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Antimicrobial Drug Resistance: "Prediction Is Very Difficult, Especially about the Future"[1]

Patrice Courvalin*

Evolution of bacteria towards resistance to antimicrobial drugs, including multidrug resistance, is unavoidable because it represents a particular aspect of the general evolution of bacteria that is unstoppable. Therefore, the only means of dealing with this situation is to delay the emergence and subsequent dissemination of resistant bacteria or resistance genes. Resistance to antimicrobial drugs in bacteria can result from mutations in housekeeping structural or regulatory genes. Alternatively, resistance can result from the horizontal acquisition of foreign genetic information. The 2 phenomena are not mutually exclusive and can be associated in the emergence and more efficient spread of resistance. This review discusses the predictable future of the relationship between antimicrobial drugs and bacteria.

Over the last 60 years, bacteria and, in particular, those pathogenic for humans have evolved toward antimicrobial drug resistance. This evolution has 2 key steps: emergence and dissemination of resistance.

Humans cannot affect emergence because it occurs by chance and represents a particular aspect of bacterial evolution. Emergence can result from mutations in housekeeping structural or regulatory genes or from acquiring foreign genetic information. However, much can be done to delay the subsequent spread of resistance. Dissemination can occur at the level of the bacteria (clonal spread), replicons (plasmid epidemics), or of the genes (transposons). These 3 levels of dissemination, which coexist in nature, are not only infectious but also exponential, since all are associated with DNA duplication. Clonal dissemination is associated with chromosome replication, plasmid conjugation with replicative transfer, and gene migration with replicative transposition (1). The spread of resistance has repeatedly been shown to be associated with antimicrobial drug use (2), which stresses the importance of the prudent use of

these drugs; a notion reinforced by the observation that resistance is slowly reversible (3,4).

Therefore, attempting to predict the future of the relationship between antimicrobial drugs and bacteria is conceptually challenging and potentially useful. For the sake of convenience, the examples will be taken mainly from the work carried out in the author's laboratory, although numerous other examples can be found in the literature.

The clinically relevant predictable resistance types are listed in the Table. Although they have not yet been reported, they may exist in nature; their apparent absence is, at least for some of them, rather surprising. For example, streptococci, including pneumococci and groups A, C, and G, can easily acquire in vitro conjugative plasmids from enterococci and stably maintain and phenotypically express them (5). Therefore, it is all the more surprising that genes commonly found on plasmids in the latter bacterial genus, such as *bla* for penicillinase production and *aac6′-aph2″* for resistance to nearly all commercially available aminoglycosides, have not yet emerged in streptococci. The situation is even more unusual for *Listeria* spp., which remain susceptible to most antimicrobial drugs even though they can acquire plasmids from both enterococci and staphylococci (6). However, the obligate intracellular existence of *Chlamydia* spp. likely protects them from contact with foreign DNA and accounts for their retained susceptibility to antimicrobial drugs.

How To Anticipate Resistance

One should distinguish "natural" antimicrobial drugs (e.g., kanamycin), which are produced by microorganisms from the environment, from semisynthetic (e.g., amikacin) and entirely synthetic compounds (e.g., quinolones), which are produced, at least in part, by humans. The microorganisms that produce natural antimicrobial drugs

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Table. Predictable resistance types

Organism	Resistance phenotype or mechanism
Streptococcus pneumoniae	Penicillinase, gentamicin, glycopeptides
Streptococcus groups A, C, G	Penicillins
Listeria monocytogenes	Penicillins, gentamicin
Legionella pneumophila	Macrolides, fluoroquinolones
Salmonella enterica serovar Typhi	Third-generation cephalosporins
Haemophilus influenzae	Third-generation cephalosporins
Neisseria meningitidis	Third-generation cephalosporins
Brucella spp.	Tetracyclines, rifampin, streptomycin
Clostridium difficile	Glycopeptides
C. perfringens	Penicillinase
Chlamydia spp.	Tetracyclines

have to protect themselves from the products of their own secondary metabolism. To ensure their survival, these organisms have developed self-protection mechanisms similar to those found in resistant human pathogens (7); this observation led to the idea that the producers constitute the pool of origin of certain resistance genes (8). Therefore, the study of resistance in the strain used for the industrial production of an antimicrobial agent could allow a strong prediction about the mechanism that will be found later in bacteria pathogenic for humans. For example, the study of glycopeptide producers would have allowed the elucidation, long before it actually occurred, of the mechanism by which enterococci and, more recently, staphylococci could become resistant to these drugs (Figure 1).

As already noted, bacteria are resistant to antimicrobial drugs after horizontal DNA transfer or mutations. Thus, another prediction that can be made is that bacteria will transfer to susceptible species, resistance determinants already known in other bacterial genera, for example, the recent acquisition of glycopeptide resistance by *Staphylococcus aureus* from *Enterococcus* spp. (9). However, this prediction is limited since it refers to mechanisms that have already been explained. In addition to being antimicrobial agent producers, the commensal bacteria of mammals, particularly those in the gut, also represent a pool of origin for resistance genes. When infections are treated with an antimicrobial agent, all bacteria in the host are affected, including the commensal flora, which could result in the selection of resistant commensals, particularly in children who are administered oral antimicrobial drugs too frequently. Large numbers of these resident bacteria are present in the digestive tract where they are often in transient, but intimate, contact with exogenous microorganisms that are in various developmental states, including competence. These conditions favor the transfer of genes by transformation and by conjugation. Including

antimicrobial drugs in animal feed also leads to the selection of a pool of resistance genes that can be transferred to commensal bacteria in the human digestive tract and thus ultimately to human pathogens, even when selective pressure is absent (10).

In the case of mutations, predictions can be supported by 2 types of experimental approaches: in vivo with intact bacteria or in vitro by using DNA. Mutations resulting in resistance can be obtained in an accelerated fashion by using hypermutators, that is, bacteria deficient in the DNA repair system (11). Mutations are also accumulated by using continuous cultures, preferably in chemostats under suitable selective pressure. A similar enhanced rate of evolution can be obtained by (saturated) DNA mutagenesis, followed by transformation into an appropriate host. This technique, for example, was used successfully to study the extent of variations in penicillinase genes that generate extended-spectrum β-lactamase agents (12).

Pathways to Resistance

Modulation of Gene Expression

In addition to developing mutations in structural genes for drug targets, bacteria can become resistant after mutational events in motifs for gene expression, such as promoters (13), in regulatory modules, such as 2-component regulatory systems (14), or positioning upstream from a gene of a mobile (15,16) or stable (17) promoter. Enhanced expression of genetic information can also be caused by alterations in translation attenuation (18). The DNA regions involved in gene regulation are not always adjacent to the target gene. This factor makes finding regulatory mutations more complicated and makes detecting resistance by this mechanism generally impossible by genotypic techniques (19).
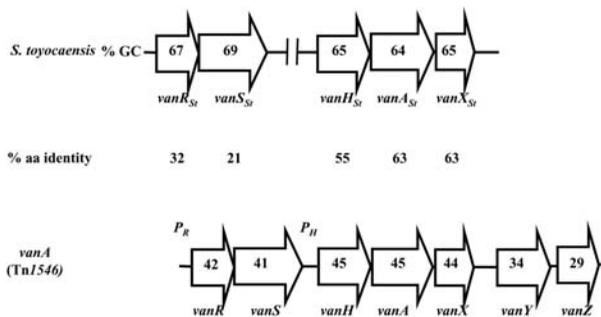
Figure 1. Comparison of the *van* gene cluster from the glycopeptide producer *Streptomyces toyocaensis* (32) and of the *vanA* operon (33) from gram-positive cocci. Open arrows represent coding sequences and indicate direction of transcription. The guanosine plus cytosine content (% GC) is indicated in the arrows. The percentage of amino acid (aa) identity between the deduced proteins is indicated under the arrows.

Dissemination by Transformation

Dissemination by transformation is more likely in spontaneously transformable bacterial species such as *Streptococcus pneumoniae*, *Acinetobacter* spp., and *Neisseria* spp. These bacteria can easily acquire, integrate, and express stretches of DNA. Since the latter can include portions of foreign chromosomes, this process renders chromosomal mutations infectious (20).

Combination of Mechanisms

Because of increased activity or the expanded spectrum of certain drug classes (e.g., β-lactam agents and fluoroquinolones) or of local therapy (e.g., extremely high concentrations in the gut after oral administration of glycopeptides that do not cross the digestive barrier) bacteria need to combine mechanisms that confer resistance to the same class of molecules. This process is necessary to achieve high-level resistance (21) or expand the substrate range provided by a single resistance mechanism (22). An example is provided by gram-negative bacteria and β-lactam agents. Extended-spectrum β-lactamase agents are point mutants of "old" penicillinases (23). Generally, the biologic price to pay for extending the substrate range of this enzyme is hypersusceptibility to β-lactamase inhibitors. However, the presence in certain enterobacteria of the gene for a penicillinase on a small multicopy plasmid, which results in production of large amounts of the enzyme and confers resistance to β-lactamase inhibitors by trapping (24). The net result of this combinatorial approach is the production of gram-negative bacteria that are resistant to all β-lactam agents, except carbapenems and cephamycins, which are not substrates for the enzymes.

Two Mechanisms Involved in Resistance Are Increasingly Frequent

Impermeability

No antimicrobial agent is active against all bacteria. In fact, the intrinsic (natural) resistance of bacteria, which is better designated as insensitivity, defines the spectrum of activity of a drug, usually because the antimicrobial drug does not penetrate the bacteria. However, microorganisms can become resistant to nearly all drug classes, including those that act at the surface of the bacteria (e.g., β-lactam agents, bacitracin), by impermeability. This resistance can be secondary to 2 distinct pathways: passive, which involves alterations of outer membrane proteins, the porins, which decrease the rate of entry of antimicrobial drugs into the bacteria by diminution of the pore size (25), and active, which involves overexpression of an indigenous efflux pump that exports the antimicrobial drug outside the cell after a regulatory mutation (26).

Trapping

The mechanism of trapping, already mentioned in the case of resistance to β-lactam agents by a combination of β-lactamases, allows titration of the drugs, an alternative to impermeability, for lowering the intracellular concentrations of the antimicrobial drugs. This mechanism also works against aminoglycosides in bacteria that overproduce an enzyme that has affinity for a drug they cannot inactivate since it lacks the modification site (Figure 2) (27,28). This mechanism has also been proposed to account for low-level resistance to glycopeptides in staphylococci by overproducing target sites in the outer layers of the peptidoglycan; thus, the antimicrobial drug does not reach the important target sites where the wall is assembled on the outer surface of the cytoplasmic membrane (29).

Prediction at the Genetic Level

Genes from gram-positive cocci can be transferred by conjugation (of plasmids or transposons) not only among these microorganisms but also to gram-negative bacteria (30). The reverse is not true because of limitations in heterologous gene expression. Consequently, one can confidently predict further dissemination of the resistance gene pool of gram-positive to gram-negative bacteria.

We have been aware for a long period that "everything that exists in the universe is the result of chance and necessity" (Democritus, 460–370 BC), which holds true for antimicrobial drug resistance. Most unfortunately, and for various reasons, it is extremely difficult to think like a bacterium. In other words, predicting the emergence of resistance to a drug class by a precise molecular mechanism is nearly impossible (e.g., glycopeptide resistance in enterococci or plasmid-mediated resistance to fluoroquinolones). We also cannot anticipate, among all the conceivable mechanisms of resistance (31), which will emerge first under natural conditions. However, based on the understanding during recent decades of the physiology (genetics and biochemistry) of bacterial resistance to antimicrobial drugs, impressive progress has been made in the tech-
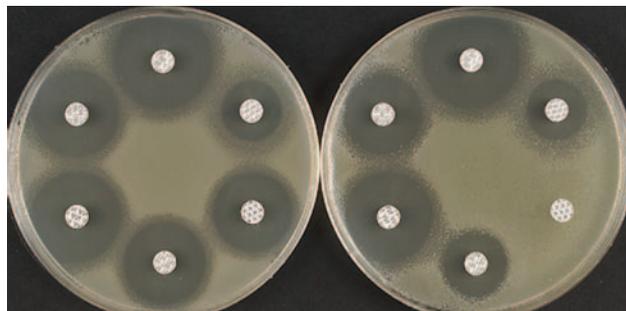
Figure 2. Disk susceptibility test results of *Escherichia coli* BM694 (left) and of strain BM694 harboring plasmid pAT346, which confers tobramycin resistance by trapping (right) (27).

niques for in vitro detection and for elucidation of resistance. This progress should, in turn, be helpful in delaying the second step of resistance: dissemination.

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Emerging Foodborne Trematodiasis

Jennifer Keiser* and Jürg Utzinger*

Foodborne trematodiasis is an emerging public health problem, particularly in Southeast Asia and the Western Pacific region. We summarize the complex life cycle of foodborne trematodes and discuss its contextual determinants. Currently, 601.0, 293.8, 91.1, and 79.8 million people are at risk for infection with *Clonorchis sinensis*, *Paragonimus* spp., *Fasciola* spp., and *Opisthorchis* spp., respectively. The relationship between diseases caused by trematodes and proximity of human habitation to suitable freshwater bodies is examined. Residents living near freshwater bodies have a 2.15-fold higher risk (95% confidence interval 1.38–3.36) for infections than persons living farther from the water. Exponential growth of aquaculture may be the most important risk factor for the emergence of foodborne trematodiasis. This is supported by reviewing aquaculture development in countries endemic for foodborne trematodiasis over the past 10–50 years. Future and sustainable control of foodborne trematodiasis is discussed.

Foodborne trematodiasis, which is caused by liver flukes (*Clonorchis sinensis*, *Fasciola* spp., *Opisthorchis* spp.), lung flukes (*Paragonimus* spp.), and intestinal flukes (*Echinostoma* spp., *Fasciolopsis buski*, heterophyids), is an emerging public health problem. In China, clonorchiasis infections have more than tripled over the past decade; ≈15 million people were infected with *C. sinensis* in 2004 (1).

The epidemiology of foodborne trematodiasis has changed in recent years. In some settings, the prevalence of foodborne trematode infections decreased significantly, which can be explained by factors such as social and economic development, urbanization, adequate food inspections, health education campaigns, use of chemical fertilizers, and water pollution (2–5). In many other areas, however, higher frequencies and transmission dynamics have been observed, which is probably the result of expansion of aquaculture for production of freshwater fish and crustaceans and improved transportation and distribution systems to bring these aquatic foods to local and international markets (5,6).

The contribution of aquaculture to global fisheries increased from 5.3% in 1970 to 32.2% in 2000 (7). By 2030, at least half of the globally consumed fish will likely come from aquaculture farming (8). Total global registered aquaculture production in 2000 was 45.7 million tons, of which 91.3% was farmed in Asia (7). Freshwater aquaculture production has increased at a particularly high rate; currently, it accounts for 45.1% of the total aquaculture production. For example, the global production of grass carp (*Ctenopharyngodon idellus*), an important species cultured in inland water bodies and a major intermediate host of foodborne trematodes, increased from 10,527 tons in 1950 to >3 million tons in 2002, accounting for 15.6% of global freshwater aquaculture production (http://www.fao.org). The major producer of grass carp is China, where it is traditionally eaten raw as sushi or *yusheng zhou* (1).

As the world's population continues to grow, efforts to increase annual fish production are essential to maintain food with a high protein value. To meet the projected demand, global production of aquatic products needs to double over the next 25 years (9). Because wild stocks are being increasingly overfished, ≈50% of marine fisheries are being used at maximum capacity, the aquaculture sector must expand to meet future needs (8,9). Aquaculture production is expected to grow at an annual rate of 5% to 7% at least until 2015 (10). Aquaculture development will provide employment and spur economic growth, both important factors for reducing poverty. However, this expansion and intensification of aquaculture should be monitored carefully in countries where foodborne trematodes are endemic because their frequencies might increase, leading to more subclinical and clinical disease.

To our knowledge, no comprehensive analysis of the relationship between occurrence of foodborne trematodiasis and development of water resources has been conducted. This situation motivated us to update estimates of people at risk for the major foodborne trematodes, to quantify the changes in freshwater fish and crustacean production in the past 10–50 years in trematodiasis-endemic countries, and to examine the relationship between

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proximity of human habitation to freshwater bodies and infections with liver, lung, or intestinal flukes. Our work will contribute to strengthening and expanding the current evidence base of contextual determinants of water-related, vectorborne diseases, including malaria (11), lymphatic filariasis (12), and Japanese encephalitis (13).

Life Cycle

The complex life cycle of foodborne trematodes has been summarized in recent publications (1,5). Briefly, parasite eggs from infected humans or animals reach freshwater bodies through contaminated fecal matter, e.g., through nonhygienic defecating habits of humans or the use of human feces for fertilizer (night soil) (4). Foodborne trematodes have widespread zoonotic reservoirs. Cats, dogs, foxes, pigs, and rodents are definitive hosts for *C. sinensis*, and domestic ruminants serve as reservoirs for *Fasciola hepatica* infections (1,14). Once eggs have reached a suitable body of fresh water, they develop and release a miracidium. It enters an aquatic snail, which acts as first intermediate host. Inside the snail, within several weeks, the miracidium transforms into cercariae. They are released into the freshwater environment and attach, penetrate, and encyst as metacercariae in susceptible second intermediate hosts. Infection with foodborne trematodes is accomplished through ingestion of metacercariae by eating raw or insufficiently cooked freshwater fish (*C. sinensis*, *Opisthorchis* spp., *Echinostoma* spp., heterophyids, *Metagonimus* spp.), freshwater crab or crayfish (*Paragonimus* spp.), aquatic plants (*Fasciola* spp., *Fasciolopis buski*), snails or tadpoles (*Echinostoma* spp.), or by drinking contaminated water (*Fasciola* spp.).

Contextual Determinants

Figure 1 depicts the contextual determinants of foodborne trematodiasis. The most important epidemiologic features responsible for transmission of foodborne trematodes include 1) ecologic and environmental factors, 2) behavioral factors, and 3) socioeconomic and cultural factors.

Population dynamics of the first intermediate host snails are affected by several environmental factors, particularly the quality, current, and temperature of the fresh water. For example, *Fossaria cubensis* and *Pseudosuccinea columella,* first intermediate hosts of *F. hepatica*, were studied in Cuba. While the former snail was more abundant in polluted habitats, the latter snail prefers clean water (15). Rainfall or evapotranspiration also show a correlation with intermediate host snail populations. In many countries, a seasonal distribution of facioliasis affected by temperature and rainfall has been observed (16).

More than 100 fish species are secondary intermediate hosts for *C. sinensis* and 35 are secondary intermediate hosts for *Opisthorchis* spp. (17). More than 50 species of
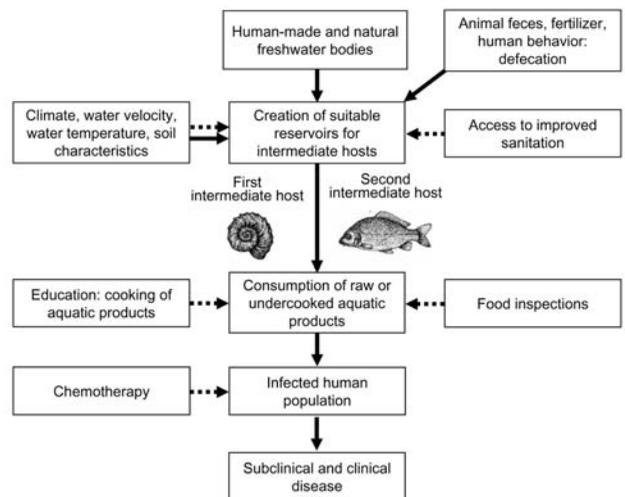
Figure 1. Contextual determinants of foodborne trematodiasis. Solid arrows, negative impact; dashed arrows, positive impact.

crustacean have been identified as secondary intermediate hosts for *Paragonimus* spp. (17). With the exception of the second intermediate host of the heterophyids (mullets, perches, and gobies), which thrive in brackish water (4), the second intermediate hosts of the other foodborne trematodes are confined to stagnant or slow-flowing fresh water (Table 1). Irrigation schemes, particularly those for rice growing, are also highly suitable reservoirs for the intermediate hosts.

