonia (6,9–11). The isolation rates of *S. maltophilia* from the respiratory tracts of patients with cystic fibrosis and from cancer and HIV-infected patients with opportunistic infections is increasing (4,12,13). Adhesion of these bacteria to abiotic surfaces such as those of medical implants and catheters suggests the development of a biofilm that protects bacteria from natural immune defenses or from the action of antimicrobial compounds. Biofilms are made up of a community of bacteria immobilized and embedded in an organic polymer matrix composed of polysaccharides and proteins of bacterial origin (14–16). Management of infection and successful clinical outcome by means of antimicrobial therapy are complicated by the intrinsic resistance of the bacteria to multiple antimicrobial agents, including carbapenems, and to the natural protection that biofilms confer to the enclosed bacteria (8,14). Besides

the ability to adhere to plastic, to survive and multiply within total parenteral nutrition and other types of intravenous infusions, and to produce extracellular enzymes (4,8), little information is available regarding virulence factors associated with the pathogenesis of these bacteria. Production of a protease and elastase appears to be important in the pathogenesis of *S. maltophilia*-associated infections (5,17).

While for some bacteria the expression of flagella does not clearly relate to pathogenesis, for a variety of bacterial pathogens, such as *Proteus mirabilis*, *Salmonella enterica*, and *Yersinia enterocolitica*, the participation of flagella in adherence and invasion has been documented (18–20). In addition, the role of flagella in the formation and development of biofilm has recently been investigated in *Pseudomonas*, *Escherichia coli*, and *Vibrio cholerae* (21–24). Jucker et al. reported that nonspecific adhesion and biofilm formation by *S. maltophilia* to glass and Teflon may be attributed to the net positive surface charge of the bacteria (23). As with a variety of microorganisms, other surface determinants may confer the adhesive attributes necessary for *S. maltophilia*-specific adhesion. Although biofilm formation by *S. maltophilia* has been documented, no surface molecule or structure such as flagella or fimbrial adhesins implicated in adherence to plastic or eukaryotic cells has yet been characterized in detail (4,23,25). To characterize the surface appendages produced by *S. maltophilia*, we purified flagella from a clinical isolate and used specific anti-flagella antibodies to test for the presence of these structures in a collection of clinical isolates. In addition, we studied the kinetics of adhesion and performed ultrastructural studies by scanning electron microscopy of bacteria adhering to plastic. These studies showed structures resembling flagella, suggesting that these structures may be important for the adherence phenomenon.

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Materials and Methods

Bacterial Strains and Growth Conditions

We included in this study 46 clinical isolates of *S. maltophilia* obtained from patients admitted to four institutions in the City of São Paulo, Brazil: Instituto Dante Pazzanese de Cardiologia, Hospital das Clínicas, Laboratório Fleury, and Hospital Universitário (Universidade de São Paulo). Most of these clinical strains were isolated from respiratory tract secretions obtained from intubated patients with pneumonia; in most cases, *S. maltophilia* was the only infectious agent found (25). *S. maltophilia* ATCC 13637 is a reference strain also used in our studies. For expression of flagella, bacteria were grown on trypticase soy agar supplemented with 5% defibrinated sheep blood (Oxoid, Basingstoke, England) at 37°C for 48 h.

Transmission and Scanning Electron Microscopy

We analyzed the presence of flagella by negative staining and transmission electron microscopy. Bacteria were negatively stained for 2 min with 1% phosphotungstic acid (pH 7.4) on carbon-Formvar (Electron Microscopy Sciences, Fort Washington, PA) copper grids as previously described (26,27). For ultrastructural analysis, bacterial specimens were fixed in 2% formalin and processed for scanning electron microscopy. Briefly, glass or plastic coverslips containing the adherent bacteria were postfixed with 1% osmium tetroxide, dehydrated by sequential ethanol concentrations, dried to critical point, and coated with a mixture of gold and palladium (27). The specimens were examined in a high-resolution Hitachi (Tokyo, Japan) scanning electron microscope.

Isolation of Flagella

For purification of flagella, clinical isolate *S. maltophilia* SMDP92 was grown on 100 blood agar plates and harvested in 100 mL of 10 mM phosphate-buffered saline (PBS), pH 7.4. The flagella were detached from the bacterial cells by vigorous shaking, and the supernatant containing the sheared flagella was separated by centrifugation at 8,000 x g for 30 min (26). The flagella were separated from outer membrane proteins and other contaminants by precipitation with 60% saturation of ammonium sulfate for 18 h at 4°C. After centrifugation at 12,000 x g for 30 min, the flagella were resuspended in PBS, and insoluble contaminants were removed by a similar centrifugation step. The supernatant was subjected to a second cycle of 20% ammonium sulfate precipitation (26). After dialysis with distilled water to remove excess salts, the purity of the preparations was monitored by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (28) and electron microscopy (26).

Western Blotting and N-Terminal Amino Acid Sequence Analysis

For SDS-PAGE and Western blot, whole bacterial cell extracts or flagellar extracts were denatured and separated in

14% polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Bedford, MA) (27). The blot was reacted with anti-flagella antibodies and secondary anti-rabbit immunoglobulin (Ig) G conjugated to horseradish peroxidase (Sigma Chemical Co., St. Louis, MO). The reaction was developed with a mixture of diaminobenzidine and 30% hydrogen peroxide (Sigma). A 38-kDa protein band of interest was excised from the PVDF membrane and subjected to N-terminal amino acid sequence analysis at the Instituto de Química, Universidade de São Paulo. Sequence analysis and homology studies with published flagellin sequences were performed by using the EMBL/GenBank (BLAST of National Center for Biotechnology Information, Bethesda, MD) software.

Anti-Flagella Antibodies

Antibodies against *S. maltophilia* flagella were raised by immunization of New Zealand rabbits with the flagellin protein (38-kDa band) excised from Coomassie-blue stained gels. The bands were dried and homogenized in complete Freund's adjuvant for the first dose and in incomplete adjuvant for the subsequent three weekly doses. Blood was collected at each immunization, and the presence of antibodies was monitored by Western blot. Antibodies against flagella obtained from *E. coli* E2348/69 (O127:H6), *Shigella flexneri*, and *S. sonnei* were available from previous studies (26,27,29).

Adhesion to Inert Surfaces

Adhesion to abiotic surfaces was studied at different times by using polystyrene 24-well plates (Nunc, Naperville, IL) with or without glass coverslips (21). Twenty microliters of an overnight culture of bacteria grown in Luria-Bertani broth was added to the wells containing 1 mL of Dulbecco's minimal essential medium (D-MEM), supplemented with high glucose, and incubated for 15 and 30 min and 1, 2, 3, 4, 5, 6, 18, 48, and 72 h. After three washes with PBS, the bound bacteria were fixed with methanol, stained with crystal violet, and visualized under a light microscope. Replica samples were fixed with 2% formalin for scanning electron microscopy as described. All experiments were conducted in triplicate and were repeated at least three times.

To quantitate bacterial adherence over time (from 30 min to 72 h), we performed an adherence assay in 96-well plates as described and measured the uptake of crystal violet staining by reading optical density at 620 nm (22).

Results

Characteristics of *S. maltophilia* Flagella

Growth of the bacteria in blood agar plates at 37°C resulted in a condition favorable for flagella expression. Analysis by electron microscopy demonstrated that while some organisms had only one polar flagellum, others had several flagellar structures (Figure 1). The flagella filaments, ~45 nm in width and >15 µm long, are indistinguishable from other

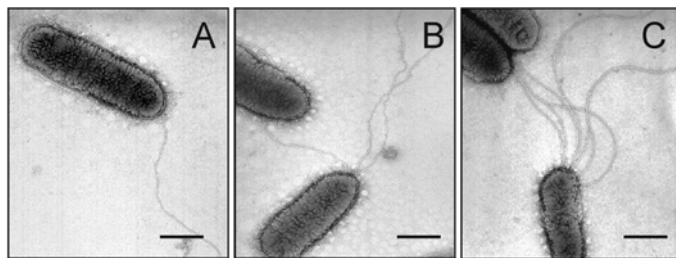


Figure 1. Electron micrographs showing expression of flagella by SMDP92. *Stenotrophomonas maltophilia* strains can have one (A) to several flagella (B,C). The flagella on these bacteria show a polar disposition. Bars, 0.5 μ m

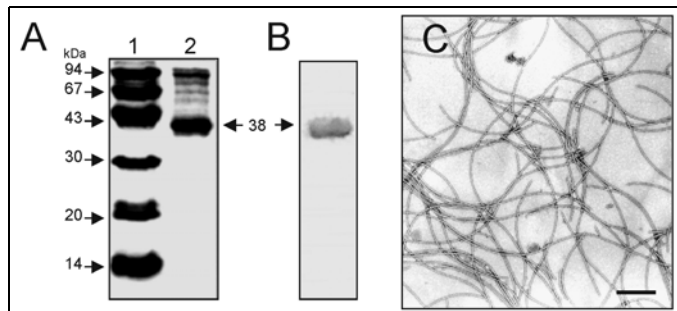


Figure 2. Analysis of flagella purified from SMDP92. (A) sodium dodecyl-sulfate polyacrylamide gel electrophoresis of purified flagella, showing the 38-kDa flagellin subunit. Lane 1, molecular weight standards; lane 2, purified SM_{FliC}. (B) Immunoblotting and reactivity of purified flagella with anti-SM_{FliC} antibodies. The 38-kDa flagellin is indicated by an arrow. (C) Electron microscopy of purified flagella visualized by negative staining. Bar, 0.37 μ m.

unsheathed flagella such as those produced by *E. coli* or *Salmonella* (30,31). After purification of flagella by repeated ammonium sulfate precipitations, a peptide band that migrated with an apparent mass of 38 kDa was visualized in SDS-PAGE Coomassie blue-stained gels (Figure 2A). Antibodies obtained against the excised 38-kDa putative flagellin reacted with this polypeptide in immunoblots (Figure 2B). The flagella preparation was rich in flagellar filaments as determined by negative staining and electron microscopy (Figure 2C).

Sequence and Antigenic Relatedness of *S. maltophilia* Flagellin to Other Flagellins

These results suggested that the 38 kDa is the major structural component (FliC) of the flagella filament. Thus, this polypeptide was subjected to N-terminal amino acid sequence analysis, which showed that the 38-kDa protein is in fact the flagellin structural protein, which is highly homologous to other bacterial flagellins. The *S. maltophilia* FliC protein, SM_{FliC}, showed considerable identity in its first 14 amino acid residues to the flagellins of *E. coli*, *P. mirabilis*, and *Shigella sonnei* (71.4%), and 78.6% identity to the flagellin of *Serratia marcescens*. The flagella produced by *P. aeruginosa* showed the lowest level of identity (57.2%) with SM_{FliC} (Figure 3).

Because of these sequence similarities, we were then interested in determining if SM_{FliC} shared any common epitopes with the other flagellins. This antigenic cross-reactivity was investigated by using several antisera against flagellins of *E. coli*, *Shigella*, *P. aeruginosa*, and *P. mirabilis*. Among these,

only antibodies against flagella of *P. mirabilis* and anti-FlaA and anti-FlaB of *P. aeruginosa* reacted in immunoblots with the *S. maltophilia* flagellins, although to differing levels of reaction (Figure 4). Anti-SM_{FliC} antibodies reacted with the SM_{FliC} proteins produced by both *S. maltophilia* strains tested (Figure 4).

Expression of Flagella by Clinical *S. maltophilia* Isolates

We investigated SM_{FliC} in fresh isolates of *S. maltophilia*. Forty-six *S. maltophilia* clinical isolates and *S. maltophilia* ATCC 13637 were studied by immunoblot, with antibodies against SM_{FliC} of SMDP92. A preparation of purified flagella was used in all reactions as a positive control. All the isolates tested produced the ~38-kDa flagellin that reacted with antibodies against SM_{FliC} (Figure 5). However, the molecular mass of the flagellin produced by some of the isolates differed slightly, and doublet bands were seen in some cases. We also performed negative staining and transmission electron microscopy in these isolates to confirm expression of flagella (Figure 1). These results show that the production of the 38-kDa flagellin and flagella is a common feature of reference and fresh clinical isolates of *S. maltophilia*.

Kinetics of Adherence to Plastic

As early as 30 min, individual bacteria were seen attaching to the glass surface and forming small clumps (Figure 6A). As the incubation time extended to 1, 2, and 4 h, the number of attached bacteria increased throughout the abiotic surface (Figure 6B–D). At 6 h, the adhering bacterial monolayer progressed into three-dimensional bacterial clumps (Figure 6E). After 18 h, extended areas of the glass surface were covered with large bacterial clumps (Figure 6F). No obvious

<i>S. maltophilia</i>	A	Q	V	I	N	T	N	T	V	S	L	N	A	Q
<i>S. marcescens</i>	A	Q	V	I	N	T	N	S	L	S	L	M	A	Q
<i>S. sonnei</i>	A	Q	V	I	N	T	N	S	L	S	L	L	T	Q
<i>E. coli</i>	A	Q	V	I	N	T	N	S	L	S	L	I	T	Q
<i>P. mirabilis</i>	A	Q	V	I	N	T	N	Y	L	S	L	V	T	Q
<i>P. aeruginosa</i>	A	L	T	V	N	T	N	I	A	S	L	N	T	Q

Figure 3. N-terminal amino acid sequence analysis. The first 14 residues of SM_{FliC} showed considerable identity to other flagellins. The highest degree of identity was found with the *Serratia marcescens* flagellin (78.6%). *Stenotrophomonas maltophilia* flagellin also showed identity to flagellins of *Shigella sonnei*, *Escherichia coli*, and *Proteus mirabilis* (71.4%), and the lowest identity was found with *Pseudomonas aeruginosa* flagellin (57.2%). The numbers on top indicate the amino acid position.

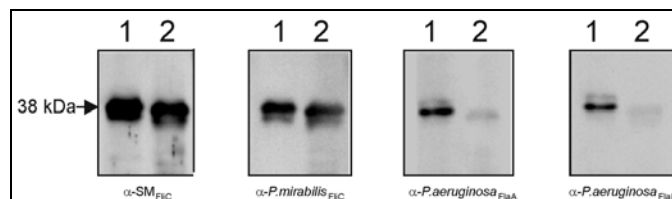


Figure 4. Reactivity of *Stenotrophomonas maltophilia* flagellin with different antibodies. Lane 1, SMDP92 strain; lane 2, ATCC 13637. Blots containing whole cells extracts of SMDP92 and ATCC 13637 were reacted with antibodies against SM_{FliC}, flagella of *Proteus mirabilis*, and anti-FlaA and anti-FlaB of *Pseudomonas aeruginosa*.

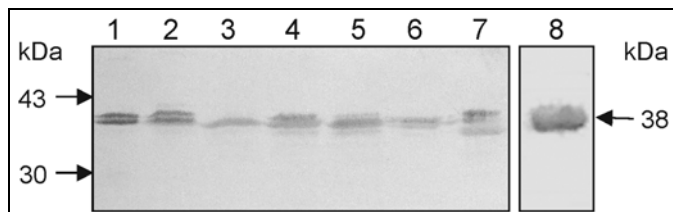


Figure 5. Identification of the 38-kDa flagellin protein SM_{FliC} in clinical isolates of *Stenotrophomonas maltophilia*. Lane 1, SMDP14; lane 2, SMDP275; lane 3, SMHC176; lane 4, SMHC181; lane 5, SMDP315; lane 6, SMDP314; and lane 7, SMHC179. Lane 8, the purified SM_{FliC}, was used as positive control. The immunoblot shows the presence of the 38-kDa flagellin protein in all the isolates. Doublet bands were seen in some of the isolates. Molecular weight standards and the 38-kDa flagellin protein are indicated by arrows.

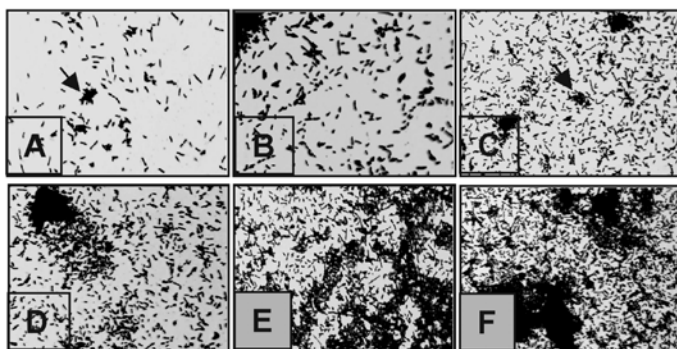


Figure 6. Kinetics of *Stenotrophomonas maltophilia* adherence to plastic. (A) As early as 30 min, individual bacteria have attached to the plastic surface and formed small clumps (arrows). (B–D) As incubation time proceeds for 1 (B), 2 (C), and 4 (D) h, the number of attached bacteria increases throughout the abiotic surface. (E) At 6 h, the bacterial monolayers progress into three-dimensional microcolonies (arrows). (F) After 18 h, the microcolonies have formed true bacterial communities. No obvious differences were noted beyond this incubation period. Magnification 400x.

differences were observed at incubation periods >18 h of infection (data not shown). The kinetics of adhesion were also monitored by quantification of crystal violet–stained-bacteria adhering to the 96-well plates. In correlation with the light microscopy micrographs shown above (Figure 6), a time-dependent adherence profile was obtained that reached a maximum level at 18 h (Figure 7), with no substantial increase in adherence beyond this period (data not shown).

Furthermore, analysis by scanning electron microscopy of SMDP92 adhering to the plastic showed structures resembling flagella on the adhering bacteria (Figure 8). These filaments were seen protruding from the bacteria, apparently forming physical bridges between them. Thus, these filaments may play some yet-undefined role in adherence to plastic. High-power magnification of adhering bacteria showed flagella-like filaments (40–50 μm in width) and thin fibrillar structures (5–7 μm in width) resembling pili interconnecting bacteria and mediating adhesion of the bacteria to the abiotic surface (Figure 8).

Discussion

Although adherence to abiotic surfaces is a property of both environmental and clinical *S. maltophilia* isolates, little information has been available to elucidate the nature of the surface factors involved in this phenomenon. Flagella have

been associated with biofilm formation in other bacteria (18,20–22), where they can perform three basic roles: a) act as an adhesin promoting intimate attachment to the surface; b) generate force to subjugate the repulsive forces between bacteria and surface; and c) promote spread of the bacteria throughout the surface (20). In 1983, Montie and Stover purified flagella from several pseudomonads, including *P. maltophilia* strain B69 (now referred to as *Stenotrophomonas maltophilia*) (32), and found that B69 produced a flagellin and had a molecular mass of 33 kDa. They found that antisera against flagella of *P. aeruginosa* and *P. cepacia* did not agglutinate *P. maltophilia* bacteria, suggesting absence of antigenic cross-reactivity between these flagella. No further biochemical characterization of *S. maltophilia* flagella has been done. In this paper, we describe the purification and characterization of *S. maltophilia* flagella; we raised specific antibodies to study the production of flagella in a collection of clinical isolates. The flagella produced by *S. maltophilia* strains are composed of a 38-kDa flagellin subunit, SM_{FliC}. The identity of this polypeptide was demonstrated by N-terminal amino acid sequencing analysis and by immunodetection assays using antibodies raised against the purified flagellin. The discrepancy between the molecular mass of SM_{FliC} and the flagellin (33 kDa) found previously in B69 could be attributed to differences in the electrophoresis conditions and molecular mass standards used, as well as to differences in the strains per se. Nevertheless, we did find molecular mass differences among flagellins produced by clinical isolates.

The comparison between the N-terminal amino acid sequence obtained from this 38-kDa polypeptide (14 residues) showed that SM_{FliC} shares important identity with several known flagellins: 71.4% identity to FliC of *E. coli*, *P. mirabilis*, and *S. sonnei*, and 78.6% identity to FliC of *Serratia marcescens*. *Stenotrophomonas* was previously considered a pseudomonad (2,3); however, the identity between the FliC of

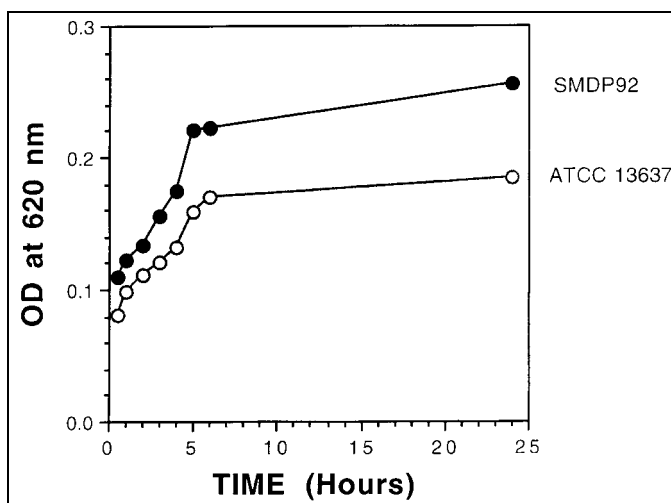


Figure 7. Graph showing kinetics of adherence by SMDP92 and ATCC 13637. Bacteria were allowed to bind to the plastic for 72 h and then were stained with crystal violet. Bacterial uptake of the dye was measured at 620 nm. Closed and open circles represent SMDP92 and ATCC13637, respectively.

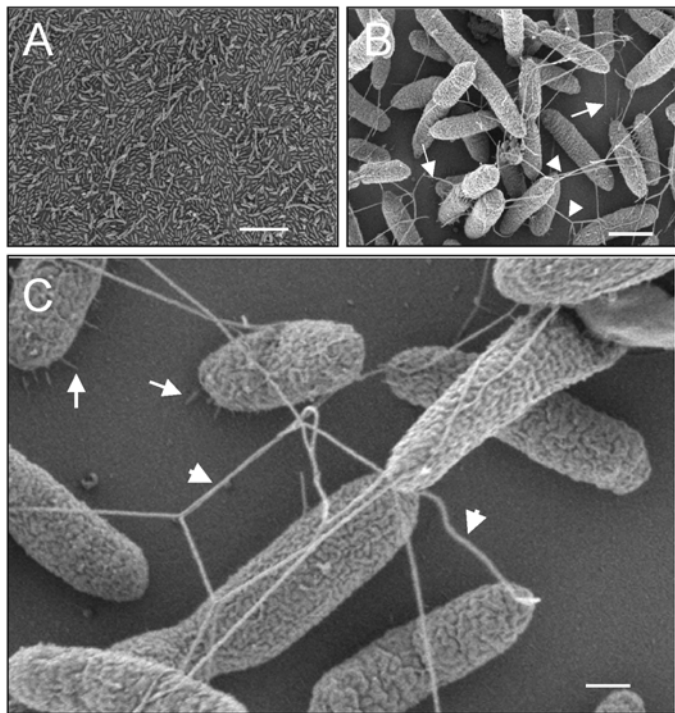


Figure 8. Ultrastructural analysis of *Stenotrophomonas maltophilia* adhering to plastic. (A) Scanning electron micrographs showing the tight adhesion of SMDP92 to the plastic surface. (B) Structures resembling flagella seem to be protruding and interconnecting bacteria (arrowheads) or connecting bacteria to the plastic (arrows). (C) In addition to the flagellalike filaments (arrowheads), high-power magnification shows the presence of thin fibrillar structures connecting bacteria to the abiotic surface. Bars: A 10 μm , B 1 μm , C 2 μm .

S. maltophilia and *P. aeruginosa* was 57.2%, which is lower than that observed with enterobacterial flagellins. In spite of the similarity between SM_{FliC} and these other flagellins, they are antigenically distinct since only antibodies against *P. mirabilis* flagellin, FlaA and FlaB of *P. aeruginosa* reacted with SM_{FliC} in immunoblots. We do not yet know the biological relevance of this finding, but based on these data, we can speculate that the flagellin gene of *S. maltophilia* was probably modified through the evolution of the organism, yielding a FliC protein with different antigenic properties but similar biological functions.

Visualization by high-resolution scanning electron microscopy of bacterial monolayers adhering to plastic showed flagellalike filaments connecting bacteria to each other and to the inert surface, suggesting that these structures are involved in adherence, along with other thin fibers, resembling pili. In *P. aeruginosa*, the flagella appear to act as structures that promote the initial interaction of the bacteria with the abiotic surface during early stages of biofilm development, as demonstrated with flagella mutants that are unable to produce biofilm (18). While the definitive role for flagella in adherence by *S. maltophilia* needs to be supported by the use of defined motility-lacking and flagella-deficient constructs, the presence of flagella at late stages of adherence on bacteria adhering to the plastic suggests that flagella may play some role in this event.

Much remains to be understood concerning the virulence mechanisms of *S. maltophilia*. The adherence of these bacteria to plastic may be important for the establishment of opportunistic infections in hospitalized and immunocompromised patients. Elucidating the surface factors that allow *S. maltophilia* to adhere to inert surfaces will contribute to the development of effective antimicrobial strategies for controlling these infections.

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Could
Malaria
Reemerge
in Italy?

Demographic Factors Associated with Hantavirus Infection in Bank Voles (*Clethrionomys glareolus*)

Gert E. Olsson,*† Neil White,*† Clas Ahlm†, Fredrik Elgh,†‡§
Ann-Christin Verlemyr,† Per Juto,† and R. Thomas Palo*†

The bank vole (*Clethrionomys glareolus*) is the natural reservoir of *Puumala virus* (PUUV), a species in the genus *Hantavirus*. PUUV is the etiologic agent of nephropathia epidemica, a mild form of hemorrhagic fever with renal syndrome. Factors that influence hantavirus transmission within host populations are not well understood. We evaluated a number of factors influencing on the association of increased PUUV infection in bank voles captured in a region in northern Sweden endemic for the virus. Logistic regression showed four factors that together correctly predicted 80% of the model outcome: age, body mass index, population phase during sampling (increase, peak, or decline/low), and gender. This analysis highlights the importance of population demography in the successful circulation of hantavirus. The chance of infection was greatest during the peak of the population cycle, implying that the likelihood of exposure to hantavirus increases with increasing population density.

The hantaviruses belong to the family *Bunyaviridae* and are the causative agents of hemorrhagic fevers with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) in humans (1,2). Each distinct form of the virus is closely associated with a single, or possibly a few, rodent species (3,4). Transmission of hantaviruses to humans occurs mainly through the inhalation of aerosols containing virus excreted from infected rodents; rodent-to-rodent transmission also may occur through biting and social grooming (5–7). Approximately 150,000 human cases of hantavirus infection are reported per year worldwide (6). Mortality in humans ranges from <0.5% in nephropathia epidemica, a mild form of HFRS (8,9), to 5% to 10% from other HFRS (6), and 45% from the more severe HPS (4).

The only hantavirus isolated in Sweden is the *Puumala virus* (PUUV) from the bank vole (*Clethrionomys glareolus*), which serves as the natural reservoir species. The bank vole is the most common and widespread rodent species in northern Sweden. In northern Fennoscandia, density fluctuations may show up to 500-fold changes from peak to decline/low phase during a 3- to 4-year cycle (10–13). In northern Sweden, the incidence of nephropathia epidemica in humans reaches an average of 40 serologically confirmed cases per 100,000 inhabitants in rodent peak years; yet up to 80% of human cases may be unrecognized (14).

The importance of factors assumed to be associated with the occurrence of hantavirus infections in natural rodent host

populations is not well understood (15). Tools need to be developed to model hantavirus transmission in the wild reservoir species to better understand the relationship between the natural circulation of the virus and incidence of the disease in human populations. Among the factors of interest, rodent age and sex are known to distinguish cohorts of high seroprevalence in the wild (16,17). These two factors represent the elapsed time of possible virus exposure and sex-biased behaviors. We have found (18, Olsson et al., ms. in prep.) that higher numbers of PUUV-infected bank voles were associated with sites of known human hantavirus exposure in peak years, suggesting an influence of the local environment on subsequent chance of PUUV exposure. Therefore, sampling sites and phase during population cycle were included in our analysis to evaluate the probability of PUUV infection. We also investigated the influence of the body condition of bank voles on their probability of being PUUV seropositive. The models we considered included a measure of body condition because either 1) malnourished bank voles would be more likely to be PUUV infected because of increased susceptibility (19), or 2) well-nourished bank voles would be more likely to be PUUV seropositive because high-quality habitats support higher or persistent numbers of bank voles (20), facilitating the spread of the virus.

The aim of our study was to relate and rank characteristics of bank voles, i.e., age, body measurements, and sex, influencing the probability of being PUUV seropositive. Complementary independent variables are sampling sites and sampling events within the population cycle. Determining which of these factors is applicable is essential to the modeling of the spread of hantaviruses within a rodent host population.

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Materials and Methods

Sampling of rodents was initiated in the fall of 1995 in the vicinity of three recently affected households in the coastal areas of Västerbotten County in northern Sweden, 63°45'–63°20' N, 20°00'–21°00' E (18); subsequently, the rodent populations were sampled twice a year, including the fall of 1999. Case sites were denoted “south,” “center,” and “north,” according to location within sampling region, and situated approximately 40 km apart (south-to-north 80 km). At these sites, nephropathia epidemica was serologically confirmed in humans 3–10 weeks before the first sampling event. Collection of animals took place within a 3-week period in May–June and again in September–October each year. Forested area sites randomly situated approximately 10 km from each case site were used as controls and denoted as paired random forest sites. At each of the six sampling sites, 30 snap-traps, baited with dry apple, were placed at 10-m intervals in each of six transects of 300-m length per site. Thus, 180 snap-traps were set during four nights on each site, constituting 720 trap-nights per site of investigation. Trap indices represent success in a sampling effort, as the number of voles captured per 100 trap nights, i.e., a reflection of the relative population density on each sampling occasion. Case sites and random forest sites comprised mainly managed, boreal coniferous forests of Scots Pine (*Pinus sylvestris*) and Norway Spruce (*Picea abies*), with a considerable cover of bilberry (*Vaccinium myrtillus*) and lingonberry (*V. vitis-idaea*).

In total, 1,568 bank voles were collected over the 5-year period 1995–1999. The phase of the population development was designated as “increase,” “peak,” or “decline/low,” depending on the relative amplitude of trap indices in consecutive years. Spring and fall samplings were combined because low spring numbers, particularly in 1997, did not allow for separate statistical analysis of seasons.

Bank voles captured in 1995 and 1996 were not available for age determination, but the age of 1,079 bank voles captured in 1997 to 1999 was determined according to criteria of molar root development and growth (21,22); each animal was subsequently assigned to one of four age classes (Table 1). Briefly, lower molar 1 or upper molar 2 was pulled from its socket, placed in a duralumin frame on a microscope slide, and measured to the nearest 0.05 mm under a stereomicroscope. Of the 1,079 specimens, 79 were not measurable on one or several of the other independent variables (Table 1); therefore, the sample size in the following analysis was 1,000 voles. Body condition of bank voles was estimated as the body mass index (BMI = weight/length², [23]), although this BMI can also be considered a measure of animal morphology. Body length measurements were obtained to the nearest millimeter (not including the tail). The total body weight was taken to the nearest 0.01 g. Weights of fetuses from pregnant females were either subtracted, when possible, or excluded from further analyses. We used enzyme-linked immunosorbent assay (ELISA) to detect PUUV immunoglobulin G antibody in bank

Table 1. Binary logistic regression variables included in the model that predicted the probability of a bank vole's being seropositive to *Puumala virus* (PUUV)

Variable	Variable description	Nominal reference level
Serostatus	Binary response variable, bank voles with PUUV-specific immunoglobulin G antibodies are denoted “1” (“success”) and seronegative bank voles “0” (“failure”)	
Age	Polytomous independent variable, age classes; 1=juvenile/subadult <3 months of age; 2 = adult born in year of sampling 3–6 months of age; 3 = overwintered adult in spring >7 months of age; 4 = overwintered adult in fall >11 months of age	Age class 1
Sex	Dichotomous independent variable for female or male	Females
BMI	Continuous variable body mass index	
Phase	Polytomous independent variable on population phase denoted as increase (1997), peak (1998), or decline/low (1999)	Decline/low
Type	Dichotomous independent variable: case site or random forest site	Random forest site
Pair	Polytomous variable on paired case- and random forest sites located south, center, or north within sampling region	South

vole sera. Details of collecting, storing, and processing serologic test results have been described (18,24).

Binary logistic regression models with serostatus as the dichotomous response variable were used to identify statistically significant factors and estimate the probabilities of the diagnostic classes, i.e., PUUV seropositive or seronegative (Table 1). We used a logit link function because we wanted to identify the influence of the independent variables separately rather than model the cumulative probability and outcome of each combination of elements. Thus, the analysis prevented confounding effects from any independent variable to the other. We present odds ratios (OR) in favor of infection in relation to reference levels for nominal variables and per unit change for the continuous variable (Table 1). We selected the most parsimonious model by using Akaike's information criterion (AIC) (25). Statistical analyses were performed by using the R statistical software package release 1.3.0 (<http://www.r-project.org>), with the methods outlined in Venables and Ripley (26) for generalized linear models of binomial data.

Results

The significantly highest trap indices of bank voles were recorded in falls of 1995 and 1998, and lower indices of bank voles were recorded during the other sampling occasions (Figure 1; Olsson et al., unpub. data). The overall PUUV seroprevalence was 15.4% in the bank voles sampled during the study period, 1995–1999.

Results from two models of logistic regression are presented (Table 2). Model I included six independent variables (age, gender, BMI, and the population phase, type, and pair),

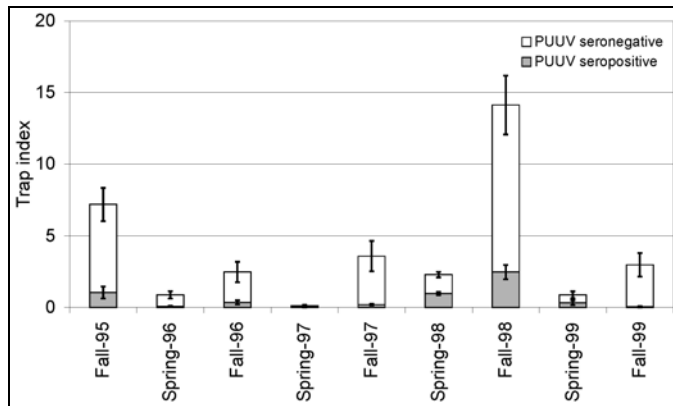


Figure 1. Mean (\pm SE) trap indices of bank voles in all six sites during a 5-year sampling period in northern Sweden. Trap indices represent success in sampling effort as numbers of voles captured per 100 trap nights. Lower, shaded part of the bars represents trap indices of *Puumula virus* (PUUV)-seropositive bank voles, and the upper part represents PUUV-seronegative voles at each sampling occasion.

and model II included all but the nonsignificant variables “type” and “pair.” The response variable in each of the models was the individual bank vole’s PUUV serostatus (seropositive 1; seronegative 0). Interactions between independent variables were examined but found to be nonsignificant. The two models were accepted as appropriate on the basis of the goodness-

of-fit test. In model II, two of the independent variables used in model I were excluded because they did not significantly contribute to the outcome of the analysis; the type of sample site denoted as “case,” “random forest,” and “pair” (north, south, and center) indicated geographic location within the sampling region. The AIC method, which suggests the direction towards selecting the best model, was used to compare and select the most parsimonious set of independent variables. AIC favored model II, which predicted 80.4% (AIC 674.999) of the responses correctly, compared with 80.7% for model I (AIC 679.897).

The importance of age as a transmission factor is shown by the high OR for the oldest age class (Table 2; Model II). The interpretation of this OR would be that the odds of being seropositive in age class 4 was 28.7 times greater than in age class 1. This value was the highest in the model, followed by that of age class 2 (OR 5.67). For age class 3, the OR was similar (4.01). The age-related increase in chance of being PUUV seropositive was also observed for seroprevalence, i.e., proportion seropositive, within each age class (Figure 2).

BMI was the second most important factor based on the rank of OR. Each unit increase in BMI led to a 4.1 times higher probability that a bank vole was seropositive. Age may indirectly play a role here because BMI within age class 1

Table 2. Outcome of the binary logistic regression models I and II predictions of risk of a bank vole’s being seropositive for *Puumula virus* under specified conditions in relation to reference levels^{a-f}

Predictor	Model I					Model II				
	Coeff.	Z ^g	p ^h	OR	95% CI	Coeff.	Z	p	OR	95% CI
Age ^a										
2	1.71	4.69	<0.001	5.55	2.71 to 11.36	1.74	4.76	<0.001	5.67	2.78 to 11.6
3	1.43	5.03	<0.001	4.18	2.39 to 7.3	1.39	5.03	<0.001	4.01	2.33 to 6.89
4	3.41	6.73	<0.001	30.27	11.22 to 81.7	3.36	6.69	<0.001	28.7	10.7 to 76.6
Gender ^b										
Male	0.61	2.72	0.007	1.84	1.19 to 2.87	0.62	2.78	0.005	1.87	1.2 to 2.9
BMI ^c	1.39	3.43	0.001	4.03	1.82 to 8.94	1.41	3.48	<0.001	4.1	1.85 to 9.07
Phase ^d										
Peak	0.88	2.57	0.01	2.4	1.23 to 4.7	0.87	2.58	0.01	2.4	1.23 to 4.65
Increase	-1.03	-2.07	0.04	0.36	0.13 to 0.95	-1.03	-2.1	0.04	0.36	0.14 to 0.93
Type ^e										
Case site	0.10	0.48	0.63	1.11	0.73 to 1.69					
Pair ^f										
Center	0.17	0.67	0.50	1.19	0.72 to 1.95					
North	0.21	0.88	0.38	1.24	0.77 to 2.0					

^aThe age class with the largest number of individuals was set as reference with which all others were compared, i.e., age class 1 (juveniles and subadults).

^bFemale bank voles were set as reference.

^cBody mass index (BMI) is a continuous variable; OR refers to unit increase.

^dThe decline/low population phase was used as reference.

^eThe sampling area type “random forest site” was set as reference.

^fSouth sampling pair was reference to other pairs on regional effect.

^gThe Z-score shows the number of standard deviations that the tested predictor class’s coefficient falls above or below the predictor’s reference level.

^hThe p value is the probability that the observed coefficient of the actual predictor class’s should be by random chance variation.

Coeff., coefficient; OR, odds ratio; CI, confidence interval

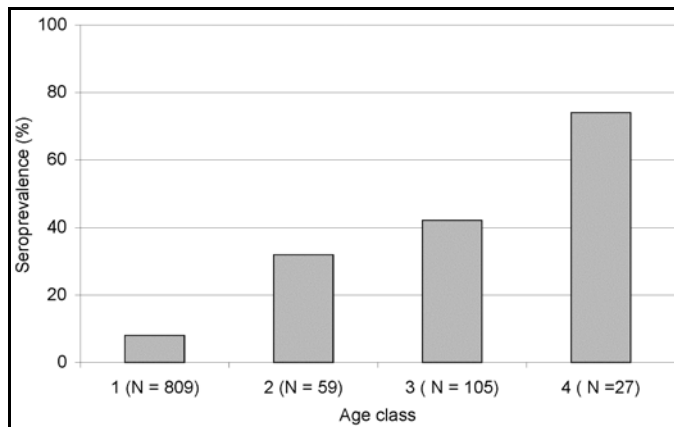


Figure 2. Seroprevalence of *Puumula virus* (PUUV)-specific immunoglobulin G antibodies within different age classes of bank voles. 1 = juvenile/subadult <3 months of age; 2 = adult born in year of sampling 3–6 months of age; 3 = overwintered adult in spring >7 months of age; 4 = overwintered adult in fall >11 months of age.

differed considerably from that in the other age classes; however, the difference between age classes ranged within 0.4 units (Figure 3).

The phase of the population development (increase, peak, or decline/low) during which the bank vole was sampled was also a significant factor (Table 2). For bank voles captured during the peak in the population cycle, the OR in favor of being seropositive were 2.4 higher than in decline/low phase ($p=0.01$). The OR during the increase phase was 0.36 ($p=0.04$), indicating that chance of being seropositive was actually higher during the decline/low phase than during the increase. The fourth independent variable in rank was the animal's gender (Table 2). Overall, the odds in favor of infection for males were 1.87 times higher than for females.

Discussion

The population abundances of bank voles varied, with 3- to 4-year fluctuations, as previously described for microtine rodents in northern Scandinavia, as did the intra-annual abundances, with higher population densities in fall than in spring (11,12). The classification of sampling occasions as increase, peak, and decline/low therefore appears to be appropriate (Table 1).

The factor with the highest influence on the probability of being PUUV seropositive was the vole age at capture. Age is an important epidemiologic parameter because chance of exposure to horizontally transmitted pathogens generally increases with age (27,28). The overwintering bank voles captured in the fall, i.e., age class 4, constituted at the most 3.6% of the total sample in the increase and peak years; however, three of four of these specimens were PUUV seropositive, constituting a small but important virus reservoir (Figure 2). No captures of that cohort were made in the decline/low years, when, on the whole, fewer seropositive specimens were captured. Our findings appear to agree with the hypothetical model of Mills et al. (16), in which the effect of consecutive years of favorable conditions on population growth and conse-

quent hantavirus transmission is considered one of the most important factors. In the model, the proportion of overwintering adults remained the same under the extended favorable conditions, but absolute numbers increased, thus facilitating the limited winter transmission to susceptible voles within the population. The bank vole population fluctuations in our study appear to be cyclic, and the increase and peak phases resemble the favorable circumstances for the hantavirus host population, as discussed by Mills et al. (16). The mass action principle of disease transmission assumes that transmission is a function of the densities of infectious and susceptible animals (29). However, because of the rapidly diminishing densities of bank vole populations after the peak in the trapping index each fall, we suggest that opportunities for hantavirus transmission decrease drastically and therefore very likely deter any time-lagged density dependence on hantavirus prevalence. In addition, as the logistic regression model shows, the studied populations were not homogeneously mixed across the demographic delimitations (e.g., age groups and gender), as the individual bank voles showed no random chance of being PUUV seropositive. Therefore, the mass action principle is not applicable to the studied system.

Communal nesting is beneficial to overwintering bank voles (30,31) and may be a facilitating factor on the successful persistence of PUUV in local populations during the non-breeding season. However, the frequent contacts between sexually mature voles during the breeding season are likely to be a situation more critical to hantavirus transmission. The difference in OR between age classes 2 and 3 was nonsignificant, as shown by the almost completely overlapping 95% confidence intervals, suggesting that transmission between overwintering specimens (living through fall to spring) was limited in the studied populations. However, the difference between age classes 3 and 4 was highly significant, as shown by the separation in confidence intervals, indicating that adult bank voles that survived the winter and thus engaged in breeding the following summer were subject to considerable hantavirus exposure during that breeding season. These patterns likely reflect the frequency and degree of social interactions among bank

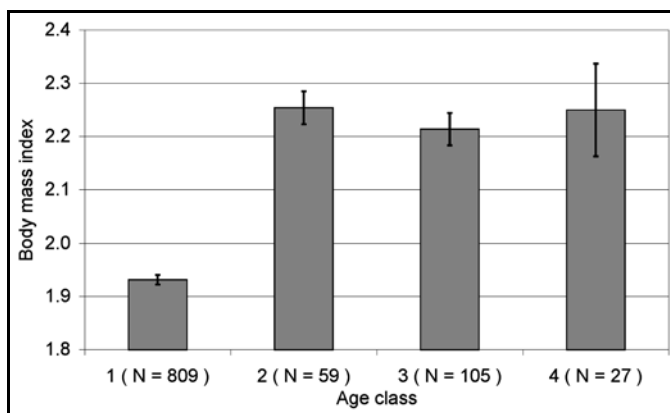


Figure 3. Mean (\pm SE) body mass index (BMI) within age classes of bank voles, where BMI separates age class 1 (juvenile/subadult) from all others; see Figure 1 for details on age classes.

voles during the winter versus the summer breeding season. The behavioral mechanism facilitating hantavirus transmission associated with sexual maturation is probably also applicable to age class 2, i.e., the specimens that likely became sexually mature during the current breeding season. This association is supported by the similar observations of Glass et al. (32) on the seroprevalence in sexually maturing Norway rats (*Rattus norvegicus*). Bernshtein et al. (17) also observed an increased rate of hantavirus transmission in bank voles during the period of high reproductive activity.

BMI, the second most influential factor, was originally developed to measure obesity in humans (23). We used BMI to identify and separate malnourished bank voles from well-nourished ones and their chances of PUUV infection. BMI reflected the distribution within age classes in that age class 1, i.e., juveniles and subadults <3 months of age, had a lower BMI than all other age classes, although the method of logistic regression rules out age per se as the confounding effect (Figure 3). One likely explanation for the differences in BMI is the changes in morphology associated with sexual maturation. As the range in BMI appeared not to overlap between the juveniles and subadults of the year (<3 months of age) and the adults born in the year of sampling (3–6 months of age), BMI may serve as a tool to distinguish the two cohorts in similar studies, when estimating the age by using the techniques discussed here may be impractical.

The effect of the peak year suggests a direct vole density-dependent chance of PUUV infection. As the chance of being PUUV seropositive in the peak of the population cycle was 2.4 times higher than in the decline/low, we conclude that this effect is caused by the density of the bank vole population. Other researchers have proposed that delayed density dependence occurs (16,33). Since the chance of infection was lower in the increase phase than the decline/low phase, we suggest that the effect was not so much a result of delayed density dependence but of having a large reservoir of infection in the population.