Behavioral determinants include unsanitary defecation habits, use of human excreta as fertilizer, and food consumption and cooking habits. In villages near the Nam Pong water resources development project in Thailand, no correlation was found between households with latrines and the extent of opisthorchiasis (21). Cooking and food consumption–related determinants are complex and include economic and sociocultural (i.e., beliefs and tradition) factors. Traditional local dishes include raw or partially cooked aquatic products. They are frequently eaten in areas endemic for foodborne trematodiasis and are part of deeply rooted cultures. Examples of typical local fish dishes include raw crab soaked in soy sauce (*ke-jang*) in the Republic of Korea, raw drunken crabs and raw grass carp in China, and raw fish (*lab-pla* and *plasom*) in Thailand (1,17). Conversely, in industrialized countries (e.g., Japan), infections are often coupled with foreign travel and eating imported aquatic foods or exotic delicacies (22).

Public health interventions, such as chemotherapy, access to improved sanitation, food inspections, and education campaigns to teach proper cooking methods for fish and other potentially contaminated aquatic foods, will affect the pool of parasites. They will also reduce the prevalence of major foodborne trematode infections.

Table 1. Geographic distribution and population at risk for major foodborne trematode infections

Foodborne trematodes	Species	Geographic distribution	Second intermediate hosts; habitats	Population at risk ($\times 10^6$)
Liver flukes	Clonorchis sinensis	China (except for Inner Mongolia, Ningxia, Qinghai, Tibet, Xinjiang), Republic of Korea, Taiwan, Vietnam*	>100 species of freshwater fish; freshwater habitats with stagnant or slow-moving waters (ponds, river, aquaculture, swamps, rice fields)	601.0†
	Opisthorchis felineus	Kazakhstan, Russian Federation, Siberia, Ukraine‡	>35 species of freshwater fish; freshwater habitats with stagnant or slow-moving waters (ponds, river, aquaculture, swamps, rice fields)	12.5§
	Opisthorchis viverrini	Cambodia, Lao People's Democratic Republic, Thailand, Vietnam‡		67.3¶
	Fasciola hepatica, Fasciola gigantica	Altiplano of Bolivia, Cuba, highlands of Ecuador and Peru, Nile delta of Egypt, northern Islamic Republic of Iran, Portugal, Spain‡	Watercress and other water plants (drinking water); irrigation channels, pastures, banks of rivers, ponds, pools	91.1#
Lung flukes	Paragonimus spp.	Southwestern Cameroon, China, Ecuador, eastern Nigeria, Peru, the Philippines, Republic of Korea**	>50 species of freshwater crab and crayfish; freshwater habitats with stagnant or slow-moving water (ponds, aquaculture)	292.8††
Intestinal flukes	Fasciolopsis buski	Bangladesh, China, India, Indonesia, Malaysia, Taiwan, Thailand‡‡	Water caltrop, water chestnut, water hyacinth, water bamboo, duckweed, water mimosa, water spinach; drainage systems of pig farms, freshwater habitats with stagnant or slow-moving waters	Not known
	Echinostoma spp.	China, Indonesia, Malaysia, the Philippines, Republic of Korea, Taiwan, Thailand‡‡	Molluscs, fish, snails and tadpoles; freshwater or brackish habitats with stagnant or slow-moving waters	Not known
	Heterophyes heterophyes	China, Egypt (Nile delta), India, Indonesia, Islamic Republic of Iran, Philippines, Sudan, Taiwan, Tunisia, Turkey‡‡	Brackish water fish (mullets, perches, gobies); brackish water habitats	Not known
	Metagonimus yokogawai	The Balkans, China, Indonesia, Islamic Republic of Iran, Israel, Japan, Republic of Korea, Spain, Taiwan‡‡	Freshwater (Cyprinid) fish; freshwater habitats	Not known

*References 1 and 18.
†Obtained by adding population at risk in China (including Taiwan) (1), Vietnam (10 million; J.Y. Chai, pers. comm.), and Republic of Korea (44% of 2005 population (17,19).
‡Reference 17.
§Obtained by adding 8% of 2005 population in Russian Federation, 1.3% in Kazakhstan, and 2% in Ukraine (17,19).
¶Obtained by adding 2005 population in the Lao People's Democratic Republic, population at risk in Vietnam (10 million; J.Y. Chai, pers. comm.), and 80% of 2005 population in Thailand (17,19).
#Obtained by adding 23% of 2005 population in Bolivia, 20.6% in Ecuador, 35.3% in Peru, 24.3% in Spain, 44.2% in Portugal, 50.7% in Egypt, 10.8% in the Islamic Republic of Iran, and total 2005 population in Cuba (17,19).
**References 4, 17, and 18.
††Obtained by adding 15.9% of 2005 population in China, 18.9% in Ecuador, 1.4% in Peru, and 14% in Republic of Korea (17,19). Population at risk in Cameroon estimated at 2.7 million (population of the South and Central province, known foci for paragonimiasis [20], estimated at 1.5 million in 1982 [http://www.absoluteastronomy.com/encyclopedia/C/Ce/Centre_Province_Cameroon.htm]), which we standardized to 2005 (19). No estimate was provided for population at risk in eastern Nigeria.
‡‡Reference 4.

Geographic Distribution and Population at Risk

Table 1 summarizes the disease endemic countries and estimated populations at risk for the major foodborne trematodes. For China and Vietnam, we used recent estimates of their at-risk population (1) (J.Y. Chai, pers. comm.). For other countries, estimates of at-risk populations have been obtained by multiplying the fraction of a previous estimate of the population at risk provided by an expert committee of the World Health Organization (17) by the most recent population figures available (19). For example, in 1995, an estimated 19 million people (44%) in the Republic of Korea were at risk for clonorchiasis. Applying the latest United Nations national population sta-

tistics (19), we estimate that 21 million people are now at risk for infection with *C. sinensis* in the Republic of Korea.

We found that 601 million people are currently at risk for infection with *C. sinensis,* of whom 570 million are in China and Taiwan. *C. sinensis* is also prevalent in Vietnam. *Opisthorchis viverrini* is endemic in Cambodia, the Lao People's Democratic Republic, Thailand, and Vietnam, and *O. felineus* is endemic in the former Soviet Union, Kazkastan, and Ukraine (17,18). An estimated 67.3 million people are at risk for infection with *O. viverrini* and 12.5 million are at risk for infection with *O. felineus.*

Human fascioliasis is a major public health problem in the Andean countries, western Europe, the Islamic

Republic of Iran, Egypt, and Cuba (16), with an estimated 91 million people at risk. This figure is half the previous estimate (17) because China has not been included in our calculation. Although *F. hepatica* is of considerable veterinary significance in China, human infections are rare (23). This finding supports our position not to include this country in the estimate. At least 292.8 million people are at risk for infection with *Paragonimus* spp., with 195 million residing in China. Paragonimiasis also occurs in the Republic of Korea and the Philippines, parts of Africa (eastern Nigeria and southwestern Cameroon), and South America (Ecuador and Peru) (17,18).

No estimates are currently available regarding populations at risk for intestinal flukes. *F. buski* is common in Bangladesh, China, India, Indonesia, Malaysia, Taiwan, and Thailand (17,18). Echinostomes have been reported in China, Indonesia, Malaysia, the Philippines, the Republic of Korea, Taiwan, and Thailand (18). Among the heterophyids, *Heterophyes heterophyes* and *Metagonimus yokogawai* are the 2 species of greatest medical importance. They are prevalent in the Balkans, China, Egypt, India, Indonesia, the Islamic Republic of Iran, Israel, Japan, the Republic of Korea, the Philippines, Spain, Sudan, Taiwan, Thailand, Tunisia, and Turkey (4).

Aquaculture Development in Trematode-endemic Countries

Aquaculture is the most rapidly growing food sector and global consumption of aquatic products has exceeded that of meat products (24). Numerous aquatic products are available at affordable prices to most population segments in the developing world. For ≈1 billion people, these foods provide more than one fourth of their total animal protein supply (24). We compiled data on the development of freshwater fish and crustacean production in the past 10–50 years with an emphasis on those countries where *C. sinensis*, *O. felineus*, *O. viverrini*, and *Paragonimus* spp. are endemic. Data were obtained from the Food and Agricultural Organization (http://www.fao.org/fi/default.asp).

Freshwater fish aquaculture has increased exponentially from an annual production of 136,000 tons in 1952 to 16.6 million tons (Figure 2) 50 years later in China, the Republic of Korea, and Vietnam. Most (97.6%, 16.2 million tons) of this fish is produced in China. In China, the amount of land used for aquaculture has increased by 75% from 2.8 million hectares in 1970 to 4.9 million hectares in 1997 (7). Freshwater crustacean production in China has increased 48-fold over the past decade from 9,509 tons in 1992 to 453,696 tons in 2002. These developments are of considerable health concern because fish and crustaceans act as second intermediate hosts of clonorchiasis and paragonimiasis, respectively.
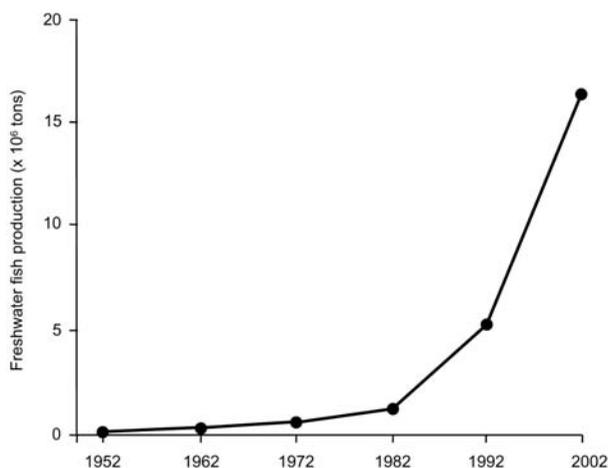
Figure 2. Development of freshwater fish production in China, 1952–2002.

Figure 3 shows that a large increase in aquaculture has also occurred in the *O. viverrini*–endemic countries of Cambodia, the Lao People's Democratic Republic, Thailand, and Vietnam. In Vietnam, freshwater fish production increased from 41,750 tons in 1962 to 390,000 tons 40 years later (a 9.3-fold increase). Conversely, fish production has decreased by 29.4% in the *O. felineus*–endemic countries of Kazakhstan, Ukraine, and the Russian Federation from 171,542 tons in 1992 to 121,032 tons in 2002.

Available aquaculture statistics are underestimated because small-scale aquaculture or rice field fisheries are not included in officially reported annual production. For example, although the officially reported annual number of inland fish produced in the Lao People's Democratic Republic in 1999 was 25,521 tons, the estimated total figure was 5.9- and 7.8-fold higher (150,000–200,000 tons) (25).
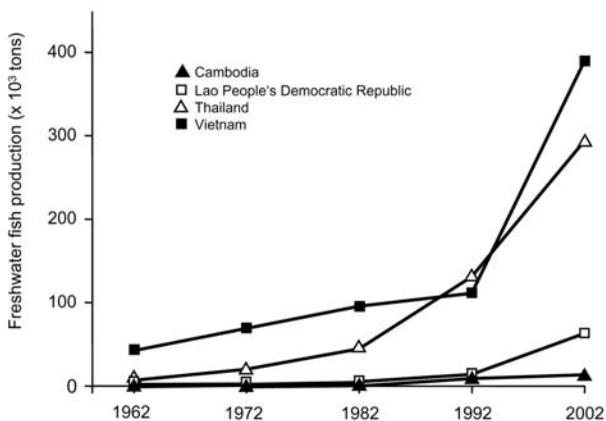
Figure 3. Development of freshwater fish production in *Opisthorchis viverrini*–endemic countries, 1962–2002.

Freshwater aquaculture is often largely dispersed, characterized by an informal nature, and usually operated in remote rural areas. Part-time fishing is the rule rather than the exception and, most importantly, the dominant share of the production is eaten within the communities where freshwater fish and other aquatic products are cultivated (25).

Relationship between Foodborne Trematodiasis and Proximity to Freshwater Bodies

We systematically reviewed the literature with an emphasis on proximity of human habitation to any form of freshwater body and its relationship to foodborne trematodiasis. Our search included the National Library of Medicine's Medline, Scielo, Biosis previews, and the Web of Science. We used the following keywords: *Opisthorchi*s, *Clonorchis*, *Fasciola*, *Echinostoma*, *Fasciolopsis*, foodborne trematodes, and foodborne trematodiasis in combination with prevalence, water, river, irrigation, dam, aquaculture, pond, and stream. Papers published in English, French, and German were considered. We also included manuscripts in Chinese, Japanese, Korean, and Russian if there was an English abstract of these papers on the aforementioned electronic databases.

Information from 12 studies on the prevalence of foodborne trematode infections in villages located close to freshwater bodies (i.e., rivers, streams, dam reservoirs, and irrigation schemes) and more distant villages is shown in Table 2. Five studies were conducted in the Republic of Korea, 4 in Thailand, 2 in Peru, and 1 in Vietnam. Five studies analyzed *C. sinensis,* 4 analyzed *O. viverrini*, and 2 analyzed *F. hepatica*. *M. yokogawai* was examined in 2 settings.

Relative risk (RR) calculations were attainable for 10 studies. We calculated RR and 95% confidence intervals (CIs) by using EasyMA software (36). A random-effects model was used for calculation of pooled RR because the interventions and conditions in these studies were expected to be heterogeneous (37). The results are summarized in Table 2 and shown as Forrest plots in Figure 4. The random summary RR measure was 2.15 (95% CI 1.38–3.36) indicating that risk for infection with foodborne trematodes in villages near freshwater bodies is 2.15-fold higher compared to that farther from the water. In 2 villages on the Khong River and Nam Pong water resources development project in northeastern Thailand, lower prevalences of *O. viverrini* were observed near the river and reservoir compared with villages not using these water sources. These observations can be explained by low snail densities in the dam reservoir (10) and the Khong River, the latter because of faster current (33).

Discussion and Conclusions

In an attempt to update the current picture of foodborne trematodiasis, we estimate that 601.0, 293.8, 91.1, and 79.8 million people are at risk for infection with *C. sinensis, Paragonimus* spp., *Fasciola* spp., and *Opisthorchis* spp., respectively. In the absence of recent national figures for at-risk populations, number of persons infected, and spatiotemporal distribution of these diseases in most trematodiasis-endemic countries, our estimates should be used judiciously.

Several issues are worth highlighting. First, estimates of persons at risk for major foodborne trematodes are considerably higher than most recent (dating back 10 years) comprehensive estimates. For example, the at-risk population for infection with *C. sinensis* was estimated to be 289 million people in the mid 1990s (17), which is less than half of the current estimate. Second, of great concern is the high number (15 million) of *C. sinensis* infections recently reported from China (1). Thus, within 10 years the number of *C. sinensis* infections has more than tripled in this country, which warrants in-depth investigations on the underlying causes. Third, it is important to juxtapose these observations with trends observed over the same period, but with an emphasis on soil-transmitted helminthiasis and schistosomiasis. In many parts of Southeast Asia, including China, the number of people infected with *Schistosoma japonicum* and the major soil-transmitted helminths (i.e., *Ascaris lumbricoides*, hookworms, and *Trichuris trichiura*) has decreased (38,39). These decreases are the result of socioeconomic development and chemotherapy-based illness control programs that largely depend on treatment with praziquantel, albendazole, and mebendazole. The issue of why there was an increase in the number of persons infected with *C. sinensis* when decreases were observed for *S. japonicum* and soil-transmitted helminthes therefore arises. We speculate that aquaculture development is the key risk factor.

Aquaculture is a rapidly growing food sector, mainly in the developing world, and particularly in Asian countries. Development of this sector is of pivotal importance for adequate supplies of food, generation of income, and employment. Different farmed aquatic products are affordable parts of the diet and essential contributors to human health in the developing world (24). However, aquaculture development results in ecologic transformations (40), and numerous aquatic animal diseases have emerged. Overcrowding and poor environmental conditions have been observed on fish farms, which lead to reduced immunity and higher susceptibility to common diseases (41). For example, massive infection with heterophyid metacercariae of aquacultured eels has been documented in Taiwan; dissection showed ≤3,762 heterophyid metacercariae in a single fish (42). In Tasmania, a higher number

Table 2. Studies comparing the prevalence of foodborne trematode infections in villages close to water bodies with distant villages*

Study site, period (reference)	Population sample	Characteristics of water body	Prevalence	RR (95% CI)
Asillo irrigation area, Peru, 1999 (26)	338 school children	500-hectare irrigation area with irrigation canals and drainage channels	*Fasciola hepatica*: 18.8%, 20.3%, 31.3% in 3 schools in irrigation scheme	NA
Kimhae county, Republic of Korea, 1974 (27)	1,809	River region	*Clonorchis sinensis*: 72.1% near riverside and 41.3% inland	1.74 (1.57–1.92)
Goyang county, Republic of Korea, 1974 (27)	578	River region	*C. sinensis*: 32.7% near riverside and 6.3% inland	5.16 (3.04–8.75)
Hadong Gun, Republic of Korea, 1978 (28)	1,163	Rivers and streams	*Metagonimus yokogawai*: 5.4%–90.8% in villages close to river and streams and 4% in village 4 km from river	7.44 (2.83–19.54)
Pohang industrial belt, Republic of Korea, 1989 (29)	3,180 employees; 200 for questionnaire analysis	Hyungsai River basin	*C. sinensis*: 52% of infected employees lived near river compared with 27.9% of uninfected employees	1.85 (1.28–2.67)
Okcheon-gun, Republic of Korea, 2000 (30)	1,081	Geum-Gang River	*C. sinensis*: 14.2% of inhabitants near river were infected compared with 3.2% of inland residents	4.51 (2.64–7.70)
			Metagonimus spp.: 8.4% of inhabitants near river were infected, compared with 1.7% of inland residents	5.01 (2.40–10.46)
Nong Wai irrigation area, Khon Kaen, Thailand, 1974–1975 (31)	627 children	Irrigation canal and channels	*Opisthorchis viverrini*: 7.3% in irrigated villages and 3.3% in nonirrigated villages	2.20 (0.87–5.51)
Nam Pong development project, Khon Kaen province, Thailand, 1977–1978 (21)	3,183	Reservoir and irrigation scheme	*O. viverrini*: 27.1% in irrigated villages and 17.2% in traditional villages (no irrigation)	1.63 (1.34–2.00)
			O. viverrini: 10.8% lakeside and 11.5% in resettlement areas	0.93 (0.66–1.31)
Chonnabot village, Khon Kaen province, Thailand, 1980–1982 (32)	4,638; 246 for incidence calculation	Wide, shallow reservoirs that remained dry in 1981–1982	*O. viverrini*: 47% in uninfected individuals becoming positive within 1 year while reservoirs were flooded and 20% during period when reservoirs were dry†	2.17 (1.42–3.29)
18 villages in Nong Khai and Loei provinces, Thailand, 1981–1982 (33)	1,259	Khong River and Huang River (flowing water)	*O. viverrini*: 51.7% and 52.6% in villages >5 km from river and 27.9% and 21.7% in villages closer to river	0.47 (0.40–0.56)
12 provinces of Vietnam, 1994–2000 (34)	>20,000	Red River delta region	*C. sinensis*: ≤31% in coastal delta region, 5% in mountainous area, and 16.3% in highlands. *O. viverrini*: highest in urban coastal areas	NA
Mantaro valley, Peru, 2000 (35)	206 children	Small streams		Odds ratio 17.22
All studies				2.15 (1.38–3.36)

*RR, relative risk; CI confidence interval; NA, not available.
†Incidence values.

of trichodinids and cilian protozoan parasites were found in fish raised on farms compared to fish caught in natural bodies of water (43).