Results from other hantavirus-rodent systems also show a sex-related bias in the odds of infection similar to our observations. This effect is likely caused by differences in behavior between males and females (16,17). Aggressive behavior, such as biting, between males has been suggested as a means of hantavirus transmission in other studies (32). However, males of *Clethrionomys* spp. do not defend territories but usually have overlapping home ranges in their competition for mating. Females do, however, compete for food and defend territories (20,34,35). Therefore, the gender effect in serostatus more likely emerges because males are more labile and subsequently have more frequent encounters with conspecifics, increasing their chance of contracting the virus.

In conclusion, long-lived bank voles appear critical to the success of hantavirus circulation and persistence within host populations. Localized absence of PUUV coincided with the absence of overwintering specimens at several sites during population decline/low. The chance of being PUUV seroposi-

tive is related to phase of population cycle and is therefore density dependent. Quantitative measures like these revealed by logistic regression are useful in developing demographic host models on the subsequent risks of exposure to humans in areas of critical rodent host dynamics.

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Behavioral, Physiologic, and Habitat Influences on the Dynamics of *Puumala virus* Infection in Bank Voles (*Clethrionomys glareolus*)

Sophie Escutenaire,* Patrice Chalon,† Florence De Jaegere,‡ Lucie Karelle-Bui,† Georges Mees,† Bernard Brochier,* Francine Rozenfeld,‡ and Paul-Pierre Pastoret†

Populations of bank voles (*Clethrionomys glareolus*) were monitored during a 4-year study in southern Belgium to assess the influence of agonistic behavior, reproductive status, mobility, and distribution of the rodents on the dynamics of *Puumala virus* (abbreviation: PUUV; genus: *Hantavirus*) infection. Concordance was high between data from serologic testing and results of viral RNA detection. Wounds resulting from biting or scratching were observed mainly in adult rodents. Hantavirus infection in adults was associated with wounds in the fall, i.e., at the end of the breeding season, but not in spring. In addition, sexually active animals were significantly more often wounded and positive for infection. Hantavirus infection was associated with higher mobility in juvenile and subadult males. Seroconversions observed 6 months apart also occurred more frequently in animals that had moved longer distances from their original capture point. During nonepidemic years, the distribution of infection was patchy, and positive foci were mainly located in dense ground vegetation.

Hantaviruses (family *Bunyaviridae*) are rodent-borne zoonotic agents responsible for human diseases called hemorrhagic fever with renal syndrome (HFRS) in Europe and Asia and hantavirus pulmonary syndrome (HPS) in the Americas (1,2). Viral transmission occurs through inhalation of aerosols from the urine, saliva, or feces of infected rodents and possibly through biting (3–5). Hantavirus infection persists in reservoir species apparently without causing clinical signs (6). In experimentally infected rodents, the virus is distributed in different organs (including lungs, kidneys, intestines, and salivary glands) and elicits the production of antibodies that may be detected lifelong, while the viremia is generally transient (4,7,8). In the wild, adult rodents are generally more often infected than younger animals. The age-dependent prevalence may result from protection of newborns by maternal antibodies and from higher risk of infection for sexually mature rodents through fighting, mating, or communal nesting (9–12). In Europe, *Puumala virus* (PUUV), which causes a mild form of HFRS in humans, is carried by bank voles (*Clethrionomys glareolus*) (13). No data are available on the dynamics of PUUV infection in bank vole populations according to behavioral patterns. Most seroconversions recorded in a capture-mark-recapture (CMR) study of PUUV transmission occurred during the breeding season and in sexually mature voles, with a prevalence bias in favor of mature males (10). Aggressive encounters in adults and the occupation of exclusive territories

by breeding females are characteristic of the breeding season in bank voles (14–16).

We studied the influence of aggressive behavior, reproductive status, and mobility of bank voles on the prevalence of PUUV infection. Along with behavioral and physiologic factors, we studied the influence of habitat on bank vole distribution. Two HFRS outbreaks were reported in Belgium in 1996 (224 cases) and in 1999 (124 cases) (17,18). Our survey was conducted from 1996 to 1999 in southern Belgium, where most patients had been reported during the epidemic years. In our trapping sites, rodent population densities were the highest in 1996 and 1999, as was the prevalence of PUUV infection, with 41 (19.2%) of 213 and 259 (39.3%) of 659, respectively, of bank voles positive (19; S. Escutenaire, unpub. data).

Materials and Methods

Study areas

From 1996 through 1999, trapping was conducted twice a year (October–November and April–May) at 21 sites distributed in five localities of southern Belgium. All sites were located in broad-leaved or mixed pine and broad-leaved forests. Four of the 21 trapping sites, at Thuin, Montbliart, Momignies, and Couvin, were selected for a CMR survey (19). Each CMR site contained mapped areas with dense or low ground vegetation. The dense ground vegetation included thickets of brambles (*Rubus* sp.), shrubs (including *Corylus avellana*, *Sambucus racemosa*, *Prunus spinosa*, *Cytisus scoparius*, *Crataegus monogyna*, *Salix caprea*, and *Lonicera per-*

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iclymenum) and dense populations of plants such as *Pteridium aquilinum* and *Epilobium angustifolium*. The low ground vegetation comprised herbs (*Carex pilulifera*, *Anthoxanthum odoratum*, *Luzula pilosa*, *Dryopteris carthusiana*, *Teucrium scorodonia*, *Scrophularia nodosa*, and *Silene dioica*) or sparse brambles located under dense spruce (*Picea abies*) or oak (*Quercus robur*, *Q. petraea*) foliage.

Sampling Procedure

On each CMR site, we constructed a 10 X 10 grid of 100 live traps (Sherman Live Trap Co., Tallahassee, FL; Tomahawk Live Trap Co., Tomahawk, WI) spaced at 10-meter intervals. Traps were set for four consecutive nights. Rodents were anesthetized with isoflurane (Forène, S.A. Abbott, Louvain-La-Neuve, Belgium), individually marked by toe-clipping, and released at their original place of capture after a blood sample was collected from the retroorbital sinus. Organs (lungs, liver, kidneys, and spleen) were also collected from any animals found dead in traps. In spring 1999, 120 traps were added to expand the CMR grids, as described (19). On the 17 trapping sites where CMR was not done, 23 to 30 live traps were placed along transects at 5-m intervals for two to four consecutive nights. The trapped animals were humanely killed, and their blood and organs were collected.

Data Collection

All rodents were examined for sex and weight. Pregnant and lactating females and males with testicles in scrotal position were considered sexually active. Weight limits to distinguish adult, subadult, and juvenile categories were inferred from the analysis of prevalence of infection and reproductive status, according to body mass of rodents. Mass classes, which differed over time (19), were <16 g (juveniles), 16–18 g (subadults), and >18 g (adults) in fall 1996, in spring 1997 and in 1999, and <13 g (juveniles), 13–15 g (subadults), and >15 g (adults) in fall 1997 and in 1998. From spring 1997 on, we recorded the presence of wounds associated with a bite or a scratch on the head (ear perforation or muzzle injuries) of bank voles.

Serologic Screening and Viral RNA Detection

Sera of rodents trapped from fall 1996 to fall 1998 were screened by an immunoglobulin (Ig) G enzyme-linked immunosorbent assay (ELISA), with PUUV CG18-20, *Hantaan virus* (HTNV) 76-118, and *Dobrava-Belgrade virus* (DOBV)–infected Vero E6 cell lysates as viral antigens (19). Sera collected in 1999 were screened by using a PUUV IgG ELISA kit (Progen Biotechnik, Heidelberg, Germany) (19).

Viral RNA was detected in ground-up lungs by means of reverse transcription (RT) polymerase chain reaction (PCR) test (19). Two nested PCRs with small (S)- or medium (M)-segment oligonucleotides were done to amplify the cDNA. The expected size of the amplified fragments was 205 base pairs (bp) (nucleotide [nt] 1033–1237) and 310 bp (nt 2463–2772) for the S and M genomic segments, respectively.

Data Analysis

The total number of rodents marked and released during a trapping session was used as an indicator of population size on each CMR site. Capture points of all rodents trapped in the grids were mapped. According to the distribution, we determined the pairs of adult voles with overlapping home ranges. Home range overlap was considered when rodents were captured at the same trap station or when the areas enclosed by the capture points overlapped (20,21). Distances between trapping points of recaptured voles were measured to estimate rodent movements.

Statistical analysis of data was done by chi-square test, Pearson correlation, and Student's *t* test. Pairs of adult rodents with overlapping home ranges were analyzed according to sex and serologic or PCR status. Data for each criteria were processed as binomial distributions.

Results

Serologic and RT-PCR Data

IgG antibodies against PUUV were detected in 318 (26.3%) of 1210 bank vole sera collected from fall 1996 through fall 1999. PUUV genomic fragments were amplified in 38 (13.7%) of 277 lung samples. Of 179 bank voles tested both by ELISA and RT-nested PCR, 169 (94.4%) had concordant results. Viral RNA was detected in the lungs of 19 (73.1%) of 26 seropositive animals and in three seronegative ones. As serum and tissues were not available for all rodents and as the concordance between results of serologic testing and PCR was high, data from both tests were pooled for further analysis. Rodents positive by ELISA or PCR or both were considered positive for PUUV infection.

Frequency of Wounds

Trapping data from spring 1997 through fall 1999 showed that the proportion of wounded bank voles was significantly (chi square 4.73, $p=0.03$) higher in autumn (150 [25.3%] of 593) than in spring (99 [19.8%] of 501). Adults were significantly ($p<0.01$) more often injured than juveniles and subadults in 1998 and 1999 (Table 1). The proportion of injured subadults (42 [18.5%] of 227) was also significantly (chi square 12.63; $p<0.01$) higher than that of wounded juveniles (20 [7.7%] of 259) in 1998 and 1999. Although no difference was observed between either sex in 1997 and 1998, the proportion of wounded animals during the 1999 epidemic year was significantly ($p=0.03$) higher in females than in males (Table 1).

Association between Wounds and Prevalence of Infection

When the fall prevalence rates were compared within mass classes, injured adults were significantly ($p<0.01$) more often positive than nonwounded ones, although no difference in prevalence was observed in the spring (Table 2). The prevalence in 1999 was significantly (chi square 12.14; $p<0.01$)

Table 1. Relationship between frequency of wounds and the age and sex of bank voles

Trapping season	J+S ^a % wounded (total no. of animals)	A % wounded (total no. of animals)	p value	M % wounded (no. of males)	F % wounded (no. of females)	p value
Spring 1997	7.7 (13) ^b	3.4 (29)		8.3 (24)	0.0 (18)	
Fall 1997	3.1 (65)	9.7 (103)		7.8 (116)	5.8 (52)	
Total 1997	3.8 (78)	8.3 (132)		7.9 (140)	4.3 (70)	
Spring 1998	0.0 (2)	35.6 (45)		33.3 (27)	35.0 (20)	
Fall 1998	14.3 (56)	58.5 (123)	p<0.01	46.7 (75)	43.3 (104)	
Total 1998	13.8 (58)	52.4 (168)	p<0.01	43.1 (102)	41.9 (124)	
Spring 1999	6.7 (223)	34.9 (189)	p<0.01	16.2 (222)	23.7 (190)	
Fall 1999	19.0 (205)	46.3 (41)	p<0.01; $\chi^2=14.15$	20.8 (125)	26.4 (121)	
Total 1999	12.6 (428)	37.0 (230)	P<0.01	17.9 (347)	24.8 (311)	p=0.03; $\chi^2=4.67$

^a J+S, juveniles and subadults; A, adults; M, males; F, females.

higher in subadults (61 [31.4%] of 194) than in juveniles ≥ 13 g (25 [15.5%] of 161). Subadults were also more often wounded.

Despite the difference in frequency of wounds between males and females in 1999, the prevalence of infection in adult females (79 [62.7%] of 126) was not higher than in adult males (71 [67.6%] of 105). During the fall of the non-epidemic years (1997 and 1998), the proportion of positive adult males (18 [14.5%] of 124) was significantly higher (chi square 4.40; $p=0.04$) than that of adult females (6 [5.9%] of 102) although no significant difference in wound frequency was observed between either sex.

Influence of the Reproductive Status of Adults

Data from spring 1997 through spring 1999 showed a correlation ($r=0.97$; $p<0.01$) between the number wounded and the number of sexually active adult males (Figure 1). The prevalence of infection was significantly higher (chi square 4.31; $p=0.04$) in sexually active males (10 [24.4%] of 41) than in other adult males (8 [10.1%] of 79) in fall but not in spring. The analysis of wounded animals also showed that in fall, sex-

ually active males still tended to be more often positive (8 [29.6%] of 27) than the other males (1 [7.7%] of 13).

Pregnant and lactating voles were significantly (chi square 5.47; $p=0.02$) more often wounded (81 [43.1%] of 188) than other adult females (18 [26.9%] of 67). The prevalence of infection was also significantly (chi square 4.77; $p=0.03$) higher in breeding females (72 [38.7%] of 186) than in non-breeding ones (16 [23.9%] of 67). Wounded animals showed no statistically significant difference in prevalence between sexually active (28 [34.6%] of 81) versus sexually inactive adult females (8 [44.4%] of 18).

Recapture of Rodents

Fifty-three bank voles were recaptured on the grids during subsequent trapping periods. For all but one animal, the recapture occurred 6 months after the first trapping. Twelve (22.6%) rodents were found positive during both capture sessions, and

Table 2. Prevalence of *Puumala virus* infection in adult bank voles, by presence of wounds

Trapping season	Wounded % positive (no. tested)	Not wounded % positive (no. tested)	p value
Spring 1997	0.0 (1)	21.4 (28)	
Fall 1997	20.0 (10)	8.6 (93)	
Spring 1998	6.3 (16)	6.9 (29)	
Fall 1998	15.3 (72)	5.9 (51)	
Spring 1999	68.2 (66)	68.3 (123)	
Fall 1999	68.4 (19)	36.4 (22)	p=0.04; $\chi^2=4.19$
Spring (1997–1999)	55.4 (83)	51.1 (180)	
Fall (1997–1999)	25.7 (101)	11.4 (166)	p<0.01; $\chi^2=9.16$

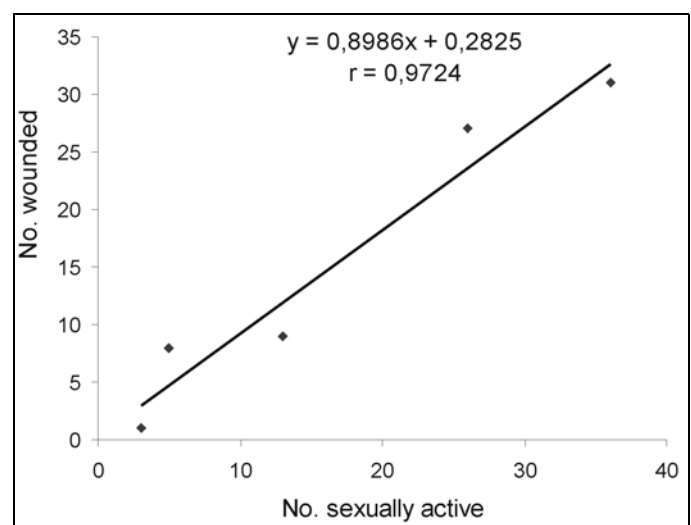


Figure 1. Linear regression in bank voles between the number wounded and the number of sexually active adult males. Significance of correlation: $p<0.01$. Data used for the analysis were collected from spring 1997 through spring 1999. The scrotal position of testicles was not inspected in fall 1999.

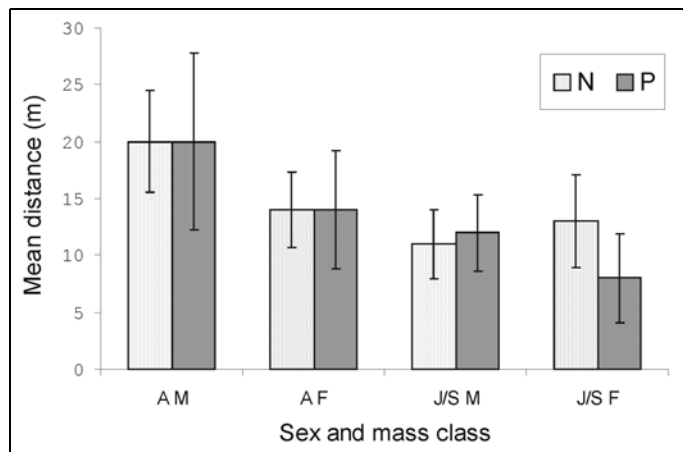


Figure 2. Mean distances (m) between capture points of positive and negative bank voles for PUUV infection in the four capture-mark-recapture (CMR) grids. N, negative; P, positive; AM, adult males; AF, adult females; J/S M, juvenile and subadult males; J/S F, juvenile and subadult females. Error bars represent 95% confidence intervals.

two (3.8%) 8-g juveniles were seropositive when first trapped and seronegative 6 months later. Twenty-two (41.5%) rodents were negative both times, and 17 (32.1%) seroconverted between the first and the second trapping. Of the 17 rodents that seroconverted, nine were juveniles or subadults at first capture and eight were adults. The proportion of animals that acquired wounds between the spring and the fall tended to be higher in rodents that seroconverted (4 [44.4%] of 9) than in the ones that remained negative (3 [25.0%] of 12), although the difference was not statistically significant ($p=0.35$).

Movement within CMR Sites

Of the rodents caught more than once during the 4-day sessions, 83 (27.5%) of 302 were trapped at one position only. Adult males were the most mobile, with a mean distance between capture points of 20 m (Figure 2). The distances covered by the positive and the negative bank voles did not differ significantly (Figure 2). However, juvenile or subadult (≥ 13 g) males that had moved distances of ≥ 20 m were significantly (chi square 3.86; $p=0.05$) more often positive than the less mobile ones with prevalence rates of 43.8% (7/16) and 19.6% (11/56), respectively. Mean distance covered was negatively correlated with the population density in adults (correlation coefficient [r] 0.84; $p<0.01$) and in juveniles and subadults (r 0.93; $p<0.01$). The rodents were the least mobile during the 1999 epidemic year.

Males that seroconverted between two trapping periods had moved a significantly (Student t value 3.16; $p=0.01$) longer distance from their original capture point (mean distance 70 m) than males that remained negative (mean distance 29 m). Furthermore, the proportion of males and females that were captured at distances ≥ 60 m from the original trapping point was significantly (chi square 6.95; $p<0.01$) higher in the group that seroconverted (9 [52.9%] of 17) than in the group that remained negative (3 [13.6%] of 22).

Distribution in the CMR Sites

The proportion of captures in the area with dense ground vegetation (Figure 3) was 83.0% (318/383) at Thuin, 87.8% (316/360) at Montbliart, 95.5% (340/356) at Momignies, and 90.8% (118/130) at Couvin. At all sites, the proportion of captures was significantly ($p<0.01$) higher in dense ground vegetation than in the other part of the grid. Comparison of prevalence of infection between both types of vegetation did not show significantly higher rates in dense ground cover (Table 3). During the nonepidemic years (1997 and 1998), however, positive animals were detected only twice in low ground cover of Thuin but were trapped seven times in dense ground vegetation of Thuin, Montbliart, and Momignies (Table 3, Figure 3).

Among adult rodents that had overlapping home ranges in the CMR sites, there were significantly more male pairs (65 of 173) and fewer female pairs (26 of 173) than expected by chance (binomial probabilities <0.01 ; proportion of males 52.1%). Statistical evidence of association was found between

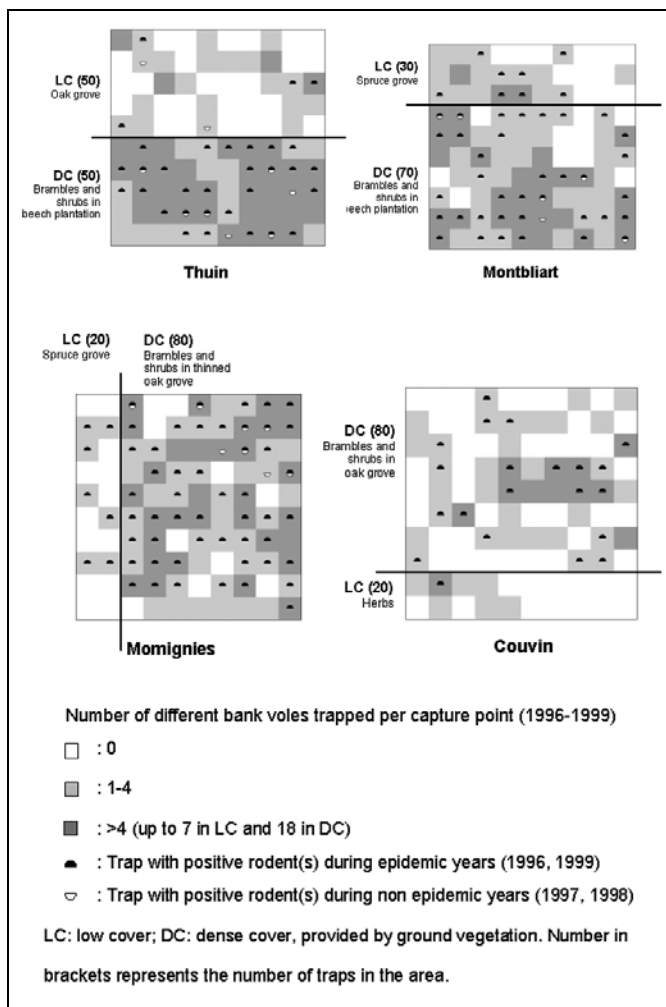


Figure 3. Distribution of trapped bank voles through 1996–1999 and representation of the vegetation cover. On each grid, the 100 live traps are represented by white, light, or dark gray squares. LC, low cover; DC, dense cover provided by ground vegetation. Number in parentheses is the number of traps in the area.

Table 3. Prevalence of *Puumala virus* infection by location and type of ground vegetation

Trapping site	1996		1997		1998		1999	
	F ^a % positive (no. tested)	S % positive (no. tested)	F % positive (no. tested)	S % positive (no. tested)	F % positive (no. tested)	S % positive (no. tested)	F % positive (no. tested)	
Dense cover								
Thuin	21.2 (66)	20.0 (10)	4.3 (23)	10.0 (10)	0.0 (29)	12.8 (47)	21.5 (65)	
Montbliart	7.7 (26)	40.0 (5)	16.7 (30)	50.0 (4)	0.0 (48)	44.9 (49)	27.2 (81)	
Momignies	17.9 (28)	0.0 (0)	0.0 (31)	0.0 (14)	15.7 (51)	64.8 (54)	28.3 (60)	
Couvin	— ^b	0.0 (1)	0.0 (19)	0.0 (9)	0.0 (17)	59.3 (27)	50.0 (18)	
Low cover								
Thuin	40.0 (5)	33.3 (3)	10.0 (10)	0.0 (0)	0.0 (6)	0.0 (13)	17.6 (17)	
Montbliart	42.9 (7)	0.0 (2)	0.0 (4)	0.0 (1)	0.0 (3)	50.0 (6)	0.0 (3)	
Momignies	50.0 (6)	0.0 (0)	0.0 (1)	0.0 (0)	0.0 (0)	50.0 (4)	33.3 (3)	
Couvin	—	0.0 (0)	0.0 (7)	0.0 (0)	0.0 (1)	66.7 (3)	0.0 (0)	

^aF, fall; S, spring.

^bTrapping in fall 1996 at Couvin was performed on a limited 0.36-ha area, where the vegetation cover was not determined.

the home range overlap and the serologic and PCR status for PUUV infection. The number of pairs including two positive animals (24/173) was higher and the number of pairs composed of a positive and a negative animal (41/173) was lower than expected by chance (binomial probabilities <0.01; prevalence of infection 22.2%).

Discussion

A widespread distribution of hantavirus infection has commonly been reported in rodent hosts during HFRS and HPS epidemics (19,22,23). Transmission of the virus is higher after sudden increases in rodent population density assumed to be related to favorable ecologic conditions (profusion of food or mild weather) (24,25). In some field studies, the prevalence of infection in rodents during non epidemic periods did not appear to be immediately related to population density (19,26–29). Social behaviors and habitat features have been proposed as additional factors influencing the dynamics of infection in the wild (29–32). In rats (*Rattus norvegicus*) and deer mice (*Peromyscus maniculatus*), which are the hosts of *Seoul virus* (SEOV) and *Sin Nombre virus* (SNV), respectively, agonistic encounters could play a role in viral transmission, as suggested by the higher prevalence of infection in wounded adults (9,32–35).

In our study, wounds resulting from biting or scratching were mainly observed in sexually mature bank voles. Wounded adults were more likely than nonwounded ones to be positive for infection in fall (i.e., at the end of the breeding season) but not in spring. Aggressive encounters would therefore play an important role in PUUV transmission during the breeding season, while behaviors such as communal nesting or mutual grooming would be determinant factors in the dynamics of infection during the winter. Aggressive behaviors begin at the onset of sexual maturity and are probably testosterone dependent in males (14). The proportion of injured animals

and the prevalence of infection were higher in adult breeding males and breeding females than in other adults. These observations underline the close relationship between aggressiveness and breeding activities, which could be associated risk factors for hantavirus infection. In contrast with results from previous studies of wild rats and deer mice (9,32,33), adult males were not wounded more often than adult females. However, the prevalence of infection was significantly higher in adult males than in adult females during the fall of the nonepidemic years (1997 and 1998). Sex-related physiologic, immunologic, or behavioral characteristics might therefore be additional factors involved in the transmission of infection. In experimentally infected rats, males were more likely than females to shed SEOV in saliva and through multiple routes (urine, feces, and saliva) (36). They were also found to shed virus in saliva and feces longer than did females (37). Although no similar studies have been performed in bank voles, the observations of SEOV in rats could indicate that fighting results in more efficient viral transmission in males than in females, who generally direct their aggression toward female intruders (14). In addition, males frequently spread small quantities of urine and feces to mark their territory and to indicate their social status during the breeding season (38). Thus, olfactory exploration of conspecific shelters and home ranges might also expose males to a higher risk of infection than females.

Movements and distribution of bank voles are also factors involved in the dynamics of PUUV infection. The association of hantavirus infection with longer distances traveled in juvenile and subadult males and in animals captured 6 months apart underlines the importance of mobility in viral transmission. The difference of prevalence between either sex in fall 1997 and 1998 may also be linked to the higher mobility of adult males compared with adult females. Our observations corroborate results from a previous study of deer mice in

which adult males living in patchy vegetative habitats were more mobile and also more often infected than adult males in dense shrub habitats (32). However, our data suggest that the mobility of bank voles was the lowest during the epidemic years. The high prevalence of infection during these years may have resulted from the high population densities, which allowed frequent encounters between rodents. Home range overlap among positive adult voles was more frequent than expected by chance. This observation could reflect a focal distribution of infected animals or may indicate that viral transmission occurs more readily between animals sharing parts of their home ranges. Direct and repeated contacts with infected conspecifics or contacts with recently shed infectious urine or feces could represent risk factors for closest neighbors within a wild breeding colony. The frequent overlap of home ranges recorded in adult males may therefore be one of the factors increasing the risk of infection in males when compared with females.

In our four CMR grids, the type of vegetation influenced the distribution of the rodents. During nonepidemic years, positive animals were more frequently trapped in dense ground vegetation where brambles were abundant. If trapping success indicates preferred habitat, then areas with dense ground cover could constitute foci for bank voles, allowing PUUV to persist during the low prevalence periods. A discontinuous distribution of the rodents could limit viral transmission, as suggested by the absence or low number of positive animals in the isolated groups from the low ground cover in Thuin (Figure 3). An uneven distribution of positive rodents related to the vegetation cover has been observed in *Peromyscus* species (28,32,33,39,40) with more restricted and well-defined focal ranges during periods of low population densities (31). A correlation was also found between prevalence of SNV infection and habitat characteristics; negative sites were associated with low and homogeneous vegetation cover (29).

We have suggested that a threshold in population density may be a determinant factor for the persistence of PUUV infection at a site (19). In this study, behavioral and physiologic factors, such as aggressiveness, mobility and reproductive status of bank voles, were shown to influence the prevalence of PUUV infection. The habitat determines the distribution of the rodents and therefore also constitutes a crucial element influencing the hantavirus enzootic cycle.

Acknowledgments

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Dr. Escutenaire completed doctoral studies in the Department of Immunology-Vaccinology, Faculty of Veterinary Medicine, University of Liège, Belgium, on the epidemiology of hantavirus infection in

wild mammals in Belgium. Her research focuses on rodent community factors involved in the dynamics of hantavirus infection.

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Instructions for Emerging Infectious Diseases Authors

Types of Articles

Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch of first author. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch of first author—both authors if only two. 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) and a brief biographical sketch of first author—both authors if only two. Report laboratory and epidemiologic results within a public health perspective. Although these reports may be written in the style of traditional research articles, they should 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) 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 1,000–1,500 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 two); and a brief biographical sketch of first author—both authors if only two. 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.

Commentary. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but should not include 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. This section includes letters that present preliminary data or comment on published articles. Letters (500–1,000 words) should not be divided into sections, nor should they contain figures or tables. References (not more than 10) may be included.

Book Reviews. Short reviews (250–500 words) of recently published books on emerging disease issues are welcome. The name of the book, publisher, and number of pages should be included.

News and Notes. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted on the journal Web page only, depending on the event date.) In this section, we also include summaries (500–1,000 words) of emerging infectious disease conferences. Summaries may provide references to a full report of conference activities and should focus on the meeting's content.

A Case-Case Comparison of *Campylobacter coli* and *Campylobacter jejuni* Infection: A Tool for Generating Hypotheses

Iain A. Gillespie,* Sarah J. O'Brien,* Jennifer A. Frost, † Goutam K. Adak,* Peter Horby,* Anthony V. Swan, ‡ Michael J. Painter, § Keith R. Neal, ¶ and the *Campylobacter* Sentinel Surveillance Scheme Collaborators¹

Preventing campylobacteriosis depends on a thorough understanding of its epidemiology. We used case-case analysis to compare cases of *Campylobacter coli* infection with cases of *C. jejuni* infection, to generate hypotheses for infection from standardized, population-based sentinel surveillance information in England and Wales. Persons with *C. coli* infection were more likely to have drunk bottled water than were those with *C. jejuni* infection and, in general, were more likely to have eaten pâté. Important differences in exposures were identified for these two *Campylobacter* species. Exposures that are a risk for infection for both comparison groups might not be identified or might be underestimated by case-case analysis. Similarly, the magnitude or direction of population risk cannot be assessed accurately. Nevertheless, our findings suggest that case-control studies should be conducted at the species level.

Campylobacters are the most commonly reported bacterial cause of acute gastroenteritis in the industrialized world (1). In the United Kingdom (UK), laboratory reports of campylobacter have increased steadily since surveillance began in 1977; in 1999, >60,000 cases were reported (incidence rate 103.7 per 100,000). However, the true population burden of campylobacter infection is thought to be much higher. For every laboratory-confirmed case reported to national surveillance in England, an additional eight cases may be unrecognized (2). This estimate suggests that in 1999, approximately half a million people in the UK became ill with campylobacter enteritis. The cost to the nation of a case of campylobacter infection has been estimated as £314.00 (at 1994–95 prices) (3); in 1999 campylobacter infection probably cost the nation >£150 million (US\$ 225 million). The clinical complications of campylobacter infection include toxic megacolon, hemolytic uremic syndrome, Reiter's syndrome, and Guillain Barré syndrome, the most common cause of acute neuromuscular paralysis in the industrialized world (4).

Although campylobacters were recognized as important pathogens >20 years ago, their epidemiology is still poorly understood (5–8). Eating poultry has long been a leading hypothesis for spread of campylobacter infection, but few case-control studies have identified it as a major risk factor

except in a commercial context (9–11). An estimated 20% to 40% of sporadic disease might result from eating chicken (12,13). Although a variety of food vehicles and other risk factors have been reported in several case-control studies, most cases in these studies remain unexplained by the risk factors identified (5–11).

A difficulty, until recently, has been the lack of routine microbiologic characterization of clinical strains (14), which has militated against systematic study of the epidemiology of the different species and subtypes of campylobacter. Control and prevention strategies cannot be developed and implemented without proper understanding of the epidemiology of campylobacter infection. On May 1, 2000, an active, population-based sentinel surveillance scheme for campylobacter infections was initiated in England and Wales (15). Its aim is to generate hypotheses for human campylobacter infection by using a systematic, integrated epidemiologic and microbiologic approach. Twenty-two district health authorities are collaborating in the scheme, working with their hospital microbiology and local environmental health departments

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¹The *Campylobacter* Sentinel Surveillance System Collaborators comprise public health, environmental health, and laboratory staff who serve the populations of the following health authorities in England and Wales: Birmingham, Bradford, Bro Taf, Bury and Rochdale, Dyfed Powys, East Kent, Enfield & Haringey, Herefordshire, Leeds, Leicestershire; Manchester, North Cumbria, North Essex, North West Lancashire; Nottingham, Salford and Trafford, South and West Devon (part), South Lancashire, Southampton and South West Hampshire, Stockport, West Pennine, and Wigan and Bolton with the PHLS Laboratory of Enteric Pathogens, the PHLS Statistic Unit and the PHLS Communicable Disease Surveillance Centre.

(Figure 1). The sentinel system covers a population of approximately 12.5 million and captures standardized information on approximately 15% of all laboratory-confirmed campylobacter infections in England and Wales. The health authorities are broadly representative of England and Wales as a whole.

We have used case-case comparisons, an adaptation of conventional case-control methods, as suggested by McCarthy and Giesecke (16), to generate hypotheses concerning risk factors for campylobacter infection. We report results from the first year of the study and discuss the strengths and weaknesses of case-case analysis.

Methods

Campylobacters isolated by National Health Service and Public Health Laboratory Service (PHLS) laboratories within the catchment area were referred to the Campylobacter Reference Unit of the PHLS Laboratory of Enteric Pathogens for speciation, serotyping, phage typing, and antibiotic resistance testing (17–20). A standard, structured clinical and exposure questionnaire was administered to each patient by the health or local authority as part of the routine investigation of foodborne infection. The questionnaire, which can be completed by the patient, captured demographic and clinical data, as well as travel history (foreign and domestic), food history (>20 exposures), milk (3 exposures) and water (8 exposures) consumption, recreational water activity, animal contacts, and other

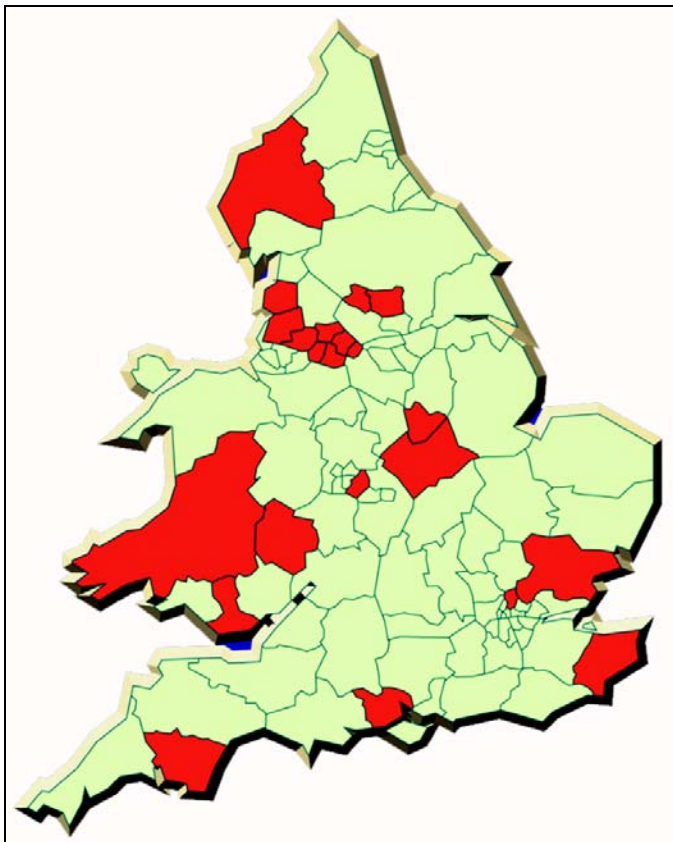


Figure 1. The health authorities in England and Wales participating in the sentinel surveillance scheme for Campylobacter.

illness (either in the household or the community) during the 2 weeks before the onset of illness. Epidemiologic exposure data and microbiologic typing information were then collated centrally by the Gastrointestinal Diseases Division of the PHLS Communicable Disease Surveillance Centre.

The combined epidemiologic and microbiologic dataset, generated through the sentinel scheme, was analyzed by Stata version seven (Stata Corporation, College Station, TX). For the case-case analysis, illness in patients infected with *C. coli* was designated a “case;” patients infected with *C. jejuni* were designated as controls. Differences in demographic and clinical data were assessed by using Pearson’s chi-square test and the Student t test. Cases were excluded from analysis if a patient was infected with more than one campylobacter subtype (133 cases) or was confirmed as infected with *C. lari* (two patients) or *C. fetus* (one patient).

The date of onset of illness for cases was used to define the month of onset and approximations of the four seasons (spring, March–May; summer, June–August; autumn, September–November; winter, December–February) were calculated. Socioeconomic group, based on occupation, was determined by standard occupational classification (21). Additional categories were generated for persons who described their occupation as unemployed, preschool child, school child, student, homemaker, retired, or part time, and for those who were unable to work because of disabilities or long-term illness. Food exposures were coded to compare those who had eaten a particular food in the 2 weeks before onset of illness (once or more than once) with those who had not. Daily water consumption was coded to differentiate no exposure from 1–4, 5–9, and >10 glasses of water drunk. Patient age was classified in 10-year age groups. Persons with missing data were omitted from the analyses using those data.

Initially, comparisons between *C. coli* and *C. jejuni* cases were performed by single-risk variable analyses. Mantel-Haenszel odds ratios (OR) were calculated for each explanatory variable. Logistic regression was applied to obtain maximum likelihood estimates of the effect of exposures on the species-specific outcome, while the data were controlled for potential confounders. Variables with a p value <0.1 from the single-risk variable analysis were included initially. Stepwise exclusion was used to simplify the model: variables were removed one at a time and tested for significance by the likelihood ratio (LR) test. Potential interactions (among the main effects included in the initial logistic regression model and age, sex, and season) were also examined by using the LR chi-square test.

Results

Epidemiologic data have been gathered for 7,360 laboratory-confirmed cases of campylobacter infection during the first year of the study (response rate 7,360 [76%] of 9,655). The median delay between onset of symptoms and completion of a questionnaire was 16 days. Case-patients ranged from <1 month to 99 years of age (Figure 2), and the overall sex distribution

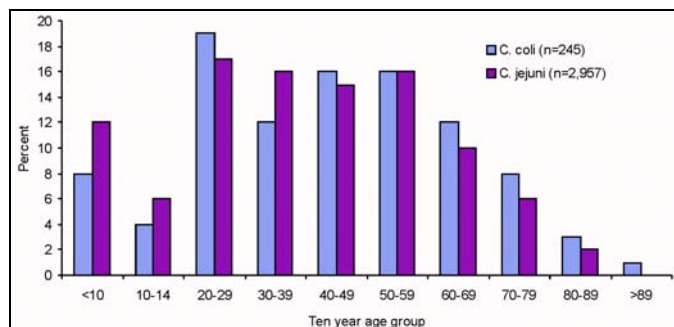


Figure 2. Age distribution of *Campylobacter coli* and *C. jejuni* cases reported to the sentinel surveillance scheme.

was even. Diarrhea (95%), abdominal pain (85%), and fever (78%) were the most commonly reported symptoms, with vomiting (35%) and bloody diarrhea (27%) reported less frequently. A total of 6,948 case-patients amassed 79,090 days of illness (mean 11), and 10% were hospitalized for an average of 5 days (range 1–42 days). Six hundred fifty-nine patients accumulated 3,048 hospital days. Five thousand one hundred seven patients reported absence from work or an inability to undertake normal activities for a total of 38,769 days (mean 8 days).

Linked epidemiologic and microbiologic data are available for 3,764 cases. *C. jejuni* accounted for 3,489 (93%) of the cases, with 272 *C. coli* (7%), 2 *C. lari* (<1%), and 1 *C. fetus* (<1%) also reported. Case-patients with *C. coli* and *C. jejuni* infection did not differ with regard to sex, clinical symptoms, or duration of illness (Table 1). However, case-patients infected with *C. coli* tended to be older (mean 42.9 years) than patients with *C. jejuni* (mean 38.5 years) ($p=0.001$).

Patients with *C. coli* infection were more likely to describe their ethnicity as Asian and to have traveled abroad in the 2 weeks before the onset of symptoms (single-risk variable analysis; Table 2). Patients with *C. coli* were also more likely to report having eaten specific types of meats (Halal meat [meat slaughtered according to Islamic law], meat pies, offal [organ meats], and pâté) and bottled water. They were less likely to have had contact with animals than were patients with *C. jejuni* infection. Persons with *C. coli* and those with *C. jejuni* infection did not differ with regard to eating chicken (89.8% vs. 90.8%; odds ratio [OR] 0.89; 95% confidence interval [CI] 0.58 to 1.36; chi square 0.59) or other types of poultry (23.6% vs. 19.7%; OR 1.26; 95% CI 0.91 to 1.74; chi square 0.16) in the 2 weeks before onset of illness.

Patients with *C. coli* infection were more likely to have drunk bottled water than persons with *C. jejuni* infection and, in general, were more likely to have eaten pâté (logistic regression analysis; Table 3). Retired persons who ate meat pies were more likely to be infected with *C. coli* than *C. jejuni*, as were Asians who had traveled abroad in the 2 weeks before illness. Case-patients with *C. coli* infection were, in general, less likely to be ill in the summer, and men who traveled abroad in the 2 weeks before illness were more likely to be infected with *C. jejuni* infection.

Discussion

To our knowledge, this population-based sentinel surveillance system for campylobacter infection is unique because we have successfully linked detailed epidemiologic exposure information with detailed microbiologic strain characterization for a large sentinel population. Campylobacters are widely distributed in the environment, and this genus is adapted to a wide range of ecologic niches throughout the food chain (22). Microbiologic data show that the prevalence of different campylobacter species and subtypes varies between different potential sources of infection, including different animal species, foods, and water (23–27). Although *C. coli* infection accounts for a small proportion of laboratory-confirmed human campylobacter cases in England and Wales, the potential for prevention is substantial if the true population burden is much higher (3). Most case-control studies have so far sought to determine risk factors for sporadic infection with campylobacter and have not sought to differentiate between species (5–

Table 1. Demographics, clinical symptoms, and severity of infections with *Campylobacter coli* and *C. jejuni*

Variable	Campylobacter species (%)		χ^2	p value
	<i>C. coli</i> (n=272)	<i>C. jejuni</i> (n=3,489)		
Mean age	42.9	38.5	-	0.001
Male	123 (45)	1,734 (50)	2.02	0.16
Female	149 (55)	1,755 (50)		
Mean length of illness	11.4	11.3	-	0.92
Diarrhea				
Yes	253 (96)	3,355 (98)	3.11	0.08
No	10 (4)	73 (2)		
Bloody stools				
Yes	73 (35)	964 (34)	0.07	0.79
No	134 (65)	1,843 (66)		
Vomiting				
Yes	87 (37)	1,249 (40)	1.00	0.32
No	151 (63)	1,885 (60)		
Abdominal pain				
Yes	236 (93)	3,013 (92)	0.13	0.72
No	19 (7)	265 (8)		
Fever				
Yes	206 (84)	2,812 (86)	1.44	0.23
No	40 (16)	440 (14)		
Seeking advice from a doctor				
Yes	260 (97)	3,345 (98)	0.65	0.42
No	8 (3)	76 (2)		
Hospitalized				
Yes	23 (9)	358 (10)	0.97	0.32
No	245 (91)	3,055 (90)		
Mean days off work/normal activities	6.7	7.6	-	0.05

Table 2. Risk exposures for *Campylobacter coli* infection, by single-risk variable analysis

Exposure	No. exposed (%)		Odds ratio	p value ^a	95% Confidence intervals
	<i>C. coli</i> (n=272)	<i>C. jejuni</i> (n=3,489)			
Summer	75 (27.6)	1,206 (34.6)	0.72	0.02	0.55 to 0.95
Dyfed Powys Health Authority	5 (1.8)	24 (0.70)	2.7	0.04	1.02 to 7.15
10-year age group (increasing)	-	-	1.10 ^b	0.001 ^c	1.04 to 1.17
Members of the armed forces	1 (0.37)	2 (0.06)	6.43	0.08	0.58 to 71.27
Retired persons	61 (22.4)	580 (16.6)	1.45	0.01	1.07 to 1.95
Preschool-aged children	14 (5.2)	288 (8.3)	0.60	0.07	0.35 to 1.05
Homemakers	16 (5.9)	131 (3.8)	1.60	0.08	0.94 to 2.73
South Asian ethnicity	21 (9.1)	168 (5.8)	1.63	0.04	1.01 to 2.61
European ethnicity	4 (1.7)	118 (4.1)	0.42	0.08	0.15 to 1.14
Travel abroad	76 (28.3)	653 (19.0)	1.68	0.0002	1.27 to 2.22
Halal meats	23 (10.7)	216 (7.3)	1.52	0.07	0.96 to 2.39
Meat pies	78 (33.9)	856 (27.9)	1.32	0.049	1.00 to 1.76
Offal (organ meat)	19 (8.7)	170 (5.6)	1.60	0.06	0.97 to 2.62
Pâté	42 (18.7)	397 (13.2)	1.51	0.02	1.06 to 2.14
Bottled water	150 (63.6)	1,646 (53.7)	1.51	0.003	1.14 to 1.98
Contact with animals	138 (51.7)	1,989 (57.8)	0.78	0.049	0.61 to 1.00

^aExposures where p<0.1 shown.

^bApproximation to the odds ratio for a one-unit increase in 10-year age group.

^cDerived from score test for trend of odds

7). This distinction is important if *C. coli* and *C. jejuni* differ in their etiology or if the contribution of similar risk factors differs between the two species. If exposures are aggregated for different pathogenic campylobacter species, the contribution of risk factors unique to or predominantly associated with *C. coli* will be masked by the predominance of *C. jejuni* (in the study population: *C. jejuni*: *C. coli* approximately 10:1). This source of bias can be overcome by comparing the exposure characteristics of cases with *C. coli* infection with those of cases with *C. jejuni* infection. The data for cases with *C. jejuni* infection are then used to contrast with, rather than dilute, any observations for *C. coli* infection. Therefore, in generating hypotheses for infection, we identified potential species differences by adopting case-case analysis.

Hypothesis: Bottled Water

Case-patients with *C. coli* infection were more likely to report bottled water consumption than were those with *C. jejuni* infection. This observation is biologically plausible. Raw water can be contaminated with *C. coli* (28,29) and, while European legislation governing the marketing of natural mineral water makes it a condition that it be free from parasites and pathogenic organisms (30), testing for campylobacters is rarely undertaken (31). As the bottled water industry is large (\$35 billion a year worldwide [32]) and expanding rapidly (consumption in the United States, which was 5 billion gallons in 2000, is predicted to increase to 7.3 billion gallons

in 2005 [32]), an accurate assessment of the risk associated with these products is required. Our hypothesis-generating questionnaire did not distinguish between types of bottled water (e.g., spring or mineral, carbonated, or still), but these issues merit further investigation by case-control study.

Hypothesis: Pâté

The finding that having eaten pâté was more likely to be reported by case-patients with *C. coli* infection than those with *C. jejuni* infection is also biologically plausible. Pork is often the main constituent of pâté, and *C. coli* is found in pigs (33). In a recent study of the occurrence of campylobacters in 400 freshly eviscerated porcine liver samples, 6% were infected with *Campylobacter* spp; most (67%) were *C. coli* (34). Pâté is a perishable comminuted meat product containing nitrite, and possibly nitrate, ascorbate, or both (35). While the use of such preservatives might deter the growth of spoilage microorganisms (assuming adequate storage conditions are maintained), vegetative pathogens might not be destroyed; therefore, the ultimate critical control point during production is likely to be effective heat treatment.

Hypothesis: Meat Pies

The fact that retired people with *C. coli* infection were more likely to report having eaten meat pies is interesting. The types of meat in the pie fillings are not known, but the finding might point to the use of cheaper cuts of meat in these products.

Table 3. Independent risk exposures for *Campylobacter coli* infection: final logistic regression model^a

Exposure	Odds ratio	p value	95% Confidence intervals
Summer	0.64	0.029	0.42 to 0.95
Summer (for participants 50–60 y of age)	3.10	0.013	1.27 to 7.59
South Asians who traveled abroad	9.70	0.006	1.89 to 49.73
Pâté	1.85	0.006	1.19 to 2.88
Pâté (for participants 50–60 y of age)	0.21	0.050	0.05 to 1.00
Meat pies eaten by retired persons	3.41	0.005	1.45 to 8.01
Bottled water	1.45	0.042	1.01 to 2.08
Men who traveled abroad	0.42	0.028	0.19 to 0.91
Male	1.05	0.804	0.72 to 1.53
Age (y)	1.00	0.586	0.99 to 1.02

^a Main effects not shown if $p > 0.05$; data were controlled for a priori confounders of age and sex.

Hypothesis: Foreign Travel

Persons from a South Asian ethnic background who had traveled abroad in the 2 weeks before onset of symptoms were more likely to have acquired a *C. coli* infection, but the reverse was true for men. This finding probably reflects the fact that travel abroad is simply a marker for activities or behavior while abroad, and a further study of the “travel cohort,” generated through the surveillance scheme, might provide a better indication of where the risks lie.

Hypothesis: Seasonality

Campylobacter infection has marked seasonality, and case-patients infected with *C. coli* were less likely to be ill in the summer than those infected with *C. jejuni*. As data accumulate, generating season-specific hypotheses might be possible, which may have implications for the time period over which analytic studies are performed.

Sources of Bias

In interpreting the results from the sentinel surveillance system, likely sources of bias should be considered. Selection bias has been minimized by including all laboratory-confirmed cases of campylobacter infection identified by PHLS and National Health Service laboratories in the participating districts. Furthermore, both groups in the case-case comparison have been subjected to the same selection process, so selection bias should not influence our analysis.

The effect of time delays in reaching the patient, and hence recall bias for reported exposures, should be limited by close collaboration between the various participants in the scheme. While the time delay reported in this study introduces some recall bias, there is no reason to believe that recall is operating differently among patients infected with different species or

among exposure groups, so that recall bias should not influence the case-case comparison.

Interpreting Case-Case Analyses

A detailed account of the pros and cons of case-case analysis is provided by McCarthy and Giesecke (16), but two important points influence the interpretation of this type of study. The first is that exposures that are a risk for infection for both comparison groups will not be identified or might be underestimated. By using patients with campylobacter infection, albeit with a different species, as “controls,” we may obscure an association with the infection of interest because the controls might share some of the risk exposures with the cases. Thus, exposures common to both infections are controlled for by the study design.

The second is that traditionally controls are selected to provide an estimate of the exposure prevalence that would be seen in the cases if there were no association between the exposure and disease. Since our controls have been differentially selected by factors that are related to certain exposures, they might not be representative of the exposure prevalence of the population group from which the cases originated. We cannot, therefore, use comparisons between our cases and controls to make statements about the magnitude or direction of population risk.

Conclusion

Our work has shown that important differences in exposures might exist for these two campylobacter species. This finding is not necessarily surprising. For example, nontyphoidal salmonellosis is well recognized to represent a large group of serotypes, each with its own distinctive epidemiology (36). Given this knowledge, conducting a case-control study with a case definition comprising *Salmonella* spp. is inconceivable. Why should the same not be true for *Campylobacter* spp.? The implications for analytic study design are that researchers should not aggregate different species, which may mask important species-specific risk factors. Thus, the comparison of two organisms thought to represent one disease with a common cause has provided new avenues for the epidemiologic investigation of human disease. Focused analytical studies, based on systematically generated hypotheses, determining etiologic fractions for the risk factors identified, will allow informed prevention strategies for human infection.

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Spatial Analysis of Human Granulocytic Ehrlichiosis near Lyme, Connecticut

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Geographic information systems combined with methods of spatial analysis provide powerful new tools for understanding the epidemiology of diseases and for improving disease prevention and control. In this study, the spatial distribution of a newly recognized tick-borne disease, human granulocytic ehrlichiosis (HGE), was investigated for nonrandom patterns and clusters in an area known to be endemic for tick-borne diseases. Analysis of confirmed cases of HGE identified in 1997–2000 in a 12-town area around Lyme, Connecticut, showed that HGE infections are not distributed randomly. Smoothed HGE incidence was higher around the mouth of the Connecticut River and lower to the north and west. Cluster analysis identified one area of increased HGE risk (relative risk=1.8, $p=0.001$). This study demonstrates the utility of geographic information systems and spatial analysis to clarify the epidemiology of HGE.

Historically, the study of the spread of diseases within populations has included a spatial component. New tools, including geographic information systems (GIS) and spatial statistics methods, enable epidemiologists to address the spatial aspects of disease rates and transmission more thoroughly and less subjectively. The emergence of tick-borne infections in the United States has been attributed to reforestation and second-growth forests, with the associated increases in reservoir and vector populations, as well as to human behavior changes including residential preferences and the increased popularity of outdoor recreational activities (1–3).

Our study used a GIS and spatial statistics to analyze the spatial distribution of a newly recognized tick-borne disease, human granulocytic ehrlichiosis (HGE). This disease was first described in a series of patients from northern Minnesota and Wisconsin in 1994 (4). The agent of HGE¹ “is most closely related to *Ehrlichia phagocytophila*, which infects sheep and cattle, and *E. equi*, which causes disease in horses. Recent research has suggested that rather than three separate species, these organisms are three variants of the same species (5–7). In the eastern and midwestern United States, the agent of HGE is transmitted to humans by the tick vector, *Ixodes scapularis*. This tick is also the vector of *Borrelia burgdorferi* and *Babesia microti*, the agents of Lyme disease and human babesiosis, respectively (8). The HGE agent is well established in vector populations in the Northeast (9–11), and infection with multiple *I. scapularis*-borne pathogens has been documented in both humans and wild mammal reservoirs (9,11–18). Since 1995, HGE has been a physician- and laboratory-reportable condition in Connecticut. In addition, an active surveillance system for HGE was established in 1997 in a 12-town area around Lyme, Connecticut (Figure 1), where Lyme disease was first described and remains highly endemic (19). This

region has a total population of 83,600 and encompasses 330.7 square miles. During the 4 years of surveillance (1997–2000), the average annual incidence of confirmed cases of HGE in the 12-town area was 42 cases per 100,000 persons. For the same 4-year period, the average annual incidence of Lyme disease in the 12-town area was 234 cases per 100,000 persons.

The use of a GIS with spatial statistics, including spatial filtering (smoothing) and cluster analysis, has been applied to other diseases, in which it is often used to analyze and more clearly display the spatial patterns of disease (20–25). Smoothing decreases the random variation associated with small case numbers and small populations, enabling disease gradients or holes to be observed that may not be apparent with raw data (20,26,27). Cluster analysis identifies whether geographically grouped cases of disease can be explained by chance or are statistically significant (23,28); it detects true clusters of disease from cases grouped around population centers (29). While many risk factors and environmental cues may be similar for Lyme disease and HGE, investigating the spatial nature of the latter in an area known to be endemic for both may increase our understanding of the epidemiology of HGE and enhance our ability to focus education and control efforts to reduce human disease risk. The goal of our study was to describe the spatial distribution of HGE within a highly endemic area and to provide the groundwork for further study to identify the environmental and landscape characteristics associated with increased risk for HGE infections.

¹Since this study was conducted, new nomenclature (*Anaplasma phagocytophila*) has been proposed; see Dumler JS, Barbet AF, Bekker CP, Dasch GA, Palmer GH, Ray SC, et al. Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia*, and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and ‘HGE agent’ as subjective synonyms of *Ehrlichia phagocytophila*. *Int J Syst Evol Microbiol* 2001;51:2145–65.

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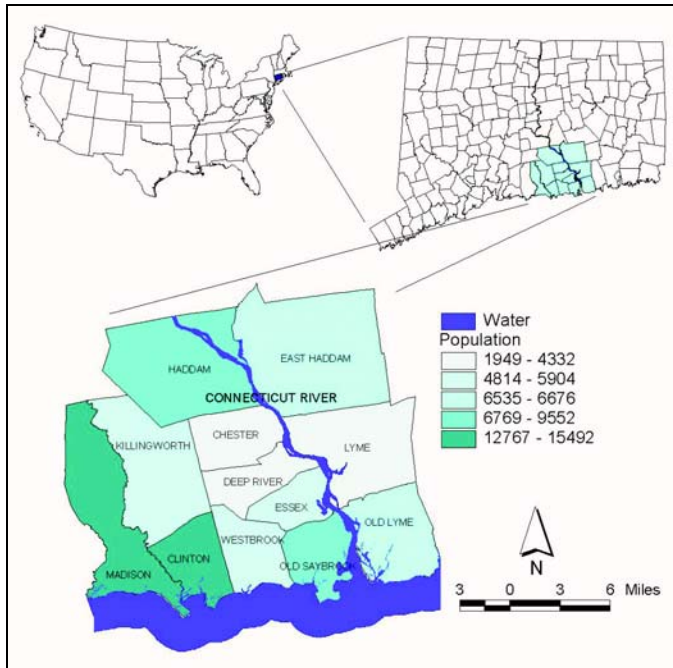


Figure 1. Surveillance area: 12-town area around Lyme, Connecticut.

Materials and Methods

Cases

The confirmed cases of HGE analyzed in this study were identified through active and passive surveillance systems described elsewhere (19). Informed consent for participation in the active tick-borne disease surveillance study was obtained from all participants or their parents or guardians, according to a protocol approved by the Yale School of Medicine Human Investigation Committee. That committee approved a waiver of consent for this analysis. Only cases detected in 1997–2000 in residents of the 12-town area around Lyme, Connecticut, were included in the analysis. A confirmed case was defined as illness in a patient who had a seroconversion or ≥ 4 -fold change in antibody titer between acute- and convalescent-phase serum specimens (by indirect fluorescent antibody or enzyme-linked immunosorbent assay), a positive polymerase chain reaction assay with primer pairs directed to genomic sequences specific to HGE, or detection of the specific 44-kDa protein band by Western blot analysis. A probable case was defined as an illness in a patient with a positive antibody titer from only a single serum sample or a < 4 -fold change in antibody titer between acute- and convalescent-phase serum samples.

Geocoding Cases

The home address was mapped for each confirmed case of HGE. We used home addresses based on the assumption that most people become infected with HGE near their homes. While peridomestic transmission has been established for Lyme disease (30), this assumption has not yet been tested for HGE. U.S. Bureau of the Census Topologically Integrated

Geographic Encoding and Referencing system (TIGER/Line) maps, which contain street segments and address ranges for the 12 towns, were obtained from the University of Connecticut Map and Geographic Information Center (MAGIC). By using geographic information system software, ArcView GIS version 3.1 (Environmental Systems Research Institute, Inc., Redlands, CA), we geocoded home addresses to individual points in a new map layer by using the TIGER/Line street data files. Addresses were matched by town to decrease the error associated with similar names in different towns. An interactive matching process was used to increase the likelihood of achieving a match for an address. In addition, some addresses were identified by street maps and then manually added as points to the map. Town boundary and population census block group maps for each town were also obtained from MAGIC and were included as themes (map layers) with the geocoded points of cases. Once cases were geocoded, they were sorted by town and population census block group. Raw annualized incidence rates were calculated by using 1990 census data.

Spatial Filtering (Smoothing)

The technique of incorporating data from surrounding areas in an image or map to define a new data value for the area of interest is called spatial filtering. Spatial filtering can involve smoothing or sharpening the data of interest. To reduce random noise in the data that comes from the high variance characteristic of small populations or small case numbers (26), we performed the smoothing type of spatial filtering. Data were exported from ArcView, and the smoothing was done in the SAS statistical analysis software package, versions 6.12 and 8.0 (SAS Institute, Inc., Cary, NC). To decrease the variance to an acceptable level, a minimum “filter” number of 10 cases per area was established to calculate the disease rate. If an area (census block group) did not meet that minimum, smoothing was performed. This involved identifying a circle around the centroid of the census block group. The circle’s radius was enlarged until it included the centroid of the next closest census block group (based on the distances between centroids), and the number of cases was recalculated. This process was continued until the total number of cases circumscribed by the circle was ≥ 10 . Then the disease rate was calculated individually for each census block group on the basis of this larger number of cases and larger population. Annualized incidence by census block group was calculated in ArcView after the data were exported back into the GIS. The technique of producing a smoothed map of disease rates allows for the display of data at a smaller geographic scale while preserving the stability of the estimated disease rates.

Cluster Analysis

Spatial cluster analysis was performed on the confirmed cases of HGE to test whether the cases were distributed randomly over space and, if not, to evaluate any identified spatial disease clusters for statistical significance (31). We applied the

“spatial scan statistic” (31) to test the null hypothesis that the relative risk (RR) of HGE was the same between any block group, or collection of block groups, and the remaining block groups. By scanning varied size areas for possible disease clusters without prior assumptions of cluster size or location, we sought to avoid preselection bias (28). SaTScan software, version 2.1 (28), designed specifically to implement this test, imposed a circular window on the map. This window moved over the area and centered on the centroid of each census block group. The area within the circular window varied in size from zero to a maximum radius, never including >50% of the total population. The SaTScan software tested for possible clusters within the variable window around the centroid of each block group. Cluster analysis was performed with the default maximum spatial cluster size of $\leq 50\%$ of the population and again with a smaller maximum cluster size of $\leq 25\%$ to look for possible subclusters. For each window of varying position and size, the software tested the risk of HGE within and outside the window, with the null hypothesis of equal risk. This procedure compensated for the inherent bias in multiple testing (31).

An additional cluster analysis was conducted by using both confirmed and probable HGE cases to address potential inclusion biases in the observed clustering of cases. These biases may have arisen because active surveillance cases were more likely to have provided both acute- and convalescent-phase samples than were cases detected through passive surveillance, and thus, had greater chance of being classified as confirmed cases and being included in analysis. Identical methods to those described above were used to perform the cluster analysis on the combined confirmed and probable cases.

Results

Two hundred forty-five cases of HGE were identified through the active and passive surveillance systems in 1997–2000. A total of 136 confirmed cases of HGE were identified; 128 (94%) of these were geocoded to points in an ArcView theme (Figure 2a). Addresses that were not geocoded consisted of two incomplete ones (street names with no number) and six post office boxes from Chester, Essex, Haddam, Lyme, Madison, and Westbrook.

Annualized incidence rates for 1997–2000 were calculated by town and census block group by using 1990 census data to show the crude distribution of HGE in the 12-town area (Figure 2b,c). Rates by town ranged from 3/100,000 in East Haddam to 156/100,000 in Lyme. Rates by census block group ranged from 0/100,000 to 187/100,000 and demonstrated a high degree of random variation because of the small population size and low case numbers.

Smoothing provided a clearer picture of the areas of increased risk on a smaller scale than by town. A filter number of 10 provided the most appropriate map of smoothed incidence rates (Figure 2d). This filter number decreased random variation and showed an increased risk of contracting HGE around the mouth of the Connecticut River with risk decreasing to the north and west.

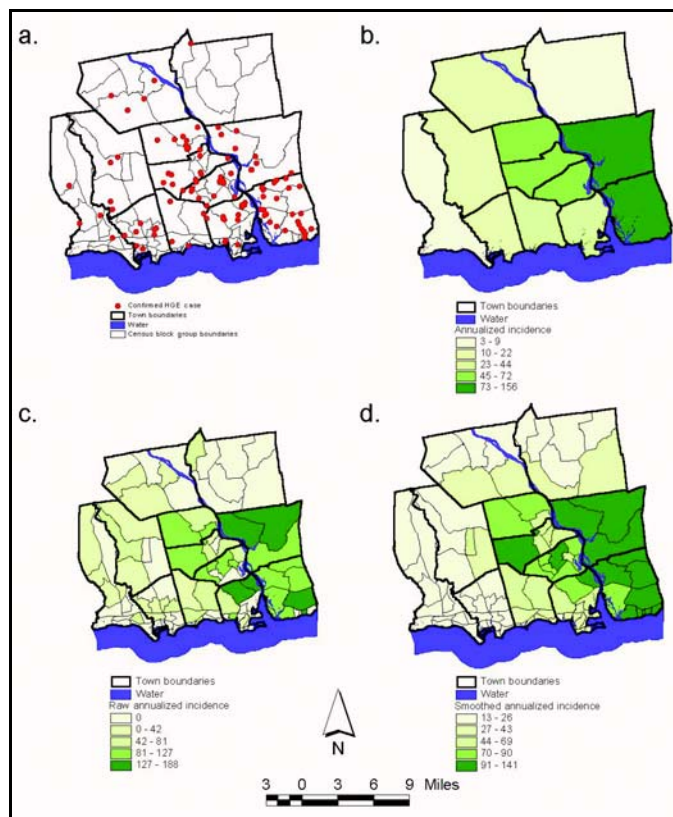


Figure 2. a. Confirmed human granulocytic ehrlichiosis (HGE) cases identified through active and passive surveillance systems, 1997–2000; b. Raw annualized incidence of confirmed HGE cases by town, 1997–2000*; c. Raw annualized incidence of confirmed HGE cases by census block group*; d. Smoothed annualized incidence of confirmed HGE cases by census block group.*
*Cases per 100,000 persons.

Using the maximum spatial cluster size of $\leq 50\%$ of the total population, the spatial cluster analysis identified a single cluster that included all census block groups in the towns of Lyme, Old Saybrook, Chester, Essex, Deep River, and Westbrook, all but one in the town of Old Lyme, and one from the town of Clinton (Figure 3a). The identified cluster contained 46.1% of the area’s total population. The overall RR within the cluster was 1.8, with an observed number of cases of 106 compared with 59 expected cases. This elevated risk within a non-random pattern of disease distribution was significant ($p=0.001$).

To investigate the possibility of smaller clusters, the same analysis was performed with a maximum spatial cluster size of $\leq 25\%$ of the total population. Two clusters were identified, one including Lyme and Old Lyme as well as parts of Essex, Old Saybrook, and Deep River (Figure 3b). This cluster contained 18.2% of the total population and had an overall RR of 2.6 ($p=0.001$), with 61 cases observed compared with an expected 23 cases. The second subcluster included areas of Deep River and Essex (Figure 3b). This cluster contained 4.2% of the total population and had an overall RR of 2.6 ($p=0.16$), with 14 cases observed compared with 5 cases expected. While the primary cluster identified in this analysis was significant and showed a higher overall RR, the larger

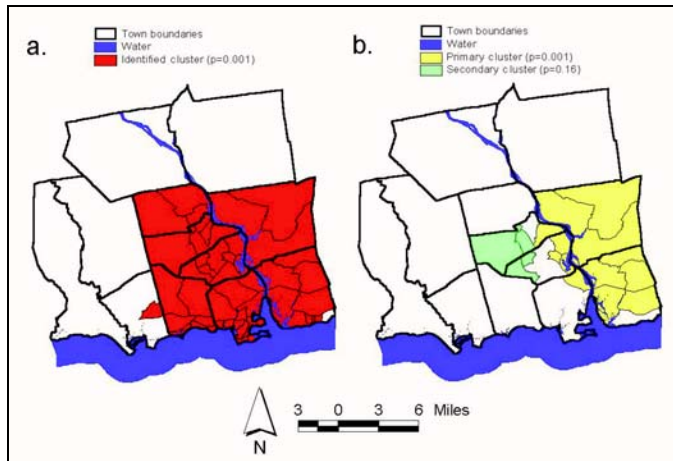


Figure 3. a. Single identified cluster of human granulocytic ehrlichiosis (HGE) cases within the 12-town area (maximum cluster size $\leq 50\%$ total population), relative risk (RR)=1.8, $p=0.001$; b. Two identified clusters of HGE cases within the 12-town area (maximum cluster size $\leq 25\%$ total population): primary cluster: RR=2.6, $p=0.001$, secondary cluster: RR=2.6, $p=0.16$.

cluster from the first analysis, as a result of the lack of pre-selection bias, better represented the areas of increased risk for infection on the basis of the spatial distribution of HGE in the 12-town area.

Cluster analyses were also performed on confirmed and probable case data. By using the $\leq 50\%$ maximal cluster size, we included identical census block groups in the cluster ($p=0.001$). In the subcluster analysis that used the $\leq 25\%$ cluster size, both subclusters identified with confirmed cases only were found to be statistically significant ($p<0.005$). The primary subcluster was geographically circumscribed when confirmed and probable cases were included in the analysis.

Discussion

Using a GIS and spatial statistics, we investigated the spatial distribution of confirmed cases of HGE and identified areas of increased risk within an area highly endemic for tick-borne diseases. Such diseases have become recognized as serious health threats in the northeast United States in the last 20 years because of increasing prevalence and heightened detection. Areas characterized by low residential density and a landscape of recently reforested deciduous forest are strongly associated with the risk for Lyme disease (30,32,33). Areas of high Lyme disease risk have been shown to also have an increased risk for HGE (19). Our study identified spatial variations in the risk for HGE in such an area. Furthermore, the analysis demonstrated that combining thorough surveillance information with spatial analysis techniques can increase understanding of the epidemiology of HGE within a highly disease-endemic area. The next step, to investigate the underlying causes of increased risk in the identified areas, will be analysis of landscape attributes and identification of the environmental variables characteristic of high-risk areas.

The spatial statistics analyses clearly yielded a nonrandom distribution of HGE within the 12-town area. Spatial filtering

(smoothing) identified areas of increased risk centered around the mouth of the Connecticut River, primarily on the eastern side of the river, in the towns of Lyme and Old Lyme. Increased likelihood of disease was seen on the western side of the river but was not as consistently high as the risk observed in Lyme and Old Lyme. Spatial cluster analysis identified a statistically significant cluster (RR=1.8, $p=0.001$) in the same area, around the mouth of the Connecticut River, including the towns of Chester, Deep River, Essex, Lyme, Old Lyme, Old Saybrook, and Westbrook. One census block group in southeastern Old Lyme was not included in the cluster, and one block group in Clinton was included. This cluster analysis was performed by using the default maximum spatial cluster size of $\leq 50\%$ of the total population. Using this default method minimizes pre-selection bias of cluster size. However, to investigate the possibility of subclusters, additional cluster analysis based on a maximum spatial cluster size of $\leq 25\%$ of the total population identified two subclusters, one significant (RR=2.6, $p=0.001$) and the second not significant (RR=2.6, $p=0.19$). The decrease in risk for HGE infection as one moves away from the coast is consistent with the results of Nicholson and Mather, who described a decreasing Lyme disease risk with increasing latitude in Rhode Island (34).

The present study analyzed the associations between human population and human disease only. Gathering and including vector population data (including population density, distribution, and infection prevalence rates) and environmental variables in the risk analysis of HGE in the 12-town area may provide a more comprehensive view of the disease risk. The relationship between Lyme disease, *I. scapularis* vectors, and landscape characteristics has been studied from remotely sensed and field-gathered data (35–37), but it is unknown whether these relationships can be applied to other tick-borne diseases, including HGE. Increased Lyme disease risk has been well correlated with increased tick abundance and prevalence of infected ticks (34,35,38). The spatial distribution of Lyme disease rates is correlated with widespread tick populations and pathogen prevalence (25). Environmental risk factors and landscape characteristics associated with Lyme disease have been identified (22,35–37). Using techniques similar to those used for Lyme disease, combined with the results of this study, future research will include investigating the landscape characteristics associated with HGE. Further, discernment of the aspects of the natural history of HGE that are not understood, especially pertaining to the reservoir host, may supply additional information that can be used to further refine areas of HGE risk.

While similar numbers of specimens were submitted for HGE testing to both the active and passive surveillance systems, the low rate of convalescent-phase specimen collection and the application of only one diagnostic test in passive surveillance resulted in fewer cases from passive surveillance being confirmed and included in the current analysis. Persons who live at the edges of the 12-town area may have been more likely to visit practitioners outside the active surveillance area.

These case-patients would have been identified through passive surveillance but would have been less likely to be confirmed. While the practices participating in active surveillance were located throughout the 12-town area and include one practice outside that area, the lower rate of confirmed cases in the passive system may have biased the results toward the center of the surveillance area. However, the similar results obtained from the spatial statistics analysis that used both confirmed and probable cases suggest that this error may not have played a large role in the observed patterns of disease.

Because of variations in testing throughout the 4 years of surveillance, analysis for temporal clusters was not possible. Retrospective testing of banked samples from previous surveillance years or continuing accumulation of surveillance data in years to come will be needed to investigate the temporal as well as spatial spread of HGE within the 12-town area. Temporal trends, combined with time series analysis of remotely sensed land cover and land-use data, may provide indications of future areas at increased risk for HGE. Concurrent analysis of the spatial and temporal distributions of other *I. scapularis*-borne diseases in this area, including Lyme disease and babesiosis, may clarify the similarities and differences in risk among these common vector-borne infections.

Our study was based on the assumption that people acquire infection with the agent of HGE peridomestically, or near their homes. Falco and Fish (30) demonstrated that most cases of Lyme disease were acquired peridomestically, but no studies have investigated whether HGE infections are similarly acquired. While the life cycle similarities of these two pathogens support the assumption that HGE transmission dynamics are similar to those of Lyme disease, additional research is needed to test this hypothesis.

This spatial analysis was limited to the described 12-town study area. This area of active surveillance was identified previously by its high rates of Lyme disease, and the towns to the east were excluded because at the time the study was initiated (1997) those towns had lower rates of Lyme disease compared with the 12 study towns. However, in this analysis, understanding of the spatial distribution of HGE would be enhanced if the towns to the east of the current study area were also included in the active surveillance, given the high rates of HGE in Lyme and Old Lyme. Stemming from this analysis, the towns to the east of the original study area (Salem and East Lyme) were added to the active surveillance area in 2002. The eastern limitation in our dataset highlights another use of spatial analysis and GIS. The tools and methods described in this study can identify areas where increased surveillance is recommended.

Human behavior is a strong predictor of tick-borne disease risk, including how people move in their environment, their outdoor activities, and the individual protection they use to prevent tick bites. Reforestation in areas previously used for agriculture results in more favorable conditions for tick and reservoir hosts, while the trend towards residential preferences in well-shaded suburban and rural areas exacerbates the tick-

human interactions. Change in human behavior concurrent with an ecologic transition further increases and alters tick-borne disease risk. Local weather variations and the periodicity of weather patterns also play a role in tick-borne disease risk. The combination of these factors results in a high variability of risk even within an area known to be hyperendemic for tick-borne diseases.

On the basis of data on peridomestic Lyme disease infections (30), prevention strategies are recommended that focus on persons' risk at home. In an area in which tick-borne diseases are highly endemic, aiming prevention strategies at areas of highest risk can potentially increase the program's effectiveness. Persons at highest risk should be informed of that risk and of the possibilities for risk reduction. Funds spent on programs might be better spent on areas where cost-effectiveness can be maximized. At this time, practical prevention advice to prevent tick-borne disease in highly disease-endemic areas is elusive.

The tools described in this article, GIS and spatial statistics, provide an opportunity to clarify and quantify the health burden from tick-borne disease within a highly endemic area and a foundation to pursue further investigation into the environmental factors resulting in increased disease burden. To implement specific and geographically appropriate risk-reduction programs, the use of such spatial analysis tools should become integral components in the epidemiologic description and risk assessment of tick-borne diseases.

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Ms. Chaput is an epidemiologist with the Delaware Division of Public Health. Her interests include employing spatial analysis tools to better understand chronic and infectious disease epidemiology, medical entomology, and global vector-borne disease control.

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Molecular Characterization of *Campylobacter jejuni* Clones: A Basis for Epidemiologic Investigation

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A total of 814 isolates of the foodborne pathogen *Campylobacter jejuni* were characterized by multilocus sequence typing (MLST) and analysis of the variation of two cell-surface components: the heat-stable (HS) serotyping antigen and the flagella protein FlaA short variable region (SVR). We identified 379 combinations of the MLST loci (sequence types) and 215 combinations of the cell-surface components among these isolates, which had been obtained from human disease, animals, food, and the environment. Despite this diversity, 748 (92%) of the isolates belonged to one of 17 clonal complexes, 6 of which contained many (318, 63%) of the human disease isolates. Several clonal complexes exhibited associations with isolation source or particular cell-surface components; however, the latter were poorly predictive of clonal complex. These data demonstrate that the clonal complex, as defined by MLST, is an epidemiologically relevant unit for both long and short-term investigations of *C. jejuni* epidemiology.

Campylobacter jejuni is the most frequently reported cause of acute inflammatory gastroenteritis in industrialized countries and a major cause of intestinal disease in children <2 years of age in developing countries (1,2). *C. jejuni* infection is widely perceived as an increasing problem; for example, the number of reported cases of *C. jejuni*-associated enteritis increased sixfold in the United Kingdom from 1977 to 2000 (3,4). The syndromes associated with *C. jejuni* infection range from mild enteritis to severe invasive disease, and sequelae can occur, including the autoimmune-mediated demyelinating neuropathies Guillain-Barré and Miller Fisher syndromes (5). The intestines of many feral and commercially reared birds, livestock, domestic pets, and animals are asymptotically colonized by this bacterium, which is widely distributed in the environment and food. Transmission to humans is thought to occur through food, drinking water, and pets (6,7).

Elucidation of the epidemiology of this zoonosis is complicated by the sporadic nature of the disease (8), along with the organism's wide distribution, high levels of genetic and antigenic diversity, and a lack of representative population samples (9). The absence of precise information on the relative importance of different sources of human infection has hindered the development of effective disease-control measures and is a major challenge in preventing human disease. The characterization of *C. jejuni* isolates has relied on serologic typing schemes since the 1980s (10,11), but the data available from these techniques have not resolved the epidemiology of

human disease. Consequently, the development of a method for the characterization of *C. jejuni* isolates that is accurate, reproducible, and comparable among laboratories has been a research priority for many years (9). While numerous phenotyping and genotyping techniques that discriminate among isolates for short-term epidemiology have been developed, generating data that are comparable among different laboratories and that accurately identify relationships among isolates from diverse sources has proven more challenging.

A multilocus sequence typing (MLST) scheme (12) has been developed and validated for *C. jejuni* (13). This approach, which exploits recent technical developments in high-throughput nucleotide sequence determination and combines them with conceptual advances in bacterial population biology, has proved to be successful for a number of bacteria (12–15). MLST is especially suitable for the investigation of diverse bacterial populations that have weakly clonal population structures (13,16,17). In common with multilocus enzyme electrophoresis, MLST indexes variation in housekeeping genes, which evolve slowly as they are under stabilizing selection for the conservation of metabolic function. The use of nucleotide sequence data directly accesses the variation in the targeted gene, and the technology employed is readily disseminated and highly reproducible (18). Further advantages are that the data are electronically portable, enabling comparison of isolates via the Internet without exchange of biological materials, and amenable to analysis by phylogenetic and population genetic techniques (19,20).

Initial analysis of *C. jejuni* populations with MLST confirmed the diverse genetic nature of the *C. jejuni* species and indicated that the population structure was likely to be weakly clonal (13,17). Weakly clonal bacterial populations comprise a

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number of clonal complexes, which correspond to “lineages,” i.e., groups of organisms presumed to derive from a common progenitor. However, the phylogenetic relationships among distinct complexes cannot in general be reconstructed because they have been disrupted by lateral gene transfer (20). The MLST approach has been used to demonstrate that a number of *C. jejuni* clonal complexes are associated with human disease and that isolates from several of these caused demyelinating neuropathies (13,21).

We examined the relationships of *C. jejuni* isolated from a broad range of sources in two countries by MLST, which was used to define clonal complexes. The results of this analysis were compared with variation observed in two cell-surface components that have been previously used for bacterial typing: the heat-stable (HS) serotyping antigen, which was investigated serologically; and the flagella, which was investigated by nucleotide sequencing of the *flaA* gene short variable region (SVR). We also examined the extent to which different isolation sources and cell-surface component variants correlated with clonal complex.

Methods

A diverse collection of *C. jejuni* isolates, mainly from the United Kingdom and the Netherlands, was investigated. Most of these isolates, which originated from human disease, food animals or products, and an environmental source (Table 1), had been characterized by HS antigen serotyping (11,22–24). For each isolate, chromosomal DNA was prepared, and MLST was performed with seven housekeeping genes as described (13). The data were deposited in the *Campylobacter* Internet-accessible database, enabling the sequence types (STs) of the isolates to be established (<http://campylobacter.mlst.net/>). In addition, the nucleotide sequence of a 321-bp region of the *flaA* gene was determined for each isolate. This sequence encompassed the SVR (25) extending from *flaA* nucleotide positions 283–603 inclusive (FlaA amino acids 95–201). Primers FLA4F (5'-GGA TTT CGT ATT AAC ACA AAT GGT GC-3') and FLA1728 (5'-CTG TAG TAA TCT TAA AAC ATT TTG-3') or FLA625RU (CAA G[AT]C CTG TTC C[AT]A CTG AAG-3') were used (25). The polymerase chain reaction amplification conditions were as follows: denaturation at 94°C for 2 min, primer annealing at 50°C for 1 min, and extension at 72°C for 2 min for 35 cycles. Nucleotide

sequence extension reactions were performed with primers FLA242FU (5'-CTA TGG ATG AGC AAT T[AT]A AAA T-3') and FLA625RU, and BigDye™ Ready Reaction Mix (Version 2, PE Biosystems, Foster City, CA) was used in accordance with the manufacturer's instructions. Reaction products were separated with an ABI prism 3700 automated DNA sequencer (PE Biosystems). The peptide sequences encoded by the SVR nucleotide sequences were deduced, and each unique amino acid sequence was assigned FlaA SVR amino acid variant number in the order of discovery. The amino acid sequence of the FlaA SVR was used to provide an indication of the phenotypic diversity of a variable cell-surface component that is known to be subject to antigenic variation. The sequences were deposited in an Internet-accessible database of FlaA SVR sequences (<http://outbreak.ceid.ox.ac.uk/campylobacter/>).

The initial step in the assignment of the STs to clonal complexes was to identify a founder or central genotype for each clonal complex. Founder STs were identified with the aid of heuristic methods such as the BURST algorithm and UPGMA cluster analysis, implemented in the computer program START (26), and split decomposition, implemented in the program SPLITSTREE (27). After the central genotypes had been identified, all remaining STs were assigned sequentially to clonal complexes. First, the STs differing at one locus from a founder were assigned, followed by those differing at two loci and finally those that differed at three loci. The clonal complexes were named after the ST of the central genotype, e.g., ST-21 complex (13). Assignment of STs, clonal complex, serotype, and FlaA SVR type permitted comparisons of these characters and their combinations with source of isolation.

For each of the clonal complexes, the significance of any association with serotype, FlaA SVR, and isolation source was independently assessed by chi square test. For the cell-surface components, the distribution of individual variants expected if they were distributed randomly among the clonal complexes was calculated. This null hypothesis was compared with the observed distribution, and the statistical significance of any observed associations was tested. For the source of isolation, we tested the null hypothesis that the distribution of clonal complexes among isolates from each source was the same as the distribution of clonal complexes found in human disease isolates.

Table 1. Sources of *Campylobacter jejuni* isolates

Source	Country of origin				Total
	United Kingdom	Netherlands	Others	Unspecified	
Human disease	370	79	25	27	501
Chickens	53	77	4	3	137
Lamb/offal	72	-	-	-	72
Beef cattle/offal	48	2	1	1	52
Sand from beaches	52	-	-	-	52
Total	595	158	30	31	814

Results

C. *jejuni* Clonal Complexes

The collection of 814 isolates contained 379 distinct sequence types. On the basis of their STs, 748 (92%) of the isolates were assigned to 1 of 17 clonal complexes (Table 2) comprising ≥ 6 members, with 318 (63%) of the 501 isolates obtained from human disease being assigned to 1 of 6 clonal complexes (ST-21, ST-45, ST-206, ST-61, ST-48, and ST-257 complexes). The STs of the remaining 66 isolates occurred either once (31 isolates, 3.8%) or in combinations of two to four identical or related sequence types (35 isolates, 4.2%). These included seven reference isolates for the Penner serotyping scheme (11) and eight isolates with one to six alleles that had divergent nucleotide sequences.

Of the 17 clonal complexes, 6 had been described previously (ST-21, ST-45, ST-22, ST-179, ST-177, and ST-52 complexes) (13), and the others (ST-206, ST-61, ST-48, ST-257, ST-353, ST-42, ST-403, ST-362, ST-354, ST-433, and ST-49 complexes) are described here for the first time (Table 2). As a result of the larger dataset and additional analysis techniques used in our study, several STs that had been provisionally described as members of ST-17, ST-65, ST-125, and ST-51

complexes (13) were shown to be members of ST-257, ST-433, ST-206, and ST-353 complexes, respectively, and the provisional names were discarded.

In 14 of the 17 clonal complexes, the central genotype was distinct from that of any other complex. The three exceptions were ST-21 complex, ST-48 complex, and ST-206 complex, which shared combinations of identical alleles at four of the seven loci (Table 2). These clonal complexes were described as separate entities with their own founder genotype for two reasons. First, each complex had an abundant central genotype, which was present in multiple isolates of the collection, and second, each central genotype had many single-, double-, and triple-locus variants. Isolates belonging to each of these three clonal complexes differed in their association with isolation source. Members of the ST-48 complex were isolated predominantly from human disease, cattle, and sand from beaches; members of the ST-21 and ST-206 complexes were also obtained from poultry (Figure).

Variation in Cell-Surface Components

Among the 814 isolates were 46 HS antigen serotypes and 64 FlaA SVR amino acid sequence variants in a total of 215 unique combinations. At the nucleotide sequence level, 221

Table 2. Clonal complexes of *Campylobacter jejuni* isolates

Clonal complex	Isolates (n)	ST ^a of central genotype							No. of STs	No. of cell-surface component variants			Cell-surface component variants (p<0.001) ^c	
		aspA	glnA	gltA	glyA	pgm	tkf	uncA		HS serotypes ^b	FlaA SVR	Antigen combinations	HS serotypes	FlaA SVR
ST-21	271	2	1	1	3	2	1	5	98	10	8	24	1,2,4 ^c ,8,10	1,10
ST-45	93	4	7	10	4	1	7	1	48	24	15	44	6, 7, 9, 12, 15, 21, 27, 33, 38, 42, 45, 55, 57, 60	1, 2, 5, 8, 9, 12, 18, 27
ST-206	60	2	21	5	37	2	1	5	37	3	8	9	1,4 ^c	1,11
ST-61	57	1	4	2	2	6	3	17	19	2	9	5	4 ^c	6,13,14
ST-48	54	2	4	1	2	7	1	5	22	4	5	7	4 ^c	1,4
ST-257	46	9	2	4	62	4	5	6	21	4	3	4	11	12
ST-353	29	7	17	5	2	10	3	6	24	9	8	14	3,11,37	2,11
ST-42	22	1	2	3	4	5	9	3	7	5	4	5	23,36,23/36	9
ST-403	20	10	27	16	19	10	5	7	12	5	6	7	35	35
ST-52	18	9	25	2	10	22	3	6	11	7	3	7	5,11	4
ST-177	17	17	2	8	5	8	2	4	17	5	13	7	-	-
ST-354	13	8	10	2	2	11	12	6	4	5	7	8	53	1,20,33
ST-22	12	1	3	6	4	3	3	3	5	2	3	3	19	3
ST-433	11	2	59	4	38	17	12	35	11	6	3	9	1,53	-
ST-362	9	1	2	49	4	11	66	8	1	1	1	1	41	3
ST-179	10	1	6	7	2	40	32	3	9	2	2	3	-	-
ST-49	6	3	1	5	17	11	11	6	2	2	2	2	18	11

^aST, sequence type; HS, heat stable; SVR, short variable region; -, no antigen variants were associated that were statistically significant (p>0.001), but isolates were antigenically varied.

^bHS 4 complex serotypes (4c) were counted as one serotype.

^cAntigen variants that were significantly associated with a particular clonal complex by the chi-square test. Isolates containing other antigen variants were also found in each complex, as were isolates which were nontypable by HS serotyping.

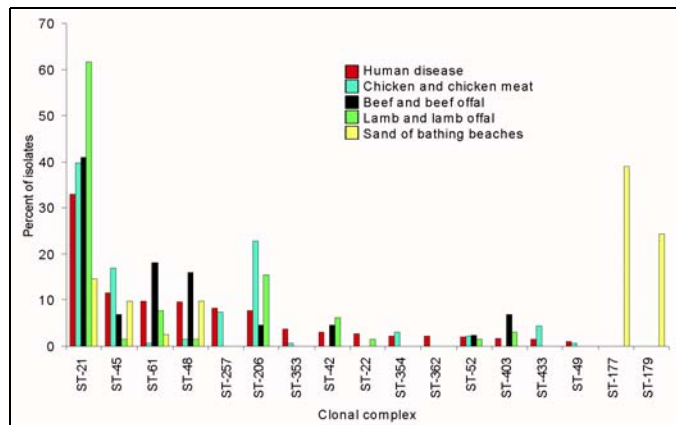


Figure. Frequency distribution of *Campylobacter jejuni* clonal complexes isolated from different sources.

distinct *flaA* SVR sequences were present. The d_N (nonsynonymous base substitutions) to d_S (synonymous base substitutions) ratio was 0.608 for the 321-bp sequence encompassing the SVR: this ratio is comparable with levels ranging from 0.036 to 0.071 for the seven housekeeping gene loci used in MLST (13).

Most of the clonal complexes contained multiple STs, HS serotypes, and FlaA SVR amino acid sequence variants (Table 2). In particular, the two most common complexes among the human disease isolates, ST-21 complex and ST-45 complex, had 24 and 43 combinations of HS serotype and FlaA SVR variant (Tables 2,3). For 15 of the 17 clonal complexes, a significant association ($p < 0.001$) with at least one surface component variant was observed (Table 2), but particular variants generally occurred in more than one clonal complex. Only three of the complexes were associated with a single HS serotype, including an association of ST-22 complex with HS serotype 19, ST-362 complex with HS serotype 41, and ST-257 complex with HS serotype 11 (Table 2). Other complexes were associated with isolates of cross-reactive or likely phase variant serotypes (22,28), including three of the most frequently represented clonal complexes in the isolate collection (ST-206, ST-48, and ST-61 complexes), which contained isolates expressing the following members of the HS serotype 4 complex: 4, 13, 16, 43, 50, and 65. Serotypes 23 and 36, which are highly cross reactive and structurally unique among the HS serotyping antigens (29,30), were only present among isolates belonging to ST-42 complex. Serotypes 27 and 57, which may be expressed by the same isolate at different times (31), were expressed by isolates assigned to the ST-45 complex (Table 3). The isolates assigned to the remaining nine complexes were either associated ($p < 0.001$) with more than one HS serotype (for example, ST-21 complex with HS serotypes 1, 2, and 4 complex) or deemed too small for meaningful statistical analysis (e.g., ST-179 complex contained 10 isolates and appeared to be associated with HS serotypes 2 and 5, and ST-49 complex contained six isolates and was associated with HS serotype 18). The ST-45 complex contained 24 different HS

serotypes among its 93 isolates (Table 3), 14 of which were found only in isolates belonging to the ST-45 complex.