In reviewing the literature, we found that residents living near bodies of fresh water have, on average, a 2.15-fold higher risk for infections with foodborne trematodes compared to inhabitants of distant villages. Unfortunately, all studies that could be included in our metaanalysis were conducted either in the Republic of Korea or Thailand, and several of these studies date back to the 1970s. Our finding is consistent with previous observations that most of the locally caught aquatic foods are eaten in the communities near freshwater bodies (17,25). However, with improving transportation and distribution systems, which allow efficient transportation of fish, the amount sold outside the local community is likely to increase considerably. Thus,
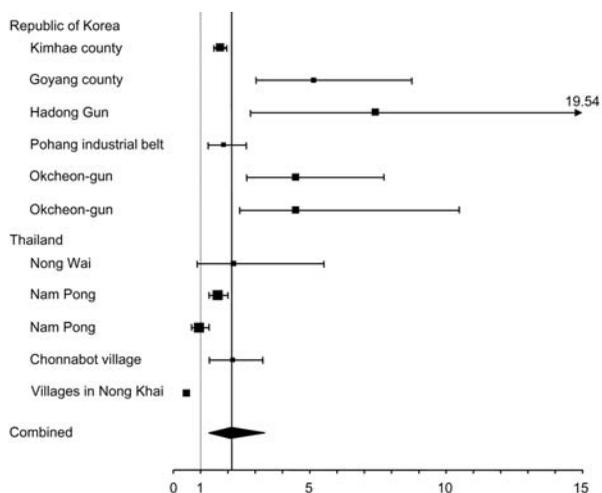
Figure 4. Metaanalysis of studies comparing the prevalence of foodborne trematode infections in villages close to water bodies with distant villages. Values on the x-axis are relative risks. Horizontal bars show 95% confidence intervals. The solid vertical line represents the mean of the combined measure. The diamond represents the combined measure.

the spatial distribution of foodborne trematodiasis will change, with an increasing prevalence of these infections in villages where no aquatic products are farmed. We suggest that future studies examine the present spatial distribution of foodborne trematodiasis in Asian countries, compare prevalence of infection in aquaculture workers with other professional groups, and determine the prevalence of parasites in fish raised in aquaculture ponds compared with natural water bodies.

This review emphasizes the important role aquaculture plays in transmitting foodborne trematodiasis. In view of the rapid growth of this food sector, strategies to reduce the current impact of these diseases and to reverse their emergence are mandatory. Safe, efficacious, and inexpensive single-dose oral drugs, such as praziquantel and triclabendazole, are available to treat foodborne trematodiasis and will remain the backbone of control (5). To enhance sustainability, chemotherapy should be used with new technologies to ensure food safety, sound health education campaigns for properly cooking aquatic foods, and access to improved sanitation.

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Evolution of H5N1 Avian Influenza Viruses in Asia

The World Health Organization Global Influenza Program Surveillance Network*1

An outbreak of highly pathogenic avian influenza A (H5N1) has recently spread to poultry in 9 Asian countries. H5N1 infections have caused $\geq$52 human deaths in Vietnam, Thailand, and Cambodia from January 2004 to April 2005. Genomic analyses of H5N1 isolates from birds and humans showed 2 distinct clades with a nonoverlapping geographic distribution. All the viral genes were of avian influenza origin, which indicates absence of reassortment with human influenza viruses. All human H5N1 isolates tested belonged to a single clade and were resistant to the adamantane drugs but sensitive to neuraminidase inhibitors. Most H5N1 isolates from humans were antigenically homogeneous and distinct from avian viruses circulating before the end of 2003. Some 2005 isolates showed evidence of antigenic drift. An updated nonpathogenic H5N1 reference virus, lacking the polybasic cleavage site in the hemagglutinin gene, was produced by reverse genetics in anticipation of the possible need to vaccinate humans.

Highly pathogenic avian influenza viruses of the H5N1 subtype are circulating in eastern Asia with unprecedented epizootic and epidemic effects (1). Nine Asian countries reported H5N1 outbreaks in poultry in 2004: Cambodia, China, Indonesia, Japan, Laos, Malaysia, South Korea, Thailand, and Vietnam (1). Between 2004 and the first 3 months of 2005, a total of 89 laboratory-confirmed human infections, 52 of which were fatal, were reported to the World Health Organization (WHO) by public health authorities in Vietnam, Thailand, and Cambodia. These records indicate that this outbreak of human H5N1 infections is the largest documented since its emergence in humans in 1997 (2). Efficient viral transmission among poultry caused the virus to spread regionally, leading to the loss of >100 million birds from disease and culling. In contrast, human-to-human transmission of the virus is exceptional but has been described, most recently in a family cluster in Thailand (3).

The 3 viral envelope proteins of influenza A virus are most medically relevant. The hemagglutinin (HA), neuraminidase (NA), and M2 are essential viral proteins targeted by host antibodies or antiviral drugs such as oseltamivir and rimantadine (4–6). The HA glycoprotein forms spikes at the surface of virions, mediating attachment to host cell sialoside receptors and subsequent entry by membrane fusion. The NA forms knoblike structures on the surface of virus particles and catalyzes their release from infected cells, allowing virus spread. The M2 is a transmembrane protein that forms an ion channel required for the uncoating process that precedes viral gene expression.

We report on phylogenetic, phenotypic, and antigenic analysis of H5N1 viruses from the 2004–2005 outbreak, focusing on these 3 genes, to address questions relevant to the public health response to the outbreak: 1) What is the genetic diversity of H5N1 viruses involved in human infections? 2) Can the relationship between human and avian H5N1 isolates help explain the source of infection? 3) Do genetic changes correlate with enhanced viral transmissibility in humans? 4) How sensitive are H5N1 isolates to antiviral drugs? 5) What is the antigenic similarity between human H5N1 viruses and current candidate vaccines? and

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6) Can candidate vaccine reference stocks be developed in time for an effective public health response?

Methods

All work involving infectious H5N1 influenza was performed in government-approved biosafety level 3–enhanced containment facilities with experimental protocols in compliance with applicable federal statutes and institutional guidelines. Influenza A (H5N1) viruses isolated in Asia and A/Puerto Rico/8/34 (PR8) (H1N1) were propagated in embryonated chicken eggs or in Madin-Darby canine kidney (MDCK) cells. The African green monkey kidney Vero cell line was from a cell bank certified for human vaccine production.

Viral RNA was extracted by using a commercial lysis solution and resin kit and amplified by reverse transcriptase–polymerase chain reaction with specific oligonucleotide primers. Nucleotide sequencing reactions were performed with a cycle sequencing kit and resolved on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). DNA sequence analysis was performed by using version 10 of the GCG sequence analysis package (7), and phylogeny was inferred by using a neighbor-joining tree reconstruction method implemented in the Phylip package (8).

Postinfection ferret antisera were prepared as previously described (9). Hemagglutination inhibition (HI) testing was performed as previously described with turkey erythrocytes (10).

Median inhibitory concentration (IC_{50}) values for oseltamivir and zanamivir were determined by using NA-Star substrate and Light Emission Accelerator IITM (Applied Biosystems, Bedford, MA, USA) as previously described (11). Biological susceptibility to rimantadine was determined by recording the yield of viral progeny in MDCK cells infected with the H5N1 strains of interest at a multiplicity of >10 median egg infectious doses in the absence or presence of 2 µg/mL rimantadine.

Plasmids with full-length cDNA from the 6 internal genes (PB1, PB2, PA, NP, M, NS) of influenza virus PR8 strain (12), flanked by human RNA polymerase I (PolI) promoter and polyadenylation site at the 3′ end and a PolI terminator as well as a PolII promoter at the 5′ end, were generated as described previously (12–14). The cDNA of N1 NA or H5 HA genes of VN/1203/2004 or VN/1194/2004 (VN/04-like) were inserted into plasmids as described above. The 4 basic amino acid codons from the cleavage site of HA were deleted by overlap extension PCR, as described previously (sequences available upon request) (13,15–17).

PR8 reassortant viruses with HA and NA from VN/04-like viruses were generated by plasmid DNA-based reverse genetics in Vero cell under good laboratory practice conditions appropriate for future human use. Candidate vaccine reference reagent reassortant viruses were generated at the National Institute of Biological Standards and Control (NIBSC), South Mimms, United Kingdom; Saint Jude Children's Research Hospital (SJCRH), Memphis, Tennessee, USA; and Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, USA. For brevity, the reverse genetics derivation method described represents a consensus of the institutions; minor unpublished protocol details unique to each laboratory were not described and are available upon request. The VN/04x/PR8 reassortant virus was recovered in embryonated eggs and identified in the allantoic fluid by HA assay. The genetic and antigenic properties of the resulting reassortant virus were determined as described previously (15,18–20). Candidate vaccine stocks were subjected to virulence studies in avian, murine, and ferret models to establish their safety (19).

Results

Analysis of HA, NA, and M2 Genes from H5N1 Viruses

Phylogenetic analyses of the H5 HA genes from the 2004 and 2005 outbreak showed 2 different lineages of HA genes, termed clades 1 and 2. Viruses in each of these clades are distributed in nonoverlapping geographic regions of Asia (Figure 1). The H5N1 viruses from the Indochina peninsula are tightly clustered within clade 1, whereas H5N1 isolates from several surrounding countries are distinct from clade 1 isolates and belong in the more divergent clade 2. Clade 1 H5N1 viruses were isolated from humans and birds in Vietnam, Thailand, and Cambodia but only from birds in Laos and Malaysia. The clade 2 viruses were found in viruses isolated exclusively from birds in China, Indonesia, Japan, and South Korea. Viruses isolated from birds and humans in Hong Kong in 2003 and 1997 made up clades 1′ and 3, respectively.

The HA genes from H5N1 viruses isolated from human specimens were closely related to HA genes from H5N1 viruses of avian origin; human HA gene sequences differ from the nearest gene from avian isolates from the same year in 2–14 nucleotides (<1% divergence). These findings are consistent with the epidemiologic data that suggest that humans acquired their infections by direct or indirect contact with poultry or poultry products (21).

Analysis of the amino acid sequences showed that both clades of H5 HAs from the 2004–2005 outbreak have a multiple basic amino acid motif at the cleavage site, a defining feature of highly pathogenic avian influenza viruses. Among all H5N1 isolates collected in east Asia since 1997, only those in clades 1, 1′, and 3 appear to be associated with fatal human infections (22,23). We compared amino acid sequences of HA from contemporary

isolates (clades 1 and 2) with those of the fatal H5N1 infections in Hong Kong in 1997 and 2003 to identify changes that may correlate with patterns of human infection (Table 1). Thirteen polymorphic sites were identified when the HA1 from the 4 consensus sequences were compared. One change in the 2004–2005 viruses is serine 129 to leucine (S129L). This change affects receptor binding because S129 makes atomic contact with cellular sialoside receptors (24). A second structural change in HA was the A156T substitution, which resulted in glycosylation of asparagine 154 and is predicted to reduce its affinity for sialosides. This change is commonly associated with viral adaptation to terrestrial poultry and increased virulence for these birds (25–27).

Because of the heightened alert due to H5N1 infections in Vietnam during the first months of 2005, we examined the HA sequences for evidence of shared amino acid changes. The HA of viruses isolated in the first 3 months of 2005 showed several amino acid changes relative to 2004 viruses (Table 1). None of the changes in the HA were common to all the 2005 viruses, which suggests that these variant viruses are cocirculating independently in poultry. The most commonly observed changes are located within short distances of the receptor-binding site. For example, positions D94, L175, and T188 may modulate the interaction of Y91, H179, and L190 with sialosides. One of the isolates from a fatal infection in 2005 showed a substitution of serine 223 to asparagine, which is predicted to facilitate binding of sialosides commonly found in mammalian species (Table 1).

The phylogenetic tree of the NA genes resembled that of the HA genes, which indicates coevolution of these 2 envelope genes (Figure 2). NA genes of isolates from Thailand seem to have diverged to form a group distinct
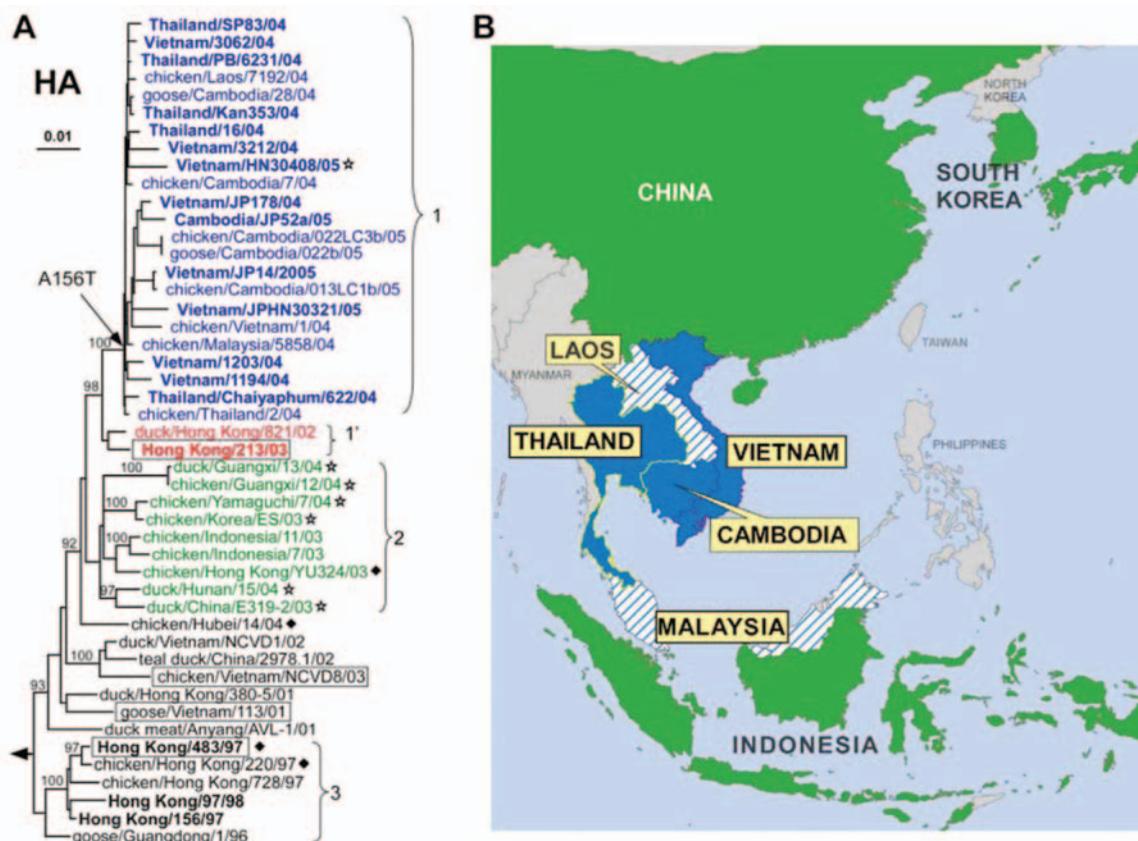
Figure 1. Phylogenetic relationships among H5 hemagglutinin (HA) genes from H5N1 avian influenza viruses and their geographic distribution. Viral isolates collected before and during the 2004–2005 outbreak in Asia and selected ancestors were included in the analysis (online Appendix Table, available from http://www.cdc.gov/ncidod/EID/vol11no10/05-0644_app2.htm). HA clades 1, 1′, and 2, discussed in the text, are colored in blue, red, and green fonts, respectively. Virus names in **boldface** denote isolates from human infections. Phylogenetic trees were inferred from nucleotide sequences by the neighbor-joining method with A/chicken/Scotland/56 genes as outgroup (not shown, denoted by arrowhead). Bootstrap analysis values ≥90% are shown. A) HA gene tree phylogeny was based on the coding region of the segment. Presence of a motif for glycosylation in HA is indicated as A156T by an arrow at the root of clade 1 and a diamond for other clades (Table 1). Stars denote absence of 1 arginine residue at the polybasic cleavage site, which starts at position 325 of HA1. Isolates to which ferret antisera were made for antigenic analyses are boxed (Table 2). B) Geographic distribution of H5N1 in east Asia: blue denotes countries reporting infections with clade 1 H5N1 in humans and birds (solid) or in birds only (hatched). Green denotes countries reporting bird infections with clade 2 H5N1 viruses.

Table 1. Amino acid differences among H5 hemagglutinins (HA1)

Clade 3	Clade 2	Clade 1'	Clade 1	H3 No.	Functional significance
N45*	D	D	D	54†	Antigenic site C
S84	N	N	N	92	Antigenic site E
A86	A	A	V	93	Antigenic site E
N94	D	D	D (1)	101	Near Y91; receptor binding?
N124	D	S	S	129	Antigenic site B
S129	S	L	L	133a	Receptor binding
L138	Q	Q	Q	142	Antigenic site A
S155	–	N155	–	159	Antigenic site B
T156‡	A	A	T	160	N154 glycosylation motif
L175	L	L	L (2)	179	Near H179; receptor binding?
T188	T	T	T (3)	192	Near L190; receptor binding?
K189	R	R	K	193	Adjacent to receptor binding, antigenic site B
E212	K	K	R	216	Antigenic site D
S223	–	N223§	– (4)	227	Receptor binding
T263	A	A	T	266	Antigenic site E
325R¶	Absent	–	–	Absent	HA cleavage efficiency

*Amino acid residue in single-letter code and position in the mature H5 HA1.
†Equivalent residue number in the mature H3 HA1 aligned with H5 amino acid sequence; –, no change from HK97 clade HA consensus.
‡A156 or S156 were found in certain clade 3 HAs; A156 was present in some HAs from clade 2. HA genes from ≈50% of isolates collected in 2005 had these substitutions present in only one isolate: 1) to N or V; 2) to M or I; 3) to A, V, or I; or 4) to N.
§Change present exclusively in isolates from humans.
¶Arginine at the start of the polybasic cleavage site, position 325.

from that of genes from Vietnam viruses. As reported previously, the NA of HK/213/03 did not co-evolve with the HA genes (28). NA genes from human and related avian H5N1 isolates from 2003–2005 as well as clade 3 isolates were characterized by deletions in the stalk region of the protein (positions 49–68 for clades 1–2 and 54–72 for clade 3) (29). Deletions in the stalk of the NA are thought to increase retention of virions at the plasma membrane (30) to balance weaker binding of sialic acid receptors by the HA with newly acquired N154 glycosylation.

Neuraminidase inhibitors are effective antiviral drugs against human influenza viruses, and preclinical studies suggest a similar effectiveness against avian influenza in humans (5,31). The IC_{50} of oseltamivir for the clade 1 and 2 NA of 2004–2005 isolates was <10 nmol/L, as compared to IC_{50} values of 85 and 1,600 nmol/L for resistant H1N1 or H3N2 mutants used as controls (Table 3). Thus, NA of H5N1 isolates is sensitive to this class of antiviral agents.

The phylogenetic tree of the M genes resembled that of

the HA genes, indicating coevolution of these genes (results not shown). The amino acid sequence of the M2 protein of clade 1 viruses as well as of HK/213/03 indicated a serine-to-asparagine substitution at residue 31 (S31N), known to confer resistance to adamantanes (including amantadine and rimantadine) (6). Clade 1 isolates from 2004 and 2005 cultured in the presence of 2 μg/mL rimantadine replicated as efficiently as in untreated cultures, whereas the replication of HK/483/97 was reduced to 1% of control values, indicating that all the currently circulating clade 1 isolates are resistant to adamantanes (data not shown).