Some of the FlaA SVR variants were associated with particular clonal complexes, for example, variant 12 with ST-257 complex; variants 6,13, and 14 with ST-61 complex; variant 3 with ST-22 and ST-362 complexes; and variant 9 with ST-42 complex. Among the 31 isolates with unique STs, 11 SVR variants were present; 5 were unique. Variant 1 was the most common of these, occurring 13 times among these isolates.

Table 3: Combinations of cell-surface components associated with ST-21 complex and ST-45 complex.

Clonal Complex	HS Serotype	FlaA SVR variant	Clonal Complex	HS Serotype	FlaA SVR variant
ST-21	1	1	ST-45 (cont.)	6	18
	1	2		7	5
	1	8		7	77
	1	10		8	5
	1	11		9	1
	1	46		9	22
	2	1		9,37	12
	2	4		12	1
	2	5		15	1
	2	8		15	24
	2	10		15	27
	4c	1		21	12
	4c	4		27	5
	4c	5		33	26
	4c	11		33	27
	5	1		38	1
	8,17	1		38	5
	9	1		40	1
	10	5		42	1
	10	8		42	27
	10	10		42	2
	11	1		44	5
	23	10		45	1
	68	1		45	23
				25	24
ST-45	2	12		53	2
	3	2		55	2
	4c	1		55	27
	4c	5		55	47
	4c	9		57	2
	5	2		57	5
	6	5		57	32
	6	8		60	1
	6	9		60	2

Certain HS serotype and SVR variants occurred together to a significant degree ($p < 0.001$, chi-square test), for example, variant 10 with HS serotype 2 and variant 8 with HS serotype 10. Many other combinations were also found but were present in insufficient numbers for statistical analysis. Clonal complexes with the same HS serotype or types did not necessarily share the same FlaA SVR variant or variants. For example, the isolates among ST-48 and ST-61 complexes both belonged to the HS serotype 4 complex but contained different SVR sequences.

Clonal Complex and Isolation Source

These results suggest differences in the frequency distribution by isolation source of isolates belonging to particular clonal complexes. The isolates assigned to the ST-21 complex were found in all sources, ranging from 32% of all isolates from human disease to >60% of all isolates from lambs; however, the ST-177 and ST-179 complexes were present only in isolates from beach sand, and ST-257 was present only in the isolates from human disease or chickens. The data also strongly suggest a nonrandom source distribution among the other clonal complexes; for example, ST-61 and ST-48 complexes appeared to be overrepresented in isolates from cattle and beef offal (Figure).

Discussion

The Kauffman-White scheme for the typing of *Salmonella enterica* provided a paradigm for the characterization of bacterial pathogens for much of the last century (32). The success of this scheme was largely a consequence of the clonal population structure of this organism, resulting in a tree-like phylogeny, which can be readily reconstructed from any of a number of characteristics (33,34). Variation in the immunologic reactivity of *S. enterica* surface polysaccharides proved to be a convenient way of identifying individual clones, permitting the rapid establishment of the epidemiology of this organism and enabling its spread to be monitored and contained. Similar serologic typing schemes have been developed for other bacterial pathogens, including *C. jejuni* and *C. coli* (11); however, a number of these bacteria, such as *C. jejuni*, engage in lateral gene transfer more frequently than salmonellae (13,17,35). These bacteria do not have a strongly clonal population structure, and loci encoding individual characteristics, such as flagella and capsular antigens, which are under diversifying selection, move by horizontal genetic exchange at relatively high frequency among *C. jejuni* isolates that do not necessarily share a recent common ancestor (36,37). Consequently, these characteristics do not provide epidemiologic information of the same quality as in salmonellae, which explains many of the difficulties in understanding the spread of *Campylobacter* disease in humans.

The MLST approach, by indexing the nucleotide sequence variation present at several housekeeping loci (which evolve slowly because they are under stabilizing selection for conservation of metabolic function), provides data that are highly

discriminatory but enable the population structure of an organism to be established. These data are not sensitive to the “genome instability” observed in *C. jejuni*, which appears to result largely from chromosome rearrangements and which adversely affects typing techniques such as pulsed-field gel electrophoresis fingerprinting (31,38,39). The sequence data presented here were consistent with the view that *C. jejuni* has a weakly clonal population structure, as suggested by previous genotypic and phenotypic studies (17,21,40). Although the isolate collection examined was diverse, most of the 379 observed STs could be assigned to 17 clonal complexes, 6 of which accounted for most of the human disease isolates from the United Kingdom and the Netherlands. Three of the clonal complexes, ST-21, ST-206, and ST-48, may form a “complex group” of related genotypes, which are widely distributed, perhaps reflecting an ability to colonize a wide range of hosts. This complex group probably corresponds to the grouping of heterogeneous yet related isolates, previously referred to as the HS serotype 1, 2, and 4 complex (40–43).

When the level of *flaA* SVR allelic diversity was assessed at the amino-acid level, a degree of discrimination between isolates was obtained comparable with that obtained with clonal complexes. Most isolates contained 1 of 15 SVR variant types (data not shown), and the number of clonal complexes identified was 17. In contrast, at the nucleotide sequence level the allelic diversity of the SVR region was so great that it was less useful for population analysis. However, the nucleotide sequences of genes under diversifying selection are useful when discriminating between related isolates, for example, in distinguishing outbreak strains.

The combination of data for the two cell-surface components, HS serotype and FlaA SVR variant, provided less discrimination than ST, and—unlike the STs—these combinations could not be categorized into larger groups. Some correspondence between the HS serotype/FlaA SVR variant type and clonal complex was observed; for example, all the isolates assigned to the ST-22 complex contained only isolates of HS serotype 19 and FlaA SVR variant 3. However, in general the variation of these molecules was an inconsistent indicator of clonal complex. Such observations have been made previously, with poor correlation detected between the HS antigens and the heat-labile (flagella) antigens (40). Many distinct combinations of HS serotype and FlaA SVR variant type occurred among the isolates assigned to the largest clonal complexes, for example, ST-21 and ST-45 complexes (Table 3), which together accounted for 49% (244/501) of the human disease isolates. A degree of overlap in the HS serotype and SVR variants was also present in a number of the clonal complexes.

The assignment of the isolates by ST and clonal complex permitted comparisons among isolates obtained from diverse sources. The fact that this classification was possible demonstrated that there was structure to the genetic diversity of *C. jejuni*. Such structure may be imposed in recombining populations by a number of forces, including clonal expansion, niche

adaptation, geographic isolation, and host immune selection (44,45). An unambiguous resolution of the evolutionary forces that shape *C. jejuni* populations will only be possible when representative samples of natural populations of this organism, i.e., those present in wild and domestic animals, are tested by multilocus nucleotide sequence-based techniques such as MLST. However, the data presented here are consistent with the view that the clonal complexes may be maintained by niche adaptation, as a number of clonal complexes were over-represented in particular isolation sources. The ST-45 and ST-257 complexes predominantly contained isolates from human disease and poultry; the ST-61 and ST-42 complexes mainly comprised isolates from human disease, cattle, and sheep; and the ST-177 and ST-179 complexes exclusively contained isolates from beach sand (Figure).

Immune selection imposed by the host may also play a role in defining the clonal complexes that are uniform in their combinations of HS serotype and FlaA SVR variant: all members of the ST-257 complex had HS serotype 11 and SVR variant 12, and all members of the ST-22 complex contained isolates of HS serotype 19 and SVR variant 3. This finding could also reflect niche adaptation if these surface components were important in the colonization of particular host species. The medical importance of such association is that the ST-22 complex is overrepresented among isolates associated with Guillain-Barré and Miller Fisher syndromes (21), probably on the basis of the lipopolysaccharide antigens that it expresses (46). However, other clonal complexes were more antigenically heterogeneous; thus, a complicating factor in exploring these relationships for all *C. jejuni* may be the organism's ability to colonize multiple hosts and its exposure to many different immune responses.

A further advantage of MLST data is that they can be used to examine bacterial species definitions more closely. Our data were consistent with the suggestion that the distinctions between different *Campylobacter* species may be complicated by interspecies genetic exchange (13). The *uncA* allele (*uncA-17*) that defined ST-61, the central genotype of fourth-largest clonal complex in this sample, was highly divergent from all other *uncA* alleles and likely to have originated in another species, perhaps *C. coli*. Ten other STs within the data set contained from one to six divergent alleles that probably originated from other *Campylobacter* species.

MLST data will enable the appropriate choice of representative isolates for whole genome analyses and studies of animal models. Comparisons of the members of complexes that are never or rarely associated with human disease, examples of which may include the ST-177 and ST-179 complexes reported here, will provide valuable comparisons with isolates from complexes often associated with human disease. Since MLST data are directly comparable and the amount of data available will expand over time, this method should be able to enable the questions of *Campylobacter* epidemiology to be addressed by the scientific community more easily.

Our study demonstrated the high throughput and cost-effectiveness achievable with nucleotide sequence-based techniques such as MLST: the 814 isolates were characterized at a consumables cost (i.e., cost of supplies, excluding time and labor) of approximately \$37 per isolate. The data presented here indicate the likely extent of diversity of *C. jejuni* isolates from humans, permitting investigations to be designed that will determine the relative importance of the food chain—and its individual components—as sources of human *Campylobacter* infection.

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The 2000 Tularemia Outbreak: A Case-Control Study of Risk Factors in Disease-Endemic and Emergent Areas, Sweden

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Johan Giesecke,† and Anders Tegnell†

A widespread outbreak of tularemia in Sweden in 2000 was investigated in a case-control study in which 270 reported cases of tularemia were compared with 438 controls. The outbreak affected parts of Sweden where tularemia had hitherto been rare, and these “emergent” areas were compared with the disease-endemic areas. Multivariate regression analysis showed mosquito bites to be the main risk factor, with an odds ratio (OR) of 8.8. Other risk factors were owning a cat (OR 2.5) and farm work (OR 3.2). Farming was a risk factor only in the disease-endemic area. Swollen lymph nodes and wound infections were more common in the emergent area, while pneumonia was more common in the disease-endemic area. Mosquito bites appear to be important in transmission of tularemia. The association between cat ownership and disease merits further investigation.

Tularemia is caused by *Francisella tularensis*, a fastidious, gram-negative rod. *F. tularensis* subsp. *tularensis*, or type A, occurs mainly in North America and is more virulent than *F. tularensis* subsp. *holarctica*, or type B, which occurs throughout the Northern Hemisphere. Type A is usually transmitted to humans by tick bites or contact with rabbits; type B is associated with water and animals living near water, and its transmission seems more complex (1–6).

In Sweden, >6,000 human cases of tularemia have been reported since the disease was first described in 1931. However, incidence varies greatly from year to year, ranging from a few cases in some years to >2,700 cases in 1967. The ulceroglandular form of tularemia is by far the most common in Sweden, except for an outbreak in the winter of 1966–67, when a large proportion of pulmonary tularemia cases occurred in farmers who processed hay contaminated by dead, infected voles (7). Apart from this outbreak, most cases in Sweden have occurred in late summer and early autumn and are thought to have been transmitted by mosquitoes (8,9).

Most cases occur within a relatively small area in the central part of Sweden, with only sporadic cases in other areas. In recent years, however, the disease seems to have spread to areas south of the disease-endemic area. This shift was apparent in the 2000 outbreak, when 187 (40%) of 464 cases were reported to have been transmitted south of the disease-endemic area. The reason for this spread is unknown.

We studied the risk factors for acquiring tularemia in Sweden, as well as the prevalence of the risk factors in the disease-endemic and the new, “emergent” areas during the outbreak of

2000. We performed a matched case-control study, using a modified questionnaire designed by a Finnish group that was studying a concurrent tularemia outbreak in Finland.

Methods

Identification of Cases and Controls

Tularemia has been a notifiable disease under the Communicable Diseases Act in Sweden since 1968. Physicians who diagnose a case, either clinically or by microbiologic means, report to the county medical officer and the Swedish Institute for Infectious Disease Control (SMI). Cases were defined as tularemia in all persons ages ≥ 18 whose illness was reported to SMI from August 1 (week number 31) to November 21, 2000 (week number 47) and who resided in any of seven counties in central Sweden, representing both disease-endemic and emergent areas (Figure 1). The cases from these seven counties represented 60% of all cases reported in Sweden during the study period; the rest were sporadic cases from other areas or infection acquired abroad (Figure 2).

Controls matched for age, sex, and place of residence were drawn from the computerized Swedish National Population Register, in which the name, date of birth, personal identifying number, and address of all citizens and residents are stored. Matching for place of residence was done on the first three digits of the five-digit postal code, since this three-digit area corresponds to a small town, village, or municipality. We chose controls whose date of birth was as close as possible to that of the patient, with a difference ≤ 12 months. Two controls were selected for each case. A questionnaire was mailed to the two controls; if neither responded within 2 weeks, a third control was chosen in the same way. If a control reported having

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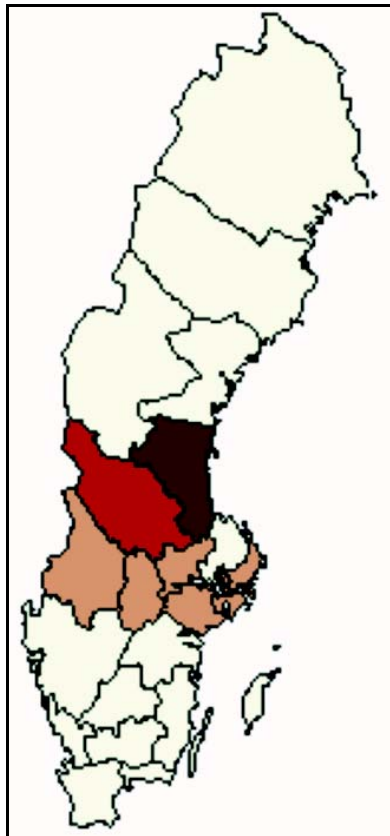


Figure 1. Map of Sweden showing the areas used in the analysis. Heavy shade marks tularemia-endemic area, medium shade the border area, and light shade the emergent area, where many cases occurred during the 2000 outbreak, but few cases were reported during the previous decade.

had fever during the period of investigation and no diagnosis other than tularemia was made, he or she was excluded from the analysis.

On the standardized questionnaire mailed to both case-patients and controls were questions on whether they had had an elevated temperature during the 4-week period 2 weeks before and 2 weeks after onset of symptoms in the patient. Additional data were collected on symptoms, medication, and referral to hospital. Participants were also asked about number of persons in the household and symptoms in other household members during the defined period.

The second part of the questionnaire contained questions on exposure to presumed risk factors for acquiring tularemia during the 4-week period preceding the reported day of onset of illness. The following exposures were recorded: owning cats, dogs, or other animals; visiting golf courses and forests; participating in farming procedures of different kinds; having contact with dead animals, with or without wearing gloves; visiting or swimming in lakes or rivers; drinking water from lakes or wells; picking berries or mushrooms; and being bitten by mosquitoes, mites, ticks, deer flies, or other insects. Respondents were also asked if they had used insect repellents. Visits to areas other than place of residence during the defined period of exposure were also recorded.

In the 2000 epidemic, cases occurred in places where tularemia had rarely been reported. To evaluate whether this spread was connected to any new risk factors, the seven counties in the study area were divided into three categories (Figure

1): the disease-endemic area, County of Gävleborg, from which cases were reported every year during the 1990s; the border area, County of Dalarna, where occasional cases and small outbreaks have been reported during the last decade; and the emergent area, which consists of the counties of Stockholm, Södermanland, Västmanland, Värmland, and Örebro, from which only one or two cases have been reported in isolated years during the 1990s, but where a large part of the cases occurred in 2000. This division was done by actual place of exposure, as could be judged by routine notifications from the period 1990–99, and not by place of residence.

The endemic group consisted of 84 case-patients and 159 controls, a total of 243 persons, while 300 persons were in the emergent group (105 case-patients and 195 controls). We chose to analyze these two groups separately for symptoms and exposures as above, but to improve the discrimination between the old and new tularemia areas, we excluded the third group, consisting of 29 case-patients and 60 controls from the border area, from this part of the analysis.

Statistics

A matched univariate analysis, with calculation of odds ratios (OR) and 95% confidence intervals (CI) by the Mantel-Haenszel method, was done in EpiInfo 6.04 (Centers for Disease Control, Atlanta, Georgia) and the Stata program (Stata Corp., College Station, Texas). Multivariable analysis was done by conditional logistic regression for matched data in the Stata program.

Results

Two hundred seventy cases fulfilled the above criteria. All the case-patients were ill during the summer or in the early autumn. No cases were reported during 2000 before the start of the study period on August 1. Of cases, 86% were confirmed with serologic testing or culture. The questionnaire was sent to all 270 case-patients and 670 controls. Replies were received from 243 (90%) of 270 case-patients and 438 (65%)

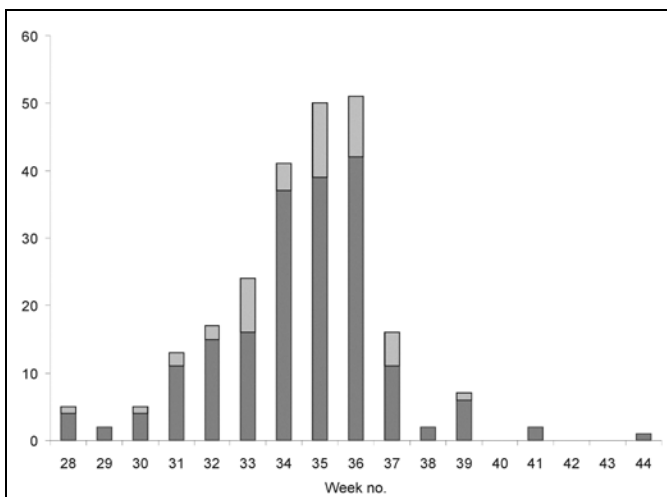


Figure 2. Week of onset of illness for cases in the tularemia outbreak in Sweden, 2000. Dark bars show cases included in this study.

of 670 controls, for a total of 681 (72%) of 940. Twenty-five cases and 21 controls were excluded because of confusion about the time periods used in the questionnaires. One control was excluded because of a documented episode of tularemia, one control was excluded because of an episode of febrile illness that could have been tularemia, and another control was excluded because the wrong person filled out the questionnaire. Thus, 218 cases and 414 controls, from which 202 matched pairs or triplets could be arranged, remained for further analysis.

With regard to symptoms, 198 (95%) of 209 case-patients reported having had a fever during their episode of tularemia. Of these, 147 (86%) of 171 reported swollen lymph nodes, 143 (79%) of 180 wound infection, 26 (20%) of 132 sore throat, 39 (28%) of 138 cough, and 10 (5.2%) of 193 pneumonia diagnosed by a physician. As a measure of severity of disease, 34 (16%) of 212 were hospitalized. The median duration of hospitalization was 4 days. No deaths were reported.

The matched univariate analysis (Table 1) gave statistically significant results for the following exposures: owning a cat, farming, visiting wooded areas, and being bitten by mosquitoes. After multivariate analysis, owning a cat, farming, and being bitten by mosquitoes remained as independent risk factors.

Sex and age distributions were similar in the endemic and emergent groups. Having had swollen lymph nodes (47 [78%] of 60 vs. 85 [96%] of 89 [$p=0.001$]) or a wound infection (48 [72%] of 67 vs. 78 [89%] of 88 [$p=0.007$]) was significantly more common in the emergent group, while having had pneumonia (7 [9.3%] of 75 vs. 1 [1.1%] of 94 [$p=0.02$]), was significantly more common in the endemic group.

The matched univariate analysis in the endemic group showed significant results for the following exposures: owning a cat, farming, and being bitten by mosquitoes. In the emergent

group, being bitten by mosquitoes and visiting woods or forests appeared as risk factors (Table 2). After multivariate analysis, owning a cat and being bitten by mosquitoes were shown to be independent risk factors for acquiring tularemia in both groups, while farming was a risk factor only in the endemic group.

Discussion

We report results from a study of a tularemia outbreak in Sweden, with a spread of the disease into new geographic areas. The use of a mailed questionnaire with matched controls from the Population Register had a high response rate even among controls.

Frequency of reported symptoms from case-patients are consistent with earlier data, showing ulceroglandular tularemia to be the dominant form in Sweden (10). A substantial part of the case-patients (16%) were hospitalized. Pulmonary tularemia was seen in only 10 (5.2%) of 193 cases, in contrast to the outbreak in the winter of 1966–67, when 11% had pneumonia symptoms.

Statistically significant independent associations were found between acquiring tularemia and the following exposures: being bitten by a mosquito, doing farm work, and owning a cat. The results for mosquito bites could have been influenced by recall bias, since this transmission route has always been thought to be the most common in Sweden and many patients might thus have been told by their physician that mosquitoes caused the infection. However, the predominance of the ulceroglandular form of tularemia and the seasonal variation of the disease support the theory that mosquito bites are the major route of transmission in Sweden.

Farming has not been connected with tularemia in Sweden since the outbreak in 1966–67. However, about pulmonary tularemia was reported among farmers in Finland in 1982 (11). In spite of detailed questions about different farming activities, no specific practice could be implicated in our study, perhaps because relatively few of the persons studied were involved in farming.

Transmission of tularemia from cat to humans, mainly caused by *F. tularensis* subsp. *tularensis*, but also by *F. tularensis* subsp. *holarctica*, has been described from North America, from both sick and healthy cats, and with or without the patient's being bitten by the cat (12–17). Furthermore, Scheel et al. reported a case of tularemia in a veterinary surgeon in Norway who cut himself while spaying a cat (18). The increased risk in cat owners could be due to direct transmission from infected cats or exposure to dead animals brought home by the cat. We found no increased exposure to dead animals among case-patients who were cat owners. The connection between cats and tularemia needs to be studied further, and a seroepidemiologic study of cats in affected areas would be of interest.

The risk for acquiring tularemia, however, is relatively small even in the disease-endemic areas, where the overall inci-

Table 1. Results of case-control analysis of risk factors for outbreak-associated tularemia, Sweden, 2000

Risk factors	Cases	Controls	Matched odds ratio	95% confidence interval
Univariate analysis				
Mosquito bites	196/202	313/392	8.3	3.3 to 21
Owning a cat	69/218	82/414	2.0	1.3 to 3.1
Farming	30/210	24/397	3.2	1.6 to 6.3
Visiting wooded areas	146/206	221/397	1.7	1.2 to 2.5
Owning a dog	32/218	73/414	0.75	0.45 to 1.3
Visiting golf courses	37/215	34/398	1.7	0.9 to 3.0
Visiting lakes and rivers	160/212	259/391	1.5	0.95 to 2.3
Multivariate analysis				
Mosquito bites	196/202	313/392	8.8	3.3 to 23
Owning a cat	69/218	82/414	2.5	1.5 to 4.2
Farming	30/210	24/397	3.2	1.4 to 7.0

Table 2. Comparison of risk factors for tularemia in disease-endemic and -emergent areas, Sweden, 2000

Risk factors	Disease-endemic areas				Emergent areas			
	Cases	Controls	Matched odds ratio	95% CI ^a	Cases	Controls	Matched odds ratio	95% CI
Univariate analysis								
Mosquito bites	78/81	119/152	8.4	1.9 to 37	93/94	150/181	9.1	2.1 to 40
Owning a cat	37/84	29/159	4.4	2.2 to 9.0	29/105	38/195	1.6	0.8 to 3.0
Farming	15/81	8/152	7.5	2.1 to 27	9/101	15/186	0.8	0.3 to 2.3
Visiting wooded areas	50/79	85/152	1.1	0.6 to 1.9	75/100	102/186	2.4	1.3 to 4.3
Multivariate analysis								
Mosquito bites			7.6	1.6 to 36			9.4	2.1 to 43
Owning a cat			4.0	1.6 to 10			2.5	1.2 to 5.5
Farming			4.9	1.1 to 22				

^aCI, confidence interval.

dence in this outbreak was approximately 66 per 100,000 population. On the basis of these findings, recommendations to the population in general about not owning a cat therefore seem unwarranted. However, informing the public about the risk of spread of tularemia from cats to humans seems reasonable.

In the separate analysis of risk factors, owning a cat and being bitten by mosquitoes appeared as independent risk factors in both groups. Farming appeared as a risk factor only in the disease-endemic area. The reason for this difference remains unknown and merits investigation. One explanation could be different farming practices, since the disease-endemic areas are heavily forested with small plots of arable land interspersed, but the emergent areas have more open, continuous farmland. No new risk factors were found in the emergent group that could explain the spread of tularemia into new areas. However, mosquito bites and spending time in the forest play a relatively bigger role in the emergent areas, but farming and contact with cats were relatively more important in the disease-endemic area (Tables 1 and 2). This finding may suggest that reservoirs in the new areas have not yet come into close contact with human settlements. The higher proportion of pneumonia in the disease-endemic area, which cannot fully be explained by greater diagnostic acumen among clinicians more familiar with the disease, could also indicate some kind of environmental contamination in areas where tularemia has long been established. The parallel of our findings with the recently described small outbreak of pneumonic tularemia in landscapers on Martha's Vineyard is intriguing (19).

This study has elucidated some of the basic epidemiology of human tularemia in Sweden. More research is needed on the epidemiology of the disease in animals. Important questions that remain unanswered are—What is the reservoir (or reservoirs)? What is the interaction between infection in different wild species? And what triggers an outbreak in humans? A follow-up field study with collection of samples from mosquitoes, water, and rodents is planned.

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First Characterization of a Cluster of VanA-Type Glycopeptide-Resistant *Enterococcus faecium*, Colombia

Diana Panesso,* Sigifredo Ospina,† Jaime Robledo,‡ María Claudia Vela,§ Julieta Peña,* Orville Hernández,† Jinnethe Reyes,* and César A. Arias*

From August 1998 to October 1999, glycopeptide-resistant enterococci (GRE) were isolated from 23 infected patients at a teaching hospital in Medellín, Colombia. Identification at the species level and by multiplex polymerase chain reaction assay indicated that all isolates were *Enterococcus faecium*. The isolates were highly resistant to ampicillin, ciprofloxacin, gentamicin, penicillin, streptomycin, teicoplanin, and vancomycin; they were susceptible only to chloramphenicol, linezolid, and nitrofurantoin. Determination of glycopeptide genotype indicated the presence of the *vanA* gene in all isolates. Molecular typing by pulsed field gel electrophoresis showed that all isolates were closely related. This study is the first molecular characterization of GRE in Colombia.

Enterococci normally colonize the intestinal tract of humans and other animals, with urinary tract infection being the most common enterococcal infection reported in humans (1). In recent years, enterococci have become important nosocomial pathogens: the organisms have been reported as the second leading cause of urinary tract infections and the third leading cause of nosocomial bacteremia in hospitalized patients (2). The most commonly identified species is *Enterococcus faecalis*, followed by *E. faecium* (3). *E. gallinarum*, *E. casseliflavus*, and *E. durans* have been reported less often (4,5).

The most important characteristics of these organisms include their inherent resistance to several antimicrobial agents and their ability to acquire resistance determinants. Resistance against such diverse groups of drugs as β -lactams, macrolides, aminoglycosides, and glycopeptides continues to evolve. The ability to grow in the presence of glycopeptides results from the change of the C-terminal residue of peptidoglycan precursors (D-Ala) to D-lactate (VanA, VanB, and VanD phenotypes) (6,7) or D-serine (VanC, VanE, and VanG phenotypes) (8–10). The change alters the affinity of the glycopeptide for its natural target (6). Six different gene clusters have been described (*vanA*-B-C-D-E-G) (6,10–12). The most predominant phenotype in *E. faecium* is VanA; VanA strains are highly resistant to both vancomycin and teicoplanin. The *vanA* gene cluster is located on transposons or related elements (6) and has also been found in nonenterococcal species such as *Arcanobacterium* (*Corynebacterium*) *haemolyticum*, *Oerskovia turbata*, *Bacillus circulans*, and *Streptococcus gallolyti-*

cus (13–16). A *van* cluster with a high degree of homology to the *vanA* cluster (designated *vanF*) has been found in the biopesticide organism *Paenibacillus popilliae* (17).

Since the initial discovery of glycopeptide-resistant enterococci (GRE) in the United Kingdom (18), nosocomial isolates of GRE have been reported from around the world (14); these isolates have also been found in healthy people in the community outside the hospital (19). In Latin America, GRE have been reported in Argentina (20) and Brazil (21). We report here the first isolation and characterization of a cluster of VanA-type glycopeptide-resistant *E. faecium* in a teaching hospital in Colombia.

Materials and Methods

Bacterial Isolates

Hospital San Vicente de Paul is a 650-bed teaching hospital providing tertiary care for Medellín, Colombia, and neighboring towns, an area with a population of 1.5 million inhabitants. From August 1998 to October 1999, we collected organisms from 23 patients. Enterococci were isolated from infected patients by classical microbiologic techniques (3). Identification at the species level was performed by the Vitek gram-positive card (bioMérieux SA, Marcy l'Etoile, France), according to the manufacturer's recommendations.

Antimicrobial Susceptibility Testing

Initial identification of resistance to vancomycin was performed by the Vitek system (bioMérieux SA). We confirmed resistance to vancomycin, determining MICs by an agar dilution method as recommended by the National Committee for Clinical Laboratory Standards (22) on Mueller-Hinton agar

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plates (ICN Pharmaceuticals Inc., Madison, WI). MICs were performed in duplicate. The following antimicrobial agents were obtained as reference powders of known potency and tested: ampicillin, ciprofloxacin, chloramphenicol, gentamicin, penicillin, streptomycin, teicoplanin, vancomycin (ICN Pharmaceuticals, Inc.), and linezolid (Pharmacia Corp., Peapack, NJ). Susceptibility to nitrofurantoin (MIC <32 µg/mL) was determined by the Vitek system (bioMérieux SA). In addition to determining MICs, high-level resistance to streptomycin was tested at concentrations of 2 mg/mL; *E. faecalis* ATCC 29212 was used as control strain. Three well-characterized strains of enterococci belonging to the genotypes *vanA* (*E. faecium* BM4147), *vanB* (*E. faecalis* V583), and *vanC* (*E. gallinarum* BM4174) were included as GRE control strains.

Polymerase Chain Reaction (PCR) for Species Identification of Enterococci and the *van* Genes

For species identification of enterococcal isolates, the genes encoding D-alanine-D-alanine ligases specific for *E. faecium* (*ddl_{E. faecium}*), *E. faecalis* (*ddl_{E. faecalis}*), *vanC-1* (*E. gallinarum*), and *vanC-2* (*E. casseliflavus*) were detected by a multiplex PCR assay, as described by Dutka-Malen et al. (23). Primers D1 (5' GCTTCTTCCTTTACGACC) and D2 (GTTC-CAGTCCTAAAAAC) for the *ddl* gene of *E. avium* were included in the multiplex mixture. A similar multiplex PCR protocol was performed separately for detection of *van* genes by using specific primers for *vanA*, *vanB*, *vanC-1*, and *vanC-2* genes (23). *E. faecium* BM4147 (*vanA*), *E. faecalis* V583 (*vanB*), and *E. gallinarum* BM4174 (*vanC-1*) were used as control strains.

Genotyping

Molecular typing was performed by pulsed-field gel electrophoresis (PFGE). Chromosomal DNA was obtained by the procedure of Antonishyn et al. (24): a loopful of bacterial colonies from a 24-h isolate was grown until A_{600} was 0.1 in brain heart infusion broth at 37°C. Bacteria were harvested by centrifugation at 4°C, and the pellet was resuspended in cell suspension buffer (1M NaCl, 10 mM Tris-HCl, pH 8.0). The suspension was embedded in 1.5% agarose and disks were made. Disks were placed in lysis buffer (6mM Tris-HCl, pH 8, 1 M NaCl, 100 mM EDTA, 0.5% Brij-58, 0.2% Na deoxycholate, and 0.5% N-lauroyl sarcosine) with additional RNase (20 µg/mL) and lysozyme (1 mg/mL) and incubated for 4 h at 37°C. The disks were washed with EDTA-sarcosine buffer (0.5 M EDTA, pH 8, and 0.1% N-lauroyl sarcosine), placed in proteinase K solution (100 µg/mL), and incubated overnight at 50°C with mild agitation. Disks were washed four times with Tris-EDTA buffer (Tris 10 mM, pH 7.5, and 1mM EDTA) for 30–60 min at room temperature on a rocker.

DNA was digested as described (25). Briefly, DNA fixed in the agarose disks was preincubated in 1 mL of buffer E (6 mM Tris, pH 8, 20 mM KCl, 6 mM MgCl₂, and 6 mM 2-mercaptoethanol) at 25°C for 30 min. Restriction was performed for 17 h in 60 µL of restriction buffer containing *Sma*I (20 U

at 25°C. The reaction was stopped by addition of 10 µL of sterile loading buffer. Gels were prepared with 1% agarose in 0.5x TBE buffer (50 mM Tris, pH 8, 50 mM boric acid, 0.2 mM EDTA). A DNA ladder (50–1000 kb) was used as the molecular size marker. Fragments were separated by electrophoresis (CHEF-DR II system, Bio-Rad Laboratories, Inc., Richmond, CA) at 6 V/cm, with switch times ramped from 1 s to 35 s over 23 h at 14°C. After staining with ethidium bromide, the restricted DNA fragments were viewed under UV light and photographed. A vancomycin-susceptible strain of *E. faecium* isolated in the same hospital was included in the PFGE protocol as the control. We interpreted the band patterns by the criteria of Tenover et al. (26).

Results

GRE Isolates and Identification

From August 1998 to October 1999, 23 GRE were collected from the same number of patients hospitalized in various wards in Hospital San Vicente de Paul. The first isolate was recovered from the pleural fluid of a patient hospitalized in the surgical ward. Isolates came from urine (35%), peritoneal fluid (22%), surgical wound (17%), intra-abdominal abscess (13%), pleural fluid (9%), and bile (4%). Molecular identification by PCR showed that all isolates were *E. faecium*, in agreement with the results of the Vitek gram-positive identification card (bioMérieux SA).

Antimicrobial Susceptibility Testing

All isolates had high levels of resistance to ampicillin (MICs 128–256 µg/mL), ciprofloxacin (>32 µg/mL), gentamicin (1,024 µg/mL), penicillin (256–512 µg/mL), streptomycin (>2,000 µg/mL), teicoplanin (>32 µg/mL), and vancomycin (512 µg/mL). The isolates were susceptible to chloramphenicol (4–8 µg/mL), linezolid (1 µg/mL), and nitrofurantoin (≤32 µg/mL).

PFGE and Glycopeptide-Resistant Genotype

Analysis of PFGE patterns obtained with the 23 *E. faecium* isolates showed that 21 isolates had the same banding pattern. The remaining two isolates had an additional band around 242 kb (Figure, lanes 2 and 15), indicating that all isolates were closely related (26). This finding suggests the presence of a bacterial clone spreading through different wards during the period of the study. The *vanA* gene was detected in all isolates, in agreement with the antimicrobial susceptibility tests (high-level resistance to both vancomycin and teicoplanin).

Discussion

The emergence of multiresistant GRE is a serious nosocomial problem with important implications for hospital infection control. Although the geographic distribution of GRE is worldwide, the epidemiology appears to differ within and across regions. For example, isolates from hospitalized patients in France were shown to be genetically unrelated

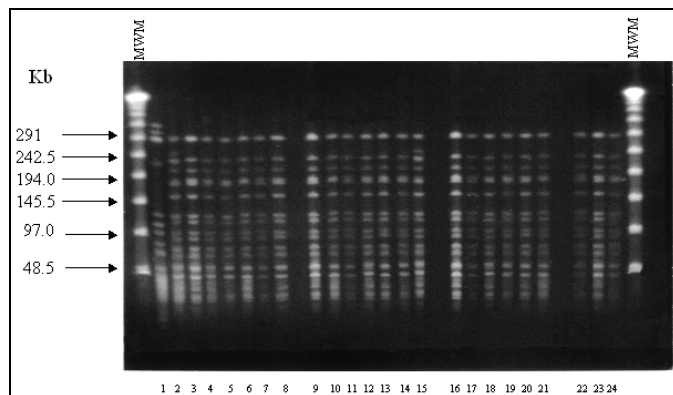


Figure. Pulsed-field gel electrophoresis restriction fragment patterns of *Smal*-digested genomic DNA obtained from glycopeptide-resistant *Enterococcus faecium* isolated at San Vicente de Paul Hospital, Medellín, Colombia. Lane 1: a susceptible isolate of *E. faecium*; lane 2–24: Restriction patterns of the 23 VanA-type *E. faecium*. MWM, molecular weight marker.

(27,28); a similar situation has been documented in the United Kingdom (29). In a study of >1,000 isolates of GRE in the United Kingdom, most were *E. faecium* and the VanA phenotype, accounting for 88% of all isolates (30). Although PFGE showed a marked genetic diversity within strains, a common clone was demonstrated in 16 hospitals. In Europe, sources outside hospitals were confirmed as the source of GRE: clonally related vancomycin-resistant enterococci strains have been identified in patients, farm animals, animal products, and the environment, including the presence of GRE in raw meat for human consumption (31–34). Avoparcin, a glycopeptide administered as a growth promoter to farm animals in Europe from 1975 to 2000 (when it was withdrawn from the market), has been implicated as an important factor for the emergence of GRE (31,35). In the United States, dissemination of clonally related strains of GRE was commonly seen in the early stages of the epidemic (14). However, a diverse set of strains has emerged (36). The increased prevalence of GRE in the United States appears to be related to the massive use of vancomycin in hospitals, which by far exceeds the use in Europe (37).

GRE have been found in other parts of Latin America (Argentina and Brazil) (20,21). Results from the SENTRY Antimicrobial Surveillance Program 1997–1999 (38) indicated a low incidence of GRE in Latin America; of 367 isolates, only three had resistance to glycopeptides (two belonged to the VanA phenotype and one VanC-type) (38). This report describes the first characterization of GRE in Colombia; our findings indicate that GRE are emerging as important nosocomial pathogens there. In fact, GRE have now become prevalent in Hospital San Vicente de Paul, and dissemination of isolates to other hospitals in the country is likely. A multicentric surveillance study carried out in 14 teaching hospitals (including five major Colombian cities) from March 2001 to March 2002 indicated that GRE have also been detected in other hospitals, mainly in the capital city of Bogotá, with a prevalence of 10% among clinical isolates of enterococci. Phenotypic characterization demonstrated the presence of both

VanA and VanB isolates (39). Of VanA-*E. faecium*, only four had resistance patterns identical to the Medellín isolates described in this study. Genotypic characterization of these isolates is currently under way.

PFGE analysis of the isolates strongly suggests the dissemination of a single clone among hospitalized patients: the emergence of GRE in Colombia is likely to follow a trend similar to the one in the United States. These data may be signaling the start of an epidemic. Factors directly related to the emergence of GRE in Colombia have not been studied properly; glycopeptides appear to be widely used in teaching hospitals, and this situation might be related to the increasing prevalence of methicillin-resistant *Staphylococcus aureus* in the last 4 years (40). Little is known about the use of antimicrobial compounds in animals for human consumption.

Strategies to control the spread of GRE in Hospital San Vicente included monitoring the stringent use of vancomycin and third-generation cephalosporins, providing education to personnel throughout the hospital (especially critical-care units), and implementing infection control measures according to the Hospital Infection Control Practices Advisory Committee (41), strongly emphasizing early detection by the microbiology laboratory of patients colonized or infected with GRE. With these measures, we have decreased the incidence of cases. However, we have not achieved total eradication; in 2001, the prevalence of GRE was 15%.

Resistance of enterococci to multiple antibiotics is common, making treatment problematic. Studies suggest that enterococci inhibited *in vitro* by ≤ 64 $\mu\text{g}/\text{mL}$ of ampicillin may be susceptible *in vivo* to high-dose ampicillin or therapy with ampicillin-sulbactam and gentamicin (if the isolate does not exhibit high-level resistance to gentamicin) (36). However, the isolates from this study exhibited high-level resistance to ampicillin (MIC 128 $\mu\text{g}/\text{mL}$), gentamicin (>1,000 $\mu\text{g}/\text{mL}$), and streptomycin (>2,000 $\mu\text{g}/\text{mL}$), which further limits the therapeutic alternatives. Ciprofloxacin is an antibiotic that has been used as an alternative for the treatment of GRE infections (42), but it was inactive against the isolates examined here.

As found by others (42–44), chloramphenicol was one of the two agents that retained *in vitro* activity against GRE in this investigation. In a retrospective study of 14 patients with clinical responses, 57% showed improvement after treatment with chloramphenicol (43). Microbiologic response was 73% in 11 patients evaluated in the same study (43). Although no lasting adverse effect related to use of the drug occurred, treatment with chloramphenicol was discontinued for two patients because of chloramphenicol-induced bone marrow suppression (43). In another study of 51 patients with bloodstream infection due to vancomycin-resistant *E. faecium*, 61% and 79% showed a clinical and microbiologic response to chloramphenicol, respectively, but no corresponding decrease in deaths occurred (45). In our study, patients with urinary tract infections (UTI) (eight cases) were initially treated successfully with nitrofurantoin (100 mg/6 h). Ampicillin (12 g/day) was used in patients with infections other than UTI. In the lat-

ter group, however, the death rate was 33%, mostly because of severe sepsis. Chloramphenicol was not used in this group of patients. Although no controlled trials have demonstrated the effectiveness of chloramphenicol for the treatment of GRE, this antibiotic could be a therapeutic alternative in Colombia.

Linezolid, a new compound from the oxazolidinone group, has just been launched in Colombia; our findings indicate that it was active against all isolates tested. Linezolid has emerged as a therapeutic alternative for multiresistant GRE in Colombia, as in other parts of the world where it is currently available. However, linezolid-resistant *E. faecium* clinical isolates have already been reported in relation to long courses of therapy (21–40 days) (46). A linezolid-resistant *E. faecium* isolated from a patient without prior exposure to an oxazolidinone has also been described (47).

In this study, we report the first isolation and characterization of a multiresistant cluster of VanA-type *E. faecium* in a Colombian hospital. The emergence of this problem and the limitation of therapeutic options require the implementation of specific infection control measures and antibiotic policies to avoid further dissemination.

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Community-Acquired Methicillin-Resistant *Staphylococcus aureus* in Institutionalized Adults with Developmental Disabilities¹

Abraham Borer,* Jacob Gilad,* Pablo Yagupsky,* Nechama Peled,* Nurith Porat,* Ronit Treffer,* Hannah Shprecher-Levy,† Klaris Riesenber,* Miriam Shipman,* and Francisc Schlaeffer*

Methicillin-resistant *Staphylococcus aureus* (MRSA) has recently been reported to emerge in the community setting. We describe the investigation and control of a community-acquired outbreak of MRSA skin infections in a closed community of institutionalized adults with developmental disabilities. In a 9-month period in 1997, 20 (71%) of 28 residents had 73 infectious episodes. Of the cultures, 60% and 32% obtained from residents and personnel, respectively, grew *S. aureus*; 96% and 27% were MRSA. All isolates were genetically related by pulsed-field gel electrophoresis and belonged to a phage type not previously described in the region. No known risk factors for MRSA acquisition were found. However, 58 antibiotic courses had been administered to 16 residents during the preceding 9 months. Infection control measures, antibiotic restriction, and appropriate therapy resulted in successful termination of this outbreak. Selective antibiotic pressure may result in the emergence, persistence, and dissemination of MRSA strains, causing prolonged disease.

Methicillin-resistant *Staphylococcus aureus* (MRSA) poses a therapeutic challenge in acute-care settings (1–4), as well as long-term skilled-nursing facilities (5–8). Recently, MRSA has also been detected in the community more often. The terms and definitions related to community-acquired MRSA remain controversial, and the “community” as a milieu for MRSA acquisition cannot be implicated with a high degree of certainty. Most studies have defined community acquisition as growth within 48–72 hours after hospital admission (9–11), which does not rule out nosocomial acquisition. Patients thought to have acquired MRSA in the community carry risk factors implicated in nosocomial acquisition (12–16).

Outbreaks of community-acquired MRSA infection are extremely rare (17–19). During 1997, we investigated an outbreak of skin and soft-tissue infection involving MRSA in a closed community of institutionalized adults with developmental disabilities. MRSA emerged and disseminated in this setting as a result of an extreme selective pressure exacerbated by heavy and continuous use of ineffective antimicrobial drugs. That such selective pressure was sufficient to promote MRSA emergence in the community underlines the threat associated with current antibiotic prescribing practices in the community.

Materials and Methods

Outbreak Setting

The outbreak occurred in a facility for persons with developmental disabilities, located in the Negev, southern Israel. The facility consists of 283 residents living in nine buildings and confined to the institution. Residents are independent with regard to activities of daily living, with minimal contact between residents of different buildings. Staff consists of 120 personnel who work exclusively in the institution and are assigned to specific buildings. Medical attention is provided by an institutional clinic. The outbreak involved a single building (number 15) inhabited by 28 residents and attended by 34 personnel.