Origin of Internal Genes of H5N1 Viruses from Asia

A complete genetic characterization of circulating H5N1 viruses is critical to identify the possible incorporation of human influenza virus genes by reassortment. To this end, we analyzed the phylogeny of the internal protein coding genes. The PB2, PB1, and PA polymerase genes from

Table 2. Antigenic analysis of H5N1 isolates from Asia

Virus antigen	Clade	Reference ferret antisera*								
		HK156	NCVD8	HK213	VN1203	VN04xPR8-rg	VN78	VN4207	VN14	VN32321
A/Hong Kong/156/97	3	**1,280**	320	640	80	320	40	80	80	80
A/ck/Vietnam/NCVD8/03	–	640	**160**	80	80	160	20	<10	160	40
A/Hong Kong/213/03	1'	1,280	1,280	**2,560**	80	640	160	160	640	640
A/Vietnam/1203/04	1	40	20	<10	**640**	320	40	160	80	40
A/Vietnam/1203/04xPR8-rg	1	80	<10	10	640	**320**	40	160	160	40
A/Vietnam/1194/04	1	40	20	10	640	320	40	160	160	40
A/Vietnam/JP178/04	1	80	10	<10	1,280	320	**80**	160	160	80
A/Vietnam/JP4207/05	1	160	40	40	1,280	640	80	**320**	160	80
A/Vietnam/JP14/05	1	20	<10	10	640	80	20	40	**80**	40
A/Vietnam/JP30321/05	1	40	40	10	<10	40	10	<10	40	**160**

*Homologous HI titers are in **boldface**.
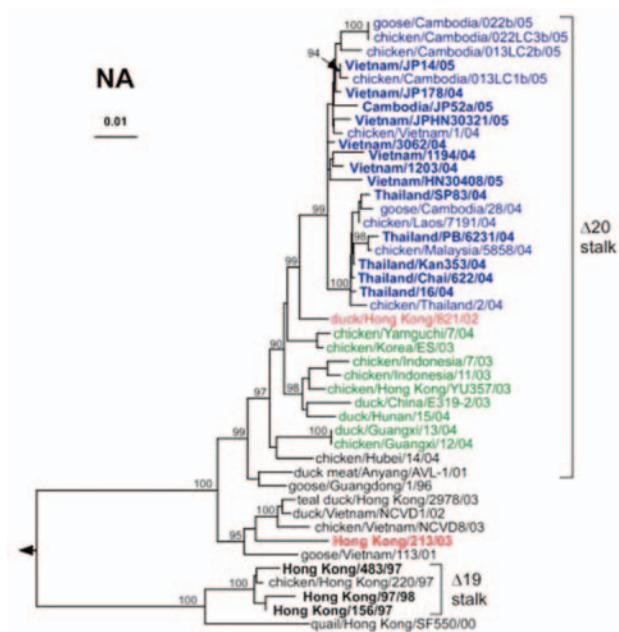
Figure 2. Phylogenetic relationships among N1 neuraminidase (NA) genes of H5N1 influenza viruses. The clade of the hemagglutinin of each of these viruses is indicated by font coloring as in Figure 1A. Brackets denote genes encoding NA protein with deletions in the stalk region; residues 49–68 for clades 1–2 and 57–75 in clade 3.

2003–2005 H5N1 isolates from humans constitute a single clade (data not shown) and have coevolved with the respective HA genes (Figure 1). No evidence of reassortment with polymerase genes from circulating H1N1 or H3N2 human influenza virus was found. The phylogenies of the NP and NS genes also supported the avian origin of these genes, indicating that all the genes from the human H5N1 isolates analyzed are of avian origin, which confirms the absence of reassortment with human influenza genes. Taken together, the phylogenies of the 8 genomic segments show that the H5N1 viruses from human infections and the closely related avian viruses isolated in 2004 and 2005 belong to a single genotype, often referred to as genotype Z (1).

Antigenic Analysis of H5N1 Viruses from Asia

Influenza vaccines whose HA are antigenically similar to circulating strains provide the highest level of protection from infection (32). H5N1 isolates collected in 2004 and 2005 analyzed by the HI test showed reactivity patterns that correlated with the 3 main clades of recent isolates identified in the HA gene phylogeny (Table 2 and Figure 1). Viruses from humans and birds in clade 1, represented by VN/1203/04, were found to constitute a relatively homogeneous and distinct antigenic group characterized by poor inhibition by ferret antisera to isolates from other clades (Table 2), in particular by the ferret antiserum raised

to HK/213/03 (64-fold reduction compared to the homologous titer). The latter isolate was previously used to develop a vaccine reference strain in response to 2 confirmed H5N1 human infections in February 2003 (15). These HI results provided the motivation for the development of an updated H5N1 vaccine that would be antigenically similar to 2004–2005 human isolates. The antigenic similarity of VN/1203/04 and the closely related VN/1194/04 to the contemporaneous H5N1 isolates from humans (data not shown) prompted their selection for vaccine reference stock development.

Antigenic analysis of human isolates from 2005 provided evidence of antigenic drift among the most recently circulating H5N1 strains (Table 2). For example, VN/JPHN30321/05 showed a reduced HI titer against VN/1203/04 reference serum. This antigenic difference is correlated with 7 amino acid differences between the HA1 domain VN/1203/04 and VN/JPHN30321/05: R53K, N84D, D94N, K140R, L175M, K189R, and V219I (Table 1 and online Appendix Table).

Development of Candidate H5N1 Vaccine Reference Stocks

Mass vaccination is the most effective approach to reduce illness and death from pandemic influenza. Inactivated influenza vaccines are manufactured from reassortant viruses obtained by transferring the HA and NA genes with the desired antigenic properties into a high-growth strain such as PR8 (33). However, reassortants with H5-derived HA with a polybasic cleavage site are potentially hazardous for animal health. Because the high pathogenicity of the H5N1 viruses in poultry, mice, and ferrets depends primarily on the polybasic cleavage site in the HA molecule, a derivative with a deletion of this motif was engineered in cloned HA cDNAs. Three high-growth reassortant influenza viruses were developed: NIBRG-14 (NIBSC), VN/04xPR8-rg (SJCRH), and VNH5N1-PR8/CDC-rg (CDC). These candidate vaccine strains, bearing mutant H5 HA, intact NA, and the internal genes from PR8, were generated by a reverse genetics approach (12,13,20,34) using Vero cells and laboratory protocols compatible with eventual use of the vaccine in human subjects (15,18). These 3 vaccine candidates were characterized genetically (nucleotide sequencing of HA and NA) and antigenically in HI assays to confirm that their antigenicity remained unchanged relative to the wildtype virus (Table 2). The candidate reference stocks had molecular and antigenic properties equivalent to parental H5N1 donor strains and lacked virulence in chicken, mouse, and ferret models.

Discussion

The growing H5N1 epizootic in eastern Asia could expand the environmental load of virus and cause more

Table 3. Sensitivity of H5N1 influenza isolates to oseltamivir

Virus	Oseltamivir IC$_{50}$*
H1N1 (H274)†	0.69
H1N1 (Y274)†	85.92
H3N2 (R292)‡	1.99
H3N2 (K292)‡	1,600.00
Hong Kong/483/97	4.86
Hong Kong/213/03	5.07
Vietnam /1194/04	2.49
Vietnam/1203/04	7.68
Chicken/VN/NCVD1/04	5.87
Chicken/VN/NCVD8/03	9.90

*Median inhibitory concentration (IC$_{50}$) of oseltamivir (nmol/L) for H5N1 influenza isolates and control H1N1 or H3N2 isolates (results for viruses shown are representatives of 31 isolates tested).
†Wildtype (H274) and resistant mutant (Y274) influenza virus A/Texas/36/91 (H1N1).
‡Wildtype (R292) and resistant mutant (K292) influenza virus A/Victoria/3/75 (H3N2).

infections in mammals (35), which would increase the probability that a highly transmissible virus will emerge in mammals. We therefore analyzed the medically relevant genes from viruses isolated from the beginning of the outbreak until March 2005 to evaluate parameters relevant to public health.

The origin of the HA genes of the 2004–2005 outbreak as well as an earlier isolate from a fatal human infection in Hong Kong in 2003 (clade 1′) can be traced back to viruses isolated in 1997 in Hong Kong (clade 3) and from geese in China (goose/Guangdong/96) (Figure 1A). The phylogeny also shows that viruses with HK/97-like HA may have circulated in avian hosts continuously after 1997, without causing any reported human infections until the 2 confirmed cases in Hong Kong in February 2003 (28).

The 2004–2005 H5N1 isolates are sensitive to 2 neuraminidase inhibitors that are recommended for prophylactic or therapeutic intervention against human infections with recent H5N1 strains. Rapidly testing potentially pandemic influenza viruses for their susceptibility to licensed drugs is essential to establish appropriate control measures.

An effective H5N1 vaccine is a public health priority and the cornerstone for pandemic prevention and control. Reverse genetics approaches allow the rapid production of high-growth PR8 reassortant viruses by engineering a virus with a homologous HA gene lacking the polybasic amino acids associated with high virulence. These candidate H5N1 pandemic vaccine viruses have been made available to vaccine manufacturers to produce pilot lots for clinical trials and are available for possible large-scale manufacturing should the need arise.

Genetic and antigenic analyses have shown that, compared to previous H5N1 isolates, 2004–2005 isolates share several amino acid changes that modulate antigenicity and perhaps other biological functions. Furthermore, our molecular analysis of the HA from isolates collected in 2005 suggests that several amino acids located near the

receptor-binding site are undergoing change, some of which may affect antigenicity or transmissibility. For example, an isolate (VN/JP12-2/05) showed a change from serine to asparagine at position 223 of the HA1 (S223N) that may affect receptor-binding specificity (36). The VN/30321/05 isolate demonstrated considerable antigenic drift from VN/04-like isolates, which have been selected as the candidate vaccine antigens. Further surveillance to determine the prevalence of such variants in poultry will be critical to determine if these variants compromise the efficacy of the candidate vaccine or increase the efficiency of transmission.

The phylogenies of the 8 genomic segments from the clade 1 and 2 isolates from 2004–2005 showed that all genes are of avian origin. All H5N1 isolates from both clades belong to 1 of the genotypes recently circulating in Eastern and Southern Asia, e.g., genotypes V and Z (1,37). The influenza virus genome has remarkable plasticity because of a high mutation rate and its segmentation into 8 separate RNA molecules. This segmentation allows frequent genetic exchange by segment reassortment in hosts co-infected with 2 different influenza viruses. No evidence has been seen that the 2004–2005 H5N1 isolates have acquired nonavian influenza genes by reassortment. However, continued surveillance is important because genetic reassortment may facilitate the evolution of viruses with increased virulence or expanded host range.

The currently circulating H5N1 viruses were reported to infect domestic or wild captive felids, such as tigers, feeding on infected bird carcasses, and the infected cats can transmit H5N1 to pen mates (38). Furthermore, circumstantial evidence indicates that tiger-to-tiger transmission of H5N1 has occurred at a zoo in Thailand (39). Recent evidence of person-to-person transmission and the clustering of H5N1 cases raise the level of concern for a pandemic of H5N1 influenza (3). Therefore, sustained and aggressive efforts to control H5N1 circulation in poultry are mandatory to avoid possible catastrophic public health consequences.

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New Measles Genotype, Uganda

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We report the first genetic characterization of wildtype measles viruses from Uganda. Thirty-six virus isolates from outbreaks in 6 districts were analyzed from 2000 to 2002. Analyses of sequences of the nucleoprotein (N) and hemagglutinin (H) genes showed that the Ugandan isolates were all closely related, and phylogenetic analysis indicated that these viruses were members of a unique group within clade D. Sequences of the Ugandan viruses were not closely related to any of the World Health Organization reference sequences representing the 22 currently recognized genotypes. The minimum nucleotide divergence between the Ugandan viruses and the most closely related reference strain, genotype D2, was 3.1% for the N gene and 2.6% for the H gene. Therefore, Ugandan viruses should be considered a new, proposed genotype (d10). This new sequence information will expand the utility of molecular epidemiologic techniques for describing measles transmission patterns in eastern Africa.

Measles virus is a negative-sense, single-stranded RNA virus in the genus *Morbillivirus* within the family *Paramyxoviridae*. Infection with this virus is typified by high fever, maculopapular rash, conjunctivitis, cough, and coryza (1). Although a vaccine-preventable disease, it still accounts for ≈770,000 deaths annually worldwide, half of which occur in Africa (2).

This virus is monotypic, but genetic variation in the hemagglutinin (H) and nucleoprotein (N) genes can be analyzed by molecular epidemiologic techniques to study transmission patterns. This molecular information, in conjunction with standard case reporting and investigation, is useful in assessing the effectiveness of vaccination programs (3–5). Genetic characterization of wildtype measles virus is a key component of laboratory surveillance activities in all phases of measles control. To facilitate genetic characterization of measles viruses, a uniform nomenclature and analysis protocol was recommended by the World Health Organization (WHO) (6). WHO currently recognizes 22 genotypes of measles virus and has established guidelines for the designation of new genotypes (5–8).

In Uganda, measles is still endemic despite the availability of a measles vaccine for almost 2 decades. This failure to completely control measles is mainly the result of inadequate coverage of measles immunization, which is a single dose of vaccine given at 9 months of age. The reported measles vaccination coverage rate in 2002 was only 74%. Given a seroconversion rate of ≈85% for measles vaccine given at 9 months of age, ≈37% of Ugandan children are expected to remain susceptible to measles. The measles vaccination coverage increased to 83% in 2003 and 2004. The number of measles cases reported to the Ugandan National Health Management Information System ranged from 57,347 in 1997 to 49,871 in 2002. Of the cases reported in 2002, 31% occurred in persons ≥5 years of age. The Ministry of Health developed a 5-year plan (2002–2006) aimed at reducing the illness and death caused by measles virus. The strategies include increasing routine immunization coverage, conducting vaccination campaigns for children 6 months to 15 years of age, providing vitamin A, and initiating a case-based measles surveillance system. In October 2003, Uganda conducted a catch-up campaign for children 6 months to 15 years of age that reached ≈13.5 million children with measles vaccine, with a national coverage of 105%. This campaign resulted in a decrease in reported measles cases to 3,522 from January to August 2004, compared with 28,072 in 2003 and 33,633 in 2002 in the same period, with no deaths among confirmed measles cases as of August 2004. Case-based measles surveillance was established in 2003, and by August 2004, >80% of districts were investigating measles cases according to the guidelines.

Some information is available on circulating measles virus genotypes in western, central, and southern Africa, but little is known about circulating strains in eastern Africa, including Uganda. The WHO Regional Measles

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Laboratory at Uganda Virus Research Institute (UVRI) undertook the present study to isolate and characterize circulating measles strains in Uganda.

Materials and Methods

Specimen Collection and Virus Isolation

Specimens for virus isolation were collected from outbreaks in different districts of Uganda, sentinel sites, and Mulago hospital, the national teaching and referral hospital, in Kampala from 2000 to 2002. Staff members from the UVRI were part of the outbreak investigation team and responsible for collecting specimens. Urine and nasopharyngeal aspirate specimens were obtained ≤7 days of onset of a rash according to WHO procedures for laboratory diagnosis of measles viral infection (9) and transported to UVRI for processing by using standard procedures.

The specimens were added onto B95a cells (10) that had been seeded into 25-cm^2 tissue culture flasks and observed daily for characteristic cytopathic effect (CPE). Infected cells were harvested when ≥75% of the culture showed CPE, and viral stocks were prepared and stored at –70°C in 0.5-mL aliquots. Specimens contaminated with bacteria or fungi were filtered through a 0.45-μm filter, and tissue culture additions were repeated. Isolates were shipped to the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia, for molecular analysis.

Polymerase Chain Reaction and Sequencing

At CDC, the isolates were passaged once in B95a cells. RNA was extracted from infected cells by using the guanidinium acid-phenol technique (11), and reverse transcriptase-polymerase chain reaction (RT-PCR) was used to amplify either the 550 nucleotides (nt) coding for the COOH terminus of N or the full-length open reading frame for H (12). PCR products were purified by using the PCR Preps DNA Purification System (Promega, Madison, WI, USA) and analyzed by agarose gel electrophoresis followed by staining with ethidium bromide. Templates were sequenced by using a cycle sequencing reaction with fluorescent dye terminators (Perkin-Elmer, Applied Biosystems Division, Foster City, CA, USA), and the reaction products were analyzed by using an ABI 3100 (Perkin-Elmer) automatic sequencer. Sequence data from multiple reactions were analyzed with version 10.1 of the Genetics Computer Group Package (Accelrys, San Diego, CA, USA). Phylogenetic analyses, including bootstrap analysis, were performed by using PAUP version 4.01 (Sinauer Associates, Sunderland, MA, USA). The sequence of the 450 nt coding for the 150 amino acids at the COOH terminus of the N gene was obtained for all the isolates, and the entire coding region of the H gene was sequenced for 2 representative isolates.

Results

Thirty-six measles virus isolates obtained from measles outbreaks (n = 8) and Mulago Hospital (n = 28) from 2000 to 2002 were selected for genetic analysis. Isolates were obtained from 6 districts within Uganda (Table 1 and Figure 1), although most were obtained from patients in Kampala. Viruses were isolated from either urine or respiratory samples collected within 7 days of rash onset. Overall, virus isolations were successful in 14% of the specimens.

Sequence data from this report are available from GenBank under accession nos. AY923185–AY923212 and AY923213–AY92321. All sequences were derived from viral isolates that were passaged once in B95a cells. The region of the N gene that is routinely sequenced for genotyping is not affected by tissue culture passage (5,6).

Table 1. Wildtype measles viruses isolated in Uganda, 2000–2002*

WHO name and genotype	Collection date
MVi/Kampala UGA/10.00-1[d10]†	3/5/2000
MVi/Kampala UGA/10.00-2[d10]	3/6/2000
MVi/Mpigi UGA/18.00[d10]†	4/27/2000
MVi/Kampala UGA/42.00-1[d10]	10/10/2000
MVi/Kampala UGA/42.00-2[d10]	10/16/2000
MVi/Kampala UGA/43.00-1[d10]	10/23/2000
MVi/Kampala UGA/43.00-2[d10]	10/23/2000
MVi/Kampala UGA/45.00[d10]	11/6/2000
MVi/Kampala UGA/46.00-1[d10]	11/8/2000
MVi/Kampala UGA/46.00-2[d10]	11/13/2000
MVi/Kampala UGA/49.00-1[d10]	11/28/2000
MVi/Kampala UGA/49.00-2[d10]	11/30/2000
MVi/Kampala UGA/50.00[d10]	12/11/2000
MVi/Kampala UGA/51.00-1[d10]‡	12/12/2000
MVi/Kampala UGA/51.00-2[d10]	12/18/2000
MVi/Kampala UGA/51.00-3[d10]†	12/18/2000
MVi/Kampala UGA/51.00-4[d10]	12/18/2000
MVi/Kampala UGA/3.01[d10]	1/15/2001
MVi/Kampala UGA/4.01-1[d10]†	1/24/2001
MVi/Kampala UGA/4.01-2[d10]	1/24/2001
MVi/Kampala UGA/6.01[d10]	2/8/2001
MVi/Kampala UGA/8.01[d10]	2/22/2001
MVi/Kampala UGA/9.01[d10]	2/27/2001
MVi/Lira UGA/12.01[d10]	3/19/2001
MVi/Kampala UGA/12.01[d10]	3/25/2001
MVi/Kampala UGA/15.01-1[d10]	4/9/2001
MVi/Kampala UGA/15.01-2[d10]	4/10/2001
MVi/Kampala UGA/15.01-3[d10]	4/11/2001
MVi/Rakai UGA/17.01[d10]	4/23/2001
MVi/Mpigi UGA/18.01-1[d10]	5/4/2001
MVi/Mpigi UGA/18.01-2[d10]	5/4/2001
MVi/Mpigi UGA/18.01-3[d10]	5/5/2001
MVi/Kampala UGA/32.01-1[d10]	8/7/2001
MVi/Kampala UGA/32.01-2[d10]	8/7/2001
MVi/Wakiso UGA/32.01[d10]	8/8/2001
MVi/Jinja UGA/8.02[d10]†	2/24/2002

*WHO, World Health Organization.
†Members of the second, smaller cluster of Ugandan viruses.
‡Reference strain for proposed genotype d10.
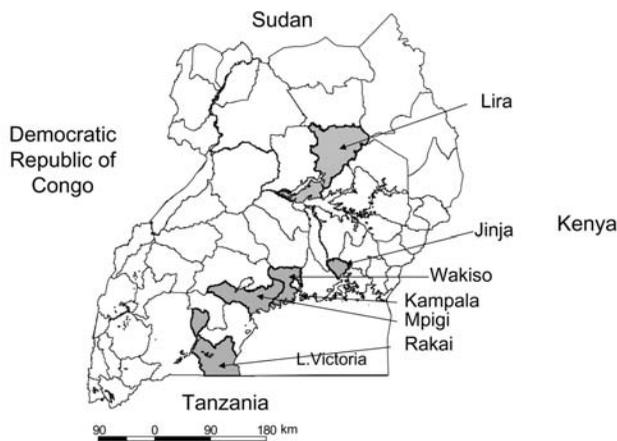
Figure 1. Map of Uganda showing districts where measles virus isolates were obtained from 2000 to 2002.

Analyses of the sequences of the N gene showed that the Ugandan isolates were all closely related and composed of 2 clusters (Figure 2 and Table 2). Most of the Ugandan sequences were in a single cluster in which the sequences differed by ≤6 nt (1.3%). Within this large cluster, 2 groups were composed of 14 and 9 viruses each that had identical N-gene sequences. Nucleotide divergence within the smaller cluster was 0.6%, and overall, Ugandan viruses differed from each other by 0–11 nt (0%–2.4%) in the N gene. Viruses from Mpigi, Kampala, and Jinja were found in both clusters, and viruses from Lira, Wakiso, and Rakai were part of the larger cluster. The sequence of the entire coding region of the H gene was obtained for 2 of the Ugandan viruses (MVi/Kampala.UGA/51.00-1 and MVi/Kampala.UGA/3.01). The Ugandan H-gene sequences differed by 21 nt (1.1%) and 6 predicted amino acids (Figure 3).

Phylogenetic analyses based on both N and H gene sequences showed that the Ugandan viruses were members of clade D. However, the sequences of the Ugandan viruses were not closely related to the sequences of any WHO reference sequences that represented the 22 currently recognized genotypes. The Ugandan sequences were closest to the sequence of the genotype D2 reference strain. The minimum nucleotide divergence between the Ugandan viruses and the D2 reference strain was 3.1% for the N gene and 2.6% for the H gene (Table 2).

Bootstrap analysis of Ugandan H-gene sequences and the WHO reference sequences showed 100% confidence in the group containing Ugandan viruses. When N-gene sequences were compared with WHO reference sequences and contemporary genotype D2 (13) and D4 (14,15) sequences, bootstrap support for the Ugandan branch was 100% (data not shown). Therefore, based on the current WHO recommendations for designating a new genotype

(7,8,16), the Ugandan viruses should be considered as a new genotype of measles. The proposed genotype that includes the Ugandan viruses is genotype d10. Mvi/Kampala.UGA/51.00-1 (GenBank accession nos. N:AY923185, H:AY923213) was chosen as the reference strain for d10 because it represents most isolates and grows to high titers in cell culture.

Discussion

This is the first report of the genetic characterization of wildtype measles from Uganda and the second that describes characterization of viruses from eastern Africa. Although this study was successful, it highlights some of the difficulties in performing virologic surveillance in developing countries. The relatively low rate of virus isolation in this study was surprising considering that all the specimens were collected within 7 days of rash onset.
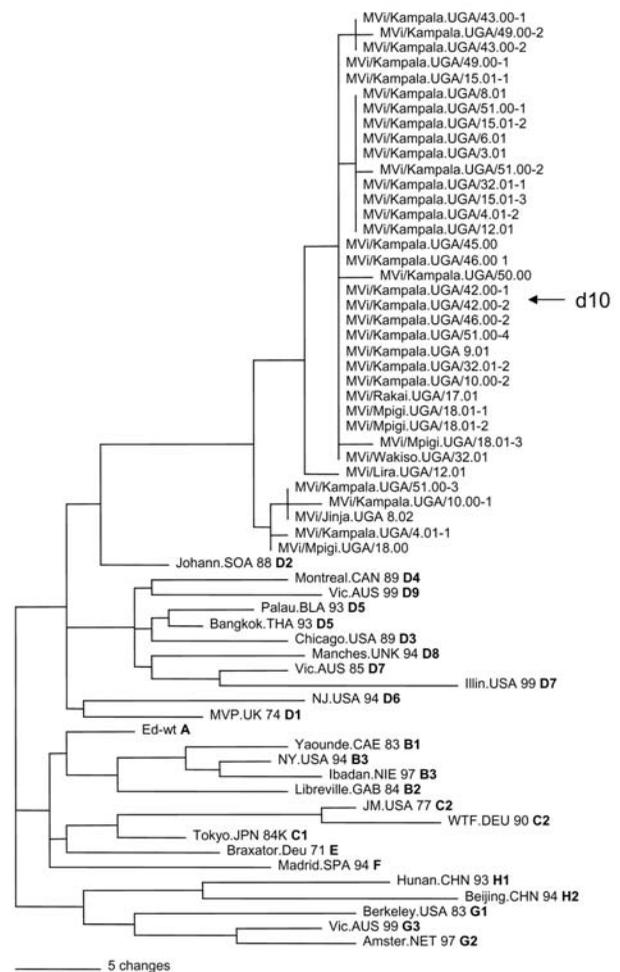
Figure 2. Phylogenetic analysis of sequences of nucleoprotein genes (450 nucleotides) of wildtype measles viruses isolated in Uganda during 2000–2002. The unrooted tree shows sequences from Ugandan viruses compared with World Health Organization reference strains for each genotype. Genotype designation is in **bold**.

Table 2. Percentage genetic distance between wildtype measles viruses from Uganda and World Health Organization reference strains

Genotype*	Nucleoprotein gene (range)†	Hemagglutinin gene‡
A	5.1–6.2	2.2
B1	6.8–8.2	3.2
B2	6.4–7.7	4.0
B3-NY USA/94	6.6–7.9	3.8
C1	5.1–5.9	2.3
C2-Erlangen Due/90	8.6–9.9	3.1
D1	4.4–5.7	2.2
D2	3.1–4.4	2.6
D3	4.8–6.4	3.5
D4	5.5–6.4	3.1
D5-Palau BLA/93	4.8–6.4	3.1
D6	5.7–7.0	2.4
D7-Illinois USA/99	7.7–9.0	3.0
D8	5.3–6.6	3.0
D9	5.7–7.3	3.0
E	5.5–6.8	2.9
F	6.2–7.5	3.0
G1	7.7–9.0	4.2
G2	7.2–8.6	4.6
G3	6.8–8.2	5.3
H1	7.9–8.8	5.5
H2	7.3–9.0	4.6

*One reference sequence was used for each comparison. For a genotype with >1 reference sequence, the indicated sequence was used for comparison.
†Range of percentage nucleotide differences between all Uganda sequences and the reference sequences for the nucleoprotein gene.
‡Since we had only 2 Ugandan hemagglutinin gene sequences, the lowest percentage difference is shown. However, both percentages did not vary by >0.1%.

However, many specimens were not collected properly, and many arrived at the laboratory in poor condition because of inadequate transportation. Better training and adequate specimen-collection equipment and supplies could prevent some of these problems. In addition, many of the specimens were contaminated, and the filtration step for removing the contaminants reduced the viral titer. Specimens that do not require reverse cold chain, such as blood spots dried onto filter paper, will improve the efficiency of virologic surveillance in countries such as Uganda.

Our results show that the percentage sequence divergence between the N and H gene sequence of the Ugandan virus isolates and the sequences of the reference strains exceeds the recommended threshold for designation of a new measles genotype, which is 2.5% and 2.0% minimum nucleotide divergence for the COOH region of the N and H genes, respectively (7,8,16). Phylogenetic analyses group these viruses in clade D. The results also showed that the Ugandan viruses had sequences that were unique among all previously characterized wildtype measles viruses and constitute a new genotype, which we propose to be genotype d10. All viruses isolated over the 2-year

period from 6 districts in Uganda belonged to this single, proposed new genotype. Therefore, d10 should be considered the endemic genotype in Uganda. The degree of genetic relatedness observed for the Ugandan viruses is surprising since these viruses were isolated during a period when measles was widespread in the country. Previous genetic analyses of wildtype measles viruses from countries with endemic measles have shown more genetic heterogeneity within a genotype, which suggested the presence of multiple chains of transmission (12,17,18).

The Ugandan viruses have a unique genetic signature that clearly distinguishes them from other African viruses. During the time when d10 viruses were isolated in Uganda, genotype D4 viruses were circulating in nearby Kenya (15) and genotypes D4 and D8 were detected in Ethiopia (14). Genotype B3, the most prevalent genotype in western Africa, has also been detected in Sudan and Democratic Republic of Congo, which are north and west,
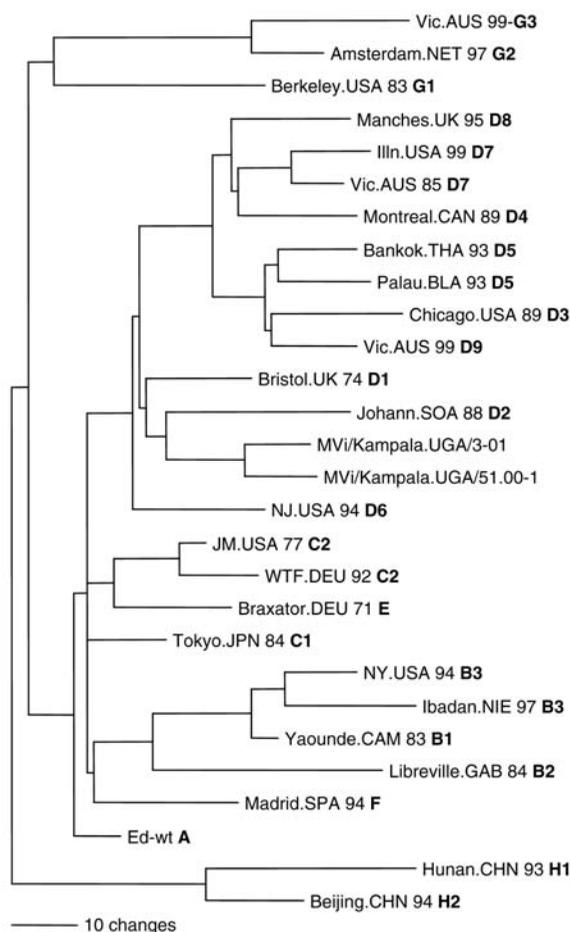
Figure 3. Phylogenetic analysis of sequences of hemagglutinin genes of wildtype measles viruses isolated in Uganda during 2000–2002. The unrooted tree shows sequences from the Ugandan viruses compared with World Health Organization reference strains for each genotype. Genotype designation is in **bold**.

respectively, of Uganda (18–23). D2 and D4 are the most frequently detected genotypes in southern Africa (24), while genotype C2 viruses were detected in northern Africa in Morocco (25). Baseline virologic surveillance has not been conducted in Tanzania, Rwanda, or Burundi, the countries that border Uganda to the south. Therefore, the exact geographic distribution of genotype d10 viruses is unknown.

The purpose of virologic surveillance is to establish the transmission pathways of measles virus. Both Uganda and Kenya have initiated accelerated measles control activities and successfully completed a baseline survey of viral genotypes. Molecular epidemiologic techniques will now be very useful in monitoring the transmission pathways in eastern Africa, contribute to the development of effective measles control strategies, and document the success of the measles vaccination program.

Acknowledgments

Mr Muwonge is a senior laboratory technologist in Uganda. His main areas of research are diagnosis, surveillance, and control of polio, measles, and rubella.

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Pyrosequencing *Bacillus anthracis*

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Pyrosequencing technology is a sequencing method that screens DNA nucleotide incorporation in real time. A set of coupled enzymatic reactions, together with bioluminescence, detects incorporated nucleotides in the form of light pulses, which produces a profile of characteristic peaks in a pyrogram. We used this technology to identify the warfare agent *Bacillus anthracis* by sequencing 4 single nucleotide polymorphisms (SNPs) in the *rpoB* gene as chromosomal markers for *B. anthracis*. In addition, 1 segment in each of the *B. anthracis* plasmids pXO1 and pXO2 was analyzed to determine the virulence status of the bacterial strains. Pyrosequencing technology is a powerful method to identify *B. anthracis*.

*B*acillus anthracis is a gram-positive, rod-shaped, spore-forming bacterium that causes the acute mammalian disease anthrax when endospores enter the body. The infection is often fatal if not treated with antimicrobial drugs before or when the first symptoms appear. The bacterium can infect livestock and humans by gastrointestinal, cutaneous, or respiratory routes. Potentially, *B. anthracis* spores can be an effective biological weapon because of their high stability. They do not divide, have no metabolism, and are resistant to drying, heat, UV light, and many disinfectants. In 2001, letters containing anthrax spores caused illness in 22 persons, leading to 5 deaths in the United States.

B. anthracis has 2 plasmids: the toxin-encoding pXO1 (182 kb) and capsule-encoding pXO2 (95 kb) (1,2). Both plasmids are required for virulence; lacking 1 of the plasmids attenuates the microorganism. The pXO1 plasmid contains genes *lef*, *cya*, and *pag*, which encode the toxin's lethal factor, edema factor, and protective antigen, respectively (3–5). The pXO2 plasmid contains the genes *capA*, *capB*, and *capC*, necessary for capsule formation (6). These genes have been used as markers to identify *B. anthracis* with polymerase chain reaction (PCR) in both environmental and clinical samples (7–9).

Differentiating between *B. anthracis* and closely related *B. cereus* and *B. thuringiensis* is difficult (10). Usually, phenotypic characteristics, such as susceptibility to β-lactam antimicrobial drugs, lack of hemolysis, lack of motility on sheep blood agar plate, and inability to ferment salicin, are used to differentiate (11,12). A variety of chromosomal markers that appear to be specific have been suggested for genotypic species determination of *B. anthracis* (13–18). We studied single nucleotide polymorphisms (SNPs) in the *rpoB* gene, described by Qi et al. (13) by using pyrosequencing technology (19). This technology can determine SNPs and short DNA stretches in real time, starting from PCR products. Biotinylated PCR amplicons that cover the region of interest are immobilized onto solid streptavidin coated beads and converted to single-stranded form. A sequencing primer is hybridized to the single-stranded DNA, and incorporation of added nucleotides is detected as light peaks by an enzymatic cascade. Enzymatic degradation of excess nucleotides allows the reaction to be performed in a single tube. When one starts from PCR products, ≤96 genetic targets can be sequenced within 1 hour.

In this study, we used the *rpoB* gene as a chromosomal marker to discriminate between *B. anthracis* and closely related bacillus species. We studied 4 *B. anthracis*–specific *rpoB* SNPs located at positions 911, 912, 913, and 914 in duplex sequencing reactions by using a unique sequencing primer for each desired SNP in a collection of 17 anthracis and 10 non-anthracis *Bacillus* strains. Simultaneously, we investigated the distribution of virulence plasmids pXO1 and pXO2 among these strains by using PCR and pyrosequencing technology to rapidly verify the amplicons.

Materials and Methods

Bacterial Strains and DNA Extraction

The *B. anthracis* reference strains used in this study were obtained from the National Collection of Type Cultures, London, England, and the Swedish Defense Research Agency. Reference strains of *B. cereus*, *B. mycoides*, and *B. thuringiensis* were obtained from the Culture Collection University of Gothenburg of Sweden.

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All bacterial strains are listed in Table 1. Bacteria were cultured on blood sheep agar at 37°C for 16 h, and genomic DNA was prepared by using a commercially available DNA extraction kit, QIAamp tissue protocol (Qiagen, Hilden, Sweden). The DNA was boiled at 99°C for 15 min, plated on blood agar, and incubated for 3 days. No growth was observed, and the DNA was removed from the biosafety level 3 laboratory. All material, including the DNA, is under the protection of our institute.

PCR

All reagents used for amplification of bacterial DNA were from Amersham Biosciences (Uppsala, Sweden) except for primers, which were from Invitrogen Life Technologies (Paisley, United Kingdom). Table 2 shows the primer sequences. The reverse primer for each PCR fragment was biotinylated. PCR primers were designed to amplify a 176-bp fragment of *rpoB*, 179 bp of the pXO1 plasmid, and 127 bp of the pXO2 plasmid. PCR was performed in 50-µL reaction mixtures containing 1× PCR buffer (10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 2.5 mmol/L MgCl$_2$), 0.8 U Taq DNA polymerase, 0.2 mmol/L each nucleotide, 0.1 µmol/L each primer and 5 µL eluate containing DNA. The reaction mixture was subjected to 95°C for 5 min and 45 cycles of 95°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s, followed by terminal extension at 72°C for 7 min.

Pyrosequencing Analysis

Two primers were designed to sequence of 30 nucleotides within the PCR amplicons generated from plasmids pXO1 and pXO2, respectively. In addition, 1 sequencing primer was designed for each of the 4 *rpoB* SNPs to be determined (Table 2). The *rpoB* primers were used in duplex sequencing reactions so that primers 911 and 912 were combined in a single reaction for sequencing of SNPs at positions 911 and 912, while primers 913 and 914 were used for combined sequencing of SNPs at positions 913 and 914. For sequencing according to the pyrosequencing technology, biotinylated PCR amplicons were immobilized onto streptavidin-coated magnetic beads and denatured to produce single-stranded DNA by using a PSQ 96 Sample Prep Tool (Biotage AB, Uppsala, Sweden). Sequencing primers were added and allowed to hybridize to the strands, after which sequencing was performed according to the manufacturer's instructions. All steps were performed at room temperature.

Results

B. anthracis was unambiguously identified by determining 4 SNPs in the *rpoB* gene by using pyrosequencing technology; thus, we were able to distinguish *B. anthracis* from the other tested bacillus species. The Figure shows

Table 1. Bacterial strains and their plasmid content*

Strain	Origin	pXO1	pXO2
Bacillus anthracis			
30	UK	+	+
57-77	UK	+	+
73-77	UK	+	+
183-78	UK	+	+
187-78	UK	+	+
188-78	UK	+	+
R81/04	Iceland	+	+
NCTC 10340	Unk.	+	+
NCTC 2620	UK	+	+
NCTC 109	UK	+	–
NCTC 1328	UK	+	–
NCTC 5444	UK	–	+
NCTC 7752	UK	–	+
NCTC 7753	UK	–	+
NCTC 08234	UK (Sterne)	+	–
ATCC 4229	Unk.	–	+
Sterne 7702	UK	+	–
B. cereus			
CCUG 7414	USA	–	–
CCUG 10781	USA	–	–
CCUG 36925	USA	–	–
CCUG 41923	Sweden	–	–
CCUG 43518A	Sweden	–	–
CCUG 46981	Sweden	–	–
CCUG 47340	UK	–	–
B. mycoides			
CCUG 26678	USA	–	–
B. thuringiensis			
CCUG 7429	Germany	–	–
CCUG 22499	UK	–	–

*Unk., unknown; NCTC, National Collection of Type Cultures; CCUG, Culture Collection University of Gothenburg.

representative output diagrams, or pyrograms, from duplex sequencing reactions of the SNPs in *B. anthracis* National Collection of Type Cultures 2026 and *B. cereus* Culture Collection University of Gothenburg 7414. The specific nucleotides 911C, 912T, 913C, and 914A were found in all *B. anthracis* strains tested and appear to be unique to *B. anthracis*. The corresponding nucleotides in all tested non-anthracis strains (*B. cereus*, *B. thuringiensis*, and *B. mycoides*) were 911T, 912C, 913T, and 914G. In duplex sequencing reactions, we could easily determine 2 SNPs in each pyrogram (Figure).

Presence of the virulence plasmids was determined by PCR and sequence verification of regions on plasmids pXO1 and pXO2 (Table 1). The assay was applied on 17 *B. anthracis* strains, 7 *B. cereus* strains, 2 *B. thuringiensis* strains, and 1 *B. mycoides* strain. Of the 17 *B. anthracis* isolates investigated, 9 isolates were positive for both pXO1 and pXO2, while the remaining 8 lacked either 1 or both of the virulence plasmids. All non-anthracis strains were negative for both plasmids. pXO1 and pXO2 PCR amplicons originating from the *B. anthracis* strains were

Table 2. Sequence of primers used in PCR and sequencing*

Target	Primer	Sequence
rpoB	PCR forward	5´-CCACCAACAGTAGAAAATGCCA-3´
	PCR reverse	b-5´-AAATTTCACCAGTTTCTGGATCTA-3´
	Sequencing 911	5´-CACCAACAGTAGAAAATGC-3´
	Sequencing 912	5´-TTCTTCGATCCAAAGC-3´
	Sequencing 913	5´-TAAAAACAGATTGTTTA-3´
	Sequencing 914	5´-AACGTTTAGCTGAAACATT-3´
pXO1	PCR forward	5´-GATATGAACCCGTACTTGTAAT-3´
	PCR reverse	b-5´-GAATCAGATGCATTTTTAATGG-3´
	Sequencing	5´-TTTATTATGAAATAGGT-3´
pXO2	PCR forward	5´-TTCGCCTTGTGATTCCGCACT-3´
	PCR reverse	b-5´-GGGCGCTGGTCGTATGTTTAG-3´
	Sequencing	5´- TGTTTGTCCACTTCC-3´

*PCR, polymerase chain reaction; b, biotin.

verified by sequencing 30 nucleotides with the pyrosequencing technology. The nucleotide sequence following the sequencing primers located within the amplified fragments was AAGATATTATCAAGGGATATTTTAAG-TAAA for all pXO1 amplicons and ACCACTCATT-

AAGTTCTTCGCACCGCTAAA for all pXO2 amplicons, which agreed with all nucleotide sequences of these regions submitted to the GenBank nucleotide database (http://www.ncbi.nlm.nih.gov), accession numbers AF065404, AE011190, AJ413934, AE017336, AJ413935,

Figure. Sequence analysis of single nucleotide polymorphisms (SNPs) in the *rpoB* gene of *Bacillus anthracis* National Collection of Type Cultures (NCTC) 2026 (A and B) and *B. cereus* Culture Collection University of Gothenburg (CCUG) 7414 (C and D). The initial addition of enzyme (E) and substrate (S) mixture and the sequential addition of nucleotides are shown on the x axis. An initial peak was generated when the substrate mixture was added due to pyrophosphate molecules remaining from the polymerase chain reaction (PCR). Incorporation of added nucleotides was detected as light peaks, measured in relative light units as shown on the y-axis. Incorporation of 2 identical consecutive nucleotides gave rise to peaks that were higher than those generated when a single nucleotide was incorporated. The 4 SNPs were determined by using the 4 sequencing primers 911–914 in 2 duplex reactions per strain. The output sequence reported by the software was a combination of the 2 unique sequences produced by each primer in the duplex reactions. The SNPs distinguishing *B. anthracis* NCTC 2026 from *B. cereus* CCUG 7414 are shown in **bold**.