Epidemiologic Investigation

The outbreak investigation began in December 1997, according to the principles of the Declaration of Helsinki. Informed consent was obtained from personnel, and consent for including residents was obtained from legal guardians and the Ministry of Health.

Information was reviewed regarding possible host risk factors (20), including age, sex, diabetes mellitus, malignancy, coronary disease, chronic lung, hepatic or renal diseases, nephrotic syndrome, congestive heart failure, obesity, debilitating

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conditions, and pressure sores, as well as therapeutic risk factors such as urinary catheters, nasogastric tubes, and other indwelling devices, steroid treatment and antibiotic therapy prescribed during 12 months preceding the outbreak. Admissions to any acute-care facility during the previous 5 years were recorded.

To confirm clustering and identify common sources of transmission, all 28 residents from building 15 were screened for both methicillin-sensitive *S. aureus* (MSSA) and MRSA carriage in both anterior nares, perineum, and secreting lesions. Nare and exudate cultures were obtained from all personnel in contact with the residents (34 persons). Additionally, nares cultures were randomly obtained from one-fifth of all residents (50 persons) residing in other buildings at the institution.

Laboratory Investigation

Cultures were obtained by rotating a moistened swab in both nares, perineum area, and secreting lesions and were processed at the Clinical Microbiology Laboratory of the Soroka University Medical Center. Identification of *S. aureus* was performed by routine methods. Methicillin resistance was determined by using a 1- μ g oxacillin disk. Susceptibility to erythromycin, clindamycin, cefuroxime, ceftriaxone, ciprofloxacin, gentamicin, fusidic acid, and vancomycin was determined by using the disc-diffusion method. Mupirocin resistance (MIC > 256 mg/L) was determined by E-test (AB Biodisk, Solna, Sweden).

Bacteriophage typing at routine test dilutions was performed at a national reference laboratory, Rabin Medical Center, Petah-Tikva, Israel. Methicillin resistance was confirmed by polymerase chain reaction (PCR) for the *mecA* gene (21). Pulsed-field gel DNA electrophoresis (PFGE) for the determination of genetic relatedness was generated by digestion with the restriction endonuclease *SmaI* as described elsewhere (22), and the banding pattern was interpreted according to current consensus (23).

Intervention

Management of the outbreak was carried out by the four-phase approach of Wenzel et al. (24), with modifications related to the setting under investigation. Basic epidemiologic measures, infection control measures, and isolation precautions were instituted, including glove use during personnel-resident contact, hand washing with 4% chlorhexidine after glove removal, reserving personal washcloths and towels for each resident, bathing daily with 4% chlorhexidine-containing soap, and changing towels, clothing, and bed sheets daily. Draining lesions were covered at all times with sterile dressings, which were promptly discarded after removal.

Treatment to eliminate nasal carriage in culture-positive persons was given after randomization, by using either intranasal mupirocin calcium 2% ointment (Bactroban, Glaxo Smith-Kline, Philadelphia, PA) or sodium fusidate 2% ointment (Fucidin, Leo Pharmaceutical, Ballerup, Denmark), twice a day for a week. Spontaneous or surgically drained lesions were

treated with the same topical antibiotic used intranasally. Systemic therapy with oral fusidic acid 500 mg twice a day (Fucidin, Leo Pharmaceutical) was reserved only for lesions surrounded by cellulitis, located around the mid-face, or in presence of systemic symptoms or signs. To limit antibiotic use, therapy other than the above was not allowed. This phase was supervised by infectious-disease specialists. Thereafter, infection control was supervised weekly by an infection control nurse and every 3 weeks by an infectious-disease specialist.

In the implicated building, follow-up cultures were obtained from all residents and personnel 1 week as well as 1 month after intervention. After 2 additional weeks, repeat cultures were obtained only from those with previous positive culture. Two years later, in March 2000, nares cultures were obtained from all residents in order to assess the prevalence of persistent carriage.

Statistical Analysis

Statistical analysis was performed with the Epi-Info software (Version 6.03; 1996, Centers for Disease Control and Prevention, Atlanta, GA), using the chi-square and Fisher's exact tests as appropriate. A p value of <0.05 was considered statistically significant.

Results

Outbreak Description

During mid-1997, an increasing number of skin and soft-tissue infections in residents of a single building were recognized by the staff. No cases were diagnosed in residents in other buildings or the remaining staff. The initial case involved an uncomplicated furuncle in a patient with dermatitis. From March 1, 1997, to December 31, 1997, 60 patient visits related to skin, soft-tissue, ear, and eye infections were recorded; 14 (23%) of these visits required surgical intervention by a local physician, but no culture material was available for analysis. No patients required referral or hospital admission.

In all, 73 infectious episodes were recorded in 20 of 28 residents in the implicated building, including 43 (59%) skin abscesses, 20 (27%) furuncles, 8 (11%) purulent conjunctivitis, and 2 (3%) external otitis. A mean of seven episodes per month (median 7, range 4–14) peaked in December 1997. The implicated organism was MRSA.

Epidemiologic Survey

The median age of residents in building 15 was 32 years (range 18–45 years), and all residents were male. The mean stay at the institution was 16.3 years \pm 6.6 years. We could not identify any known risk factor for MRSA carriage or infection. No residents had been admitted to acute-care hospitals within the 5 years preceding the outbreak, and no contact with known carriers was established. However, 58 courses of oral antibiotics, including amoxicillin, amoxicillin-clavulanate, penicillin, cefuroxime-axetil, cloxacillin, erythromycin, ciprofloxacin,

and trimethoprim-sulfamethoxazole, were administered to 16 of 20 (80%) infected residents during a 9-month period, for a total of 572 antibiotic-days (Figure 1). Excess antibiotic consumption was not observed in other buildings.

Bacteriologic Survey

The first survey was carried out in December 1997 and included 83 cultures, of which 48 were obtained from 28 residents, consisting of 28 (58%) nasal, 10 (21%) perineal, and 10 (21%) exudate cultures. Thirty-four nasal and one exudate cultures were obtained from personnel. Forty of 83 were positive, 48% grew MRSA or MSSA, for a positive culture rate of 29 (60%) of 48 in residents and 11 (32%) of 35 in personnel. Seventeen (61%) of 28 and 10 (29%) of 34, residents and personnel, respectively, were nasal carriers of *S. aureus*; 16 (95%) and 2 (20%) of the isolates were MRSA. Eighteen (29%) of 62 nasal, 2 (20%) of 10 perineal, and all 11 exudate cultures (including 1 from staff) grew MRSA (a total of 31 isolates).

All 31 MRSA isolates were susceptible to ciprofloxacin, gentamicin, fusidic acid, and vancomycin, but only 14 (45%) were susceptible to clindamycin and erythromycin. Typing showed that all MRSA isolates belonged to phage-type 29/52/54/95/47/HK2 [52A/79/75/92], an unusual type that had not been isolated before from any patient either in the community or the acute-care setting in southern Israel. All MRSA isolates from both carriers and infected persons yielded an indistinguishable PFGE pattern, except for one isolate that yielded a closely related pattern (one band difference) and was thought to belong to the outbreak strain (Figure 2).

In residents, the 17 nasal carriers had 57 episodes of skin infections during 1997 compared with 16 episodes in 11 non-carriers ($p < 0.01$). At this point, infection control measures were enforced and carriers were randomized to receive intranasal mupirocin (11 cases) or sodium fusidate (14 cases). The same agent was applied topically after drainage.

Postintervention Survey

The nasal carriage eradication rate was 11 (100%) of 11 for mupirocin and 9 (64%) of 14 for sodium fusidate ($p = 0.08$). The five carriers in whom fusidic acid failed were retreated 2 weeks later with mupirocin and complete eradication was achieved.

In January 1998, a third survey was carried out, including 72 cultures (62 persons), 8 (11%) of which were positive. Of the residents' cultures, five (two nasal and three exudate cultures) grew MRSA. Three nasal cultures obtained from personnel grew MSSA. One-fifth of the remaining residents and personnel were screened (80 persons), and none were found to be MRSA carriers. No infections were recorded outside building 15 throughout the study period.

Eleven new episodes of furuncles yielding mupirocin-sensitive MRSA were recognized postintervention, involving five residents and one personnel. Six responded to topical mupirocin, while systemic fusidic acid was clinically indicated in five (27 antibiotic days). Since March 1998, no additional episodes

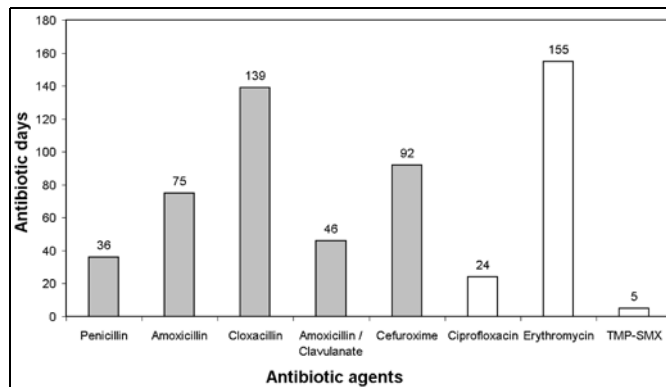


Figure 1. Systemic antibiotic use agents administered to infected residents in Building 15 between March and December 1997. Shaded bars represent beta-lactam agents.

were diagnosed in 2 years of follow-up. In March 2000, the nasal culture survey was repeated in the 28 residents. Two (7%) of 28 cultures grew *S. aureus*, MSSA in one resident and MSSA together with MRSA in another. The latter patient had recurrent colonization for 2 years despite repeated eradication efforts, even while the rest of the residents were culture negative.

Discussion

MRSA is an important pathogen in both acute-care and long-term care facilities (1–7). The complex interaction between patients admitted to chronic- and acute-care facilities is well-known, and the resulting “import” and “export” of MRSA is of great concern (25), making MRSA outbreaks problematic. Defining community-acquired MRSA with accuracy is difficult since widely used criteria seem to reflect “community existence” rather than “community acquisition.” Persistent nasal carriage, which may involve up to 35% of carriers (26,27), can further complicate any attempt to determine the exact time of acquisition.

We describe an outbreak of community-acquired MRSA infections in which the only identified risk factor was a history

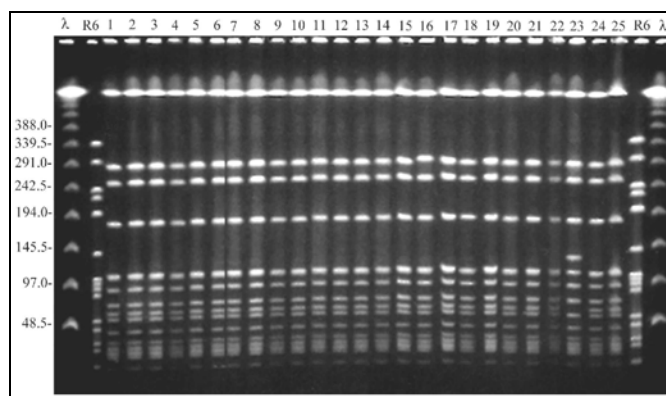


Figure 2. Pulse-field gel electrophoresis of study isolates obtained during the first survey on December 1997 from residents and staff. Lambda ladder and the DNA of the reference *Streptococcus pneumoniae* strain R6, digested by *Sma*I, were used as molecular weight markers. The gel includes 25 representative MRSA isolates. All isolates but one show an indistinguishable banding pattern, thus representing the outbreak strain. Isolate number 23 shows a closely related pattern (one band difference) and is considered to belong to the outbreak strain.

of heavy exposure to multiple antibiotics, especially beta-lactams, over a relatively short period of time in a population living under closed conditions. The first stage leading to selection of MRSA within MSSA isolates in hospitalized patients is antibiotic treatment (4,28). Such selective pressure permits the growth of a multiresistant bacterial population within a susceptible one, a process that may take <48 hours in approximately 15% of MSSA nasal carriers (4).

Thus, massive antibiotic pressure appears to be an important mechanism for the selection of community-acquired MRSA and its subsequent dissemination when favorable environmental conditions exist, as in the outbreak presented here. Although introduction of an MRSA strain from outside the facility cannot be absolutely ruled out, this possibility is less favorable owing to the epidemiologic features of the population, lack of contact with known carriers or admission to acute-care facilities, lack of risk factors except antibiotic therapy, and the uniqueness of the outbreak strain's phage type in our country. MRSA emergence in the institution was possible given that contemporary epidemic MRSA clones are actually descendents of old MSSA isolates that had received the *mecA* element and that the evolution of MRSA from of MSSA lineages appears to coincide with selective antibiotic pressure after the introduction of new antibiotics (29).

Skin and soft tissue infections are by far the commonest infections caused by *S. aureus*, and similar to other community-acquired MRSA outbreaks (17–19), recurrent abscesses, or furuncles, or both were the predominant infection in our study. A high prevalence of skin infection has been shown to be a risk factor for persistent nasal carriage of MRSA, augmenting person-to-person transmission (23). Moreover, patients with pathologic skin conditions have a higher risk not only of acquiring skin infections but also of dispersion of infecting strains (27,30,31). Failure to consider MRSA as a cause of such infections may lead to delayed diagnosis and inappropriate therapy, permitting a cycle of disease progression and widespread transmission. Fortunately, no invasive infections occurred as might have been expected in MRSA-colonized patients in nosocomial settings (32). Propagation of the outbreak could be explained by the close and continued physical contact between residents and staff, facilitating transmission, with the high rate of nasal colonization in residents serving as an independent risk factor for MRSA infection (33–35).

Elimination of carriage was achieved by intranasal antibiotics (either mupirocin or fusidic acid); the aim was to control the outbreak and prevent recurrence. We also treated the great majority of infections topically, in addition to bathing with chlorhexidine and appropriate drainage with the intention of maximally reducing systemic antibiotic use.

While mupirocin is a well-accepted eradication regimen (24), the possible emergence of mupirocin resistance during therapy (especially with skin application) should be considered. Fusidic acid, widely used in Israel, has been shown to be a convenient and risk-free method for eradication of nasal *S.*

aureus carriage (36), but currently no comparative data evaluate both agents. In our population, only five cases required systemic therapy, according to study indications, and mupirocin resistance was not detected during follow-up. However, persistent nasal MRSA carriage, 2 years after the onset of the outbreak, demonstrates a continued potential threat for both the reemergence of MRSA in the institution and perhaps, dissemination to the community.

Our essential goal was to achieve maximal infection control while maintaining selective pressure at the minimum. Critical factors, which are difficult to achieve in nosocomial settings, allowed us to achieve nearly total eradication of MRSA: 1) only one resident appeared to be a persistent nasal MRSA carrier; 2) all residents were exposed during a specific time frame without mingling between newly admitted or discharged residents; 3) enforcement of strict environmental cleansing and infection control measures; and 4) selective antibiotic pressure owing to minimal use of systemic agents (only 5 courses).

In conclusion, massive, and perhaps unjustified, systemic antibiotic use in communities, particularly those involving close interaction between members, may permit the emergence of multiresistant bacteria such as MRSA, with a high risk for disease. Implementation of antibiotic control strategies is crucial to prevent the dissemination of MRSA in the community as a whole.

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Biological Warfare at the 1346 Siege of Caffa

Mark Wheelis*

On the basis of a 14th-century account by the Genoese Gabriele de' Mussi, the Black Death is widely believed to have reached Europe from the Crimea as the result of a biological warfare attack. This is not only of great historical interest but also relevant to current efforts to evaluate the threat of military or terrorist use of biological weapons. Based on published translations of the de' Mussi manuscript, other 14th-century accounts of the Black Death, and secondary scholarly literature, I conclude that the claim that biological warfare was used at Caffa is plausible and provides the best explanation of the entry of plague into the city. This theory is consistent with the technology of the times and with contemporary notions of disease causation; however, the entry of plague into Europe from the Crimea likely occurred independent of this event.

The Black Death, which swept through Europe, the Near East, and North Africa in the mid-14th century, was probably the greatest public health disaster in recorded history and one of the most dramatic examples ever of emerging or reemerging disease. Europe lost an estimated one quarter to one third of its population, and the mortality in North Africa and the Near East was comparable. China, India, and the rest of the Far East are commonly believed to have also been severely affected, but little evidence supports that belief (1).

A principal source on the origin of the Black Death is a memoir by the Italian Gabriele de' Mussi. This memoir has been published several times in its original Latin (2,3) and has recently been translated into English (4) (although brief passages have been previously published in translation, see reference [5]). This narrative contains some startling assertions: that the Mongol army hurled plague-infected cadavers into the besieged Crimean city of Caffa, thereby transmitting the disease to the inhabitants; and that fleeing survivors of the siege spread plague from Caffa to the Mediterranean Basin. If this account is correct, Caffa should be recognized as the site of the most spectacular incident of biological warfare ever, with the Black Death as its disastrous consequence. After analyzing these claims, I have concluded that it is plausible that the biological attack took place as described and was responsible for infecting the inhabitants of Caffa; however, the event was unimportant in the spread of the plague pandemic.

Origin of the 14th-Century Pandemic

The disease that caused this catastrophic pandemic has, since Hecker (6), generally been considered to have been plague, a zoonotic disease caused by the gram-negative bacterium *Yersinia pestis*, the principal reservoir for which is wild rodents (7–11). The ultimate origin of the Black Death is uncertain—China, Mongolia, India, central Asia, and southern Russia have all been suggested (see Norris [1] for a discussion of the various theories). Known 14th-century sources are of

little help; they refer repeatedly to an eastern origin, but none of the reports is firsthand. Historians generally agree that the outbreak moved west out of the steppes north of the Black and Caspian Seas, and its spread through Europe and the Middle East is fairly well documented (Figure 1). However, despite more than a century of speculation about an ultimate origin further east, the requisite scholarship using Chinese and central Asian sources has yet to be done. In any event, the Crimea clearly played a pivotal role as the proximal source from which the Mediterranean Basin was infected.

Historical Background to the Siege of Caffa

Caffa (now Feodosija, Ukraine) was established by Genoa in 1266 by agreement with the Kahn of the Golden Horde (15). It was the main port for the great Genoese merchant ships (16–20), which connected there to a coastal shipping industry to Tana (now Azov, Russia) on the Don River. Trade along the Don connected Tana to Central Russia, and overland caravan routes linked it to Sarai and thence to the Far East (12,19,20).

Relations between Italian traders and their Mongol hosts were uneasy, and in 1307 Toqtai, Kahn of the Golden Horde, arrested the Italian residents of Sarai, and besieged Caffa. The

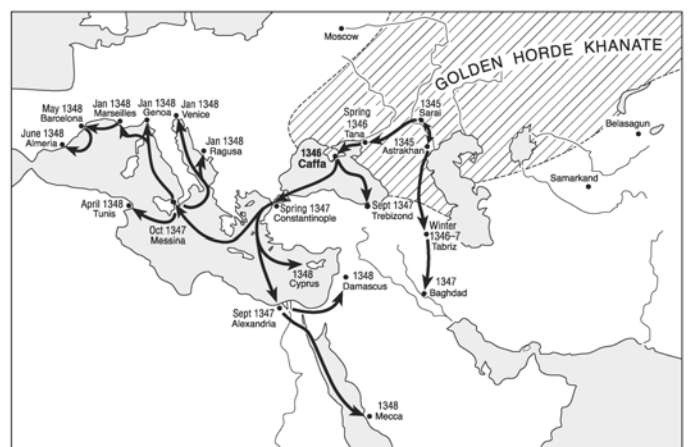


Figure 1. Tentative chronology of the initial spread of plague in the mid-14th century (12–14).

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cause was apparently Toqtai's displeasure at the Italian trade in Turkic slaves (sold for soldiers to the Mameluke Sultanate). The Genoese resisted for a year, but in 1308 set fire to their city and abandoned it. Relations between the Italians and the Golden Horde remained tense until Toqtai's death in 1312 (19).

Toqtai's successor, Özbeğ, welcomed the Genoese back, and also ceded land at Tana to the Italians for the expansion of their trading enterprise. By the 1340s, Caffa was again a thriving city, heavily fortified within two concentric walls. The inner wall enclosed 6,000 houses, the outer 11,000. The city's population was highly cosmopolitan, including Genoese, Venetian, Greeks, Armenians, Jews, Mongols, and Turkic peoples (21)

In 1343 the Mongols under Janibeg (who succeeded Özbeğ in 1340) besieged Caffa and the Italian enclave at Tana (12), following a brawl between Italians and Muslims in Tana. The Italian merchants in Tana fled to Caffa (which, by virtue of its location directly on the coast, maintained maritime access despite the siege). The siege of Caffa lasted until February 1344, when it was lifted after an Italian relief force killed 15,000 Mongol troops and destroyed their siege machines (21). Janibeg renewed the siege in 1345 but was again forced to lift it after a year, this time by an epidemic of plague that devastated his forces. The Italians blockaded Mongol ports, forcing Janibeg to negotiate, and in 1347 the Italians were allowed to reestablish their colony in Tana (19).

Gabriele de' Mussi

Gabriele de' Mussi, born circa 1280, practiced as a notary in the town of Piacenza, over the mountains just north of Genoa. Tonomi summarizes the little we know of him (3). His practice was active in the years 1300–1349. He is thought to have died in approximately 1356.

Although Henschel (2) thought de' Mussi was present at the siege of Caffa, Tonomi asserts that the Piacenza archives contain deeds signed by de' Mussi spanning the period 1344 through the first half of 1346. While this does not rule out travel to Caffa in late 1346, textual evidence suggests that he did not. He does not claim to have witnessed any of the Asian events he describes and often uses a passive voice for descriptions. After describing the siege of Caffa, de'Mussi goes on to say, "Now it is time that we passed from east to west to discuss all the things which we ourselves have seen..."

The Narrative of Gabriele De' Mussi

The de' Mussi account is presumed to have been written in 1348 or early 1349 because of its immediacy and the narrow time period described. The original is lost, but a copy is included in a compilation of historical and geographic accounts by various authors, dating from approximately 1367 (Figure 2). The account begins with an introductory comment by the scribe who copied the documents: "In the name of God, Amen. Here begins an account of the disease or mortality which occurred in 1348, put together by Gabriele de Mussis of Piacenza."

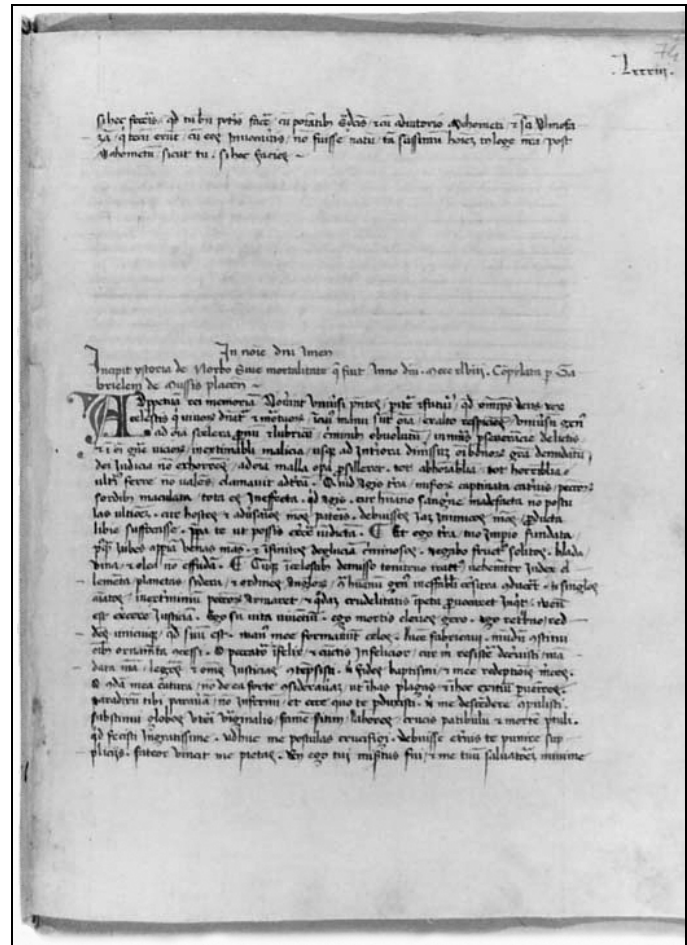


Figure 2. The first page of the narrative of Gabriele de' Mussi. At the top of the page are the last few lines of the preceding narrative; de' Mussi's begins in the middle of the page. The first three lines, and the large "A" are in red ink, as are two other letters and miscellaneous pen-strokes; otherwise it is in black ink. Manuscript R 262, fos 74r; reproduced with the permission of the Library of the University of Wrocław, Poland.

The narrative begins with an apocalyptic speech by God, lamenting the depravity into which humanity has fallen and describing the retribution intended. It goes on:

"...In 1346, in the countries of the East, countless numbers of Tartars and Saracens were struck down by a mysterious illness which brought sudden death. Within these countries broad regions, far-spreading provinces, magnificent kingdoms, cities, towns and settlements, ground down by illness and devoured by dreadful death, were soon stripped of their inhabitants. An eastern settlement under the rule of the Tartars called Tana, which lay to the north of Constantinople and was much frequented by Italian merchants, was totally abandoned after an incident there which led to its being besieged and attacked by hordes of Tartars who gathered in a short space of time. The Christian merchants, who had been driven out by force, were so terrified of the power of the Tartars that, to save themselves and their belongings, they fled in an armed ship to Caffa, a settlement in the same part of the world which had been founded long ago by the Genoese.

“Oh God! See how the heathen Tartar races, pouring together from all sides, suddenly invested the city of Caffa and besieged the trapped Christians there for almost three years. There, hemmed in by an immense army, they could hardly draw breath, although food could be shipped in, which offered them some hope. But behold, the whole army was affected by a disease which overran the Tartars and killed thousands upon thousands every day. It was as though arrows were raining down from heaven to strike and crush the Tartars’ arrogance. All medical advice and attention was useless; the Tartars died as soon as the signs of disease appeared on their bodies: swellings in the armpit or groin caused by coagulating humours, followed by a putrid fever.

“The dying Tartars, stunned and stupefied by the immensity of the disaster brought about by the disease, and realizing that they had no hope of escape, lost interest in the siege. But they ordered corpses to be placed in catapults¹ and lobbed into the city in the hope that the intolerable stench would kill everyone inside.² What seemed like mountains of dead were thrown into the city, and the Christians could not hide or flee or escape from them, although they dumped as many of the bodies as they could in the sea. And soon the rotting corpses tainted the air and poisoned the water supply, and the stench was so overwhelming that hardly one in several thousand was in a position to flee the remains of the Tartar army. Moreover one infected man could carry the poison to others, and infect people and places with the disease by look alone. No one knew, or could discover, a means of defense.

“Thus almost everyone who had been in the East, or in the regions to the south and north, fell victim to sudden death after contracting this pestilential disease, as if struck by a lethal arrow which raised a tumor on their bodies. The scale of the mortality and the form which it took persuaded those who lived, weeping and lamenting, through the bitter events of 1346 to 1348—the Chinese, Indians, Persians, Medes, Kurds, Armenians, Cilicians, Georgians, Mesopotamians, Nubians, Ethiopians, Turks, Egyptians, Arabs, Saracens and Greeks (for almost all the East has been affected)—that the last judgement had come.

“...As it happened, among those who escaped from Caffa by boat were a few sailors who had been infected with the poisonous disease. Some boats were bound for Genoa, others went to Venice and to other Christian areas. When the sailors reached these places and mixed with the people there, it was as if they had brought evil spirits with them: every city, every settlement, every place was poisoned by the contagious pesti-

lence, and their inhabitants, both men and women, died suddenly. And when one person had contracted the illness, he poisoned his whole family even as he fell and died, so that those preparing to bury his body were seized by death in the same way. Thus death entered through the windows, and as cities and towns were depopulated their inhabitants mourned their dead neighbours.” (Reproduced with permission from Horrox, pp. 16–20 [4])

The account closes with an extended description of the plague in Piacenza, and a reprise of the apocalyptic vision with which it begins.

Commentary

In this narrative, de’ Mussi makes two important claims about the siege of Caffa and the Black Death: that plague was transmitted to Europeans by the hurling of diseased cadavers into the besieged city of Caffa and that Italians fleeing from Caffa brought it to the Mediterranean ports.

Biological Warfare at Caffa

de’ Mussi’s account is probably secondhand and is uncorroborated; however, he seems, in general, to be a reliable source, and as a Piacenzian he would have had access to eyewitnesses of the siege. Several considerations incline me to trust his account: this was probably not the only, nor the first, instance of apparent attempts to transmit disease by hurling biological material into besieged cities; it was within the technical capabilities of besieging armies of the time; and it is consistent with medieval notions of disease causality (22).

Tentatively accepting that the attack took place as described, we can consider two principal hypotheses for the entry of plague into the city: it might, as de’ Mussi asserts, have been transmitted by the hurling of plague cadavers; or it might have entered by rodent-to-rodent transmission from the Mongol encampments into the city.

Diseased cadavers hurled into the city could easily have transmitted plague, as defenders handled the cadavers during disposal. Contact with infected material is a known mechanism of transmission (8–11); for instance, among 284 cases of plague in the United States in 1970–1995 for which a mechanism of transmission could be reasonably inferred, 20% were thought to be by direct contact (24). Such transmission would have been especially likely at Caffa, where cadavers would have been badly mangled by being hurled, and many of the defenders probably had cut or abraded hands from coping with the bombardment. Very large numbers of cadavers were possibly involved, greatly increasing the opportunity for disease transmission. Since disposal of the bodies of victims in a major outbreak of lethal disease is always a problem, the Mongol forces may have used their hurling machines as a solution to their mortuary problem, in which case many thousands of cadavers could have been involved. de’ Mussi’s description of “mountains of dead” might have been quite literally true.

Thus it seems plausible that the events recounted by de’ Mussi could have been an effective means of transmission of

¹Technically trebuchets, not catapults. Catapults hurl objects by the release of tension on twisted cordage; they are not capable of hurling loads over a few dozen kilograms. Trebuchets are counter-weight-driven hurling machines, very effective for throwing ammunition weighing a hundred kilos or more (22).

²Medieval society lacked a coherent theory of disease causation. Three notions coexisted in a somewhat contradictory mixture: 1) disease was a divine punishment for individual or collective transgression; 2) disease was the result of “miasma,” or the stench of decay; and 3) disease was the result of person-to-person contagion (23).

plague into the city. The alternative, rodent-to-rodent transmission from the Mongol encampments into the city, is less likely. Besieging forces must have camped at least a kilometer away from the city walls. This distance is necessary to have a healthy margin of safety from arrows and artillery and to provide space for logistical support and other military activities between the encampments and the front lines. Front-line location must have been approximately 250–300 m from the walls; trebuchets are known from modern reconstruction to be capable of hurling 100 kg more than 200 m (25), and historical sources claim 300 m as the working range of large machines (26). Thus, the bulk of rodent nests associated with the besieging armies would have been located a kilometer or more away from the cities, and none would have likely been closer than 250 m. Rats are quite sedentary and rarely venture more than a few tens of meters from their nest (27,28). It is thus unlikely that there was any contact between the rat populations within and outside the walls.

Given the many uncertainties, any conclusion must remain tentative. However, the considerations above suggest that the hurling of plague cadavers might well have occurred as de' Mussi claimed, and if so, that this biological attack was probably responsible for the transmission of the disease from the besiegers to the besieged. Thus, this early act of biological warfare, if such it were, appears to have been spectacularly successful in producing casualties, although of no strategic importance (the city remained in Italian hands, and the Mongols abandoned the siege).

Crimea as the Source of European and Near Eastern Plague

There has never been any doubt that plague entered the Mediterranean from the Crimea, following established maritime trade routes. Rat infestations in the holds of cargo ships would have been highly susceptible to the rapid spread of plague, and even if most rats died during the voyage, they would have left abundant hungry fleas that would infect humans unpacking the holds. Shore rats foraging on board recently arrived ships would also become infected, transmitting plague to city rat populations.

Plague appears to have been spread in a stepwise fashion, on many ships rather than on a few (Figure 1), taking over a year to reach Europe from the Crimea. This conclusion seems fairly firm, as the dates for the arrival of plague in Constantinople and more westerly cities are reasonably certain. Thus de' Mussi was probably mistaken in attributing the Black Death to fleeing survivors of Caffa, who should not have needed more than a few months to return to Italy (16).

Furthermore, a number of other Crimean ports were under Mongol control, making it unlikely that Caffa was the only source of infected ships heading west. And the overland caravan routes to the Middle East from Serai and Astrakhan insured that plague was also spreading south (Figure 1), whence it would have entered Europe in any case. The siege of

Caffa, and its gruesome finale, thus are unlikely to have been seriously implicated in the transmission of plague from the Black Sea to Europe.

Conclusion

Gabriele de' Mussi's account of the origin and spread of plague appears to be consistent with most known facts, although mistaken in its claim that plague arrived in Italy directly from the Crimea. His account of biological attack is plausible, consistent with the technology of the time, and it provides the best explanation of disease transmission into besieged Caffa. This thus appears to be one of the first biological attacks recorded (22) and among the most successful of all time.

However, it is unlikely that the attack had a decisive role in the spread of plague to Europe. Much maritime commerce probably continued throughout this period, from other Crimean ports. Overland caravan routes to the Middle East were also unaffected. Thus, refugees from Caffa would most likely have constituted only one of several streams of infected ships and caravans leaving the region. The siege of Caffa, for all of its dramatic appeal, probably had no more than anecdotal importance in the spread of plague, a macabre incident in terrifying times.

Despite its historical unimportance, the siege of Caffa is a powerful reminder of the horrific consequences when disease is successfully used as a weapon. The Japanese use of plague as a weapon in World War II (29) and the huge Soviet stockpiles of *Y. pestis* prepared for use in an all-out war (30) further remind us that plague remains a very real problem for modern arms control, six and a half centuries later (31).

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Respiratory Tract Reinfections by the New Human *Metapneumovirus* in an Immunocompromised Child

Gilles Pelletier,* Pierre Déry,* Yacine Abed,* and Guy Boivin*

The human *Metapneumovirus* (HMPV), a new member of the *Paramyxoviridae* family, has been recently associated with respiratory tract infections in young children. We report the case of a young, immunocompromised child who had severe lower respiratory tract infections during two consecutive winter seasons caused by genetically distinct HMPV strains.

A variety of viruses—such as the influenza viruses A and B, the *Human respiratory syncytial virus* (HRSV), the parainfluenza viruses, and the adenoviruses—cause seasonal respiratory tract infections in young children. Symptoms range from influenzalike illnesses to lower respiratory tract syndromes, such as bronchiolitis, croup, and pneumonitis (1–4). These viruses may also cause severe respiratory infections in immunocompromised patients (1,4). However, the cause of many cases of bronchiolitis and pneumonitis remains unknown despite the extensive use of sensitive diagnostic techniques.

The human metapneumovirus (HMPV) has been recently classified as a new member of the *Paramyxoviridae* family based on nucleic acid sequence, gene organization, and electron microscopy findings (5–7). This virus has been reported to cause respiratory tract infections in children <5 years of age from the Netherlands (5), as well as in elderly patients from North America (7). We describe a case of recurrent HMPV respiratory tract infections, associated with genetically distinct viral strains, in an immunocompromised child.

Case Report

A 7-month-old girl, who had acute lymphoblastic leukemia with meningeal involvement (treated with intravenous vincristine and doxorubicin, as well as intrathecal methotrexate), was brought to the hospital on March 23, 1998, for recent onset of nasal congestion and nonproductive cough. Her medical history showed that she had recent contact with family members who had a common cold. On initial physical examination, her temperature was 38.9°C with a respiratory rate of

40 per min, with no rales at lung auscultation. Initial laboratory tests showed a leukocyte count of $1,800 \times 10^9$ cells/L with 25% neutrophils and 3% band forms. The child was hospitalized and treated empirically with intravenous ceftazidime. Urine and blood cultures were negative for bacterial pathogens. The patient rapidly improved and was discharged from the hospital on day 3 after admission; no antibiotics were given, and the presumptive diagnosis was viral upper respiratory tract infection. The next day, the patient was brought to the same hospital for an exacerbation of coughing and sneezing. On physical examination, she had a temperature of 38.5°C, with bilateral wheezing at lung auscultation. The respiratory rate was 36 per min, and a clear nasal discharge was noted. The leukocyte count was $1,600 \times 10^9$ cells/L, with 33% neutrophils and 0% band forms. Blood was drawn for cultures, and a nasopharyngeal aspirate (NPA) sample was obtained for HRSV antigenic testing (Test Pack, Abbott Laboratories, Abbott Park, IL) and viral culture on Madin Darby Kidney cells (MDKC), tertiary monkey kidney cells (LLC-MK2), Hep-2, human foreskin fibroblast, Vero, Mink lung, human lung adenocarcinoma (A-549), human rhabdomyosarcoma (RD), transformed human kidney 293, and human colon adenocarcinoma (HT-29) cells. The patient was again treated empirically with intravenous ceftazidime. Three days after admission her condition had improved, and she was discharged from the hospital. The final diagnosis was bronchiolitis, and antibiotics were not given. Bacterial blood cultures and the rapid antigenic test for HRSV were negative. However, after 17 days of incubation, the viral culture from the NPA showed a nonhemagglutinating virus (isolate 1) growing in LLC-MK2 cells. Immunofluorescence assays with antibodies against common respiratory pathogens (influenza viruses A and B, HRSV, adenoviruses, and parainfluenza viruses 1–4) were all negative.

The next year, on January 18, 1999, during a scheduled medical appointment for administration of intravenous (daunorubicin and vincristine) and intrathecal (methotrexate, cytarabine, and hydroxyurea) chemotherapy in addition to oral dexamethasone, the now 17-month-old girl again had nasal congestion and nonproductive cough. Her father mentioned that he had an upper respiratory tract infection 2 weeks earlier. An NPA sample was obtained for viral culture and HRSV antigenic detection. The patient did not appear ill and was allowed to go home the same day after chemotherapy. The HRSV antigenic test came back negative, but the viral culture showed an unidentified cytopathic effect only apparent in LLC-MK2 cells after 14 days of incubation (isolate 2). Immunofluorescence assays were again negative for all common respiratory viruses. The patient received another course of intravenous chemotherapy (daunorubicin and vincristine) on January 25 at the outpatient clinic, and her upper respiratory tract symptoms were then treated with an oral antibiotic (axetil cefuroxime).

Five days later, on January 30, 1999, the child was admitted to the hospital with a fever (39.9°C), persistent dry cough, and clear nasal discharge while on oral antibiotic. On physical

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examination, her respiratory rate was 28 per min with fine bilateral crackles at lung auscultation. An otoscopic exam showed bilateral otitis. The leukocyte count showed neutropenia (0.400×10^9 cells/L), and ceftazidime plus vancomycin were initiated. The blood cultures taken on the first day of hospitalization indicated a coagulase-negative staphylococcus (1 of 2 bottles were positive). On the fourth day of hospitalization, the patient's clinical condition deteriorated with desaturation (pO_2 , 0.88) and tachypnea (respiratory rate, 60–70 per min). A chest x-ray showed bilateral infiltrates compatible with pneumonitis. The next day, an NPA sample was obtained for a viral culture and direct immunofluorescent assays against typical respiratory viruses. The results were negative. Intravenous erythromycin was administered for empirical coverage of atypical pathogens. On day 10 of hospitalization, the patient was transferred to the pediatric intensive-care unit and was intubated. A bronchoscopy was performed, and a bronchoalveolar lavage (BAL) sample was obtained for cultures of fungal, mycobacterial, viral, and bacterial pathogens, as well as for specific staining procedures for *Pneumocystis carinii*, fungi, bacteria, and mycobacteria. At that point, ceftazidime was replaced by trimethoprim/sulfamethoxazole, and an amphotericin B lipidic complex formulation (Abelcet, Liposome, Canada) was added. All cultures and specific staining procedures performed with the BAL sample were negative. Direct immunofluorescence assays on the BAL sample also were negative for influenza A and B, parainfluenza 1–4, and HRSV. On day 13 of hospitalization, the patient's respiratory signs deteriorated markedly, with bilateral pulmonary infiltrates compatible with acute respiratory distress syndrome. Four days later, because of free air in the pericardium and peritoneum (the patient was on high ventilatory pressure), drainage tubes were placed, resulting in a temporary improvement of pulmonary gas exchange. New blood cultures drawn on day 27 were negative. The next day, shock developed, and vasopressor drugs were started. Because of her irreversible condition, all treatment was stopped on day 30, and the patient died shortly thereafter. No autopsy was performed.

Two years after the child's death, reverse transcription-polymerase chain reaction assays for HMPV with infected LLC-MK2 cell culture supernatants of the two unidentified isolates were performed and found to be positive. On the basis of partial published sequences, a set of primers was designed for amplification of the F gene of HMPV (5,7). The forward primer sequence was 5'-ATGCTTGGAAAGTGGTG-3', and the reverse primer sequence was 5'-TCTTCTTACCATTG-CAC-3'. Amplified products were sequenced by an automated DNA sequencer (ABI 377A [Perkin-Elmer Applied Biosystems, Foster City, CA]) and the 759-bp nucleotide sequences of the two viral isolates from consecutive seasons were aligned with the Clustal W software (8). The two viral isolates differed by 114 nt in the F gene sequence, which resulted in a change of 10 amino acids (Figure).

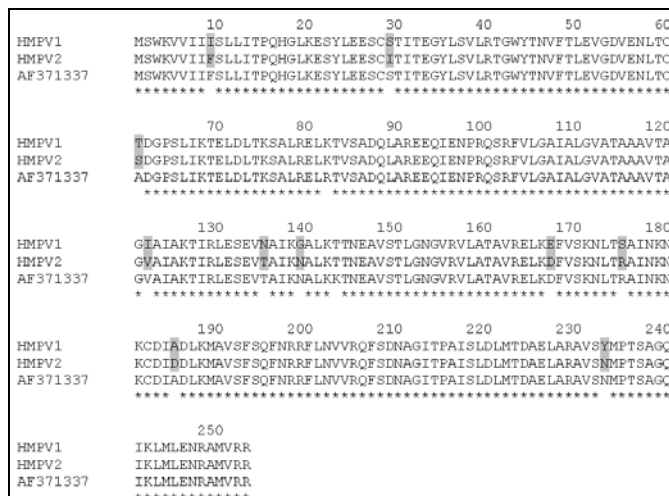


Figure. Comparison of the partial amino acid sequences of Human *Metapneumovirus* (HMPV) isolates 1 (recovered in 1998) and 2 (recovered in 1999) for the fusion protein (residues 1 to 253). The sequences were aligned with the reference sequence from the Netherlands (GenBank accession no. AF371337). Asterisks denote identical residues; the shaded boxes highlight different amino acids between the two HMPV isolates from this study.

Conclusions

We describe the case of a young immunocompromised child who had respiratory tract infections during two consecutive seasons, probably due to HMPV, a newly discovered paramyxovirus. On the basis of sequence analysis of the viral F gene, we found that the child was infected by two genetically distinct HMPV strains.

In the first clinical episode (occurring at the age of 7 months), the initial symptoms resembled a common cold and progressed to bronchiolitis, followed by a complete resolution of symptoms within a few days. HMPV was the only organism isolated in culture from an NPA sample, and the HRSV rapid antigenic test was negative. Ten months later, the child had a second respiratory tract infection, again with common cold symptoms. HMPV was again the sole pathogen isolated in an NPA sample. In the next weeks, however, the patient's clinical condition deteriorated, with development of bilateral pneumonitis and respiratory failure and ultimately death. The BAL sample obtained showed no bacterial, viral, or fungal pathogens by culture, antigenic testing, or special staining procedures. The exact cause of respiratory failure in this immunocompromised child remains uncertain, because autopsy was not performed. An opportunistic infection or a neoplastic infiltrate was the most probable cause. HMPV could have led to the acute respiratory distress syndrome in this immunocompromised patient. We suggest that the initial upper respiratory tract infection may have progressed to pneumonitis after the patient received intensive chemotherapy. The fact that HMPV was not recovered in the last BAL sample does not rule out such viral infection since paramyxoviruses are very labile (1), and the culture may not have been incubated long enough for a cytopathic effect to be observed.

The two HMPV isolates from this study had many differences at the nucleotide level (similarity 85.0%) but much less at the amino acid level (similarity 96.0%) in the gene coding for the fusion (F) protein. Isolate 2, recovered in 1999, had greater amino acid similarity with the prototype sequence from the Netherlands (5) compared with isolate 1 (98.0% vs. 96.0% similarity). Furthermore, the two strains seem to belong to the two different HMPV lineages previously reported by Dutch and North American groups (5,7). We suggest that many HMPV strains may cocirculate in a specific population, and such viral diversity, coupled with waning immunity, as found in elderly and immunocompromised patients, may lead to multiple reinfections similar to HRSV (2,9–12).

Future studies are needed to evaluate the full clinical spectrum associated with HMPV infection, the groups at risk for severe complications, and the potential therapeutic options. Our data suggest that HMPV should be added to the list of pathogens associated with severe respiratory tract infections in immunocompromised patients.

Dr. Pelletier is a postdoctoral fellow in infectious diseases at Laval University. His research interests include the molecular detection and epidemiology of respiratory viruses.