M29081, M30210, AF188935, AE011191, and AE017335. *B. anthracis* was successfully identified by using pyrosequencing technology for genotyping 4 SNP positions of the *rpoB* gene, which appear to be specific for *B. anthracis*, and 2 fragments of virulence plasmids pXO1 and pXO2.

Discussion

In biologic warfare, speed and accuracy are in high demand for identifying and characterizing microbial species. In this study, we investigated the possibility of using pyrosequencing technology to rapidly identify and characterize strains of *B. anthracis* and distinguish them from related non-anthracis *Bacillus* strains. This method has been used to analyze multiple targets that are important in microbial infections (20,21).

By determining 4 SNPs in the *rpoB* gene, *B. anthracis* strains were successfully identified. This chromosomal marker can discriminate between *B. anthracis* and other closely related species from the *Bacillus* genus. The 16S rRNA gene cannot be relied upon to differentiate *B. anthracis* from its close relatives; therefore, we did not include this target in the assay (22). By careful design of nucleotide dispensation order, multiple SNPs may be analyzed in 1 single sequencing reaction by using a unique primer for each desired SNP. To save time and reduce reagent cost, we analyzed the *rpoB* SNPs in duplex pyrosequencing reactions. The resulting pyrograms of overlapping sequences were easily resolved by the accompanying software (Figure). Using 1 well for all 4 SNP positions may further optimize the method.

This technology validates PCR-based assays by qualitatively verifying that a positive PCR result is not the effect of nonspecific amplification, as shown here by sequence verification of PCR amplicon generated from virulence plasmids pXO1 and pXO2. The risk of false-positive results is thereby minimized.

We illustrate for the first time how pyrosequencing technology can identify *B. anthracis*. Using this technology in diagnostic laboratories is advantageous because it is rapid, simple, nonradioactive, inexpensive, and automated. It is a powerful method to rapidly determine genetic targets; as many as 96 samples can be analyzed in 40 minutes. Genetic analysis with pyrosequencing technology could make selecting antimicrobial drug treatment easier and potentially complement typing methods and time-consuming, traditional microbial identification, such as biochemical testing, phage lysing assays, and immunologic assays.

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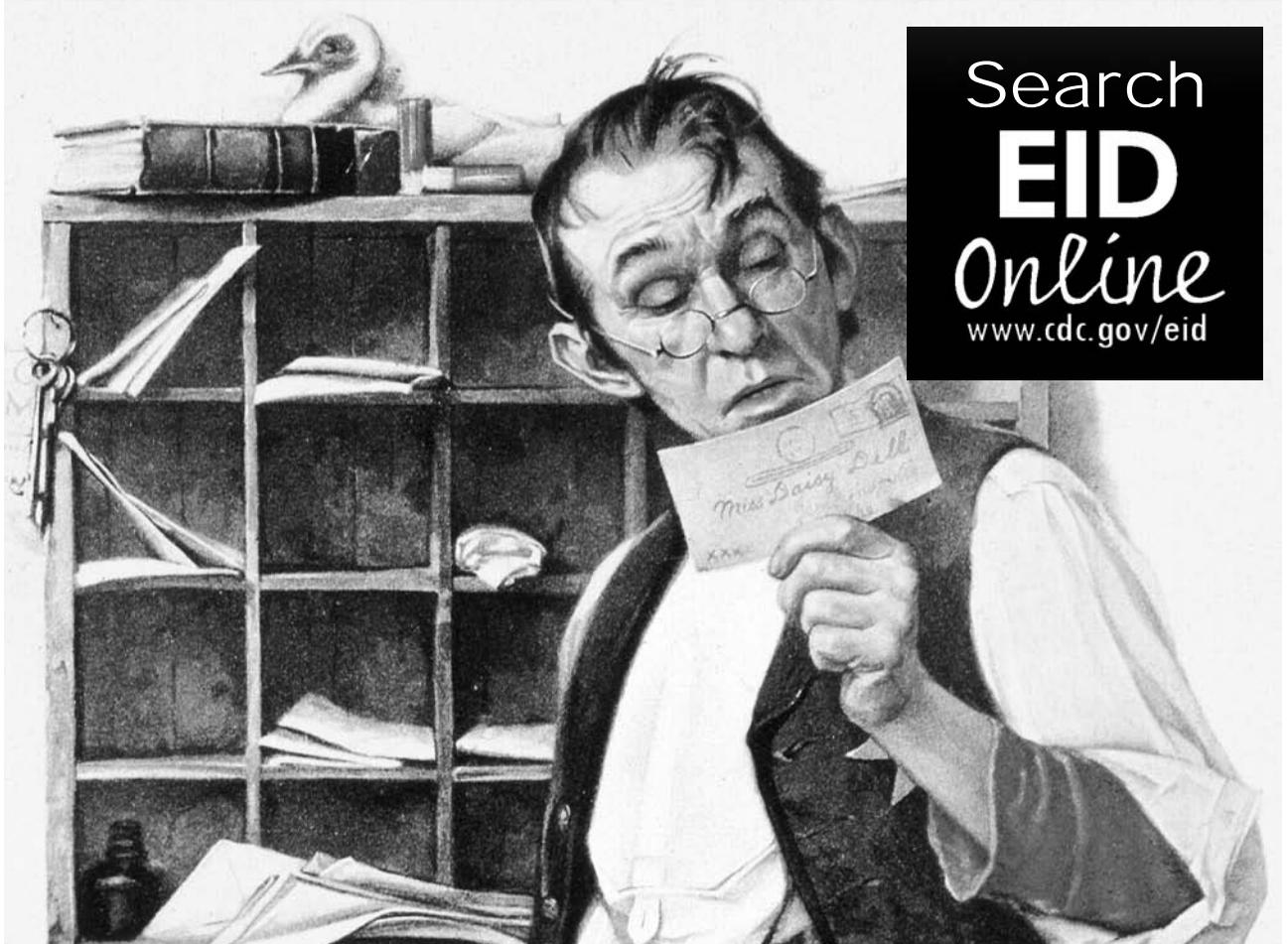
Community-associated Methicillin-resistant *Staphylococcus aureus*, Minnesota, 2000–2003

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We compared characteristics of community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) skin and soft tissue infections (SSTIs) and CA-MRSA invasive disease identified in Minnesota from 2000 through 2003. A total of 586 patients with SSTIs and 65 patients with invasive disease were identified. Patients with invasive disease were more likely to be smokers (p = 0.03), and report a history of immunosuppressive therapy (p = 0.03), emphysema (p = 0.011), or injection drug use (p = 0.020) than were SSTI patients. Invasive disease isolates were less likely to be susceptible to ciprofloxacin (p = 0.002) and clindamycin (p = 0.001) and more likely to have healthcare-associated pulsed-field gel electrophoresis subtypes than SSTI isolates (p<0.001). Patients with invasive disease may have had healthcare exposures that put them at risk of acquiring healthcare-associated MRSA, and which were not exclusion criteria in the CA-MRSA case definition. Continued surveillance of MRSA is needed to better characterize CA-MRSA infections.

Methicillin-resistant *Staphylococcus aureus* (MRSA) was first reported in 1961 and was recognized as a nosocomial pathogen by the late 1960s (1,2). Known MRSA risk factors include recent surgery or hospitalization, residence in a long-term care facility, presence of a percutaneous device or indwelling catheter, or recent dialysis (3).

In the 1980s, MRSA infections were reported in persons who lacked traditional MRSA risk factors. These infections appeared to be acquired in the community and are now known as community-associated (CA) MRSA infections. These infections have been reported worldwide (4–18). Outbreaks have occurred in many settings and among different populations (10,16,19–23).

Previous studies have demonstrated significant differences between MRSA isolates from persons with health-care exposures and persons without these exposures in both antimicrobial susceptibility results and pulsed-field gel electrophoresis (PFGE) subtypes (5,7). Studies have also demonstrated demographic differences between CA-MRSA cases and healthcare-associated (HA) MRSA cases regarding age, race, and income (5,7).

The most common clinical manifestations of CA-MRSA are skin and soft tissue infections (SSTIs) such as abscesses or cellulitis (5,7,9,24). Less commonly, CA-MRSA can cause severe diseases such as necrotizing pneumonia, osteomyelitis, and septicemia (6–9). Most CA-MRSA infections resolve, but deaths from invasive CA-MRSA disease have been reported (8).

Although invasive disease caused by CA-MRSA has been described in the literature, no research has been published that evaluates possible patient and isolate differences between CA-MRSA SSTIs and CA-MRSA invasive disease. A subanalysis of CA-MRSA invasive disease patients and SSTI patients and isolates was conducted by using data collected from CA-MRSA prospective sentinel surveillance in Minnesota from 2000 through 2003.

Methods

Facility Enrollment

In 2000, 12 sentinel hospitals in Minnesota (6 in the 7-county Minneapolis–St. Paul metropolitan area and 6 in greater Minnesota) began reporting all cases of MRSA isolated in their respective microbiology laboratories to the Minnesota Department of Health (MDH). Characteristics of these sentinel sites have been described elsewhere (7).

Case Enrollment

Infection control practitioners from each hospital completed a case report form for patients with a positive MRSA culture obtained during 2000–2003. Patient medical records were reviewed to determine the type of

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infection, history of underlying illness (injection drug use, diabetes, malignancy, chronic heart or lung conditions, chronic skin conditions), or immunosuppressive therapy (defined as long-term systemic steroid use, excluding topical creams, steroids used only for short-course treatment, and inhaled steroids used for asthma) and any history of patient healthcare exposures as defined in the CA-MRSA case definition. The hospital laboratories submitted CA-MRSA isolates to MDH.

All patients with cultures obtained during 2000–2002 who met the CA-MRSA case definition based on medical record review were interviewed to confirm their classification (patient culture dates 2000–2002) and to assess possible CA-MRSA risk factors (patient culture dates 2001–2002). Patients identified at 4 of the 12 sentinel sites during 2003 who had no exclusionary healthcare exposures noted on medical record review were contacted to confirm CA-MRSA classification and conduct risk factor interviews. In addition, a random sample of 2003 patients from the remaining 8 sentinel sites were interviewed to confirm CA-MRSA classification. Informed consent was obtained from all patients before telephone interview.

US Census data from 2000 were used to provide median income by zip code (25) as a proxy for case household income. The University of Minnesota and MDH Institutional Review Boards reviewed and approved the study.

CA-MRSA Case Definition

The Centers for Disease Control and Prevention (CDC) Active Bacterial Core Surveillance Program defined a CA-MRSA case as a patient with an MRSA infection and no history of the following: surgery, hospitalization, or residence in a long-term care facility within the year before infection, presence of a percutaneous device or indwelling catheter, dialysis within the previous year, hospitalization >48 h before MRSA culture, or previous MRSA infection or colonization.

Patients were classified as confirmed CA-MRSA case-patients if the medical record review and interview did not show any of the above healthcare risk factors. Patients were classified as probable CA-MRSA case-patients if the medical record review did not show any healthcare risk factors, but the interview was not completed (because of patient refusal, inability to locate, or language barriers).

Subanalysis Inclusion

CA-MRSA patients identified from prospective sentinel surveillance with culture dates in 2000 and 2003 were included in this subanalysis if they had an SSTI (e.g., abscess, cellulitis, folliculitis, wound infection [nonsurgical]) or infection in a normally sterile site caused by CA-MRSA. CDC's Active Bacterial Core Surveillance

Program definition of sterile site infections was used to define cases of invasive CA-MRSA disease. This definition defines a normally sterile site as a portion of the body in a healthy state in which no microorganisms are found and includes the following: blood, cerebrospinal fluid, pleural fluid, peritoneal fluid, pericardial fluid, bone, joint fluid, internal body site (lymph node, brain, heart, liver, spleen, vitreous fluid, kidney, pancreas, or ovary), or other normally sterile site. Although cases of necrotizing pneumonia caused by CA-MRSA have been reported (26), CA-MRSA specimens isolated only from sputum were not included in our subanalysis because sputum was not defined as a sterile site.

Isolate Characterization

All MRSA isolates submitted to MDH were tested to confirm *Staphylococcus aureus* identification by using a tube coagulase test (27) (Difco Laboratories, Detroit, MI, USA). Testing for antimicrobial susceptibility was performed by using a broth microdilution panel (PML Microbiologicals, Wilsonville, OR, USA) containing the following 11 antimicrobial agents: ciprofloxacin, gentamicin, trimethoprim/sulfamethoxazole, clindamycin, tetracycline, erythromycin, rifampin, linezolid, mupirocin, vancomycin, and oxacillin. Clinical and Laboratory Standards Institute (CLSI, formerly National Committee for Clinical Laboratory Standards) breakpoints were used to determine levels of resistance for all antimicrobial agents except mupirocin, for which no CLSI breakpoints exist (28). A standard of <4 µg/mL was used as a breakpoint for susceptibility to mupirocin (29).

Molecular Characterization

Molecular subtyping of MRSA isolates was performed by PFGE and digestion with the restriction endonuclease *Sma*I (30). Patterns were evaluated both visually and with BioNumerics software (Applied Maths, Kortrijk, Belgium) by using the dice coefficient. Indistinguishable patterns must visually appear identical, and the DNA patterns must differ by <1.5% with respect to molecular weight. MRSA isolates were considered part of a CA-MRSA pulsed-field type (PFT) if they were ≥80% similar to the USA300 or USA400 reference strains based on Dice coefficients. MRSA isolates were considered part of an HA-MRSA PFT if they were ≥80% similar to USA100, USA200, or USA500–800 reference strains (30).

Statistical Analysis

The Yates continuity corrected chi-square test was used to test for trends with EpiInfo version 6.2 (CDC, Atlanta, GA, USA), and univariate analysis of the data was performed with EpiInfo 2000 (CDC). Multivariate logistic regression was used to evaluate the association of the type

of MRSA infection (SSTI versus invasive disease) with microbiologic and molecular features of the MRSA isolates. Demographic characteristics associated with the type of infection in the univariate analysis were controlled for in the multivariate analysis model. An $\alpha \leq 0.05$ significance level was required for predictors to remain in the model. Multivariate analysis was accomplished by using SAS version 8.0 for Windows (SAS Institute, Cary, NC, USA).

Results

A total of 738 CA-MRSA infections were identified from January 1, 2000, to December 31, 2003. SSTIs accounted for 79% (586/738) of all infections reported, and invasive disease accounted for 9% (65/738) of all CA-MRSA infections reported. The proportion of CA-MRSA infections that were invasive did not differ significantly over the study period. The most common site of invasive disease was the bloodstream (50%), followed by joint or bone (32%). Clinical information was available for 511 (87%) of 586 SSTI patients. The most common clinical conditions reported for SSTIs were abscesses (49%) and cellulitis (33%) (Table 1).

Case Demographics

Invasive disease patients were more likely to be male than SSTI patients (66% vs. 51%, odds ratio [OR] 1.89, 95% confidence interval [CI] 1.10–3.24). No difference in median age was found between the 2 groups. Race information was available for 54 (83%) of 65 invasive disease patients and 477 (81%) of 586 SSTI patients. No difference was shown between the 2 groups when race was analyzed in terms of white and nonwhite race categories (Table 2).

Patient hospitalization status was available for all of the invasive disease CA-MRSA patients and 562 (96%) of 586 SSTI patients. As expected, invasive disease patients were more likely to be hospitalized for their infection than were SSTI patients (OR 6.89, 95% CI 3.81–12.4). Results remained significant after controlling for age and sex (p<0.001). No differences were observed between median income of CA-MRSA invasive disease patients and SSTI patients (Table 2).

History of underlying medical conditions was obtained for 58 (89%) of 65 invasive disease patients and 515 (88%) of 586 SSTI patients. Invasive disease patients were more likely to report a history of underlying illness than were SSTI patients (OR 2.08, 95% CI 1.05–4.20). Invasive disease CA-MRSA patients were more likely to have a history of immunosuppressive therapy (OR 9.31, 95% CI 1.87–47.2), solid organ malignancy (OR 9.16, 95% CI 1.27–66.3), or emphysema/chronic obstructive pulmonary disease (COPD) (OR 13.9, 95% CI 2.29–85.5) than SSTI

Table 1. Community-associated methicillin-resistant *Staphylococcus aureus* invasive infection sites and skin and soft tissue infection clinical manifestations

Site or manifestation	No. (%)
Invasive infection site (n = 65)	
Bloodstream infection	
Without focus	25 (38)
With skin focus	6 (9)
With respiratory focus	2 (3)
Pleural fluid	3 (5)
Peritoneal fluid	2 (3)
Joint/bone	21 (32)
Other*	6 (9)
Skin and soft tissue clinical manifestation (n = 511†)	
Abscess	251 (49)
Cellulitis	171 (33)
Folliculitis	28 (5)
Wound infection	27 (5)
Impetigo	11 (2)
Other‡	62 (12)

*Other invasive isolate sources included brain tissue (1), lymph nodes (2), pancreatic aspirate (1), kidney abscess aspirate (1), and internal tissue (1).
†A total of 411 skin and soft tissue infection patients had clinical manifestations reported. Results include multiple clinical manifestations per patient.
‡Other skin clinical manifestations included psoriasis, mastitis, cystic acne, furuncles, carbuncles, insect/spider bites, and eczema.

patients. Invasive disease CA-MRSA patients were also more likely to be current smokers (OR 2.18, 95% CI 1.09–4.67) or injection drug users (OR 5.56, 95% CI 1.29–23.9) than SSTI patients. History of underlying illness (p = 0.007), immunosuppressive therapy (p = 0.003), emphysema/COPD (p = 0.012), current smoking (p = 0.028), and injection drug use (p = 0.021) remained significant in a multivariate model that controlled for age and sex (Table 2).

Isolate Characteristics

We received isolates from 60 (92%) of 65 invasive disease patients and 525 (90%) of 586 SSTI patients. Tests for antimicrobial drug susceptibility were completed on 57 (95%) of 60 invasive disease isolates and 517 (98%) of 525 SSTI isolates. All isolates were susceptible to linezolid and vancomycin. Compared with SSTI isolates, those from invasive infections were less likely to be susceptible to ciprofloxacin (OR 2.79, 95% CI 1.54–5.04) and clindamycin (OR 3.34, 95% CI 1.67–6.69). When ciprofloxacin and clindamycin susceptibilities were analyzed in a model that controlled for sex and age, the results remained significant (p = 0.002 and p = 0.001, respectively) (Table 3).

Initial antimicrobial therapy information was documented for 41 (63%) of 65 patients with invasive disease whose isolates were available and for 415 (71%) of 586 patients with SSTI whose isolates were available. For 27 (66%) of 41 invasive disease patients and 333 (80%) of

Table 2. Community-associated methicillin-resistant *Staphylococcus aureus* invasive disease and skin and soft tissue infection (SSTI) patient demographics*

Characteristic	Invasive disease patient (n = 65)	SSTI patient (n = 586)	OR (95% CI)
Median age, y	28	24	NS
Sex (male), no. (%)	43 (66)	296 (51)	1.89 (1.10–3.24)
Race, no. (%)			
White†	33 (51)	254 (43)	NS
Unknown	10 (15)	105 (18)	
Nonwhite†	21 (32)	223 (38)	NS
Black	14 (21)	125 (21)	
Native American	6 (9)	82 (14)	
Asian	1 (0.5)	4 (0.7)	
Other	1 (0.5)	12 (0.2)	
Median income (US)‡	$38,237	$38,237	NS
Patients hospitalized, no. (%)§	49 (75)	173 (31)	6.89 (3.81–12.4)
	(n = 58)	(n = 515)	
Presence of underlying condition, no. (%)	31 (53)	183 (36)	2.08 (1.21–3.60)
Immunosuppressive therapy,¶ no. (%)	3 (5)	3 (0.6)	9.31 (1.83–47.2)
Solid organ malignancy, no. (%)	2 (4)	2 (0.4)	9.16 (1.27–66.3)
Diabetes, no. (%)	9 (16)	41 (8)	NS
Current smoker, no. (%)	12 (21)	55 (11)	2.18 (1.09–4.37)
Emphysema/COPD, no. (%)	3 (5)	2 (0.4)	13.9 (2.29–85.5)
Injection drug use, no. (%)	3 (5%)	5 (1)	5.56 (1.29–23.9)

*OR, odds ratio; CI, confidence interval; NS, not significant; COPD, chronic obstructive pulmonary disease.
†Race was calculated by using the number of cases with known race as the denominator.
‡Based on the mean household income in the zip code of each case-patient (source: 2000 US Census).
§Calculated by using the number of cases with known hospitalization status. Twenty-four SSTI patients had unknown hospitalization status.
¶Defined as long-term systemic steroid use, excluding topical creams, steroids used only for short-course treatment, and inhaled steroids used for asthma.

415 SSTI patients, the initial antimicrobial agent prescribed was of a class to which the organism was resistant. Invasive disease patients were more likely to be empirically treated with an antimicrobial drug to which their MRSA isolate was susceptible than were SSTI patients (OR 2.10, 95% CI 1.05–4.20). Results remained significant after controlling for age and sex (p = 0.015).

All available isolates received were characterized by PFGE. Fifty-three (88%) of 60 invasive disease isolates and 501 (95%) of 525 SSTI isolates had PFGE subtypes that could be categorized into PFTs that have been associated with HA-MRSA disease (USA100, USA200, USA500–800) or CA-MRSA (USA300 and USA400) (30). Compared with PFGE subtypes from SSTI isolates, PFGE subtypes from invasive disease isolates were more likely to be associated with HA-MRSA PFTs (OR 3.63, 95% CI 2.03–6.50). This result remained significant after controlling for age and sex (p<0.001) (Table 4). When invasive disease and SSTI case isolate susceptibility to ciprofloxacin and clindamycin were analyzed in a multivariate model that controlled for CA or HA-MRSA PFT and sex, no difference in susceptibility patterns was found between the 2 groups (Table 3).

Confirmed CA-MRSA Analysis

Three hundred two (52%) of 586 SSTI patients and 36 (55%) of 65 invasive disease patients were confirmed (through patient interview and medical record review, as opposed to medical record review alone) to meet the CA-MRSA case definition. Confirmed CA-MRSA patients and isolates underwent the previously described analysis regarding differences in underlying conditions and isolate antimicrobial susceptibility. Underlying condition information was available for 30 (83%) of 36 invasive disease patients and 273 (90%) of 302 SSTI patients. Confirmed invasive disease CA-MRSA patients were more likely than confirmed SSTI patients to report a history of underlying illness (OR 2.36, 95% CI 1.09–5.10), history of immunosuppressive therapy (OR 10.0, 95% CI 1.92–52.0), solid organ malignancy (OR 19.4, 95% CI 1.71–221), or to be a current smoker (OR 3.06, 95% CI 1.25–7.50). Hospitalization information was available for all invasive disease patients and 299 (99%) of 302 SSTI patients. Confirmed invasive disease patients were more likely to be hospitalized for their infection than were confirmed SSTI patients (OR 5.94, 95% CI 2.75–12.8). Isolates were available for 30 (83%) of 36 invasive disease patients and 265 (88%) of 302 SSTI patients. Confirmed CA-MRSA invasive disease isolates were less likely to be susceptible to ciprofloxacin (OR 5.02, 95% CI 2.11–12.0) and clindamycin (OR 5.75, 95% CI 2.58–12.8). Twenty-eight (93%) of 30 invasive disease isolates and 257 (85%) of 302 SSTI isolates could be categorized into PFTs that have been associated with HA-MRSA or CA-MRSA. Invasive disease isolates were more likely to have HA-MRSA PFTs than were SSTI isolates (OR 4.24, 95% CI 1.90–9.43). When invasive disease

Table 3. Community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) invasive disease patient and skin and soft tissue infection (SSTI) patient isolate characteristics*

Antimicrobial agent	Invasive disease isolates (n = 57) No. (% susceptible)	SSTI isolates (n = 517) No. (% susceptible)	OR (95% CI)	p value†	p value‡
Oxacillin (methicillin)	0	0	NA		
Ciprofloxacin	37 (65)	433 (84)	3.34 (1.67–6.69)	0.002	0.23
Clindamycin	44 (77)	475 (92)	2.79 (1.54–5.04)	0.001	0.20
Erythromycin	21 (37)	201 (39)	1.09 (0.62–1.92)		
Gentamicin	56 (98)	509 (98)	1.14 (0.14–9.25)		
Linezolid	57 (100)	517 (100)	NA		
Mupirocin	56 (98)	508 (98)	1.14 (0.14–9.23)		
Rifampin	56 (98)	515 (99)	4.60 (0.41–51.5)		
Tetracyline	52 (91)	469 (91)	0.94 (0.36–2.46)		
Trimethoprim-sulfamethoxazole	56 (98)	514 (99)	3.06 (0.31–2.99)		
Vancomycin	57 (100)	517 (100)	NA		

* OR, odds ratio; CI, confidence interval; NA, not applicable.
† Refers to the probablility that the percentage susceptible for invasive CA-MRSA isolates differed from SSTI CA-MRSA isolates after controlling for sex and age.
‡Refers to the probability that the percentage susceptible for invasive CA-MRSA isolates differed from SSTI CA-MRSA isolates after controlling for sex and pulsed-field type associated with healthcare-associated MRSA or CA-MRSA.

and SSTI isolate susceptibility to ciprofloxacin and clindamycin were analyzed in a multivariate model that controlled for CA- or HA-MRSA PFT and sex, invasive disease isolates were still more likely to be resistant to ciprofloxacin than were SSTI isolates (p = 0.04).

Discussion

This report compares CA-MRSA invasive disease patients and their isolates with those of SSTI patients. Invasive disease patients were more likely to be male and more likely to have a history of underlying conditions (immunosuppressive therapy, emphysema/COPD, injection drug use, and smoking) than were SSTI patients. Invasive disease isolates were similar to HA-MRSA isolates in that they were resistant to additional antimicrobial drugs (clindamycin and ciprofloxacin) and were more likely to belong to a PFT usually associated with HA-MRSA (7). These similarities suggest that invasive CA-MRSA patients may have had healthcare exposures that put them at risk of acquiring HA-MRSA, even though they are classified as CA-MRSA by the current CDC case definition.

The results of the ciprofloxacin and clindamycin multivariate analysis, including PFT association with both confirmed and probable CA- or HA-MRSA, showed no difference in susceptibility patterns between invasive disease and SSTI isolates. This suggests that the initial differences in susceptibility were not due to more resistant CA-MRSA strains causing invasive disease, but rather that more of the invasive disease isolates classified as CA-MRSA were actually HA-MRSA strains, which are typically resistant to more antimicrobial agents. However, when this same analysis was conducted by using confirmed CA-MRSA cases only, invasive disease isolates were still more likely to be resistant to ciprofloxacin. More research is needed to determine whether invasive disease

CA-MRSA isolates are more resistant to antimicrobial drugs than CA-MRSA isolates that cause SSTI.

Invasive disease patient characteristics identified in this analysis were similar to results from other studies, which found that CA invasive disease patients had underlying conditions such as diabetes, smoking, and cardiovascular disease (31,32). The underlying conditions identified in the *S. aureus* and MRSA patients in these studies do not disqualify them from meeting the current CDC CA-MRSA case definition, yet these conditions may have put them at risk of acquiring HA-MRSA.

One possible explanation for some of the results of this analysis could be the likelihood that invasive disease patients had more healthcare exposures than did SSTI patients. This hypothesis is supported by the fact that invasive disease patients reported serious underlying illnesses that would imply a history of extensive healthcare contacts. During these healthcare contacts, invasive disease patients may have been colonized by HA-MRSA strains. A recent study found that in 50% of patients nasally colonized with MRSA subsequent infection developed over the

Table 4. Distribution of HA-MRSA PFTs among invasive disease and SSTI community-associated MRSA isolates*

HA-MRSA PFTs	Invasive disease isolates (n = 25), no. (%)	SSTI isolates (n = 99), no. (%)
USA100	17 (28)	43 (8)
USA200	3 (5)	4 (0.8)
USA500	5 (8)	40 (7.6)
USA600	0	1 (0.2)
USA700	0	1 (0.2)
USA800	0	10 (2)
Total†	25 (42)	99 (19)

*HA, healthcare-associated; MRSA, methicillin-resistant *Staphylococcus aureus*; PFTs, pulsed-field types; SSTI, skin and soft tissue infection.
†Odds ratio 3.63, 95% confidence interval 2.03–6.50, p<0.001.

next 18 months (33). Although we were unable to determine the colonization status of our patients for this analysis, patients have been found colonized with MRSA for up to 40 months (34).

This study has several limitations. Although the hospital laboratories were selected to reflect state population demographics, the study was not population based. Therefore, generalizing the findings to entire state population is not possible. Also, some HA-MRSA patients may have been misclassified as CA-MRSA patients because of incomplete ascertainment of HA risk factors. However, since no major differences were found in results when analysis was restricted to confirmed CA-MRSA patients, misclassification bias as a result of incomplete ascertainment of HA risk factors that are exclusion criteria for the current CA-MRSA case definition is unlikely to be a large factor. In addition, the sample size, particularly of invasive disease cases, limited the ability to detect small statistical differences between the 2 groups. Finally, complete data on all cases were not available for all factors analyzed in this report. These missing data could have biased the results of this analysis.

Underlying conditions or healthcare exposures not currently included as exclusion criteria in the CA-MRSA case definition may put patients at risk of HA-MRSA colonization and infection. In addition, persons with underlying conditions may also be at greater risk of invasive disease caused by MRSA. Clinicians should be aware of possible serious MRSA infections in persons without previously recognized HA-MRSA risk factors. Continued surveillance of CA-MRSA is needed to further define the epidemiology of invasive disease and SSTI and to develop recommendations for the prevention and control of this emerging public health threat.

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Ms Buck is an epidemiologist with the Minnesota Department of Health Acute Disease Investigation and Control Unit. Her primary research interests are methicillin-resistant *Staphylococcus aureus* and infection control issues.

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Methicillin-resistant *Staphylococcus aureus* and Vancomycin-resistant Enterococci Co-colonization[1]

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We assessed the prevalence, risk factors, and clinical outcomes of patients co-colonized with vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) upon admission to the medical and surgical intensive care units (ICUs) of a tertiary-care facility between January 1, 2002, and December 31, 2003. Co-colonization was defined as a VRE-positive perirectal surveillance culture with an MRSA-positive anterior nares surveillance culture collected concurrently. Among 2,440 patients, 65 (2.7%) were co-colonized. Independent risk factors included age (odds ratio [OR] 1.03, 95% confidence interval [CI] 1.01–1.05), admission to the medical ICU (OR 4.38, 95% CI 2.46–7.81), male sex (OR 1.93, 95% CI 1.14–3.30), and receiving antimicrobial drugs on a previous admission within 1 year (OR 3.06, 95% CI 1.85–5.07). None of the co-colonized patients would have been identified with clinical cultures alone. We report a high prevalence of VRE/MRSA co-colonization upon admission to ICUs at a tertiary-care hospital.

Methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) cause nosocomial infections and are associated with increased rates of illness and death (1,2). Both organisms are now endemic in many healthcare institutions, particularly in intensive care units (ICUs) (3). Vancomycin is commonly used to treat infections caused by MRSA; however, recent emergence of *S. aureus* infections with high-level resistance to vancomycin call into question the future effective-ness of vancomycin for these nosocomial infections (4). All known vancomycin-resistant *S. aureus* (VRSA) isolates reported thus far have possessed the *vanA* gene, which confers resistance to vancomycin and is believed to have been acquired when an MRSA isolate conjugated with a co-colonizing VRE isolate (5–10). Thus, patients simultaneously co-colonized with MRSA and VRE are likely at increased risk for colonization or infection by VRSA.

Patients in the ICU and other critically ill patients are at high risk for co-colonization with MRSA and VRE and, possibly, VRSA, since both organisms are endemic and associated with increased illness severity (11,12). Despite the high risk, epidemiologic risk factors associated with co-colonization by MRSA and VRE in patients admitted to ICUs have not been described. In addition, previous studies in this population have been limited by the use of clinical cultures as markers for colonization, which underestimate the true proportion of patients colonized with these resistant organisms (13–15).

To our knowledge, this study is the first to assess independent risk factors and outcomes for patients co-colonized with VRE and MRSA. The aim of this study was to estimate the prevalence, risk factors, and clinical outcomes of patients who are co-colonized by VRE and MRSA upon admission to the medical and surgical ICUs of a tertiary-care facility.

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Methods

Study Design and Patient Population

This study was approved by the institutional review board of the University of Maryland, Baltimore. This study utilized a prospective cohort of adult patients admitted to the medical ICU (MICU) and surgical ICU (SICU) of the University of Maryland Medical Center (UMMC) between January 1, 2002, and December 31, 2003. UMMC is a tertiary-care facility in Baltimore, Maryland. The MICU is a 10-bed, private room unit providing care to patients who have acute or potentially life-threatening medical conditions including hematologic and other malignancies. The SICU is a 19-bed, private room unit providing care to adult patients with solid organ transplantation and abdominal, genitourinary, orthopedic, and otolaryngologic surgery.

During the study period, routine surveillance cultures of the anterior nares for MRSA and perirectal area for VRE were obtained from patients within 48 hours of admission to both ICUs for infection control purposes. Cultures were obtained from an average of 80% of admitted patients. Patients from whom both cultures were not obtained upon ICU admission or had an ICU length of stay <5 hours were excluded. Patients may have had multiple admissions during the study period, and all eligible admissions were included in this analysis.

Data Collection and Variables

All data were abstracted from the UMMC central data repository that contains the patients' electronic medical records. The validity of these data was assessed by randomly sampling 10% of the patients' electronic data records and comparing them to the original paper medical records. The positive and negative predictive values of this assessment exceeded 99% for both validity measures, which was similar to values seen in previous studies with this same data source (16–19).

Co-colonization by VRE and MRSA upon ICU admission was defined as a positive surveillance culture of the perirectal area for VRE and a positive surveillance culture of the anterior nares for MRSA within 48 hours of admission to either the MICU or SICU. All coexisting conditions were defined by using International Classification of Diseases, 9th Revision and the Charlson Comorbidity Index (20).

Laboratory Methods

All media and reagents were from BD Biosciences (San Jose, CA, USA) unless otherwise noted. *S. aureus* was isolated from both anterior nares and perirectal cultures. Nares swabs were plated on tryptic soy agar with 5% sheep blood to isolate *S. aureus*. Plates were examined at 24 and 48 hours for creamy, golden β-hemolytic colonies typical of *S. aureus*. Perirectal swabs were plated on both tryptic soy agar with 5% sheep blood and phenylethyl alcohol agar (Remel, Lenexa, KS, USA) and cultured in Baird *Staphylococcus* Enrichment broth (Merck, Darmstadt, Germany). Presumptive *S. aureus* colonies were confirmed by positive catalase and Murex Staphaurex (Remel) reactions. MRSA were identified by growth on Mueller Hinton agar with 4% NaCl and 6 µg/mL oxacillin.

Enterococci were isolated by plating perirectal swabs on Columbia Modified CNA agar and examined at 24 and 48 hours. Presumptive enterococci colonies that were gram-positive cocci, catalase negative, and pyrrolidonyl-β-naphthylamide positive were plated on vancomycin screening agar and motility agar. Vancomycin-resistant, nonmotile enterococci were identified as VRE.

Statistical Analyses

Three risk factor analyses were conducted: 1) VRE/MRSA co-colonized patients were compared to all other ICU patients, 2) patients colonized with VRE alone were compared to patients not colonized with VRE, and 3) patients colonized with MRSA alone were compared to patients not colonized with MRSA. Patients co-colonized with MRSA or VRE were excluded for analyses 2 and 3 above that compared solitary VRE or MRSA colonization to noncolonized patients.

Student t, chi-square, Fisher exact, and Wilcoxon rank sum tests were used for descriptive analyses to assess bivariable differences between groups. All variables that were significant ($\alpha = 0.1$) in the bivariable analyses were included in the initial (full) multivariable logistic regression model. In each of the multivariable analyses performed, variables not significantly associated ($\alpha = 0.05$) with the outcome (VRE/MRSA co-colonization and VRE or MRSA solitary colonization upon admission to the ICU) were removed from the model. Each of the removed variables was then reinserted into the model to assess if its presence altered the regression coefficient by $\geq 20\%$. If so, this risk factor was included in the final model. The resulting multivariable logistic regression model was considered the final model and was used to calculate odds ratios and 95% confidence intervals for the remaining risk factors.

Differences in patients' clinical outcomes after assessing colonization status were also assessed. These variables included length of stay after the ICU admission culture, subsequent positive clinical cultures for VRE or MRSA, in-hospital death rate, and readmission to the index hospital or transfer to another healthcare facility. Subsequent positive clinical cultures were limited to sterile sites, and thus only blood, cerebrospinal fluid, or urine cultures were considered.

Results

During the 2-year cohort period (January 1, 2002–December 31, 2003), 3,090 patients were admitted to the MICU or SICU for a >5-hour stay. Of these, 2,440 patients (79.0%) had both anterior nares and perirectal admission cultures collected. Sixty-five patients (2.7%) were co-colonized with VRE and MRSA, 247 patients (10.1%) were colonized with VRE alone, and 175 patients (7.2%) were colonized with MRSA alone. Of the 57 MRSA/VRE co-colonized patients with perirectal samples available for additional analysis, 23 patients (40.4%) were perirectally colonized with MRSA in addition to perirectal VRE and MRSA nasal colonization. Results of the bivariable analyses for VRE/MRSA co-colonization are displayed in Table 1. Because of space constraints, bivariable results for colonization by MRSA or VRE alone are not shown. These results suggest that patients colonized with VRE, MRSA, or both were significantly more likely to have been admitted to the MICU, have had previous hospital admissions, and have antimicrobial exposures within 1 year of current admission (p<0.05 for all). Among co-colonized patients, 58% had been admitted to the index hospital in the previous year, and 51% had received antimicrobial drugs during an admission in the previous

year. In addition, ≈48% of MRSA/VRE co-colonized patients had a previous positive culture (clinical or surveillance) for MRSA before the study period, and ≈28% had a previous positive culture for VRE.

Independent risk factors identified with logistic regression for the different colonization states (co-colonization, VRE only, or MRSA only) were markedly different (Table 2). Table 3 displays clinical outcomes of VRE/MRSA co-colonized patients. Approximately 25% percent of co-colonized patients died during the current admission; however, mortality was not significantly higher compared to non–co-colonized patients (p = 0.15). Three percent of co-colonized patients had MRSA-positive clinical cultures, and 3% of co-colonized patients had VRE-positive clinical cultures on the current or subsequent admissions (within 6 months). None of the co-colonized patients had clinical cultures positive for both organisms. Thirty-two percent of VRE/MRSA co-colonized patients were transferred to another hospital upon discharge from the index hospital compared with 15% of non–co-colonized patients (p<0.01).