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Detection by Enzyme-Linked Immunosorbent Assay of Antibodies to West Nile virus in Birds

Gregory D. Ebel,* Alan P. Dupuis II,* David Nicholas,* Donna Young,* Joseph Maffei,* and Laura D. Kramer*

We adapted an indirect immunoglobulin G enzyme-linked immunosorbent assay to facilitate studies of *West Nile virus* (WNV) and evaluated its application to taxonomically diverse avian species. Anti-WNV antibodies were detected in 23 bird species, including many exotic species, demonstrating its value in studies of WNV epizootiology.

West Nile virus (WNV) is transmitted in an enzootic cycle between *Culex* spp. mosquitoes and their avian hosts (1–4). Sentinel birds have long been used in arbovirus surveillance (5–9), and serologic surveys of wild and captive birds are valuable in determining whether an arbovirus is present in a particular locality (10). While plaque-reduction neutralization tests (PRNT) are the standard for arbovirus serologic testing, they are frequently unavailable in many laboratories for several reasons: They generally require high levels of biocontainment; they are time-, labor-, and cost-intensive; and they require specialized technical expertise. A rapid serologic diagnostic assay suitable for screening large numbers of specimens and posing minimal biohazard would facilitate large-scale avian-based serologic surveillance for WNV. Accordingly, we sought to determine whether an indirect enzyme-linked immunosorbent assay (ELISA) designed to detect seroreactivity against *St. Louis encephalitis virus* (SLEV) and *Western equine encephalomyelitis virus* (WEEV) (11) could be modified to detect anti-WNV antibodies in taxonomically diverse wild-caught and captive avian species.

To produce ELISA antigen, Vero cells were infected with WNV and processed into antigen as described (12), with New York–derived reference stocks of WNV (31000365; see Ebel et al. [13] for source and sequence information). Fifty microliters of antigen diluted 1:100 in fresh coating buffer (0.015M Na₂CO₃, 0.035M NaHCO₃, pH 9.6) was applied to each well of Immulon 1 (Dynatek Laboratories Inc, Winooski, VT) ELISA plates. Negative antigen (uninfected Vero cell lysate produced as described above) was placed in every third column of the plate (i.e., columns 1, 4, 7, 10), and positive anti-

gen was placed in the remaining columns. The plate was then placed in a humid chamber, and antigen was allowed to bind overnight at 4°C. In the morning, antigen-containing solution was discarded, the plate was washed three times with phosphate-buffered saline (PBS) with 0.05% Tween, 100 µL blocking buffer (PBS with 0.05% Tween and 2.0% Casein) was added, and the plates were placed in a humid chamber in a 37°C incubator for 1 h. Following incubation, blocking solution was discarded and test samples, diluted 1:100 in PBS with 0.05% Tween, and 0.5% bovine albumin (PBS-T-BA), were applied to one negative and two positive antigen-containing wells. Plates with test specimens were returned to a humid chamber and incubated at 37°C for 1 h. Following incubation, plates were removed, washed as above, and 50 µL of horseradish peroxidase–conjugated goat anti-wild bird immunoglobulin (Ig) G (Bethyl Laboratories, Inc., Montgomery, TX), diluted 1:1000 in PBS-T-BA, was applied to each well. After incubation and washing as above, plates were developed with 50 µL of tetramethylbenzidine (TMB)-peroxidase substrate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) for 7 min. The reactions were stopped with 50 µL of 1:20 H₂PO₄, and the optical density (OD) of each well was read at 450 nm. Blank (no test sera), positive, and negative controls were included on each plate. To compute the positive/negative (P/N) value of each sample, we divided the mean OD of positive antigen-containing wells by the OD of the negative antigen-containing wells. Samples with a P/N value ≥ 2 were considered positive and were tested further by PRNT(14). Specimens were confirmed positive if their 90% neutralization titer against WNV was at least fourfold greater than against SLEV, a closely related flavivirus that may cross-react with WNV antigens in screening assays (15,16).

Optimum concentrations of antigens for the ELISA were determined by applying known positive and negative chicken samples to wells containing serial twofold dilutions of antigen. Optimal concentrations were defined as those yielding the highest mean P/N value for known positive samples and P/N values closest to unity (one) for known negative samples. Generally, a 1:100 dilution of the crude antigens was optimal. Using a similar strategy, we then determined the optimal serum dilutions for pigeon and wild bird sera.

Specimens for testing were either donated from the collection at the Bronx Zoo or collected during an avian surveillance project conducted in New York City during 2001. Avian blood samples were collected as whole blood and stored at 4°C, centrifuged for 10 min at maximum speed in a microcentrifuge, and serum was separated. In some cases, samples were collected and heparinized, and plasma was separated and stored as described previously.

PRNT testing was conducted according to standard protocols (14). Briefly, where sample quantities permitted, test sera were serially diluted from 1:5 through 1:160 in BA-1 diluent (M199H, 0.05M Tris pH 7.6, 1% bovine serum albumin, 0.35g/L sodium bicarbonate, 100 units/mL penicillin, 100 µg/mL streptomycin, and 1 µg/mL fungizone) and 100 µL was

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incubated overnight at 4°C with 100 µL of virus containing approximately 200 PFU of WNV (strain 31000365) or SLEV virus strain no. 59268 (Parton). If insufficient sample was available, higher starting dilutions (usually 1:10) were used. In the morning, 100 µL of each serum-virus mixture was added onto confluent monolayers of Vero cells and allowed to adsorb at 37°C for 1 h. Following incubation, a nutrient-agar overlay was added, and the plates were returned to the incubator. PRNT testing for WNV used a single basal medium Eagle-based overlay containing neutral red, while SLEV testing required application of a double overlay, the first without and the second with neutral red, applied 3 days after the first. Plaques were counted on the 3rd (WNV) or 5th (SLEV) day after the test was initiated. The highest dilution of serum neutralizing 90% of the inoculum as determined by back-titration was considered the neutralizing titer.

All statistical analyses were done with Microsoft Excel (Microsoft Corp., Redmond, WA).

The predictive value of a positive test (PVP) and of a negative test (PVN) were determined by using the sera of birds caught in mist nets during a WNV serologic survey conducted during the summer of 2001 (manuscript in preparation). Of 3,581 specimens tested, 233 (7%) were ELISA positive. Of these positive specimens, 163 (70%) were also positive by PRNT, for a PVP of 70%. Five additional ELISA-positive specimens yielded indeterminate results: although neutralizing antibody was detected by PRNT, a fourfold difference between WNV and SLEV titers was not detectable. To determine the PVN of the ELISA, 110 ELISA-negative specimens were tested for neutralizing antibody by PRNT. All ELISA-negative specimens were also negative by PRNT, yielding a PVN of 100%.

To determine whether this protocol detects antibodies against WNV in a wide range of bird species, we used our ELISA to test known positive (PRNT-confirmed) serum specimens from 23 different avian species. The indirect ELISA protocol detected anti-WNV antibody in all 23 species, representing 12 avian orders. All PRNT-positive specimens contained ELISA antibody to WNV (Table). Species that were negative by ELISA were uniformly negative by PRNT. One domestic chicken that had been experimentally infected with SLEV had a positive P/N ELISA result and a positive PRNT result. The infection was confirmed as SLEV since the SLEV titer on this specimen was fourfold greater than the WNV titer (data not shown). P/N values were not correlated with either PRNT titer (coeff.=0.44) or with the natural log of the PRNT titer (coeff.=0.30) (data not shown).

Conclusions

The PVP of this assay appears to be somewhat lower than that of another reported ELISA protocol (17) and some other flavivirus serologic assays, such as PRNT, but is higher than that reported for the assay from which it was derived (11). The PVP of our test might have been higher had we more stringently evaluated our ELISA-positive specimens: a number of specimens had P/N values ≥ 2 because one of the two positive antigen wells was highly reactive. None of these specimens were confirmed by PRNT. The high values in the reactive well may have occurred as a result of technical error (e.g., splashing). Alternatively, the ELISA may be more sensitive than neutralization and may detect anti-WNV antibodies that PRNT does not. We always performed a confirmatory test to resolve true from false positives; nonetheless, this ELISA dramatically reduced the number of confirmatory tests we conducted during WNV surveillance in 2000 and 2001. Use of the ELISA described here yielded substantial cost reduction and time savings compared with screening specimens by PRNT.

This ELISA detected anti-WNV antibody in a taxonomically diverse array of captive and wild birds. In 23 species from 12 avian orders, IgG antibodies were detectable by using commercially available anti-wild bird horseradish peroxidase-conjugated antibodies. The breadth of the reactivity of this conjugate was surprising, given that it was generated by using IgG isolated from the sera of four species representing only four avian orders: Passeriformes, Columbiformes, Galliformes, and Anseriformes (11). Although this protocol has been documented to react broadly in an ELISA to detect SLEV antibody in 13 species representing seven orders (11), known positive sera from three orders (Ciconiiformes, Gruiformes, and Charadriiformes) were not detected. We obtained positive results for each of these orders. The reasons for this discrepancy in our results are not clear but may be related to differences in the antibody titer of the specimens we tested or to general differences in the immune response to WNV compared with SLEV. Alternatively, some of the measures we took to optimize our test (e.g., the substitution of tetramethylbenzidine peroxidase substrate for 2,2'-azino-di-[3-ethylbenzthiazoline-6-sulfonate]) may have increased the assay's sensitivity, allowing detection of fewer bound conjugated antibodies, as may occur with test sera derived from divergent avian species. The lack of correlation between P/N values with PRNT titers is not surprising given that the P/N value was obtained from a single serum dilution and does not represent an endpoint titer. Although this serologic method should be evaluated for each avian order tested, our results demonstrate that this testing protocol is appropriate for WNV serologic surveys of free-ranging and captive sentinel birds.

Table. Comparison of serologic assay results with reactivity across a range of avian orders and species

Common Name	Species	Order	Indirect ELISA P/N ^a	PRNT titer
House Sparrow # 1	<i>Passer domesticus</i>	Passeriformes	3.0	40
House Sparrow # 2	<i>P. domesticus</i>	Passeriformes	3.0	20
House Sparrow # 3	<i>P. domesticus</i>	Passeriformes	7.2	80
Northern Mockingbird	<i>Mimus polyglottos</i>	Passeriformes	2.1	>10
European Starling	<i>Sturnus vulgaris</i>	Passeriformes	3.0	40
American Crow #1	<i>Corvus brachyrhynchos</i>	Passeriformes	2.8	80
American Crow #2	<i>C. brachyrhynchos</i>	Passeriformes	2.0	20
Rock Dove #1	<i>Columba livia</i>	Columbiformes	2.9	80
Rock Dove #2	<i>C. livia</i>	Columbiformes	2.5	320
Rock Dove #3	<i>C. livia</i>	Columbiformes	7.6	10
White-naped Crane	<i>Grus vipio</i>	Gruiformes	5.3	>640
Waldrapp Ibis #1	<i>Gerontica eremita</i>	Ciconiiformes	9.4	320
Waldrapp Ibis #2	<i>G. eremita</i>	Ciconiiformes	11.4	640
Waldrapp Ibis #3	<i>G. eremita</i>	Ciconiiformes	4.0	80
Black Crowned Night Heron	<i>Nycticorax nycticorax</i>	Ciconiiformes	5.2	160
Flamingo #1	<i>Phoenicopterus chilensis</i>	Phoenicopteriformes	3.7	160
Flamingo #2	<i>P. chilensis</i>	Phoenicopteriformes	4.2	>640
Malay Great Argus	<i>Argusianus argus</i>	Galliformes	7.3	40
Kenya Crested Guineafowl	<i>Guttera edouardi</i>	Galliformes	6.7	80
Wild Turkey	<i>Meleagris gallopavo</i>	Galliformes	6.0	320
Bulwer's Pheasant	<i>Lophura bulweri</i>	Galliformes	3.5	>640
American White Pelican	<i>Pelecanus erythrorhynchos</i>	Pelecaniformes	5.7	>640
Guanay Cormorant	<i>Phalacrocorax bougainvillii</i>	Pelecaniformes	2.1	80
Brown Pelican	<i>Pelecanus occidentalis</i>	Pelecaniformes	3.8	160
Domestic Goose	<i>Anser sp.</i>	Anseriformes	3.6	40
Trumpeter Swan	<i>Cygnus buccinator</i>	Anseriformes	4.0	160
Barred Owl	<i>Strix varia</i>	Strigiformes	2.6	160
Ostrich	<i>Struthio camelus</i>	Struthioniformes	3.9	640
Magellanic Penguin	<i>Spheniscus magellanicus</i>	Sphenisciformes	4.3	>640
Black-Necked Crane	<i>Grus nigricollis</i>	Gruiformes	2.4	80
Laughing Gull	<i>Larus atricilla</i>	Charadriiformes	6.9	320
Domestic Duck	<i>Anas sp.</i>	Anseriformes	1.1	<10
Canada Goose	<i>Branta canadensis</i>	Anseriformes	1.2	<20
Domestic chicken	<i>Gallus gallus</i>	Galliformes	0.9	<10
SLEV-positive chicken	<i>G. gallus</i>	Galliformes	2.5	20
WEEV-positive chicken	<i>G. gallus</i>	Galliformes	1.3	<20

WNV-negative specimens shown below bold line

^aELISA, enzyme-linked immunosorbent assay; P/N, positive/negative ratio; PRNT, plaque-reduction neutralization tests; SLEV, St. Louis encephalitis virus; WEEV, Western equine encephalomyelitis virus.

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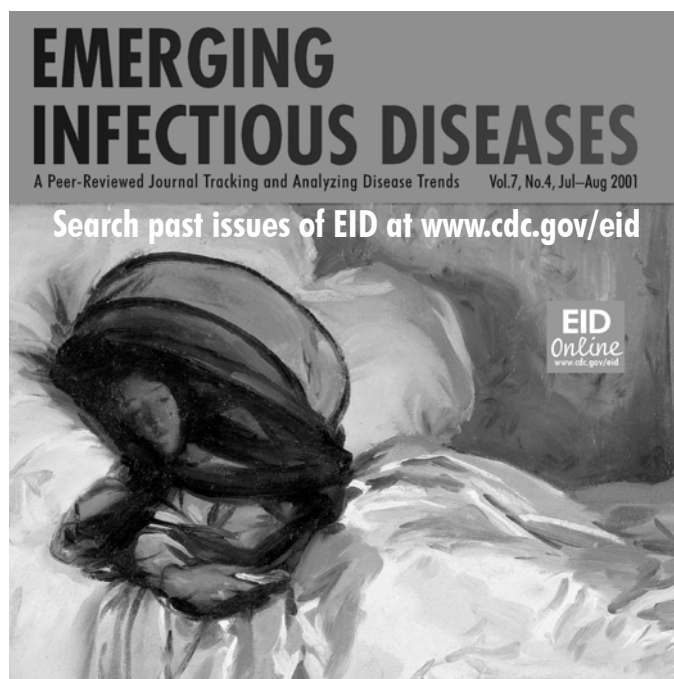
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First Detection of Spotted Fever Group Rickettsiae in *Ixodes ricinus* from Italy

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and Claudio Genchi*§

Ixodes ricinus from Italy were examined for the first time to detect whether rickettsiae were present. Using molecular methods, we detected three different spotted fever group rickettsiae, including *Rickettsia helvetica*. Our results raise the possibility that bacteria other than *R. conorii* are involved in rickettsial diseases in Italy.

The genus *Rickettsia* comprises obligately intracellular, gram-negative bacteria. Before sequence-based classification methods were introduced, the genus was divided into two groups: the typhus group (TG), which included *R. prowazekii*, *R. typhi*, and *R. canada*, and the spotted fever group (SFG), which comprised all others. Recent phylogenetic studies of genes such as *gltA*, *ompA*, “gene D,” and that encoding the 17-kDa protein (hereafter referred to as “17kDa”) have shown that these two groupings are not consistent with species relationships; consequently, they have been modified (summarized in [1]). The TG now comprises only *R. prowazekii* and *R. typhi*, while the SFG contains seven divergent lineages: the *R. rickettsii* group, *R. japonica*, *R. montana*, the *R. massiliae* group, *R. helvetica*, *R. felis*, and the *R. akari* group. The AB bacterium, *R. bellii*, and *R. canada* cluster outside both the TG and SFG in most analyses (1).

Members of the SFG rickettsiae are usually associated with ixodid ticks, which transfer them to vertebrates via salivary secretions and between themselves transtadially and transovarially. Several tick-borne rickettsiae are causative agents of human or animal diseases. The prevalences of these diseases are primarily dependent on the geographic distribution of host ticks, which act as both vector and reservoir. Among rickettsiae found in Europe, *R. conorii* is probably the most well known. This bacterium, transmitted by *Rhipicephalus sanguineus*, causes “boutonneuse” or Mediterranean spotted fever (MSF), an endemic disease in several countries. Until recently, MSF was thought to be the only rickettsial disease prevalent in Europe, but in recent years some new human rickettsioses have been attributed to bacteria previously consid-

ered of unknown pathogenicity (2). An example is *Rickettsia helvetica*, which was originally isolated in 1979 from *Ixodes ricinus* but was shown to have pathologic relevance only in 1999, when it was associated with fatal perimyocarditis in two Swedish men (3). In addition, *R. helvetica* stimulated a specific antibody response in a man in France who had low-grade fever, headache, and myalgia (4).

In Italy, the only rickettsia isolated from humans and ticks thus far has been *R. conorii*. Since its host, *Rh. sanguineus*, favors warm climates, MSF is more common in central and southern Italy (5,6). In the years 1992–1998, approximately 8,500 cases of human rickettsioses presumed to be MSF were reported to the Italian Ministry of Health. Regarding the distribution of cases in different parts of Italy, some central (Lazio) and southern (Sardinia, Sicily, and Calabria) regions of the country have a particularly high morbidity rate, reaching an average of 11.9 cases for every 100,000 inhabitants in Sardinia, compared with the national average of 2.1.

The diagnosis of MSF in Italy usually depends on clinical evidence supported by serologic confirmation, mainly by the microimmunofluorescence (MIF) technique. A major limitation of MIF is cross-reactivity, which renders it unable to differentiate between various SFG rickettsiae (4). Thus, some cases of MSF in Italy, especially where the disease is not endemic, may in fact be due to other rickettsiae.

I. ricinus is found with high prevalence in the Italian Alps and Apennines (reaching 96% of all ticks collected in some areas) and in almost all other Italian regions that contain humid, forested habitats (7). While all life stages of *Rh. sanguineus* are mainly associated with dogs, *I. ricinus* can feed on >200 host species, primarily wild rodents and ruminants. In a survey in Liguria of ticks recovered from people, most ticks (89.3%) were *I. ricinus*; *Rh. sanguineus* was recorded less frequently (9.8%) (8).

To date, no studies have been conducted on potential rickettsiae in Italian ticks, other than *Rhipicephalus* spp. Recently, various *Rickettsia* species have been found in *I. ricinus* from other European countries, including *R. helvetica* in Switzerland, France, Sweden, Slovenia, and Portugal (4) and *Rickettsia* spp. IRS3/4 in Slovakia (9). To check whether such bacteria are also present in Italian *I. ricinus*, we studied specimens from three regions. We used molecular-sequence-based identification techniques, which offer high sensitivity and specificity compared with serologic tests and circumvent the need for bacterial culturing.

The Study

A total of 109 *I. ricinus* specimens were collected in northern and central Italy (Figure 1), identified by using standard taxonomic keys, and stored at –20°C. Specifically, 89 ticks (70 adults and 19 nymphs) were collected by dragging vegetation in different parts of Trentino Province in April–October 1997 and 1999, and 10 ticks (7 adults and 3 nymphs) by dragging in Feltre (Veneto Region) in March 2000. Ten more ticks (7 adults and 3 nymphs) were collected from a patient at the

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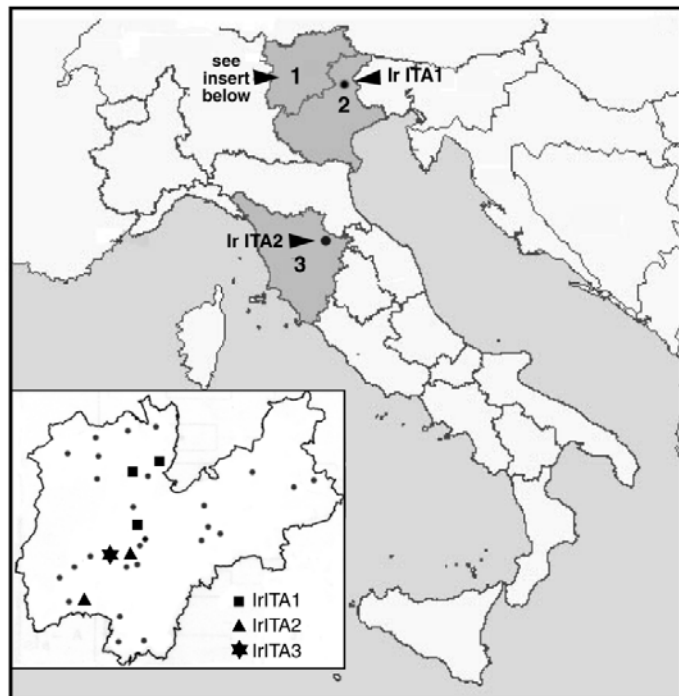


Figure 1. Location of *Ixodes ricinus* collection sites and detection of spotted fever group rickettsiae. 1). Trentino Province. Dots represent places where ticks were not found to have rickettsiae; different shapes represent the places where IrITA1 (Terlago, Denno, Vervò), IrITA2 (Molina di Ledro, Drena) and IrITA3 (Drena) were detected. In Feltre (2; Veneto Region) only IrITA1 was detected, while in Parco Nazionale delle Foreste Casentinesi (3; Toscana Region) only IrITA2 was detected.

Ospedale di Careggi in Firenze in May 1997. The man had been bitten in Parco Nazionale delle Foreste Casentinesi (Toscana Region; see Figure 1) a number of hours earlier but did not display any illness. MIF tests with *R. conorii* antigens were performed on his arrival at the hospital and again 4 weeks later; results were negative in each instance. Tick samples were placed in 50 μ L of 10 mM Tris-HCl (pH 8.0), heated at 90°C for 10 min, crushed with a sterile plastic homogenizer, and treated with 10 μ g of proteinase K at 50°C for 3 h. Polymerase chain reaction (PCR) of a 341-bp portion of *gltA* was performed by using the primers Rp CS.877p and Rp CS.1258n under conditions previously described (10). These primers were chosen for an initial screening because they are known to amplify all rickettsiae (11).

One hundred nine PCRs were performed, and nine positives (two adult females, three adult males, and four nymphs)

were found. An initial estimate of the overall prevalence in Italian *I. ricinus* is thus 8.25%. To better establish intrageneric relationships, the nine positive samples were subjected to further PCR analysis with the primer pairs Rr 17.61p/Rr 17.492n and Rr 190.70p/Rr 190.602n (10), which amplified 394-bp and 488-bp portions of 17kDa and *ompA*, respectively. PCR bands for all three genes were then sequenced directly by using an ABI PRISM sequencer (Perkin-Elmer, Foster City, CA). To compare the sequences obtained during this study with those of other rickettsiae, sequences present in GenBank were selected by means of BLAST as well as on the basis of previous reports (1,12). Sequences were converted to their putative amino acid sequences and aligned by using the program Clustal X (<ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/>). Based on these alignments, nucleotide alignments were performed manually, and phylogenetic relationships were inferred by maximum likelihood (ML). The appropriate model of sequence evolution was determined by Modeltest 3.06 (http://zoology.byu.edu/crandall_lab/modeltest.htm), and trees were produced using the program TreePuzzle 5.0 (www.tree-puzzle.de), which provides branch lengths as well as quartet puzzling support values at each node with >50% support.

Comparisons of the sequences identified with those from closely related SFG *Rickettsia* spp. are shown in the Table; Figure 2 shows the results of phylogenetic analysis. *gltA*-based results (Figure 2a) show that all strains detected are SFG rickettsiae. For 17kDa (Figure 2b), no identical sequences for IrITA2 and IrITA3 were present in GenBank, and they clustered with *R. cooleyi* (isolated from *I. scapularis* in Texas [13]). *ompA* was the most variable of the three genes analyzed (Figure 2c) and could only be amplified from IrITA2 and IrITA3. Consistent with the results from *gltA*, *ompA* from IrITA2 was 100% identical to IrR/Munich; however, two substitutions were found between these two sequences and that of IRS4. Notably, for *ompA*, the cluster to which IrITA2 and IrITA3 belong also contains a strain detected in Spain (14). This finding suggests that these bacteria may be widespread in Europe. On the basis of *ompA* (and 17kDa) sequences, the clade containing IrITA2 and IrITA3 was closest to a clade containing *R. cooleyi* and an endosymbiont (10), both hosted by *I. scapularis*. All previous attempts to amplify *ompA* from *R. helvetica* by using various primers have failed, which suggests that the gene is either absent or too variable to work with primers designed from other SFG bacteria (12). This would explain

Table. Locations and numbers of ticks infected with spotted fever group (SFG) *Rickettsia* spp., and similarities between three genes in Italian SFG *Rickettsia* spp.^a with those from closely related bacteria from elsewhere in Europe^b

Sequence	Location (and no.) of infected <i>I. ricinus</i> specimens	SFG <i>Rickettsia</i> spp. with highly similar sequences present in GenBank	% Identity		
			<i>gltA</i>	17 kDa	<i>ompA</i>
IrITA1	Trento Province (3) Feltre (2)	<i>R. helvetica</i>	100	99	–
IrITA2	Trento Province (2)	IrR/Munich	100	–	100
	Toscana (1)	IRS4 (Slovakia)	100	–	99
IrITA3	Trento Province (1)	IRS3 (Slovakia)	100	–	99

^aThe number of ticks examined from Trento, Feltre, and Toscana were 89, 10, and 10 respectively.

^b*I.*, *Ixodes*; *R.*, *Rickettsia*.

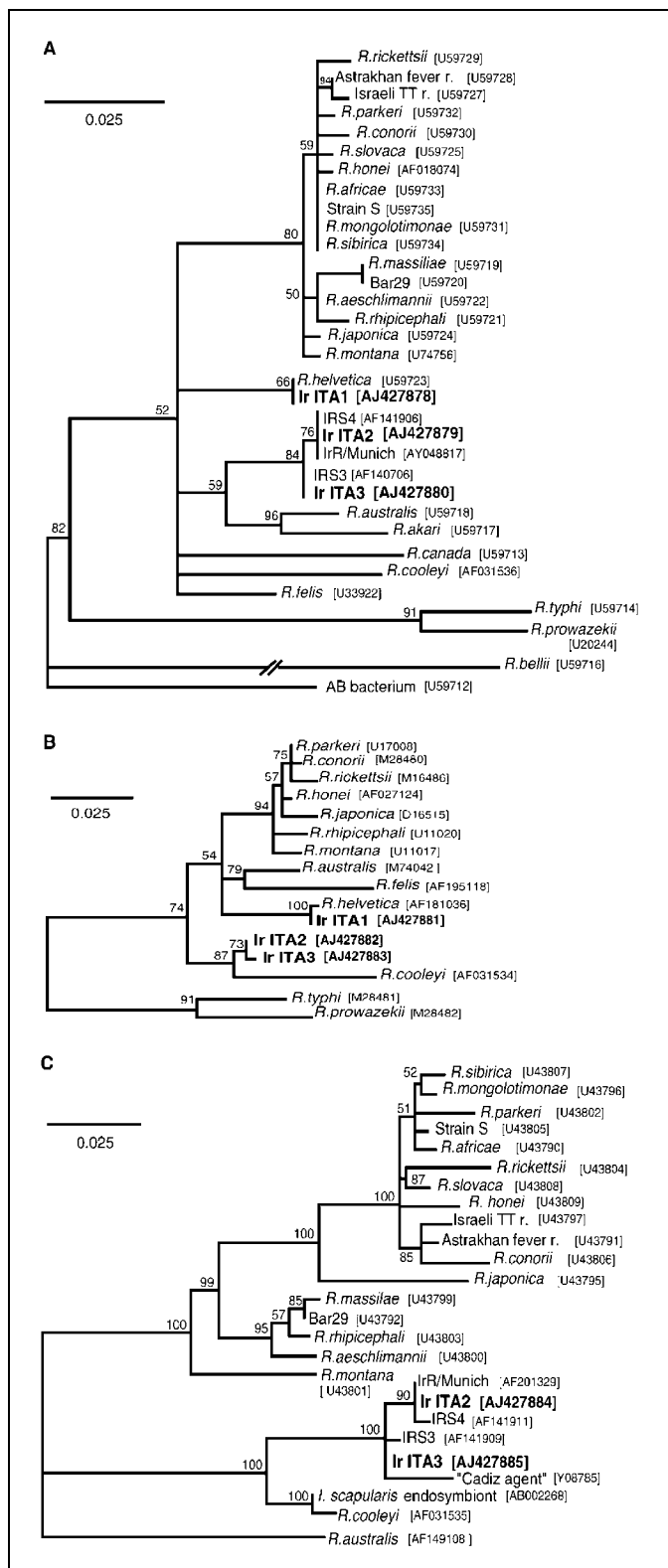


Figure 2. Phylogenetic analyses of rickettsiae based on *gItA* (A), 17 kDa *ompA* (B) and *ompA* (C). Based on analyses in Modeltest 3.06, the models of substitution chosen for analysis in Treepuzzle 5.0 were TrN+G for *gItA* and 17kDa, and HKY+G for *ompA*. GenBank accession numbers for each sequence, including those found in this study (IrITA1-3), are shown adjacent to each strain. Numbers near each node represent quartet puzzling support values. Scale bars represent number of inferred substitutions at each site.

why we were unable to amplify *ompA* from IrITA1. Taken together, the results from the three genes indicate that the clade containing IrITA2 and IrITA3 represents a lineage divergent from the seven described previously (1).

Conclusions

Our results represent the first demonstration of rickettsiae in Italian *I. ricinus* and the first use of molecular-sequence-based methods to identify rickettsiae in Italy. One bacterium, *R. helvetica*, occurs in several parts of Europe and has been implicated as a human pathogen. The other two strains have only recently been discovered in *I. ricinus* from Slovakia and Germany. Whether they are pathogenic is not known, but since other rickettsiae of previously unknown pathogenicity have subsequently been shown to be associated with disease (*R. helvetica* and *R. slovaca* [15]), these new strains warrant attention.

Several studies on rickettsioses in Italy have been published in the last two decades, and they all report *R. conorii* as the causative agent. As MSF is the only known rickettsiosis in Italy, diagnostic tests use *R. conorii* as the only antigen for serologic assays (16,17). However, since SFG rickettsiae cause cross-reactions, confusion about the source of the illness may occur. Although antibiotic therapy is generally effective for all SFG-related diseases, a better understanding of how different rickettsiae cause different symptoms will only come with their correct identification. During 1996–1999 in the regions we sampled, 23 rickettsioses (assumed to be MSF) were reported from Veneto, 42 from Toscana, and 3 from Trentino Province (Italian Ministry of Health, unpub. data). While many were likely to be MSF cases, the possibility exists that some were caused by other SFG (perhaps *R. helvetica*).

Unlike most studies, one serosurvey in northeastern Italy (18) used the complement-fixation test, which is less prone to cross-reactions (19); none of the sera tested was found positive for antibodies to rickettsiae. This finding may be explained by the use of *R. conorii*, *R. rickettsii*, *R. typhi*, and *R. akari* as the only antigens. Serosurveys such as these could therefore benefit from the use of antigens from the bacteria identified in our study. *I. ricinus* is one of the most abundant tick species in Italy, having a very low host specificity and a record of attacking large numbers of humans (8). The results reported here add SFG rickettsiae to the list of potentially dangerous pathogens that Italian *I. ricinus* carry.

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Antibodies to Nipah-Like Virus in Bats (*Pteropus lylei*), Cambodia

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Travis Clemins,* Joe Walston,§
and Thomas G. Ksiazek†

Serum specimens from fruit bats were obtained at restaurants in Cambodia. We detected antibodies cross-reactive to Nipah virus by enzyme immunoassay in 11 (11.5%) of 96 Lyle's flying foxes (*Pteropus lylei*). Our study suggests that viruses closely related to Nipah or Hendra viruses are more widespread in Southeast Asia than previously documented.

A large outbreak of encephalitis among swine farmers in Malaysia occurred from October 1998 to April 1999. Initially, *Japanese encephalitis virus* (JEV), a mosquito-borne pathogen endemic to the region, was suspected as the causative agent. However, a new paramyxovirus, Nipah virus, which is closely related to Hendra virus (HeV), was later implicated as the cause. Unlike JEV, Nipah virus predominated in adults rather than children. Nipah virus cases clustered in members of the same household, suggesting a high attack rate; in contrast, JEV causes symptomatic encephalitis in approximately 1/300 infected persons. A high proportion of Nipah virus patients had direct contact with pigs, unlike others in the same neighborhood who did not have the virus (providing evidence against a mosquito-borne disease); in addition, many of the pigs belonging to affected farmers had an associated history of illness (1–5).

Clinically and epidemiologically, the Nipah virus cases in humans also differed from the few reported HeV infections (6). HeV is transmitted from horses, and two of three patients with HeV infections had severe respiratory involvement; only one patient had severe meningoencephalitis. In contrast, Nipah virus infections involved direct contact with pigs and had predominant central nervous system disease, with only mild or undiagnosed clinical or radiologic evidence of pulmonary involvement. Incubation periods were ≤ 1 month. The main symptom was headache with fever, followed by rapid deterioration in consciousness (1,4).

Nipah virus infection in pigs was frequently asymptomatic or, alternatively, occurred as an acute febrile illness with tem-

peratures $\geq 40^{\circ}\text{C}$, accompanied by signs of respiratory and neurologic disease. Respiratory signs included open-mouth breathing, increased or forced respiration, and a harsh, nonproductive cough. Neurologic signs included head pressing, agitation and biting at bars, tetanic spasms, trembling, and muscle fasciculations (7,8).

Comprehensive studies of domestic animals and wildlife showed that a substantial proportion of Malaysian fruit bats (genus *Pteropus*) had neutralizing antibodies to Nipah virus (7,9). Nipah virus was recently isolated from urine of Malaysian small flying foxes (*Pt. hypomelanus*) (10). HeV was detected in the four *Pteropus* spp. that occur in Australia, with a moderate (20%–25%) prevalence of HeV-neutralizing antibody (11). In addition, HeV was isolated from the grey-headed flying fox (*Pt. poliocephalus*) and black flying fox (*Pt. lecto*) (12). In preliminary studies in Indonesia, antibodies to Nipah-like viruses have been detected in other *Pteropus* spp. (T. Ksiazek, pers. comm.).

The Study

To further investigate the distribution of this new group of viruses, we investigated the prevalence of virus antibodies in other members of the genus *Pteropus* in Cambodia. In restaurants where bats are eaten in Phnom Penh, we collected 2-mL blood specimens from each bat as it was prepared for food. The restaurant owners purchased bats from a hunter who trapped them in Kampong Cham Province and transported them alive to restaurants in Phnom Penh. We stored the whole blood on wet ice for as long as 48 h, then transported it to the U.S. Naval Medical Research Unit No. 2, National Institute of Public Health Laboratory, in Phnom Penh on wet ice, and centrifuged it to separate the serum from the clot. Serum specimens were pipetted into screw-capped plastic vials and frozen at -20°C . Frozen serum specimens were sent from Cambodia to the Special Pathogens Branch, Division of Viral and Rickettsial Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia. On arrival, the serum specimens were tested for antibodies to Nipah virus by enzyme immunoassay (EIA).

Of 96 serum specimens from the fruit bat (*Pt. lylei*), 11 (11.5%) were positive ($\geq 1/10$) for Nipah virus antibodies by EIA. All 11 were confirmed by serum neutralization test. Nine additional sera were found positive (low titers) only by neutralization assay. We also screened sera (when sufficient quantities were available) by neutralization test against HeV. In general, results were equivalent between the two tests. No sera were found positive for HeV and negative for Nipah virus. Our results suggest that the virus circulating in Cambodia is neither Nipah nor HeV, but another closely related virus.

Conclusions

Several species of the genus *Pteropus* show serologic evidence of Nipah or HeV infection. Attempts by several groups to recover virus from tissues of serologically positive bats have been unsuccessful, as have immunohistochemical tests to detect the infection in tissues (9). Several possible reasons

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may account for the inability to recover virus from serologically positive bats. Antibody-positive bats may represent the portion of those infected that survived and cleared the virus. Experimental inoculation of a small number of Australian *Pteropus* bats with a related paramyxovirus resulted in findings that the virus replicates, causes microscopic lesions, and is shed; the virus appears to clear as the antibody response appears (13,14). We did not attempt to isolate virus from blood, and our attempts to detect virus antigen in tissues by immunohistochemical tests in one bat were unsuccessful.

We observed no evidence that HeV (15) or Nipah viruses move directly from bats to humans. However, during the outbreak of Nipah virus encephalitis in Malaysia, several laboratory-confirmed Nipah cases that lacked exposure to infected pigs were identified (P. Kitsutani, pers. comm.). In Cambodia, the distribution of *Pt. lylei* is limited to sites where they are protected from hunting, including urban areas and temples, where the human-bat interaction may be increased. The fact that these large bats are caught and used for food further increases the risk for exposure and infection in humans.

Future studies should include an evaluation of the risk of Nipah virus infection among populations intensely exposed to bats, such as those who capture, transport, slaughter, and butcher bats, as well as bat rehabilitators, animal caretakers, and wildlife conservationists. We suggest that future studies also include a cross-sectional survey of swine in Cambodia. Unlike workers on the large, commercial swine production farms of Malaysia, typical swine farmers in Cambodia raise several swine for their own use and for the local market. The potential for amplification of the virus, unlike that observed in the large concentrated pig population in Malaysia, remains very limited. Finally, a systematic study of encephalitis causes may show whether Nipah virus causes disease in humans in Cambodia and elsewhere in the region.

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Cerebral Aspergillosis Caused by *Neosartorya hiratsukae*, Brazil

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Anna Karenina, †‡ Josepa Gené,* Alberto Stchigel,*
and Arnaldo Lopes Colombo†

We report the first case of infection by *Neosartorya hiratsukae*, an ascomycete in which the conidial state resembles *Aspergillus fumigatus*. The fungus caused a brain infection in a Brazilian woman, who died despite itraconazole treatment. Diagnosis was established by direct microscopic examination, computed tomographic scan, and magnetic resonance imaging of the brain, and repeated cultures from the lesions. The in vitro antifungal susceptibility of the isolate is provided.

Aspergillus fumigatus is the most common filamentous fungus to cause opportunistic infections in humans. Two close relatives of *A. fumigatus*, classified in the ascomycetous genus *Neosartorya*, have been documented to cause occasional opportunistic infections (1). These species are *N. fischeri* and *N. pseudofischeri*. The former has been reported on two occasions as causing systemic infection in transplant recipients (2, 3), as well as a mixed pulmonary infection in a patient with myeloma (4). *N. pseudofischeri* has been reported to cause different localized and invasive infections (5–9). The conidial states of these species are morphologically very similar to that of *A. fumigatus*.

We describe the first cerebral infection caused by another species of *Neosartorya*, *N. hiratsukae*. This taxon has been described only in Japan, where it was isolated from air and from pasteurized aloe juice (10).

Case Report

A 75-year-old Brazilian woman was admitted to the Hospital do Servidor Público Estadual de São Paulo on May 5, 1999, with progressive memory loss, confusion, and involuntary movements in the upper right arm after a fall 1 year earlier. She had also developed gait disorder, with short steps and constant loss of balance that led to a diagnosis of Parkinson's disease in a neurology consultation in March 1999; however, the condition did not respond to the usual treatment. On April 30, a computed tomographic (CT) scan of the brain showed multiple lesions in both brain hemispheres, after which the

patient was referred to the hospital. Past clinical history showed an evaluation of productive cough in 1996, with bloody sputum, night sweats, and intermittent fever; she underwent bronchoscopy with pathologic examination, which showed vascular congestion and focal intra-alveolar edema, but no specific pathogen was identified.

On examination, the patient appeared chronically ill, mildly pale, disoriented, and confused, although she was able to follow simple commands. The lungs had decreased sounds in both lower thirds, with rales. The upper arms moved slowly and repetitively, with a loss of strength. Tendon reflexes were normal. The patient underwent surgical exploration, with drainage of the frontal and occipital lesions. Four samples of a yellowish, dense liquid were collected. Laboratory examination did not show neoplastic cells or neutrophils in the liquid. Direct microscopic examination showed septate hyphae in all the samples. Cultures were negative for aerobic and anaerobic bacteria and mycobacteria, and two samples were positive for a fungus, tentatively identified as *Aspergillus* sp. The patient was initially treated for 21 days with ceftriaxone, oxacillin, and metronidazole without major improvement. When the fungus was isolated, she was treated with amphotericin B and underwent postdrainage magnetic resonance imaging (MRI) of the brain, which showed multifocal brain abscesses, including subtentorial and supratentorial lesions (Figure 1). A chest x-ray and a CT scan of the thorax showed a small, bilateral pleural effusion and a left pulmonary cavitory lesion. Left pleural drainage obtained 80 mL of a clear yellow pleural effusion with pH 7.5, 19,870 leukocytes/mL and 970 erythrocytes/mL, with negative cultures for mycobacteria, aerobic and anaerobic bacteria, and fungi. Refractory hypokalemia developed after the patient received a total dose of 1 g of amphotericin B,

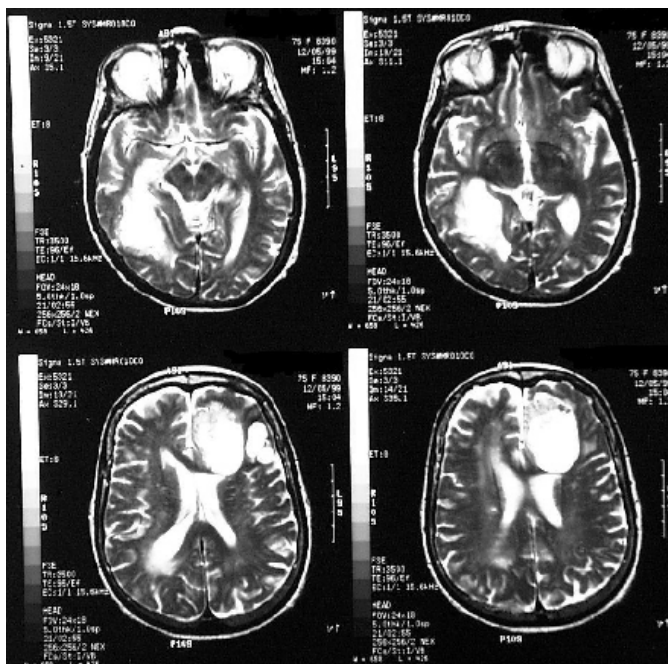


Figure 1. Magnetic resonance imaging of the brain obtained after first drainage.

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which led to discontinuation of this drug. A course of itraconazole (400 mg/day) was initiated.

On the first day of itraconazole therapy, another MRI of the brain was performed, which showed an increase in the volume of the abscesses. During the next few days, the patient's mental state worsened, and she had another cerebral drainage 8 days later. A yellowish opaque liquid, with a white granular deposit, was obtained from the frontal and parietal abscesses. A cerebral fragment was obtained for analysis, which showed only reactional brain tissue. All cultures were negative for bacteria but positive for the same fungus that had been isolated previously. Her clinical and neurologic status improved, and she was discharged to a nursing home on July 20, 1999; itraconazole (400 mg/day) treatment was continued.

The patient was again admitted to the hospital on August 8, and she stayed for 21 days after being diagnosed with urinary sepsis caused by *Escherichia coli*. She was successfully treated with intravenous ceftriaxone. Her neurologic condition had been stable since her previous hospital discharge. She was last seen at the outpatient clinic on September 29, with an unchanged neurologic condition. During the hospital stay, another MRI of the brain showed a decrease in the size of all lesions, which was interpreted as a good response to the itraconazole therapy. Because of the difficulties of maintaining home care, she was admitted to another nursing hospital. Her clinical state deteriorated, and she died in November 1999 from multiple organ failure. Clinical information on this last hospital admission is very limited, and a postmortem examination was not performed.

Cultures obtained on the two occasions yielded molds with identical morphologic features, and one isolate was referred to the Medical School of Rovira i Virgili University in Reus, Tarragona, Spain, for identification purposes.

The isolate was subcultured on Czapek agar and malt extract agar (MEA), and incubated at approximately 25°C in the dark. After 14 days, the colonies on Czapek agar were very restricted (12 mm–14 mm in diameter), velvety, irregularly folded, umbonate, and white to yellowish white, with a pale yellow reverse. Sporulation was absent. On MEA, the colonies developed rapidly, attaining a diameter of 40 mm–46 mm in 14 days. They were velvety, radially folded, white to greenish white, with a pale yellow reverse. Ascospores and conidial heads developed throughout the culture. The fungus grew restrictedly at 45°C.

The microscopic features of ascospores and conidial heads were examined from wet mounts prepared in lactic acid under light microscopy. Ascospores were non-ostiolate, superficial, white to light cream colored, globose or subglobose, measuring 120 µm–600 µm in diameter, and covered with a white aerial mycelium. The peridium was thin and membranous. The asci were eight-spored, more or less globose, and measured 11 µm–15 µm in diameter. The ascospores were hyaline, one-celled, and lenticular, with two closely appressed equatorial crests. They measured 6 µm–7.5 µm x 4 µm–5 µm, including the crests, and their convex walls showed a fine reticulate

ornamentation. Numerous conidiophores of an *Aspergillus* sp. were intermixed with the ascospores. The conidiophores consisted of green to bluish green, uniseriate, short columnar conidial heads over hyaline to light green, smooth and thick-walled stipes, which measured 100 µm–170 µm x 3 µm–4 µm. The conidia were pale greenish, globose or subglobose, 2 µm–2.5 µm in diameter and with smooth or delicately roughened walls.

On the basis of the above characteristics, and especially taking into account the ascospore ornamentation observed under scanning electron microscopy (Figure 2), we identified the isolate as *N. hiratsukae* (10,11). The isolate was morphologically compared with the type strain of *N. hiratsukae* (NHL 3008) and was proven to be the same species (Figure 2A,B). In addition, *N. hiratsukae* is the only species of *Neosartorya* with reticulated ascospores that grow restrictedly on Czapek agar, a characteristic also shown in the case isolate. *N. pseudofischeri*, the most common *Neosartorya* species involved in human infections, is easily distinguished because its ascospore walls are ornamented with raised flaps of tissue resembling triangular projections or long ridge lines (Figure 2C). Living cultures of the case strain are deposited in the Centraalbureau voor Schimmelcultures, the Netherlands (CBS 109356) and in the Institute of Hygiene and Epidemiology, Belgium (IHEM 18438).

The case isolate was tested to determine its susceptibility to five antifungal drugs. Tests were carried out by a microdilution method described previously (12) and adapted from the reference method for molds recommended by the National Committee for Clinical Laboratory Standards (13), with RPMI 1640 medium buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid, an inoculum of 9.1×10^5 CFU/mL, an incubation temperature of 30°C, a second-day reading (48 h), and an additive drug-dilution procedure. MICs and minimum fungicidal concentrations (MFC) were as follows:

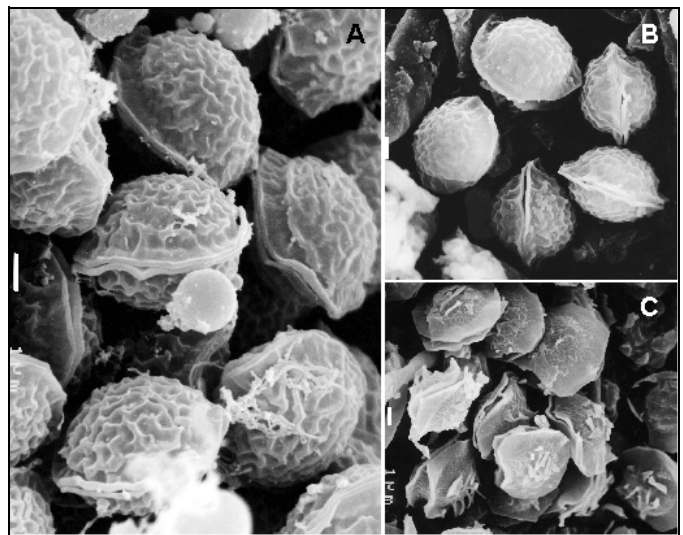


Figure 2. Ascospores of *Neosartorya hiratsukae*, CBS 109356 (A) and NHL 3008 (B), and of *N. pseudofischeri*, NRRL 3496 (C), under scanning electron microscopy. Bars A, B, C = 1 µm.

amphotericin B 1 and >16 µg/mL, flucytosine 64 and >64 µg/mL, itraconazole 0.25 and 0.25 µg/mL, voriconazole 0.25 and 0.5 µg/mL, and UR-9825 0.06 and 0.5 µg/mL, respectively. Results demonstrated good activity of the four-azole derivatives tested. UR-9825, a novel triazole not yet licensed, showed the lowest MIC. MFCs of amphotericin B and flucytosine were very high, indicating the ineffectiveness of these drugs. These data correlate with our clinical results since a total dose of 1 g of amphotericin B was unable to reduce the brain abscesses, while the patient responded well to itraconazole at daily doses of 400 mg.

This case report is important because such clinical isolates of *Neosartorya* spp. that produce white colonies and do not become green, like colonies of *A. fumigatus*, are often discarded as contaminants. Therefore, the real incidence of aspergillosis caused by *Neosartorya* species could be underreported. Many *Neosartorya* species, other than *N. fischeri* or *N. pseudofischeri*, are thermotolerant and can grow at temperatures above 37°C, showing their inherent ability to invade the brain.

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Cyclosporiasis Outbreak in Germany Associated with the Consumption of Salad

Peter C. Döller,* Karl Dietrich,† Norbert Filipp,†
Stefan Brockmann,‡ Caroline Dreweck,‡
Reinhard Vonthein,§ Christiane Wagner-Wiening,‡
and Albrecht Wiedenmann¶

This outbreak is the first foodborne cyclosporiasis outbreak reported from central Europe. The illness was reported in 34 persons who attended luncheons at a German restaurant. The overall attack rate was 85% (34/40). The only foods associated with significant disease risk were two salad side dishes prepared from lettuce imported from southern Europe and spiced with fresh green leafy herbs ($p=0.0025$).

Cyclospora cayetanensis, a protozoan parasite, which was named and classified by Ortega et al. in 1994 (1), is endemic in geographic regions with warm or tropical climates (2,3). Cyclosporiasis typically has onset after an incubation period of approximately 1 week and is characterized by protracted and often relapsing gastroenteritis. Treatment is with trimethoprim-sulfamethoxazole (4). After two nationwide outbreaks of cyclosporiasis linked to raspberries imported from Guatemala (5) occurred in the United States and Canada in 1996–97, reports speculated that imported food could also cause outbreaks or sporadic infections in other regions with a temperate climate, such as central Europe (6,7).

The Study

On December 13 and 14, 2000, four independent parties of 6, 7, 7, and 20 persons attended luncheons in a restaurant in southwest Germany. From December 29, 2000, to January 18, 2001, some of these persons contacted local health authorities because of protracted, sometimes relapsing gastroenteritis symptoms. After several stool specimens were negative for routine bacteriologic, virologic, and parasitologic tests, the patients who were still having gastrointestinal symptoms were referred to the outpatient department of a tropical medicine clinic for an examination for intestinal protozoa. *C. cayetanensis* was detected with a modified Ziehl-Neelsen technique in stool smears of 9 of 19 persons (8 attendees of the luncheons

and the owner of the restaurant). The first laboratory-confirmed diagnosis was made 27 days after the peak of the outbreak, when the number of excreted oocysts still detectable in the stool smears was moderate or low. Confirmatory tests (epifluorescence microscopy, differential interference contrast, and object measurement with an electronic image analysis system) were performed at the State Health Office in Stuttgart and the Institute for General and Environmental Hygiene of the University of Tübingen (Figure 1).

All 40 attendees of the luncheons were asked to complete a questionnaire that included questions about age, gender, travel history, food items and beverages consumed at the luncheons, onset and duration of symptoms, physician consultation, examination of stool samples, antibiotic treatment, and days absent from work.

Using the criteria established by Herwaldt et al. (5), we defined cases of clinical cyclosporiasis as illness in persons who began to have at least one gastrointestinal symptom (diarrhea, flatulence, weight loss, nausea, abdominal cramps, or vomiting) 12 hours to 14 days after the event. Patients in whom typical oocysts were detected in at least one stool sample were defined as having laboratory-confirmed cases.

The statistical analysis was performed with the software packages Epi Info Version 6.04 (Centers for Disease Control and Prevention, Atlanta, GA) and StatXact 3.0.2 (Cytel Statistical Software, Cambridge, MA). Univariate relative risks with exact 95% confidence intervals and two-tailed p values of the unconditional test were calculated according to the procedure described in the manual of StatXact 3.0.2. (8).

According to initial reports from the four groups who had attended the luncheons, the overall attack rate was 85% (34 of 40 persons). Thirty of these 40 persons participated in the retrospective cohort study. Twenty-six persons had clinical cases; eight had laboratory-confirmed cases; and four did not become ill. The attack rate in the study participants was 87% (26 of 30 persons), i. e., the ratio of ill to non-ill persons in the retrospective cohort study was not substantially distorted. All participants were adults 22–65 years of age; 12 persons were men and 18 were woman.

The epidemic curve of the outbreak is illustrated in Figure 2. The median incubation time was 8 days (range 5–14 days). Symptoms occurred with the frequencies listed in the Table. The median duration of symptoms was 25 days (range 15–42

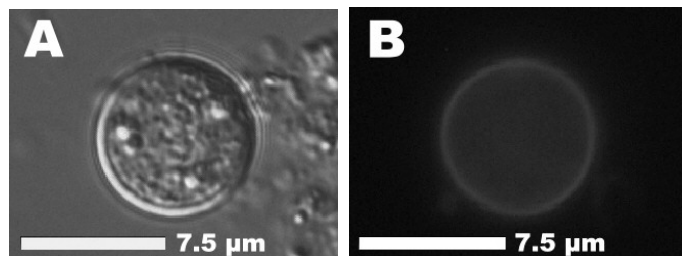


Figure 1. Unsporulated oocyst of *Cyclospora cayetanensis* in an unstained stool preparation. A) Differential interference contrast. B) Same oocyst with typical blue autofluorescence (Filter sets: 365-nm excitation, 395-nm dichroic mirror, 420-nm suppression).

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Table. Symptoms associated with 26 cases of cyclosporiasis during a foodborne outbreak, Germany, December 2000

Symptom	No. with symptom / no. with available data (%) ^a
Diarrhea ^b	24/25 (96.0)
watery	23/24 (95.8)
mucus	11/18 (61.1)
bloody	0/18 (0.00)
Flatulence	21/22 (95.5)
Weight loss	23/25 (92.0)
Nausea	22/24 (91.7)
Abdominal cramps	19/24 (79.2)
Vomiting	10/19 (52.6)
Fever	9/21 (42.9)

^aThe denominators for symptoms vary because some respondents reported not being sure whether a symptom occurred.

^bDiarrhea was defined as >3 bowel movements per day.

days; n=19). The outbreak caused a total of 80 days off work (range 2–24 days; n=12). Only four patients who consulted a physician before January 18, the day the first case was laboratory confirmed, had already received antibiotic treatment. At least one of them received a drug (amoxicillin) without documented effectiveness against *Cyclospora*. All patients with a laboratory-confirmed diagnosis of cyclosporiasis received a 7-day treatment of trimethoprim-sulfamethoxazole, starting immediately after diagnosis had been made.

Frequencies, relative risks, and 95% confidence intervals were calculated for 12 main courses, 3 side dishes, 12 beverages, and 2 desserts; all items had been consumed by the 30 study participants. The only food item that showed a clear, statistically significant association with disease (relative risk [RR]=5.0 [1.4<RR<204], p=0.0045) was a salad side dish (side dish 1). All 25 persons who ate this salad became ill. The salad consisted of several sorts of vegetables (butterhead let-

tuce, mixed lettuce, red cabbage, white cabbage, carrots, cucumbers, and celery). Mixed lettuce (German: "Mixsalat") was a designation for a commercially available mix of four varieties of green and red lettuce (lollo rosso, lollo bianco, oak leaf, and romaine lettuce). The other available salad (side dish 2) had been prepared with only two components: butterhead lettuce and mixed lettuce. Eight participants had chosen salad 2 and only one was not affected. Six of them had eaten salad 1 as well. Accordingly, one participant was affected who had only eaten salad 2. The three persons who did not eat any salad did not become ill. Therefore, the vehicle for the transmission of *Cyclospora* oocysts must have been one of the components common to both salad 1 and salad 2, i.e., one or more of the lettuce varieties or the fresh green leafy herbs (dill, chives, parsley, green onions) used to spice both salads. Chervil, which was also used for flavoring, had been stewed and can therefore be excluded. The pooled data for side dishes 1 and 2 (consumption of salad 1, salad 2, or both) showed a highly significant association with disease (p=0.0025; [1.4<RR<∞]). As none of the participants who had not eaten any of the two salads became ill, the exact RR risk for either salad is not defined (denominator is zero). Neither of the two available dressings was associated with the disease. The hypothesis that all the main courses were uniformly contaminated, e.g., by the cook, was also tested, but no significant association was found. Only one participant had a history of traveling abroad in the 2 weeks before the luncheons.

The sources of implicated food items were traced through the analysis of invoices and delivery notes in the files of the restaurant owner and of retailers, wholesalers, and importers (for two lettuce batches, dill, parsley, and green onions) or by verbal report (chives).

The batches of butterhead lettuce and mixed lettuce went through four separate trading stages from the producer to an exporter/importer, a wholesaler, and a retailer before being bought by the restaurant proprietor. The butterhead lettuce batch had been grown in southern France, while the mixed lettuce batch had been grown in the province of Bari in southern Italy. Dill, parsley, and green onions were also grown in southern Italy (Naples, Eboli); only the chives had been grown in a greenhouse in Germany. Samples of the implicated batches were no longer available for microbiologic examination.

Conclusions

To our knowledge, this foodborne outbreak of cyclosporiasis is the first reported from central Europe. Mesclun salad mix (also known as spring mix, field greens, or baby greens—a mixture of various types of baby lettuce leaves) and fresh basil have been involved in outbreaks in the United States (9,10). *Cyclospora* oocysts have also been isolated from lettuce in Peru (11), and Egypt (12) and from green leafy vegetables in Nepal (13). As *Cyclospora* must sporulate for at least 7 days at 25°C–30°C in the environment to become infectious (14), direct person-to-person or person-food-person transmission in a restaurant is nearly impossible. During the winter season in

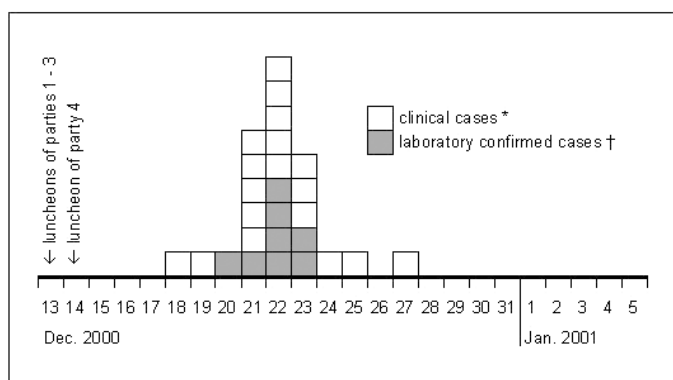


Figure 2. Epidemic curve of an outbreak of cyclosporiasis occurring in attendees of four luncheons in a restaurant in Germany in December 2000. Results for the 26 cases in a retrospective cohort study. *Defined as having reported any of these symptoms: diarrhea (>3 bowel movements per day), loss of appetite, weight loss, flatulence, abdominal cramps, nausea, vomiting. †Laboratory confirmation by detection of *Cyclospora* oocysts in at least one stool sample by a modified Ziehl-Neelsen technique.

Germany, domestic sewage, surface water, and drinking water would not meet the temperature requirements for the sporulation process.

Therefore, the most probable routes of contamination of one or more of the implicated vegetables are fertilization with human waste or fecally contaminated water used to irrigate crops, prepare pesticides, or freshen or clean produce at their origin (15). In addition to contamination of field crops through the water route, we considered that seasonal field workers often do not have access to appropriate sanitary facilities.

The outbreak we described may represent a much larger problem. Public health offices and laboratories, general practitioners, and medical microbiology labs should be alerted to the fact that *Cyclospora* infections in central Europe can no longer be regarded as solely travel related. Physicians should be aware of the typical symptoms, the diagnostic methods, and the medical treatment of this emerging pathogen.

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Seroepidemiology of Human Enterovirus 71, Singapore

Eng-Eong Ooi,* Meng-Chee Phoon,† Baharudin Ishak,† and Soh-Ha Chant†

Human enterovirus 71 has caused outbreaks in many parts of the world, especially Southeast Asia, with some fatal cases. The epidemiology of this viral infection is not well understood. We conducted a serologic survey in Singapore children, and the results indicate that infection occurs largely in preschool settings.

Human enterovirus 71 (HEV71) is an emerging concern in many parts of the world. It has caused several large outbreaks, occasionally associated with many deaths in children (1–3). In September and October 2000, a large nationwide outbreak of hand, foot, and mouth disease (HFMD) caused by HEV71 occurred in Singapore (4). Most of the cases were in children <6 years of age; four cases were fatal.

The epidemiology of HEV71 infection in Singapore and most parts of the world has not been well studied. A few reports suggest that HEV71 infection is common and mostly subclinical (5,6). Differences in the DNA sequences of the HEV71 isolates do not appear to play an important role in clinical outcome (7,8). Ho and colleagues (3) cited preliminary evidence indicating that more than half the adult population in Taiwan had been exposed to HEV71 before the 1998 epidemic.

The settings where most HEV71 transmission occurs are, however, uncertain. During the 2000 outbreak of HEV71-associated HFMD in Singapore, a decision was made to close all preschool centers nationwide in an effort to break the chain of transmission of this virus (4). The effectiveness of this control measure is unclear. HFMD was made a legally notifiable disease on October 1, 2000, which coincided with both the middle of the HEV71-associated HFMD outbreak and the closing of the preschool centers in Singapore (4). Although the closure of preschool centers was thus temporally associated with a decline in reported HFMD cases, no comparable data were available before and after the implementation of the preschool closure to allow assessment of the impact of this measure.

To devise appropriate preventive measures, the transmission of this virus in the natural setting needs to be determined. A serologic survey would be useful for this purpose. Such a study in children had not been conducted in this region. We report the findings of an HEV71 serologic survey in Singapore children.

The Study

We surveyed 856 children ≤ 12 years of age. Serum samples were collected, with informed parental consent, during the 18-month period of July 1996 to December 1997 at a pediatric clinic at the National University Hospital, which serves the entire country. All children who were born at the hospital or who were brought for routine visits and vaccinations during this period were included. The children had no sign of disease at the time of sample collection.

The serum samples, which had been used in a previous study of dengue (9), were divided and stored at -20°C and inactivated at 56°C for 30 min before use. Neutralizing antibody against HEV71 (EV71/7423/MS/87), an isolate that had been characterized previously (10), was detected by using a neutralization test by microtechnique as previously described, with modifications (11). Sample dilutions of 1:8 to 1:2,048 were assayed. Twenty-five microliters of 100 tissue culture infective dose (TCID₅₀) virus was mixed with 25 μL of the appropriate serum dilution and incubated for 2 h at 37°C in a CO₂ incubator, followed by the addition of 100 μL of rhabdomyosarcoma cell suspension at a concentration of 2×10^4 cells/0.1 mL. Each dilution was tested in duplicate. Readings were taken visually with an inverted microscope after 6 days of incubation at 37°C in 5% CO₂. The antibody titer for the sample is the highest dilution that prevents the development of cytopathic effects in both wells. An antibody titer of ≥ 8 was considered positive. The geometric mean titer (GMT) and 95% confidence intervals (95% CI) were also calculated. Statistical analysis was done by Student *t* test.

The results showed that 44.0% of mothers had antibodies to HEV71 (deduced from antibody prevalence in cord blood), which waned rapidly so that after 1 month, none of the children tested had maternal antibodies to HEV71 (Figure 1). Only 1 (0.8%) of the 124 samples from children ages 1–23 months had anti-HEV71 antibodies. From 2 to 5 years, the seropositive rate increased at an average of 12% per year. In samples from children ≥ 5 years old, the age-specific seroprevalence reached a steady state at approximately 50%.

Figure 2 shows the age-specific GMT in seropositive samples by age group. Formal schooling in Singapore begins at 6

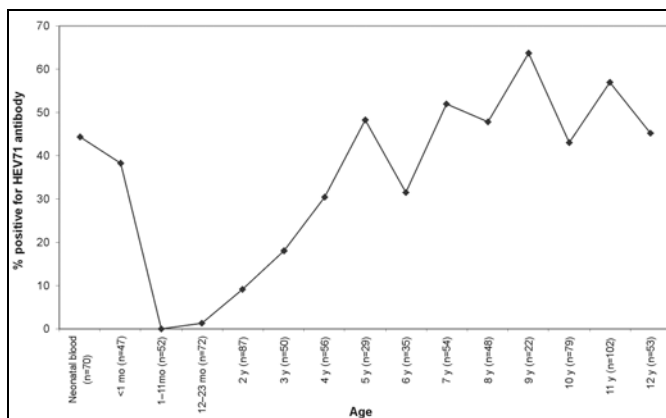


Figure 1. Seroprevalence rate of anti-Human Enterovirus 71 (HEV71) antibodies.

*National Environment Agency, Singapore; and †National University of Singapore, Singapore

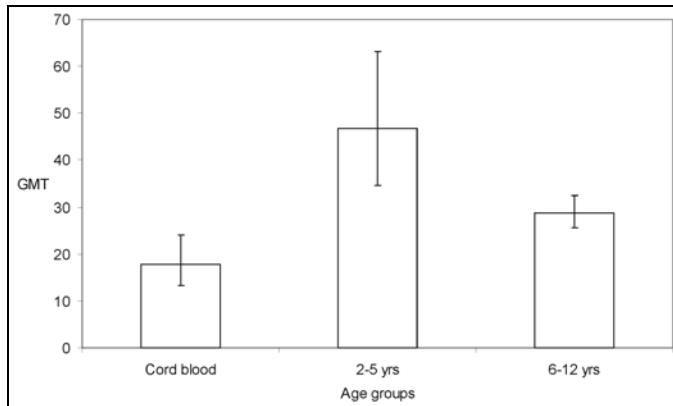


Figure 2. Geometric mean titer (GMT) of anti-Human Enterovirus 71 (HEV71) antibody with 95% confidence intervals in children of different age groups.

years of age. From 2 to 5 years, however, most children receive preschool education in child-care centers or kindergartens. The GMT of the anti-HEV71 antibodies was higher in preschool children (GMT 46.8; 95% CI 34.7 to 63.1) than in those of formal school age (6–12 years old) (GMT 28.8; 95% CI 25.7 to 32.4). The difference in these two means was statistically significant (t test = 3.07; df = 240; p =0.002). Furthermore, the GMT titer of maternal antibody in neonatal blood (GMT 17.8; 95% CI 13.2 to 24) was also significantly lower than in the preschool children (t test = 4.02; df = 78; p =0.0001).

Conclusions

This report is the first detailing the seroepidemiology of HEV71 in Singapore children. The results indicate that most infection occurs in preschool-aged children. The concentration of this susceptible group of the population in classrooms, along with the sharing of toys and other teaching tools, are factors contributing to HEV71 transmission.

The differences in GMT in the different age groups indicate that infection outside these preschool years is uncommon. This finding is supported by the following observations: 1) the small proportion of children <2 years old who were seropositive; 2) the proportion of seropositive children reached a steady state after 5 years of age; and 3) the GMT of anti-HEV71 antibody declined with age. If substantial levels of transmission occurred at home, GMT would be expected to increase with age, as reexposure to HEV71 would boost the antibody levels in both children and mothers. Transmission at home would also result in high maternal antibody titers that last for at least 6 months, as observed in our previous study of dengue (9). Furthermore, a fair proportion of children <2 years old would also be expected to be seropositive. Instead, the results of this study indicate that HEV71 is transmitted in preschool centers, where older children and adults are less likely to spend substantial amount of time. The control of HEV71 infection should thus be specifically focused at such places. The cost-effectiveness of implementing preventive measures such as strict observation of handwashing in a preschool setting in Singapore should also be evaluated.

For this study, we used serum samples collected during 1996–1997. In 1997, an outbreak of HEV71 in children occurred in Malaysia that resulted in several deaths (1,2). In Singapore at that time, HFMD was monitored on the basis of volunteer reporting from the operators of preschool centers. This surveillance allowed 12 localized clusters of HFMD to be identified (12). Virus isolation studies were also carried out on samples from the children who attended these centers. Coxsackie virus A16 was isolated in three of these clusters, and HEV71 was isolated in one. No viruses were isolated in the other clusters. No HEV71-related deaths were recorded during this period. These observations indicate that HEV71 was present during or before the time of sample collection, although no nationwide outbreak was observed during that time.

This study is confined in scope to Singapore children. To support the hypothesis that HEV71 transmission occurs largely in the preschool setting, a serologic study of preschool teachers and directors would be very useful. Furthermore, as the mode of transmission of enteroviruses is essentially the same, by the fecal-oral route, the serologic profile for the other enteroviruses may be the same as that observed in this study. No such data are currently available.

In conclusion, HEV71 infection is common in Singapore children and is acquired largely in the preschool years. Transmission of this virus is lower in the other age groups. This finding suggests that HEV71 transmission occurs mainly in places where preschool children congregate, and public health measures to control the spread of this virus should focus on these places.

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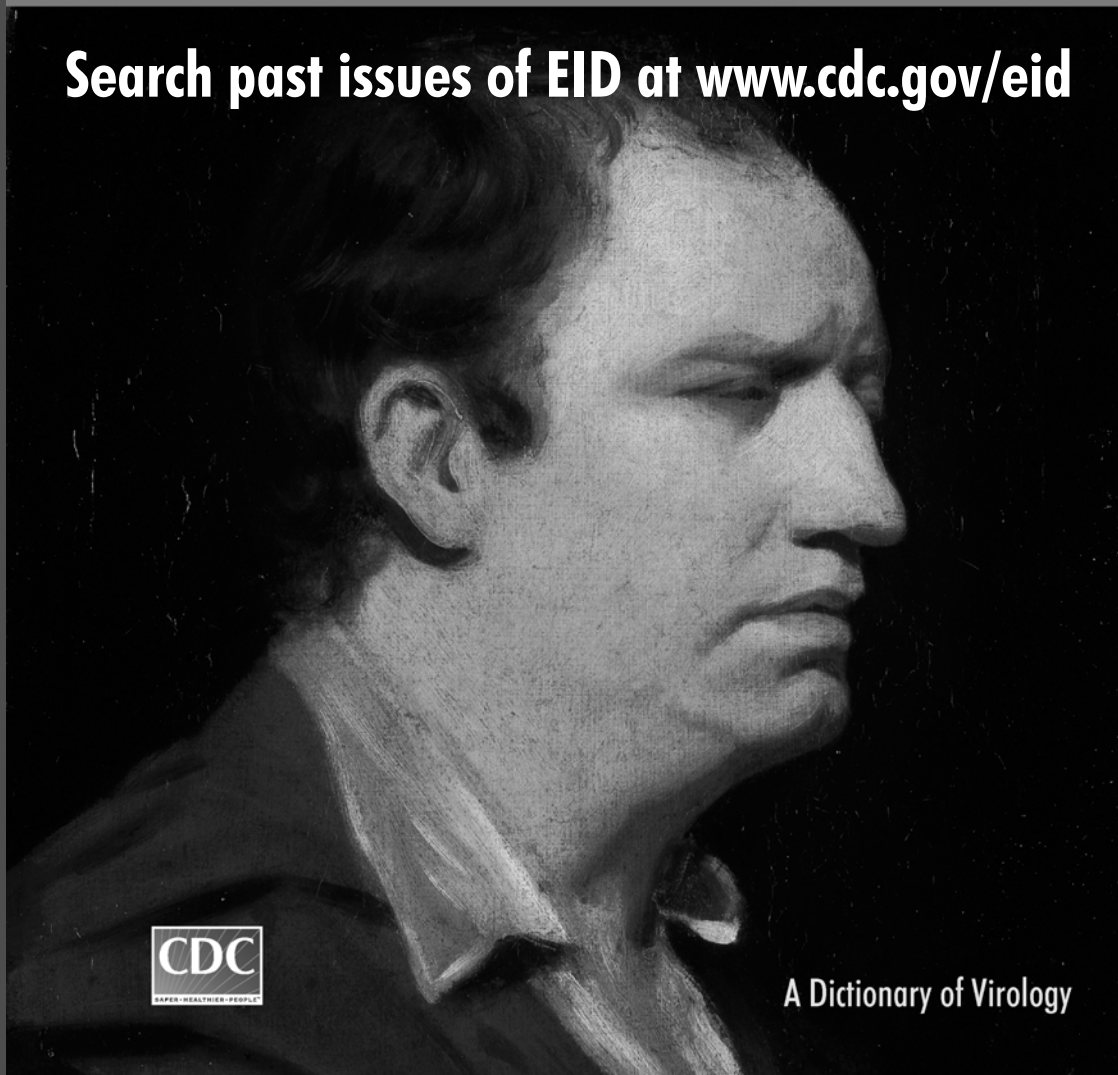
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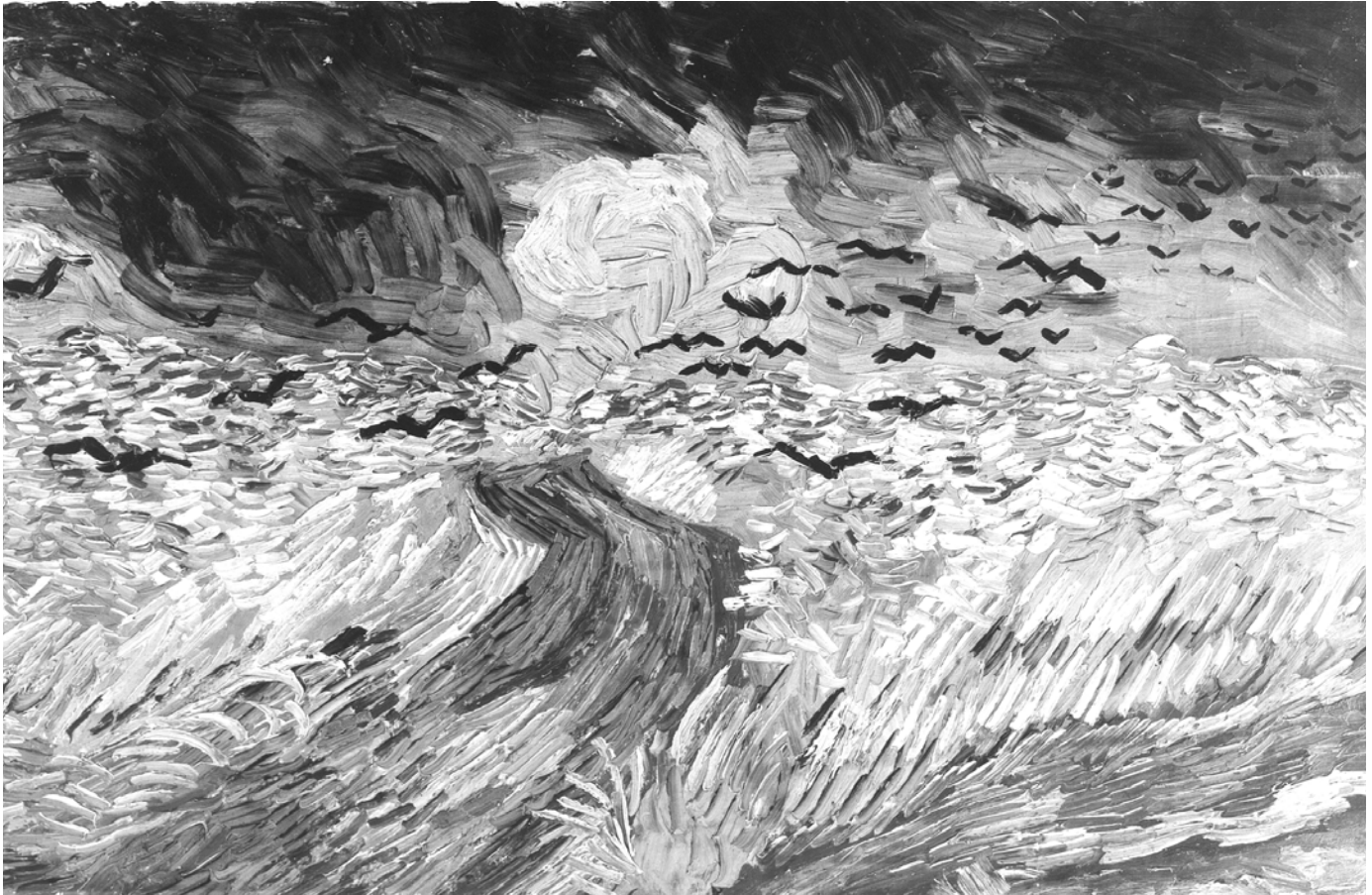
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A Dictionary of Virology



Vincent van Gogh (1853-1890) "Crows in the Wheatfields" 1890. Oil on canvas, 50.35 cm x 103 cm. Amsterdam, Van Gogh Museum (Vincent van Gogh Foundation)

Madness

Gerald N. Callahan

There are clouds in the painting, of course. Almost any one of us would have included those clouds, thick with electricity and rainwater. And there is the wheat field, smudged out like an empty palm, orange beneath the storm-stricken sun. Surely, many of us would have insisted on the wheat as well. Through the middle of the wheat, a rutted road slices to the horizon and disappears beneath the clouds. Even I, a scientist, would have included the road. A storm like that demands a road. Without the road, there is no hope at all. But then there are the crows—the one true hint of what had been and what was to come—fistfuls of them, flung into the swirls beneath the angry wet anvils. All that the painter had lost, irretrievably lost, he put inside those crows.

Van Gogh died because of an instant (or a lifetime) during which the portrait of his life appeared worse than the portrait of his death. Died because his pictures filled up with crows. We call that a behavioral disorder because we imagine healthy people don't see the crows, healthy people don't choose death over life. And we say that behavioral disorders are caused by

"mental" diseases to distinguish them from "real" diseases— infections, tumors, broken bones, burst blood vessels, polio. Real diseases are diseases of the *body*.

We do that—razor medicine off at the neck—because people such as René Descartes and Pope Urban VIII contended that the human soul resides in the mind, and human disease resides in the body. Sometimes because of that contention, we believe people with mental diseases are less genuinely ill than people with somatic diseases. Sometimes we even believe that people with mental diseases and behavioral disorders suffer more from weakness of spirit and flaws of character than from genuine disease. Beneath our collective breath, we say that the crows are inside their heads, and having said that, we imagine that the crows are not real.

My Uncle Henry had a habit of leaving his fly unzipped, completely unzipped, regardless of who might be around to notice. My mother, his sister, hated that. Henry's underpants were usually urine-stained, his shirt tails hung out of the opening in his pants, and he had a propensity to yell "shit" and to

spit for no apparent reason. Mother hated that too. And because she imagined that Henry's eccentric behavior was concrete evidence of his total disregard for others, especially her, mother raised up a little hatred for Henry himself. Over the years, that hatred blossomed inside her and bore seed.

I don't think Henry ever noticed how much mother despised him. At times, I'd watch him look at her with his sea-blue eyes, and I'd see something back there in the hollows. Whatever it was, though, it wasn't shame, resentment, anger, or even understanding. Henry's been dead now for more than 30 years, but mother still gets angry whenever I mention his name. I don't understand that. Henry, I imagine, tried mother's patience at times. But I'm certain he never intended to haunt her for 3 decades after his death. In fact, I don't think Henry intended much of anything, at least not toward the end.

Mother, though, is still tied to Henry, years after he cut his ties with everything and was laid to rest. And if she could explain today why she so despises Henry, mother would tell you it is because he was dirty and crazy. What she wouldn't tell you (but you might discern if you listened to her for a moment or two) is that she believed that had Henry cared to, he could have stopped being crazy, just as easily as he could have stopped being dirty. Filth and craziness were just his ways of getting to her and making her life difficult.

Instructional Film—Scene I

A bucolic panorama somewhere in, say, Nigeria. The sky is first-morning blue, and a breeze tinged with wood smoke is ruffling the tall grass. There are cattle in the grass. A few are grazing, but most are standing or lying down lazily swatting flies with their tails. At first, the cattle are the only animals we see. But as the camera zooms in, we notice that the grass where the cattle sit or graze is home to brown ants and a few land snails, which at first glance seem in danger of being eaten by the cattle. As we watch longer though, we see that both ants and snails stay low enough in the grass to evade the foraging ruminants—idyllic, mutualistic Nigeria. Fade to black.

Instructional Film—Scene II

The camera's eye reopens a few miles to the north. The sun has fallen beyond the horizon, and the breeze wrestling with the grass has cooled. Most of the cattle here are grazing. As the camera closes in, we notice something oddly different from Scene I. Below the cattle, many of the ants have worked their way up the shafts of grass and appear to be waiting for something. As we watch in cinematic magnification, a pink tongue, large as a python, wraps itself around several insect-encrusted blades of grass, and brown ants (lots of them) suddenly disappear behind a huge set of cud-scarred teeth. Again, fade to black.

Let's consider, for a moment, what we have just witnessed. In Scene I, the ants are cautious, responsible, and sane. In Scene II, the ants are none of those things. We are tempted, briefly, to say that the ants in Scene II are mentally ill. But we don't say that, because we don't imagine that ants have

minds—at least not like human minds—so by definition, ants cannot be “mentally” ill. True or not, our belief serves a purpose; it cuts away some of the gauze that surrounds behavioral diseases in humans. That's useful because the ants in Scene II clearly *are* crazy, whether we are comfortable with the words or not. They have climbed where they know they shouldn't have and remain there in reckless disregard of the danger. They have lost the ability to care for themselves and seem to no longer value life more highly than death. These ants are insane, deranged, imbalanced, nutso. Men and women who behave similarly line the lavatory-colored halls of our country's mental institutions from Passaic to Seattle.

Still, we don't look for history of child abuse in ants, discuss ant toilet training, or accuse ants of character flaws and laziness. And we don't imagine that ants are crazy or that their problems are all in their heads because, after all, these are ants. But if “mentally ill” isn't accurate, what should we call them? Surprisingly, the answer to that question has come, not from psychologists or psychiatrists but from microbiologists, specifically parasitologists. And tedious dissections, not discussions, showed the way.

In Scene II (the one with the self-destructive ants), there was another actor (one we couldn't see) at work inside every character portrayed. Because it is a very small actor, it is perhaps understandable that it escaped our attention. Everyone in Scene II was infected with a microscopic, lance-shaped fluke, a trematode called *Dicrocoelium dendriticum*.

D. dendriticum is a parasitic flatworm. Parasitism is one of the oldest and most venerated ways of life on this planet. Living things have evolved to parasitize nearly all other living things—plants, animals, or microorganisms. Parasites themselves can be parasitized by smaller, but equally devious, life forms. And the animals and plants that do the parasitizing are as varied as their hosts. All things that parasitize animals fall into two groups, protozoa and helminths. Protozoa are single-celled animals such as *Plasmodium falciparum*, which infects nearly one-third of the world's population and causes malaria. Helminths are worms—round, flat, and tape. *D. dendriticum* is a flatworm (or fluke as flatworms are sometimes called).

In spite of their amazing variations, parasites have one characteristic in common: they cannot reproduce themselves outside their hosts. Parasites have lost the ability to perform one or more vital functions usually related to collecting or digesting food. Inside an animal's body, the host takes care of these functions for them; outside the hosts, the parasites often die. Many parasites have evolved complex life cycles that help meet this uniform need in ways only parasites understand. These cycles involve several hosts and stages of development and necessarily feed back on themselves, so parasites end up in the same hosts where they began the cycles, and the whole process starts over.

D. dendriticum is like that. In fact, among parasites, *D. dendriticum* has one of the more interesting life cycles. Life begins for this fluke in the bile ducts of grazing cattle. This is where the adult flukes lay their eggs. Bile is produced in the

liver and is transported, via the bile duct, to the intestine, where the bile aids in the digestion of dietary fats. In infected cattle, when the bile moves from the liver to the intestine, it takes *D. dendriticum* eggs along with it. A short while later, the eggs (along with the cow's or bull's feces) find their way onto the grasses underfoot. There, snails with inexplicable tastes ingest the cattle's feces and the parasite's eggs. Inside the snails, the eggs of *D. dendriticum* hatch, pass through two sporocyst stages of their lives, transform into another life stage called cercaria, and migrate to the respiratory chambers of the snails. Inside their respiratory chambers, snails make slime balls to aid movement across the fields. When the slime balls are secreted onto the snails' feet, so are the cercaria. As the snails make their way toward whatever it is that draws snails, the slime balls are left behind in the grass.

Slime balls are ant food. When ants eat them, they also eat the cercaria of *D. dendriticum*. Inside the ants, most of the cercaria encyst in the walls of the abdomen, but one or two migrate to the head and encyst in the subesophageal ganglion, a part of the brain. Here the cercaria transform into another life stage called metacercaria. Unlike the metacercaria left behind in the abdomen, these never become infective. These metacercaria do something else. They drive their hosts mad.

As evening approaches and the air temperature drops, ants infected with *D. dendriticum* do not return to the colony along with their fellow workers. Instead, the infected ants climb to the tops of surrounding grasses, clamp their mandibles into the grass blades, and remain there, immobile, until the morning sun warms them again. When that happens, the ants (at least those who survive) resume their normal behavior—until the following evening.

Temporary insanity. "Temporary" because it lasts only as long as the sun is down. "Insanity" because the timing of the ants' indiscretions corresponds exactly to the feeding cycles of the grazing cattle who feed most vigorously during the late evenings and early mornings. But here, the grasses are filled up with mad beings that suffer not from poor toilet training or moral and spiritual turpitude but from an infectious disease. Parasitic madness. Madness with a past and a purpose.

Each night beneath the African moon, crazy ants perch atop the grasses of Nigeria and wait for the cracked molars of hungry cattle to end a mad ritual. When the madness is complete and the ants are finally eaten, *D. dendriticum* completes its complex life cycle, and the arduous trip from cow to ground to cow closes once more. Inside the cow, digestive juices strip ant from parasite, and while the scene fades to black, life begins again, minus a few crazy ants.

Elsewhere in Nature

In the jungles of South America, there is another dance between ants and parasites. The parasite is a mushroom. Beneath tropical canopies, spores of *Cordyceps*, a mushroom, are whipped about on warm equatorial breezes and spun between tree trunks and twisting vines until they land in the spiracles of black ants. Spiracles (holes in the tough exoskele-

ton of ants) allow ants to breathe. *Cordyceps* uses the spiracles to get beneath ants' skin. Once inside the ants, the fungus attaches to the soft tissues and begins to raise a family. For a few days, everything is fine, but the fungus knows that it will need more than the ants can provide. Soon, deep within the ants, the fungus will achieve sexual maturity, and it will be time to sporulate. Once again the infected ants, driven by the new-found energy of sudden acrophilia, leave the relative safety of the earth, climb atop the grass, clamp their mandibles onto the tips of the green shoots, and hang there. The fungus then consumes the ants' brains and sprouts through the emptied skulls. Bathed in sunlight, once again, the fungus sporulates. At the grass tops, where the wind blows freely, the spores are quickly spread, sometimes for miles and always to other ants. The fungus has lifted itself from the primordial slime, gathered itself upon the wind, and set off, once more, for a new life. Other varieties of *Cordyceps* mushrooms parasitize and alter the behavior of caterpillars, mealybugs, and beetles. Fungal madness. Infectious insanity.

And then there's the odd story of *Wolbachia*. Most of us, I think, believe that genetics and evolution pretty much pre-terminate how we will reproduce ourselves. It seems unlikely that we have a choice about whether we procreate by mating with members of the opposite sex (as humans do) or by occasionally splitting ourselves in two (as bacteria usually do). But it turns out that though an animal's reproductive behavior may not be of his or her own choosing or a matter of genetics, evolution, or physiology either.

Some time ago, entomologists studying wasps and wood lice (which most of us call sow bugs) noticed that some species of these insects reproduce parthenogenetically, that is, without males (in fact without mating) and produce only (or mostly) female offspring. The entomologists concluded that these wasps and wood lice had evolved this method of reproduction to gain some advantage beyond our current understanding of biology—not to mention pleasure.

The entomologists were wrong. This sort of sexual behavior in wasps and wood lice isn't normal. It's a disease, an infectious disease. Entomologists leapt to the wrong conclusion because of something they couldn't see, something hidden inside the wasps and the wood lice. *Wolbachia pipientis*, a bacterium and obligate intracellular parasite, lives in the ovaries and testes of many insect species. As many as 16% of insects (some 2 to 5 million species) may be infected with one strain or another of *Wolbachia*. But the bacterium is only transmitted vertically (mother to offspring), so only female insects can transmit the infection. To limit the number of male offspring, *Wolbachia* has developed ways to manipulate its host's sex life.

Wolbachia interferes with the production and function of hormones and changes infected male wood lice into female wood lice. In other insects, the bacterium induces a state of "cytoplasmic incompatibility" between males and females, which prevents males and females from any productive mating. And in some wasps, it has completely eliminated males

from the species. For these wasps to survive, the females must resort to parthenogenesis, and under these conditions, they can produce only female offspring. *Wolbachia* isn't the only bacterium capable of this sort of sex selection by elimination of males or alteration of male behavior. At least five other species of bacteria similarly eliminate males from insect species to accelerate bacterial transmission in the wombs of females.

Males turned into females, entire species of sexually inept insects, species of insects in which males have disappeared altogether—all this aberrant behavior, all of these “behavioral disorders,” can be cured with antibiotics, eliminated completely by any of a number of drugs that destroy bacteria. Yes, but that's ants and wood lice. Bugs. Mammals are a lot more complex than ants and wood lice, aren't they?

Rats are intermediate hosts for another parasite, a single-celled protozoan called *Toxoplasma gondii*. *T. gondii* begins and ends its life cycle in domestic cats. The immune response that cats mount against this parasite forces the parasite into very tough cysts that are shed in cats' feces. The cysts survive in soil for years waiting for an intermediate host, a rat, to eat them. Inside the rats, *T. gondii* resumes its life cycle. The ultimate goal of the parasite is to complete the cycle by returning to its primary host, the cat. Cats do not have a great fondness for dead animals. So *T. gondii* doesn't kill its rodent host. But rats have a general fear of cats and avoid the scent (and urine) of cats at all costs, which slows the transmission of *T. gondii*. To overcome this bottleneck, the parasite has learned to make rats crazy. Rats infected with *T. gondii* show no fear of cat urine. This is not because they have no sense of smell, because some of these rats develop an attraction—an often fatal attraction—to cat urine. They go mad and seem to invite their own deaths. Again, no dysfunctional family, birth defect, or blow to the head made these animals crazy. This madness has an infectious cause.

T. gondii infects people too. Gardening in cyst-infested soil, handling infected meat, or emptying litter boxes used by infected cats can result in infection. In fact, nearly half the people in this world have *T. gondii* cysts in the brain. *T. gondii* has never figured out a way to make humans palatable to cats, but that doesn't mean people are unaffected by the parasite. In psychological tests, women with *T. gondii* cysts in the brain were more outgoing and warm-hearted than uninfected controls, and men infected with the parasite were more jealous and suspicious than uninfected men—behavior with a twist, a protozoan twist.

No one knows what drove Vincent van Gogh to take his own life; depression was part of it, certainly. But depression alone seems an insufficient explanation. In the year before his death, the painter enthusiastically brought Paul Gauguin to join him in Arles. Less than 2 months later, van Gogh attacked his guest with a straight razor, then in remorse, cut off his own ear and offered it to a local prostitute. In the same year, van Gogh painted “Sunflowers,” a celebration of yellows and browns, and “Starry Night,” a tableau of haunting blues and swirling stars—a portrait of the abyss itself. Within a few months of

one another, he painted the inviting “Bedroom at Arles” and a brutal self-portrait that leaves the viewer nowhere else to look. Later that same year, he painted “Starlight over the Rhine” and “Wheatfield with Crows.”

Those dramatic swings from euphoria to abject despair suggest bipolar disorder, manic-depression. At the height of his mania, van Gogh painted “Sunflowers.” In the pit of depression, he painted his last work, “Wheatfield with Crows.” Bipolar disorder is a “behavioral” disorder, a mental disease of unknown cause. Many of us imagine that in the bipolar patient, the crows are all inside the mind. We may be wrong. Perhaps it was all in van Gogh's head, all in his mind. But even if we are unwilling to change our thoughts about what “mind” means, we may have to change our thoughts about what “all” means.

Rats, tree shrews, and monkeys (mammals like us—some much like us) infected with Borna disease virus behave much like humans with bipolar disorder. These animals exhibit periods of apparent mania and periods of obvious depression. They are more anxious, less sexually active, less interested in food, and have a greater desire for salt—just like manic-depressive humans. All because of a virus, another obligate intracellular parasite. And because of that virus, infected animals have abnormalities that mimic a devastating behavioral disorder of humans.

Humans also become infected with Borna disease virus, and those infected appear more susceptible to certain “behavioral” disorders. At autopsy, nucleic acid from Borna disease virus has been found in the brain of a disproportionately high number of patients with bipolar disorder, severe depression, and schizophrenia. Viral madness?

Obsessive-compulsive disorder (OCD) manifests as the inability to resist or stop continuous abnormal thoughts; fears; or ritualistic, repetitive, and involuntary behavior. People with OCD may not be able to stop washing their hands, stop hoarding things, stop checking if they've turned the stove off, or stop driving around the block to look for accidents and their victims. OCD is a mental disorder, experts tell us, a behavioral anomaly.

Unexpectedly, though, a substantial number of children with obsessive-compulsive disorders have first signs of illness a few weeks after streptococcal infection—strep throat. Streptococci are infectious bacteria, the same bacteria that cause scarlet fever, rheumatic fever, glomerulonephritis, and other diseases. Apparently after strep infections, our immune systems may mistake our own cells for our enemies. In some patients with OCD, the immune system's enemy appears to be part of the brain. These patients' immune systems produce antibodies that attack the cells of the brain, and almost overnight, the “craziness” that we call OCD can develop. Antibiotics, which kill streptococci, often relieve the symptoms.

Here an infectious disease is amplified somehow by a person's own immune system, and abruptly someone we once called “sane” can't get it out of her head that she is going to harm her own children, can't stop counting the silverware,

can't stop scrubbing her hands, can't stop thinking about murdering her husband.

And then there's my Uncle Henry, the one who disgusted mother. His cursing, personal habits, and appearance were odd, I'll admit. All of us, I suppose, imagined he was a little crazy. Mother imagined it more than most. And you remember, she resented his craziness most of all. As it turned out, though, there was more to Henry than met our eyes. Henry had syphilis, an infectious disease that's been around since at least the 16th century when it was named the "great pox." A bacterium, a spirochete called *Treponema pallidum*, causes syphilis. *T. pallidum* is transmitted through small breaks in the skin that occur during sexual intercourse. Within a few months, the bacteria spread from the point of entry to the lymphatics, the joints, and the skin. And, in 10% of people who go long enough without treatment, bacteria spread to the brain and spinal chord. Once inside the brain, the spirochetes cause some paralysis and progressive dementia. Apparently, Henry was among that 10%.

By 1941, when Alexander Fleming finally got around to developing penicillin to the point of clinical application, it was too late for Henry. Before then, Paul Ehrlich and others treated syphilis with "Salvarsan," a compound that contained, among other things, arsenic. Sometimes it worked, and sometimes it didn't. But often the infected understood so little about the disease that if they sought treatment at all, it was usually too late. And even now, antibiotics are of little use after the spirochete is in the brain. Then, there is only an unstoppable, progressive, "mental" disease. So for many like Henry, uneducated about the dangers of casual sex and infected before Fleming's penicillin, there was only a final madness.

In spite of that, mother still resents Henry, and, I'm sure, the nature of his infection. I doubt that mother ever connected the two, ever imagined that the fire beneath the boiler of Henry's craziness was being stoked by bacteria. She never mentioned Henry's disease until I was much older. Old enough, I guess, that she thought I might understand, even though *she* never did. And she never forgave him, never believed that all of it wasn't Henry's fault—the disease, the craziness, the indiscretions. She discounted the disease and laid the blame squarely on Henry. She hated him for that. She hated him too, I'm sure, for the way his craziness freed him from all responsibility and left her to bear his shame.

Now my mother has Alzheimer's, another disease with no known cause. Some believe an infectious agent is involved—

maybe so, maybe not. As the disease progresses, a protein called amyloid is deposited in mother's brain, and parts of her brain are slowly disappearing. At autopsy, brains from Alzheimer's patients often resemble wispy growths of pale coral with deep fissures and frail fins. Everything I believed was mother is slowly yielding to the disease. Her poverty is nearly complete, the disease nearly crystalline, and the craziness fulminant. Now, she rarely recognizes my father (her husband of more than 60 years) or any of her four children. Long-term memory seems more resistant. The mention of Uncle Henry still causes her to twist her lip into a sneer and curse him for his craziness.

We are told that van Gogh killed himself because he was depressed or crazy or both. But who knows? Who knows what truly tortured this man or what more lay beneath the lurid oils and feverish brush strokes? Regardless, for at least one moment, the picture of his death seemed less terrifying to him than the picture of his life. And though he may have tried to prevent it, that dark picture finally crawled out of the end of his brush and onto the canvas. It was all suddenly there in the crows. He saw that, and when he was finished, he stared long and hard at what he had done, covered the painting, and left the studio. A week or so later, he shot himself, perhaps repeatedly. Two days after that, he died.

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EMERGING INFECTIOUS DISEASES

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JOURNAL BACKGROUND AND GOALS

What are “emerging” infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as “emerging.” These diseases, which respect no national boundaries, include

- ★ New infections resulting from changes or evolution of existing organisms.
- ★ Known infections spreading to new geographic areas or populations.
- ★ Previously unrecognized infections appearing in areas undergoing ecologic transformation.
- ★ Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

Why an “Emerging” Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and *West Nile virus* infection. CDC’s efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC’s efforts against the threat of emerging infections. However, even as it addresses CDC’s interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

What are the goals of Emerging Infectious Diseases?

- 1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal
 - ★ Investigates factors known to influence emergence: microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.
 - ★ Reports laboratory and epidemiologic findings within a broader public health perspective.
 - ★ Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.
- 2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal
 - ★ Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
 - ★ Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
 - ★ Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

Lethal Leptospiral Pulmonary Hemorrhage: An Emerging Disease in Buenos Aires, Argentina

To the Editor: In the Buenos Aires metropolitan area, 40–100 cases of human leptospirosis are reported annually. Occasional epidemic outbreaks have been characterized by mild leptospiral illness. Severe illness with acute renal failure and extensive cutaneous and visceral hemorrhages (always accompanied by jaundice) has been observed only rarely. A review of our data for 1990–1999 showed that 276 human cases were diagnosed; 43 of these were characterized by pneumonia alone or associated with another syndrome. No severe pulmonary hemorrhage due to leptospirosis was detected in these cases (Table), and the case-fatality rate was <1% (1).

Rodents and dogs are considered major reservoirs for this zoonotic illness. *Rattus norvegicus* (78%) and *R. rattus* (22%) are the most widely distributed and predominant species. Rodent abundance has been estimated by the Hayne's Index¹ as 0.414–0.465. Prevalence of leptospiral infection as measured by kidney culture of captured rodents ranges from 25% to 40% (1). Antibody prevalence in dogs in Buenos Aires can be as high as 60%. Canine infection is mainly related to the presence of stagnant water and time spent outdoors (2).

Statistically, the most important sources of infection are leisure activities (31.4%); certain types of work, including garbage collection, sewer and construction work, and gardening (26.1%); and floods (16.1%) (3). During 2000–2001, a total of 93 cases

¹Hayne's Index (Hayne D. Two methods for estimating population from trapping records. *J Mammal* 1949;30:399–411.): relative abundance index = rodents/m². In urban areas it does not represent the actual number of individual animals.

Table. Clinical findings in human leptospirosis, Hospital F.J. Muñiz, Buenos Aires, 1990–1999

Year	Cases	J ^a	N	M	P	H	IL
1990	130	37	37	26	15	14	19
1991	27	12	9	2	7	7	4
1992	25	16	13	7	7	4	0
1993	29	10	7	0	5	3	10
1994	12	4	3	0	4	1	2
1995	12	4	1	0	0	1	1
1996	7	4	1	1	0	1	0
1997	12	8	5	0	3	0	2
1998	14	6	5	2	1	0	1
1999	8	3	3	1	1	1	1

^aJ, jaundice; N, nephritis; M, meningitis; P, pneumonia; H, hemorrhages; IL, influenza-like.

were reported in this area. An outbreak that included 47 cases took place in March 2001, in Quilmes in the suburban area (Informe de Epidemiología de Quilmes, Buenos Aires, unpub. data). Four patients died with suspected leptospiral illness; three of these patients had confirmed cases. We describe two cases with lethal pulmonary hemorrhage.

On July 2000 and March 2001, two women, ages 28 and 34, who lived in urban slum settlements, became ill. A high abundance of rodents inside their houses and in the neighborhood was reported in both cases. After 7–10 days of unspecific febrile illness, a severe pneumonia developed in both women. No jaundice, renal involvement, or thrombocytopenia was observed. When the patients were admitted to the critical-care unit, electrocardiograms were normal for both.

For one of the patients, empiric treatment was begun with 4 g of ceftriaxone plus 1 g of erythromycin daily. In the other, 800 mg/day of ciprofloxacin replaced the erythromycin. Endoscopic examination showed no lesions within the bronchial lumen, and abundant hemorrhagic secretions were obtained by aspiration. Both patients were mechanically ventilated and remained stable for the first 48 hours. Between the second and third day of ventilation, they became hypoxemic with acidosis and hypotension. Except

for pulmonary hemorrhages, no other sign of bleeding was observed. Both patients died with cardiovascular collapse 10–11 days after onset of illness.

The microagglutination test with 10 serovars was positive for leptospirosis, as well as macroagglutination and enzyme-linked immunosorbent assay (ELISA) with leptospiral antigen, for immunoglobulin (Ig) M. Blood, urine, and bronchoalveolar lavage culture were negative for leptospira, as well as for other bacteria. IgM-capture ELISA (Andes serotype) for hantavirus was negative. Pathologic studies performed in one of the patients showed severe hemorrhage inside the pulmonary alveoli, with few interstitial lymphocytes; some septum tissue showed minimal enlargement. Warthin-Starry staining was negative for leptospira.

Rodents were captured near one patient's house, and their kidneys were cultured in Ellinghausen-McCullough Johnson-Harris medium. Three strains of *Leptospira interrogans* serovar *icterohaemorrhagiae* were isolated and characterized; laboratory guinea pigs were injected with the strains and several died 8–10 days later. Tegumentary jaundice was present, as well as abdominal hemorrhage foci. Pulmonary hemorrhages were observed bilaterally. Pericardial hemorrhages are remarkable as a possible cause of cardiopulmonary collapse. *Leptospira* were recovered from the liver and the

kidneys, although brain and lung cultures were negative.

Another group of guinea pigs that had also been injected with *Leptospira* was humanely killed as soon as symptoms appeared. Necropsy showed primary lung injury. Lungs were pale with hemorrhages widely spread over the surface. Lesions were similar to those observed in one of the patients. Neither jaundice nor renal damage was found. *Leptospira* was isolated from kidneys, lungs, and brain. Jaundice has been reported in severe forms of human disease. Thrombocytopenia has been associated with renal failure and death in human patients.

Respiratory involvement in leptospirosis could be classified as a) mild to moderate (20% to 70% of patients), with pulmonary infiltrates commonly associated with jaundice and minimal alteration of renal function; b) severe, with jaundice, nephropathy, hemorrhages (severe Weil's syndrome) (4), and occasional death due to renal failure, myocarditis, or massive hemorrhages with cardiovascular collapse; and c) pulmonary hemorrhage which is frequently fatal, without jaundice, nephropathy, or other hemorrhages.

In the past two decades, an increasing number of cases of leptospiral pulmonary hemorrhages have been reported, especially from South-east Asia (5). In a review of leptospirosis in Brazil, death was associated with renal failure in 76.2% of fatal cases, while 3.5% were related to pulmonary hemorrhages (6). In the epidemic outbreak in Nicaragua in 1995, this form was considered the cause of death in the 40 fatal cases reported (7).

The two cases reported here were associated with pulmonary hemorrhage. This clinical form has not been previously reported in the Buenos Aires metropolitan area. Environmental and social factors, the prevalence of infection in reservoirs, and the virulence of the isolated strains must be considered in primary or critical-care

units in the diagnosis of new cases, whether or not associated with an outbreak.

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First Documentation of Human Crimean-Congo Hemorrhagic Fever, Kenya

To the Editor: On October 21, 2000, a previously healthy 25-year-old male farmer was admitted to a mission hospital in western Kenya with an acute hemorrhagic illness. Four days before admission, the patient had rapid onset of fever, headache, nausea, vomiting, severe muscle pains, and diarrhea, which became bloody. On admission his temperature was 36.4°C, pulse was 60/minute, respiratory rate was 20/minute, and blood pressure was 90/40 mm Hg. In addition to the signs and symptoms listed above, the only other abnormal finding on admission was neck stiffness. The differential diagnoses included bacterial dysentery and meningitis. Results of a blood smear for malaria parasites and Widal test for typhoid were negative, and cerebrospinal fluid and urine examinations were normal.

The patient was treated with doxycycline, cotrimoxazole, metronidazole, and intravenous fluids. On the day after admission, the patient's vomitus became blood stained and blood was passed rectally. The patient was isolated and strict barrier nursing implemented on the suspicion of viral hemorrhagic fever (VHF). Progressive hypotension developed, resistant to resuscitation efforts with intravenous fluids and corticosteroids, and later massive bleeding from the nose, mouth, and upper and lower gastrointestinal tract occurred. The patient died on the second day of admission, 6 days after onset of illness. A serum sample was sent to the Arbovirus and Viral Hemorrhagic Fever Reference Laboratory in Nairobi for diagnostic screening.

Serologic tests in Nairobi were negative for yellow fever, dengue, West Nile, Chikungunya, and Rift Valley fever (immunoglobulin [Ig] M—

capture enzyme-linked immunosorbent assay) and reverse transcriptase-polymerase chain reaction (RT-PCR) tests for flaviviruses, alphaviruses, and Bunyamwera serogroup bunyaviruses were also negative. RT-PCR for *Crimean-Congo hemorrhagic fever virus* (C-CHFV) was positive. Tests for anti-C-CHFV-specific IgM antibody by indirect immunofluorescence were negative. Virus isolation attempts were then terminated because the cultivation of C-CHFV (the presumptive cause) requires biosafety level 4 facilities. The specimen was submitted to the Special Pathogens Unit in Johannesburg for confirmation of the result. The sample was positive by RT-PCR for C-CHFV and was IgM and IgG antibody negative. No isolation of the virus could be made from the serum sample, possibly because it was received by the Johannesburg laboratory 8 days after initial collection and following freeze-thaw conditions. The specimen was insufficient to attempt C-CHFV antigen detection assays. Sequencing of the RT-PCR amplicon confirmed C-CHFV.

C-CHFV is a tick-borne virus of the genus *Nairovirus*, family *Bunyaviridae*, and is widely distributed throughout eastern Europe and the Crimea, to the Middle East and western China, Pakistan, and Africa. Natural hosts for this virus are varied (including wild and domestic animals and birds) and may reflect the feeding preferences of the host tick (1). While C-CHFV infections are rare in humans, the virus is notorious for nosocomial outbreaks of VHF, typically following admission of an index case to a health-care facility where VHF was not suspected, with mortality rates up to 40%.

Previous evidence for C-CHFV in Kenya is limited and based on serology (human and bovine) and two isolations of C-CHFV from non-human sources (1,2). This report represents the first documented case of acute human C-CHFV infection in Kenya. The hospital concerned belongs to a VHF surveillance network serving to

increase awareness and preparedness within Kenyan health-care facilities. In this case suspicion of VHF was raised, and the patient was immediately isolated, noninvasive procedures were instigated, and barrier nursing was implemented to prevent nosocomial transmission. No family or hospital staff member who had close contact with the patient became ill. Although VHFs are rare, this report stresses the need for health facilities in Kenya and East/Central Africa to include VHFs in their differential diagnosis of unexplained fever with hemorrhagic tendencies, as well as the utility of the surveillance network. The causative agents of Ebola hemorrhagic fever, Marburg hemorrhagic fever, C-CHFV, Rift Valley fever, and yellow fever are all endemic in East and Central Africa, and sporadic cases, as well as outbreaks, are likely to continue to occur in this region (3–5).

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¹Dr. Kazooba-Voskamp, the attending physician in this case, has requested that the hospital's identity remain anonymous.

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Preparing at the Local Level for Events Involving Weapons of Mass Destruction

To the Editor: The use of hijacked airplanes in the attacks on the World Trade Center and the Pentagon on September 11, 2001, clearly illustrated the immediate and massive destruction that can result from a well-orchestrated, long-planned, and purposeful terrorist act. Weapons of mass destruction (WMD) events (i.e., biological, nuclear, or chemical attacks) present different challenges than other incidents involving mass casualties (e.g., chemical spills, transportation mishaps, or natural disasters). Persons involved in a biological weapons attack, for example, may take days to develop symptoms and seek medical care (1); a large geographic area may be affected, or persons may travel long distances and unwittingly infect others, including hospital personnel (2). Furthermore, traditional hazardous materials and emergency medical procedures may be inadequate to respond to a WMD event (3–5). As events of September 11 and its aftermath make clear, medical public health systems were not optimally prepared. An effective response to a WMD event focuses on two key areas: joint efforts between the medical community and public health agencies and better trained and coordinated first responders (i.e., law enforcement, public safety, hospital personnel, and public health officials) (1–3).

In early 2001, telephone interviews with West Virginia county health directors (CHDs) or their equivalent were conducted to ascer-

tain the level of collaboration between their departments and local hospitals in regard to WMD preparedness and a coordinated medical and public health response. Forty-four (90%) of 49 CHDs completed the interview. One of the 49 responding CHDs is responsible for a six-county area, thus accounting for the state's 55 counties.

Fewer than half (20 of 44) of the respondents have provided contact information to local hospitals, and barely 20% have reciprocal information. Twenty-one percent were either unaware of a policy for WMD preparedness or reported that it was being handled by another agency. Although 72% of CHDs had attended WMD training, only 14% of the training was in conjunction with hospitals. While nearly two thirds rated their communication with hospitals as moderate to strong, a similar proportion stated they had no protocol for communicating with hospitals about a WMD event. Eighty-six percent of CHDs reported that no new collaborative efforts were directed towards the early identification of new or emerging infectious diseases possibly related to bioterrorism. However, approximately one third of the CHDs thought they should take initiative in this matter. Over 60% indicated that primary responsibility for identifying biological agents rested in another agency or was not the sole responsibility of the CHD. Further, 20% indicated they were weak or untrained in this area and thought that development and implementation of policies, procedures, and training were needed. While 93% of CHDs felt joint

training with hospitals would be beneficial, particularly in defining their respective roles in a WMD scenario, many cited manpower and scheduling constraints for such joint training sessions. Overall, CHDs reported weak relationships with area hospitals, but thought that development or improvement of policies and procedures through regular meetings and training would help prepare and plan for a WMD event.

The results of this survey suggest that before September 11, West Virginia CHDs and local hospitals had little collaboration in preparing to respond to a WMD event. Despite the recent terrorist activities, local health departments and hospitals may still be reluctant to spend resources in preparation for events with a low probability of occurring, such as WMD incidents. The local health departments and hospitals think that other pressing programs will be jeopardized (6–8). Many federal and state initiatives are under way to enhance the public health infrastructure and its preparation and response to bioterrorism. Improving on programs to meet daily operational challenges, as well as those presented by a WMD event, must include the expertise of local health departments and hospitals and encourage the creation of innovative, cost-effective preparedness programs at the local level (9,10). Future research should be conducted in areas of resource education and training, allocation and sharing, personnel, and policy. This research will indicate if existing programs should be improved

and if new programs should be instituted.

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Correction, Vol. 8, No. 5

In “Phylogenetic Analysis of a Human Isolate from the 2000 Israel *West Nile virus* Epidemic” by Thomas Briese et al., errors occurred in the text and figure legend. On page 529, right column, line 25, and in the figure legend on page 530, the host species for ISR-00PigC is pigeon. Additionally, in the figure legend, the GenBank accession no. for ISR-00PigC is AF380671, and the GenBank accession no. for WNV-ROM96(0334)-1996 is AF205879.

The online article at <http://www.cdc.gov/ncidod/EID/vol8no5/01-0324.htm> has been corrected.

We regret any confusion these errors may have caused.

Guidelines for Letters. Letters discussing a recent *Emerging Infectious Diseases* article (400–500 words, 5–10 references) should be received within 4 weeks of the article's publication. Letters reporting preliminary data (500–1,000 words, 10 references) should not duplicate other material published or submitted for publication, should not be divided into sections, and should avoid figures or tables. All letters have the same authorship, financial disclosure, and acknowledgment requirements as full articles and should include a word count. For more guidance on manuscript preparation, see *Emerging Infectious Diseases Instructions to Authors*. Send letters to the Editor, *Emerging Infectious Diseases*, Centers for Disease Control and Prevention, 1600 Clifton Rd., MS D 61, Atlanta, GA 30333, USA, or e-mail: eideditor@cdc.gov.
Letters Editor: Patricia M. Quinlisk, Des Moines, Iowa, USA

Conference Summary

Global Infectious Disease Policy

At the International Conference on Emerging Infectious Diseases 2002, held in Atlanta, the Centers for Disease Control and Prevention (CDC) released a document entitled, "Protecting the Nation's Health in an Era of Globalization: CDC's Global Infectious Disease Strategy," which describes plans for controlling infectious diseases worldwide. The document outlines global partnerships and measures for improving capacity for disease surveillance and outbreak response and for applying proven public health tools to the control of emerging infectious diseases over the next decade. In addition, the document calls for strengthening global initiatives for disease control, conducting applied research on diseases

of international importance, and building public health training and capacity worldwide.

International Emerging Infections Programs in various parts of the world will support the activities outlined in the global strategy document. The International Emerging Infections Program (IEIP), Thailand, is the first site in the network of IEIPs proposed in the plan. Through the IEIP network, modeled after the U.S. Emerging Infections Program, specialists will work with local ministries of health to support laboratory-enhanced, population-based surveillance for infectious diseases. Data from this surveillance will allow ministries of health to prioritize diseases, evaluate targeted interventions, and support global efforts to prevent and control disease. IEIPs will train local scientists and CDC personnel, provide diagnostic and epidemiologic resources when outbreaks occur, and serve as platforms for regional infectious disease control activities.

In December 2001, IEIP Thailand and the Southeast Asia Regional Office of the World Health Organization hosted a training course on anthrax, attended by 64 participants from 16 countries. In 2002, IEIP Thailand initiated an investigation of an increase in reported leptospirosis cases through a study of hospitalized patients with febrile illness. IEIP Thailand is planning studies of respiratory illness and encephalitis later this year; a second IEIP site will be launched soon.

The Global Infectious Disease Strategy document is available at <http://www.cdc.gov/globalidplan>. Supplementary materials are available at <http://www.cdc.gov/ncidod>. For a print copy of the strategy, send a request by e-mail to ncid@cdc.gov; or by fax to 404-639-4194; or contact NCID, Office of Health Communications, 1600 Clifton Road, NE, Mailstop C14, Atlanta, GA 30333 USA.

Eric D. Mintz

Conference Summary

Fifth Annual Conference on New and Re-Emerging Infectious Diseases

The fifth annual Conference on New and Re-Emerging Infectious Diseases was hosted on April 18–19, 2002, by the College of Veterinary Medicine, University of Illinois at Urbana-Champaign (UIUC). The conference featured 8 speakers and 35 poster presentations.

Recent Infectious Diseases

Beatrice Hahn (University of Alabama, Birmingham, AL) opened the conference with a presentation on the search for the origins of HIV. The evidence indicates that two simian immunodeficiency viruses (SIV), one from chimpanzees (SIVcpz) and the other from sooty mangabeys (SIVsm), crossed the species barrier to humans,

generating HIV-1 and HIV-2, respectively. Dr. Hahn stressed the importance of characterizing the prevalence, geographic distribution, and genetic diversity of naturally occurring SIV infections to investigate whether humans continue to be exposed to SIV and if such exposure could lead to additional zoonotic transmissions.

William Hueston (University of Minnesota, St. Paul, MN) gave a personal account of how bovine spongiform encephalopathy (BSE), appeared in Europe and how chronic wasting disease (CWD,—another transmissible spon-giform encephalopathy that affects elk and deer) is spreading across North America. The disease seriously affects the elk industry. CWD causes emaciation and eventually death. The disease has been endemic for decades in elk and wild deer populations in southeastern Wyoming, northeastern Colorado, and a small part of Nebraska. That infections on elk farms could spread the disease to wild populations of elk and deer is of concern and may affect the

hunting industry, especially in eastern states, which have large populations of white-tailed deer.

Bioweapons

Edward Eitzen (U.S. Army Medical Research Institute of Infectious Diseases [USAMRIID] Fort Detrick, MD) recounted the history of state-sponsored biological weapons programs and the emergence of bioterrorism by non-state participants in recent years. The various ways biological agents can be used as weapons and the potential routes of exposure were discussed as prelude to the medical effects of these agents and their effects on the health-care system. Medical countermeasures and other important responses to attacks with biological agents were highlighted, including priorities for the nation to be better prepared. After the anthrax attacks in Florida, New York, New Jersey, and Washington, DC, the threat of biological warfare became much more real; however, these attacks were not the first in the United States. Dr. Eitzen

described USAMRID's role in detecting contaminated material and helping in the clean-up effort in the response to the anthrax attack.

Cholera and Multidrug-Resistant Tuberculosis

A filamentous bacteriophage (CTX ϕ) integrated in the *Vibrio cholerae* chromosome encodes the cholera toxin, and Matthew Waldor (New England Medical Center, Boston, MA) described another phage (RS1) that flanks the CTX ϕ prophage in the bacterial chromosome and is important for the CTX ϕ prophage propagation. RS1 relies upon CTX ϕ -encoded proteins for packaging and secretion of its genome; however, RS1 is not simply a parasite, as it can aid the CTX ϕ prophage while exploiting it. The unique RS1-encoded protein RstC is an antirepressor that counteracts the activity of the CTX ϕ repressor, RstR. RstC and RstR appear to form intracellular aggregates that prevent the repressor from binding to its operators. Inactivation of RstR results in increased transcription of CTX ϕ genes and increased transmission of both RS1 and CTX ϕ .

Tuberculosis remains a major global health burden: an estimated one third of the world is infected with *Mycobacterium tuberculosis*. The successful spread of this slow-growing airborne bacteria continues to be a public health challenge. Current problems are further complicated by the rise of multidrug-resistant (MDR) strains, the failure to develop new anti-mycobacterial drugs, and the deadly marriage between HIV and TB. Two genotyping networks have generated data on >60,000 clinical isolates of *M. tuberculosis*, as indicated by Barry N. Kreiswirth (Public Health Research Institute, New York, NY). Unique genetic markers were identified to distinguish various branches of the *M. tuberculosis* genetic lineage that are associated with large MDR outbreaks, such as the drug-resistant W strain that spread through New York State prisons and New York City

hospitals. Outbreaks with related clones were also identified in regions of the United States, Russia, South Africa, and in several Asian countries. Preliminary evidence indicates that members of this lineage grow better in macrophage cell lines and are hypervirulent, causing early death in immunocompetent mice.

Re-Emerging Parasitic Diseases

Through vigorous efforts made in the past two centuries, public health workers have succeeded in developing vaccines, antibiotics, and chemotherapeutics, and as a result most infectious diseases have been brought under control in industrialized countries. However, in developing countries, infectious diseases have been harder to contain, and the increase in migration and movement of populations in the last two decades has made national boundaries disappear as far as the transmission of infection is concerned. Some diseases, such as malaria, have been eradicated from industrialized countries mainly through extensive work on vector control, but their presence in developing countries has increased because of neglect or drug resistance. Donald Goldberg (Washington University, St. Louis, MO) showed how the Malaria Genome Project helped to find novel proteases, several of which appear to function in hemoglobin metabolism. One of these proteases (Histidine Aspartic Protease, HAP) is homologous to three other aspartic proteases involved in hemoglobin metabolism but has a histidine in place of one of the two aspartic acids involved in catalysis. Despite this change, HAP is an active protease with distinct properties, and together with a series of cysteine and metalloproteases and a dipeptidyl peptidase, provides attractive focus for antimalarial drug development.

African trypanosomiasis has reached epidemic proportions in recent years, and its etiologic agents have become noteworthy among molecular biologists for their ability to use genomic rearrangements to

change their major surface protein (variant surface protein, VSG). John Donelson (University of Iowa, Iowa City, IA) reviewed antigenic variation in *Trypanosoma brucei* and recent findings on the use of an extranuclear body containing RNA polymerase I to regulate expression of VSG genes. Donelson provided examples of how the African Trypanosome Genome Project has helped to elucidate the sequence of new expression site-associated genes (ESAGs) and how the RNA interference technique has contributed to identifying essential roles of ESAGs in the *T. brucei* life cycle.

Leishmaniasis has recently emerged as an opportunistic infection after the advent of the AIDS epidemic. Kwang-Poo Chang (Chicago Medical School, Chicago, IL) proposed a hypothetical model to account for nontoxigenic microbial virulence involving two groups of different molecules of parasite origin. One group is responsible for invasion and evasion of mammalian hosts by *Leishmania* to achieve infection but does not directly cause the disease or virulence phenotype. For example, repeated injections of animals with these molecules, such as gp63 and LPG, do not result in any visible disease signs. The other group is formed by immunoreactive epitopes whose interactions with the host immune system result in immunopathology, accounting for the clinical symptoms. The proposed examples include B-cell epitopes specific to *Leishmania* identified by a number of laboratories working with visceral *Leishmania* spp. All have been found in the parasite's cytoplasm often as complex proteins, i.e., ribosomes, nucleosomes (histones), chaperonins, structural proteins (tubulins, kinesin), glycosomes (triose phosphate isomerase). These epitopes are unique to *Leishmania* molecules not shared with those found in autoimmune diseases. One example is the anti-K39 antibodies in kala-azar. K-39 is referred to as 39 aa repetitive peptides found in a kinesin-like gene (5 kb), which is expressed only by the

amastigotes of visceral *Leishmania* spp. The anti-K39 antibodies in Indian kala-azar, for example, reach a titer as much as 1:1,000,000. This antibody and other specific anti-*Leishmania* antibodies are nonprotective, as they cannot reach the intracellular antigens within amastigotes inside macrophages but contribute to the hematologic disorders of kala-azar, such as albumin: immunoglobulin G ratio reversal, and hyperplasia of B-cell populations in the lymphoid organs.

The poster section highlighted recent work on several emerging infectious diseases. Using a cell line in which tubulin was labeled with the fluorescent indicator green fluorescent protein (GFP) and immunofluorescence assay, G.W. Gant Luxton and Kevin Tyler (Northwestern University [NWU], Chicago, IL) demonstrated a rapid association of host tubulins with the plasma membrane at the site of *Trypanosoma cruzi* contact. Juan Leon, from the same laboratory (NWU), showed that captopril, an antifibrotic angiotensin-converting

enzyme inhibitor, is effective in ameliorating experimental autoimmune myocarditis and experimental Chagas heart disease. Ileana Cuevas from the Daniel Sanchez Laboratory (University of General San Martin, San Martin, Argentina) reported the presence of a farnesylated protein tyrosine phosphatase in *T. cruzi*. Carlos Lopez-Estraño (NWU) reported experiments using truncated versions of histidine-rich protein II with green fluorescent protein to investigate how they are transported to the malaria-infected erythrocyte cytosol.

N-acetylglucosamine-1-phosphate transferase gene is a potential marker for genotyping Old World *Leishmania* isolates, as reported by Kayoko Waki (Chicago Medical School, Chicago, IL). A thioredoxin and a thioredoxin-glutathione reductase are present in *Schistosoma mansoni*, as reported by David Williams (Illinois State University, Normal, IL). Thioredoxin is present in egg-secretory products and is a novel B-cell antigen in schistosome-infected mice. Schistosomes

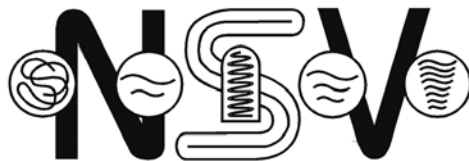
appear to be the first example of an organism with a redox system based exclusively on thioredoxin-glutathione reductase.

Ibulaimu Kakoma's (UIUC, Urbana) and Byeong-Kirl Baek's (Chonbuk, Korea) laboratories reported the use of polymerase chain reaction to verify vertical transmission of *Theileria sergenti* in cows, an important problem for the control of theileriosis. Kakoma and Baek also reported the characterization of the protective response in rats against homologous challenge infections with *Strongyloides venezuelensis*. Finally, Anna M. Schotthoefer (UIUC, Urbana) reported that infection with the larval trematode *Ribeiroia ondatrae* could be responsible for limb malformations in tadpoles and could explain the observed increase in the frequency of these malformations within natural frog populations.

In summary, this interdisciplinary conference generated stimulating discussions on various aspects of emerging and re-emerging infectious diseases. Since two SIV viruses were the cause of AIDS in humans, other SIVs can potentially infect humans and cause disease. The spread of chronic wasting disease from elk farms to wild populations of elk and deer in North America is of great concern. Genetic markers have been a great resource to identify MDR strains of *M. tuberculosis*. The interaction of the cholera toxin-encoding phage with an additional phage in the genome of *V. cholerae* has unexpected consequences for their transmission. Non-state-sponsored bioterrorism has changed our appreciation of bioweapons. Parasitic diseases, such as malaria, African trypanosomiasis, and leishmaniasis have re-emerged in recent years and the study of their agents has provided potential focus for their chemotherapy and insights into microbial virulence.

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About the Cover

Claude Monet (1840–1926) “Nymphéas” (Water Lilies) 1916–1919.
Oil on canvas, 200 cm x 180 cm.
Musée Marmottan, Paris/Bridgeman Art Library, New York

“Until then I knew only naturalist and, to tell the truth, almost exclusively Russian naturalist art...I believed that no one had the right to paint so imprecisely. I vaguely felt that the object (the subject) was missing in this work. But with astonishment and confusion, I observed that not only did it surprise, but it imprinted itself indelibly in the memory and that before your eyes it recomposed itself in the smallest details. All this remained muddled in me, and I could not yet foresee the natural consequences of this discovery. But what clearly came out of it is the incredible power, a power I had never known, of a palette that outstripped my wildest dreams. The object used as an indispensable element in my work unconsciously lost some of its importance to me. In short, there was already a little bit of my enchanting Moscow on this canvas.”

Wassily Kandinsky, one of the most original and influential artists of the 20th century, on impressionism and Claude Monet

During his studies in Paris, Claude Monet met Renoir, Sisley, and Bazille. He admired Manet and worked with Courbet and Trouville. In London in 1871, he discovered Turner and began to collect Japanese paintings. His works were exhibited in 1874, 1876, 1877, and 1882, alongside works by painters known as “impressionists” (from the word “impression” used in the 1872 Monet painting “Impression, soleil levant”). Cézanne, Renoir, Pissarro, Matisse, John Singer Sargent, and many other painters and prominent critics were friends and admirers of Monet and his work (1).

In 1883, Monet discovered Giverny, a village northwest of Paris that became his home for 43 years and a major force in his art. At Giverny, he purchased a small island, “île aux Orties,” and planted an elaborate garden, which he would paint in all kinds of light and weather and from which he may have derived some of the “energy and truth” many saw in his paintings. It has been said that the enchanted natural environment Monet created at Giverny was itself one of his major masterpieces. Monet’s home and gardens at Giverny are now a living museum, where the visitor can stroll through the flower paths that inspired the expansive version of reality found in his paintings.

In his effort to capture just the right amount of light and dark, Monet always worked on several canvases at once and furiously followed the changing daylight. He painted intently, disregarding all the topical trends (the Nabis, Pointillists, Fauvists, Cubists), and declared to his astonished contemporaries, “The subject is not important to me; what I want to reproduce is what exists between the subject and me.” Near the end of his life, as a result of his intense efforts to place what he painted in the proper light and shade, he banished the subject from his paintings, bringing about the birth of abstract art (1).

Monet became famous for his “Séries” of paintings on various subjects, from cathedrals and bridges to clouds and flowers. The painting depicted on the cover of this issue of Emerging Infectious Diseases comes from a series of large decorative panels of water lilies, called the “Décorations des Nymphéas,” in which shapes (present in some early panels) eventually give way to explosions of color under various lights, which take the viewer far beyond the quaint pond with its floating flowers.

The dazzling complexity of color and light in the “Nymphéas” panels opens the viewer’s eyes to the incredible diversity of nature and to the depth and mystery of the life it sustains. Monet’s water is teeming with possibilities, all of them interconnected in an elaborate and thoroughly harmonious plan. The plants, or the shades representing the plants, exist only in connection with each other and with the light and darkness that surround them. Monet’s “Nymphéas,” illustrates for the viewer why bacterial, viral, parasitic, and all life cannot but continuously evolve and reemerge.

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Polyxeni Potter

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Cascais, Portugal

Contact: Joe Bernosky (303) 347-6209

E-mail: jbernosky@awwa.org

September 27—30

Interscience Conference on Antimicrobial Agents and Chemotherapy (42nd)

San Diego, California

Contact: Meetings Dept. (202) 942-9248

Website: <http://www.asmusa.org>

October 22—23 (tentative dates)

American Society of Tropical Medicine and Hygiene (ASTMD) Courses and Meetings, 2002

Intensive Review Course in Clinical Tropical Medicine and Travelers' Health
Chicago, Illinois

Contact: ASTMH (847) 480-9592

E-mail: astmh@astmh.org

Website: <http://www.astmh.org>

October 24—25

Europa and Argentina in Medicine and Science

XVIIth Meeting of Argentine

Society of the History of Medicine

Buenos Aires, Argentina

Contact: Jaime E. Bortz

Fax: 54-11-4307-9791

E-mail: historiadelamedicina@arcoap.com.ar

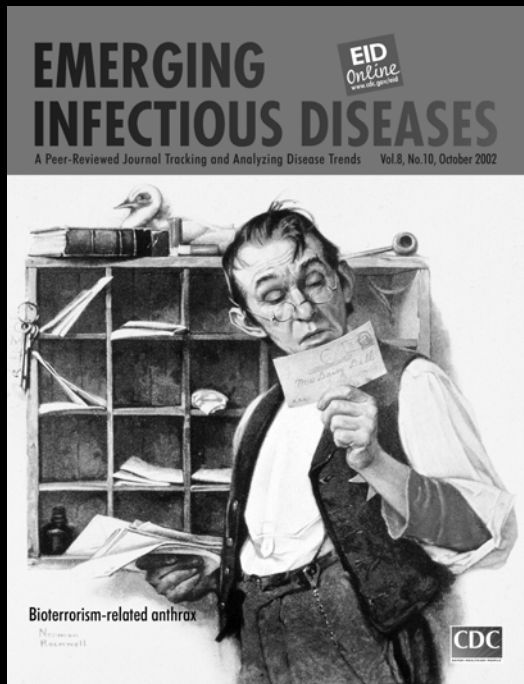
October 24—27

40th Annual Meeting of the Infectious Diseases Society of America

Chicago, IL

Information: (703) 299-0200

Website: <http://www.idsociety.org/ME/AM2002/ToC.htm>



Upcoming Issue on Anthrax

For a complete list of articles included in the October issue, and for articles published online ahead of print publication, see <http://www.cdc.gov/ncidod/eid/upcoming.htm>

Look in the October issue on Anthrax for the following topics:

Epidemiologic Findings from a National Investigation of Bioterrorism-Related Anthrax, United States, 2001

First Bioterrorism-Related Anthrax Case Identified in the United States—Palm Beach County, Florida, 2001.

The Public Health Response and Epidemiologic Investigation Related to the Opening of a *Bacillus anthracis*-Containing Envelope, on Capitol Hill, Washington, D.C.

An Outbreak of Inhalational Anthrax in Postal Workers, Washington, D.C., 2001

Epidemiologic Investigation of Bioterrorism-Related Anthrax, New Jersey, 2001

Use of Call-Tracking Data to Evaluate Response to Bioterrorism-Related Anthrax

Anthrax Postexposure Prophylaxis in Postal Workers, Connecticut, 2001

Comparison of Surface Sampling Methods for *Bacillus anthracis* Spore Contamination

Collaboration Between Public Health and Law Enforcement: The Constitutional Challenge



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Editorial Policy and Call for Articles

Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal has an international scope and is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, demography, sociology, and other disciplines. Inquiries about the suitability of proposed articles may be directed to the Editor at 404-371-5329 (tel), 404-371-5449 (fax), or eideditor@cdc.gov (e-mail).

Emerging Infectious Diseases is published in English and features the following types of articles: Perspectives, Synopses, Research Studies, Policy and Historical Reviews, Dispatches, Commentaries, Another Dimension, Letters, Book Reviews, and News and Notes. The purpose and requirements of each type of article are described in detail below. To expedite publication of information, we post journal articles on the Internet as soon as they are cleared and edited.

Chinese, French, and Spanish translations of some articles can be accessed through the journal's home page at <http://www.cdc.gov/eid>.

Instructions to Authors

Manuscript Preparation. For word processing, use MS Word. Begin each of the following sections on a new page and in this order: title page, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, figure legends, appendixes, and figures. Each figure should be in a separate file.

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 e-mail 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. Printed manuscript should be single-sided, beginning with the title page. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

Tables and Figures. Create tables within MS Word's table tool. Do not format tables as columns or tabs. Send graphics in native, high-resolution (200 dpi minimum) .TIF (Tagged Image File), or .EPS (Encapsulated Postscript) format. Graphics should be in a separate electronic file from the text file. For graphic files, use Arial font. Convert Macintosh files into the suggested PC format. Figures, symbols, letters, and numbers should be large enough to remain legible when reduced. Place figure keys within the figure. For more information see EID Style Guide (http://www.cdc.gov/ncidod/EID/style_guide.htm).

Manuscript Submission. Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch) and verifying that the final manuscript has been seen and approved by all authors. Submit an electronic copy (by e-mail) to the Editor, eideditor@cdc.gov.

Types of Articles

Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch of first author. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch of first author—both authors if only two. 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) and a brief biographical sketch of first author—both authors if only two. Report laboratory and epidemiologic results within a public health perspective. Although these reports may be written in the style of traditional research articles, they should 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) 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 1,000–1,500 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 two); and a brief biographical sketch of first author—both authors if only two. 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.

Commentary. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but should not include 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. This section includes letters that present preliminary data or comment on published articles. Letters (500–1,000 words) should not be divided into sections, nor should they contain figures or tables. References (not more than 10) may be included.

Book Reviews. Short reviews (250–500 words) of recently published books on emerging disease issues are welcome. The name of the book, publisher, and number of pages should be included.

News and Notes. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted on the journal Web page only, depending on the event date.) In this section, we also include summaries (500–1,000 words) of emerging infectious disease conferences. Summaries may provide references to a full report of conference activities and should focus on the meeting's content.