Discussion

Increasing VRSA prevalence in the healthcare setting

Table 1. Characteristics of the study population by co-colonization status*			
Characteristic	MRSA/VRE co-colonization (n = 65)	No co-colonization (n = 2,375)	p value
Demographic variable			
Mean age, y (SD)	61.1 (15.1)	55.7 (16.0)	<0.01
Male sex, n (%)	43 (66.2)	1,277 (53.8)	0.05
Variables from current admission			
Type of ICU (MICU), n (%)	49 (75.4)	1,014 (42.7)	<0.01
Transfer from another hospital, n (%)	10 (15.4)	711 (29.9)	<0.01
Comorbidities			
HIV/AIDS, n (%)	3 (4.6)	77 (3.2)	0.54
Malignancy, n (%)	6 (9.2)	423 (17.8)	0.07
Cardiac disease, n (%)	20 (30.8)	486 (20.5)	0.04
Diabetes mellitus, n (%)	14 (21.5)	486 (20.5)	0.83
Liver disease, n (%)	2 (3.1)	182 (7.7)	0.17
Renal disease, n (%)	3 (4.6)	74 (3.1)	0.50
Mean Charlson comorbidity score (SD)	2.1 (2.2)	2.3 (2.3)	0.37
Antimicrobial drugs before culture			
Vancomycin, n (%)	14 (21.5)	226 (9.5)	<0.01
Piperacillin-tazobactam, n (%)	17 (26.2)	398 (16.8)	0.05
Imipenem, n (%)	2 (3.1)	72 (3.0)	0.98
Cephalosporins, n (%)	7 (10.8)	426 (17.9)	0.14
Aminoglycosides, n (%)	4 (6.2)	109 (4.6)	0.55
Fluoroquinolones, n (%)	11 (16.9)	223 (9.4)	0.04
Variables from previous admissions			
Previous MRSA colonization/infection	31 (47.7)	145 (6.1)	<0.01
Previous VRE colonization/infection	18 (27.7)	178 (7.5)	<0.01
Hospital admission (<1 y), n (%)	37 (57.9)	751 (31.6)	<0.01
ICU admission (<1 y), n (%)	17 (26.2)	309 (13.0)	<0.01
Antimicrobial drugs during admission (<1 y), n (%)	33 (50.8)	606 (25.5)	<0.01

*MRSA, methicillin-resistant *Staphylococcus aureus*; VRE, vancomycin-resistant enterococci; SD, standard deviation; ICU, intensive care unit; MICU, medical ICU.

Table 2. Components of final logistic regression models*

Characteristic	MRSA/VRE co-colonization, OR (95% CI)	VRE colonization, OR (95% CI)	MRSA colonization, OR (95% CI)
Age	1.03 (1.01–1.05)		
Male sex	1.93 (1.14–3.3)		
Admission to MICU	4.38 (2.46–7.81)	1.84 (1.38–2.46)	
Antimicrobial drugs during prior admission (<1 y)	3.06 (1.85–5.07)	3.38 (2.54–4.51)	1.66 (1.20–2.30)
Diabetes mellitus		1.39 (1.00–1.83)	1.83 (1.30–2.58)
Liver disease		1.64 (1.04–2.58)	
Renal disease		2.28 (1.26–4.1)	
Vancomycin		1.54 (1.03–2.31)	
Piperacillin-tazobactam		1.77 (1.27–2.47)	
Imipenem		2.47 (1.40–4.36)	
Fluoroquinolones		2.01 (1.37–2.96)	
HIV/AIDS			2.74 (1.46–5.14)

*Only variables significantly associated with the outcome are included in the final models. MRSA, methicillin-resistant *Staphylococcus aureus*; VRE, vancomycin-resistant enterococci; OR, odds ratio; CI, confidence interval; MICU, medical intensive care unit.

could result in considerable illness and death (21). To our knowledge, we present the first estimates of the prevalence, risk factors, and clinical outcomes of patients with VRE and MRSA co-colonization. As has been previously reported, co-colonization with VRE and MRSA has always preceded VRSA colonization or infection, and patients admitted to ICUs are likely a high-risk population (11–13,15). We show that among patients who had admission cultures taken, 4.6% admitted to the MICU and 1.2% admitted to the SICU of a tertiary-care facility during a 2-year period were co-colonized with MRSA and VRE, with an overall co-colonization proportion of 3% in both ICUs together. In addition, the observation that ≈40% of MRSA/VRE co-colonized patients were perirectally colonized with both organisms suggests the potential risk for the exchange of genetic material, which could result in the emergence of VRSA.

None of the 65 VRE/MRSA–co-colonized patients had positive clinical cultures for both VRE and MRSA on current or subsequent admissions to the index hospital. Thus, none would have been identified as co-colonized without the active surveillance culturing program in place at our institution. As has been suggested by previously reported cases of VRSA, early identification of these patients is recommended to minimize antimicrobial selective pressure and enhance infection control efforts to reduce the poten-

tial for patient-to-patient transmission (5,7).

In this study, <25% of co-colonized patients died, and nearly 35% were discharged to other hospitals or rehabilitation facilities, where the risk of transmitting these organisms to other patients is substantial. Considering the potential for colonization, infection, and transmission of VRSA, treating physicians, hospital staff, and infection control personnel at receiving institutions must be adequately prepared to isolate and treat these patients (22,23). Previous studies of transmission from vancomycin-intermediate *S. aureus* (VISA)- or VRSA-colonized or infected patients show that these organisms can be transmitted to close contacts or other hospitalized patients (7,21,24).

Previous studies have also shown that carriage of VRE or MRSA may be persistent, which would increase the potential for co-colonized patients to transmit either or both of these organisms to other patients (25,26). A mathematical model of the transmission dynamics of VRE has suggested that persistent colonization is the most important factor for increasing the endemic prevalence of this organism in the hospital (26). Furthermore, the potential for prolonged co-colonization could increase the likelihood that these patients would experience sufficient selective pressure for emergence of VRSA.

Recent reviews by Reuf, Fridkin, and Cosgrove et al. have summarized risk factors associated with clinical cul-

Table 3. Outcomes of patients co-colonized with VRE and MRSA*

Outcome	MRSA/VRE co-colonization, n = 65	No co-colonization, n = 2,375	p value
Death, n (%)	16 (24.6)	420 (17.7)	0.15
Mean length (d) of hospital stay (SD)	12.0 (16.5)	14.0 (17.8)	0.37
Subsequent MRSA-positive clinical culture, n (%)	2 (3.1)	34 (1.4)	<0.01
Subsequent VRE-positive clinical culture, n (%)	2 (3.1)	39 (1.6)	0.38
Discharge location			
Home or self-care, n (%)	27 (41.5)	1,467 (61.8)	<0.01
Hospital, n (%)	21 (32.3)	368 (15.5)	<0.01
Rehabilitation facility, n (%)	1 (1.5)	62 (2.6)	0.59
Unknown, n (%)	0	18 (0.8)	0.48

*VRE, vancomycin-resistant enterococci; MRSA, methicillin-resistant *Staphylococcus aureus*; SD, standard deviation.

ture positivity for VRSA and VISA (4,22,23). However, with only 3 reported cases of VRSA and ≈20 reported cases of VISA, the precision of statistical associations has been limited. Recent exposure to vancomycin and recent isolation of MRSA were identified as risk factors for VISA in addition to concurrent colonization or infection with VRE and MRSA. Among co-colonized patients in this study, ≈21% received vancomycin, 26% received piperacillin-tazobactam, and 17% received a fluoro-quinolone before culture results.

This study is the first to report the prevalence, risk factors, and clinical outcomes of patients with VRE/MRSA co-colonization upon admission to the ICU. We report several risk-factors for co-colonization, including that the odds of co-colonization for patients admitted to the MICU were >4 times greater than those of patients admitted to the SICU and that patients who received antimicrobial drugs within 1 year of admission had 3 times the odds of co-colonization as patients who had not received antimicrobial drugs during a previous admission.

Ray et al. assessed the prevalence of gastrointestinal *S. aureus* colonization among a convenience sample of 37 patients colonized with VRE and reported that 20 (54.1%) of these patients were also colonized with MRSA (27). However, comparing these results to those presented here is difficult, given the stark differences between patient groups. The study by Ray et al. included only hospitalized patients from whom a minimum of 3 stool samples (collected weekly) were obtained (i.e., inpatients with extended lengths of stay). We believe that ICU patients co-colonized with VRE and MRSA are at risk of acquiring and transmitting VRSA because they generally are exposed to greater antimicrobial selective pressure, have extended lengths of stay, greater likelihood of indwelling devices, greater severity of illness, and are more likely to have a history of previous hospitalization and related exposures than patients admitted to general medical wards. Despite these differences, perirectal colonization of both organisms was similar between the patients in the study by Ray et al. and this study (54.1% vs. 40.3%, respectively). Other studies have also suggested a prevalence of VRE/MRSA co-colonization or co-infection ranging from 9.5% to 28.6%; however, these studies relied upon clinical cultures to provide these estimates (14,15).

A limitation of this study is that investigators were unable to determine the species of the VRE isolates. Historically, *Enterococcus faecalis* has been more likely to be associated with conjugation events and subsequent VRSA colonization or infection compared with *E. faecium* (7,28). However, only 3 VRSA cases are known, and we are not aware of any biologic explanation for why *E. faecium* would be less likely to be involved in transmission of vancomycin resistance to MRSA compared with

E. faecalis. Still, these data would have been useful and informative. A previous, hospitalwide study at UMMC suggested that among isolated VRE, the prevalence of *E. faecium* and *E. faecalis* were 87%, and 13%, respectively (29).

In summary, these data describe a high prevalence of patients co-colonized with VRE and MRSA on admission to an ICU at a tertiary-care hospital, none of whom would have been detected by clinical culture. Risk factors for VRE and MRSA co-colonization are also described. Given that many of these patients were discharged to other institutions, treating physicians and infection control personnel must be cognizant of the risks for VRSA colonization and infection and use appropriate precautions. Appropriate methods to rapidly detect co-colonized patients must be identified to suppress the emergence of VRSA; limit patient-to-patient transmission of MRSA, VRE, and VRSA; and prevent endemic VRSA colonization in health-care institutions.

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Mallards and Highly Pathogenic Avian Influenza Ancestral Viruses, Northern Europe

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Outbreaks of highly pathogenic avian influenza (HPAI), which originate in poultry upon transmission of low pathogenic viruses from wild birds, have occurred relatively frequently in the last decade. During our ongoing surveillance studies in wild birds, we isolated several influenza A viruses of hemagglutinin subtype H5 and H7 that contain various neuraminidase subtypes. For each of the recorded H5 and H7 HPAI outbreaks in Europe since 1997, our collection contained closely related virus isolates recovered from wild birds, as determined by sequencing and phylogenetic analyses of the hemagglutinin gene and antigenic characterization of the hemagglutinin glycoprotein. The minor genetic and antigenic diversity between the viruses recovered from wild birds and those causing HPAI outbreaks indicates that influenza A virus surveillance studies in wild birds can help generate prototypic vaccine candidates and design and evaluate diagnostic tests, before outbreaks occur in animals and humans.

Wild birds, predominantly ducks, geese, and shorebirds, form the reservoir of influenza A viruses in nature (1,2). Influenza A viruses are subtyped on the basis of the antigenic properties of the hemagglutinin (HA) and neuraminidase (NA) glycoproteins, expressed on the surface of virus particles. To date, 16 HA and 9 NA subtypes have been detected in wild birds and poultry throughout the world (3,4). Viruses containing HA of subtypes H5 and H7 may become highly pathogenic after introduction in poultry and cause outbreaks of highly pathogenic avian influenza (HPAI, formerly termed fowl plague) (1,2). The switch from a low pathogenic avian influenza (LPAI) phenotype, common in wild birds and poultry, to the HPAI phenotype is achieved by the introduction of basic amino

acid residues into the HA0 cleavage site (5). HPAI isolates have been obtained primarily from commercially raised birds such as chickens, turkeys, quail, guinea fowl, and ostriches (6). In the last decade, the frequency of detected HPAI outbreaks has increased, with outbreaks of avian influenza A viruses of subtype H5N2 in Mexico (1994); Italy (1997) and Texas (2004); H5N1 in Hong Kong (1997) and Southeast Asia (ongoing since 1997); H7N3 in Australia and Pakistan (1994); H7N4 in Australia (1997); H7N1 in Italy (1999); H7N3 in Chile (2002) and Canada (2003); and H7N7 in the Netherlands (2003) (7–15).

Influenza A viruses of subtypes H5 and H7 have been frequently detected in mammals (16). H7N7 viruses have been endemic in horses for some time (17), were transmitted from seals to humans in the United States in 1980 (18,19), and were isolated from humans in the United Kingdom in 1996 (20) and the Netherlands in 2003 (12,13). H7N2 and H7N3 influenza A viruses were isolated from humans in the United States in 2003 (21,22) and Canada in 2004 (23), respectively. HPAI H5N1 viruses circulating in Southeast Asia since 2003 have been detected in at least 108 human cases of respiratory illness, of which 54 were fatal (24). In addition, these H5N1 influenza A viruses have been detected in pigs (25), cats, leopards, and tigers (26–29) in Southeast Asia. As a consequence of the relatively frequent zoonoses caused by influenza A viruses of subtypes H5 and H7, these virus subtypes are given high priority with respect to pandemic preparedness.

Wild birds harbor the LPAI ancestral viruses of HPAI strains of poultry (and mammals). In our influenza A virus surveillance studies in wild birds in northern Europe, we detected numerous influenza A viruses of subtype H5 and H7 in Mallards (*Anas platyrhynchos*). We show that for each of the HPAI outbreaks that occurred in Europe since 1997, we have found close LPAI relatives in Mallards. Our observations indicate that influenza A virus surveillance in

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wild birds provides opportunities for pandemic preparation; the prototype influenza A viruses obtained from wild birds may guide production of vaccines as well as reagents to develop and validate diagnostic tests.

Materials and Methods

Specimens

In our ongoing influenza A virus surveillance studies in wild birds in northern Europe (30), Mallards were trapped with duck traps in Lekkerkerk and Krimpen aan de Lek in the Netherlands and Ottenby Bird Observatory on the southernmost point of the island Öland in Sweden (Figure 1). Cloacal samples were collected with cotton swabs and stored in transport media consisting of Hanks' balanced salt solution, 10% vol/vol glycerol, 200 U/mL penicillin, 200 μg/mL streptomycin, 100 U/mL polymyxin B sulfate, and 250 μg/mL gentamicin (MP Biomedicals, Zoetermeer, the Netherlands) at –70ºC.

RNA Isolation and Virus Detection

RNA isolation and reverse transcription–polymerase chain reaction (RT-PCR) were performed as described previously (32) for samples obtained until 2002. Beginning in 2003, RNA was isolated by using a MagnaPure LC system with the MagnaPure LC Total nucleic acid isolation kit (Roche Diagnostics, Almere, the Netherlands), and influenza A virus was detected by using a real-time RT-PCR assay (33). To ensure efficient influenza A virus detection, the published probe sequence was changed to 6-FAM-TTT-GTG-TTC-ACG-CTC-ACC-GTG-CC-TAMRA-3′, based on the avian influenza A virus sequences available from public databases. Amplification and detection were performed on an ABI7700 machine with the TaqMan EZ RT-PCR Core Reagents kit (Applied Biosystems, Nieuwerkerk aan den IJssel, the Netherlands) by using 20 μL eluate in an end volume of 50 μL. Pools of 5 individual samples were prepared and processed in parallel with several negative and positive control samples in each run. Upon identification of influenza A virus–positive pools, RNA isolation and RT-PCR procedures were repeated for the individual samples within each positive pool; individual RT-PCR–positive samples were subsequently used to isolate virus.

Virus Isolation and Characterization

For influenza A virus RT-PCR–positive samples, 200 μL original material was injected into the allantoic cavity of 11-day-old embryonated hens' eggs. The allantoic fluid was harvested 2 days after injection, and influenza A virus was detected by using hemagglutination assays with turkey erythrocytes. When no influenza A virus was detected on the initial virus isolation attempt, the allantoic

fluid was passaged once more in embryonated hens' eggs. Virus isolates were characterized with a hemagglutination inhibition (HI) assay with turkey erythrocytes and subtype-specific hyperimmune rabbit antisera raised against all HA subtypes (4,34).

Sequence Analysis and Phylogenetic Trees

NA subtypes of influenza A virus isolates were characterized by RT-PCR and sequencing. RT-PCR and sequencing of the HA and NA genes were performed essentially as described by others (35). PCR products were purified from agarose gels with the Qiaquick Gel Extraction kit (Qiagen, Leusden, the Netherlands) and sequenced with the Big Dye terminator sequencing kit version 3.0 (Amersham Pharmacia Biotech, Roosendaal, the Netherlands) and an ABI PRISM 3100 genetic analyzer (Applied BioSystems), according to the instructions of the manufacturer. All primer sequences are available upon request. Nucleotide and amino acid sequences were aligned by using the ClustalW program running within the BioEdit software package, version 5.0.9 (36). We first generated trees for H5 and H7 by using all full-length HA1 amino acid sequences available from public databases. Amino acid sequence alignments were bootstrapped 100 times, and distance matrices were generated by using Kimura parameters. The trees were generated by using the UPGMA (unweighted pair-group method with arithmetic mean) clustering method of the Neighbor program of PHYLIP version 3.6 (37). The consensus of 100 UPGMA trees was calculated, and the branch lengths of this consensus tree were recalculated by using the Fitch program of PHYLIP 3.6.

For selected influenza A virus isolates of European origin, DNA maximum-likelihood trees were generated by using full-length HA nucleotide sequences from which the sequences encoding the HA cleavage site were excluded. Alignments were bootstrapped 100 times by using the Seqboot package of PHYLIP version 3.6, and trees were constructed with the Dnaml package, using 3 jumbles. The consensus tree was calculated by using the Consense package of PHYLIP 3.6; this tree was used as usertree in Dnaml to recalculate the branch lengths from the nucleotide sequences. Finally, the trees were rerooted at midpoint by using the Retree software of PHYLIP 3.6. Trees were visualized with the Treeview 1.6.6 program distributed with BioEdit version 5.0.9. All nucleotide sequences presented here are available from GenBank under accession numbers AY684894, AY338460, AY995883–AY995898, and AY999977–AY999991.

Serology

HI assays were performed to compare the antigenic properties of influenza A virus strains by using postinfection ferret antisera and hyperimmune rabbit antiserum

generated against the following influenza viruses: A/Tern/South Africa/61 (H5N3), A/Duck/Hong Kong/205/77 (H5N3), A/Hong Kong/156/97 (H5N1), A/Equine/Prague/1/54 (H7N7), A/Seal/Massachusetts/1/80 (H7N7), A/Mallard/Netherlands/12/00 (H7N3), A/Netherlands/033/03 (H7N7), and A/Netherlands/219/03 (H7N7), as described previously (4,34). HI assays were performed in duplicate. All serum samples were treated overnight with receptor-destroying enzyme at 37ºC and subsequently incubated at 56ºC for 1 hour. Twofold serial dilutions of each antiserum, starting at a 1:20 dilution, were tested for their ability to inhibit the agglutination of horse erythrocytes by 4 hemagglutinating units of influenza A virus. Serum dilutions were made in phosphate-buffered saline (PBS) containing 0.5% vol/vol bovine serum albumin (BSA, fraction V, Gibco, Breda, the Netherlands). Horse erythrocytes were stored in PBS containing 0.5% vol/vol BSA. In the HI assay, 50 µL of a 1% vol/vol horse erythrocyte dilution was added to each well (38).

Results

Avian Influenza A Virus in Wild Birds in Europe

Of 172 virus isolates obtained within this study period, 33 contained HA genes of subtypes H5 or H7, 6 were of subtype H5N2, 2 were H5N3, 1 was H5N6, 8 were H5N9, 1 was H7N3, 14 were H7N7, and 1 was H7N9. All H5 and H7 influenza A viruses were isolated from samples collected from Mallards during fall migration at marshalling sites in the Netherlands (1 H5 isolate from October 1999 and 1 H7 isolate from December 2000) and Sweden (all other H5 and H7 isolates collected from September to January 2002) (Figure 1).

Characterization of H7 Influenza A Viruses

Sequence analyses of the HA open reading frames (ORFs) of the 16 H7 influenza A viruses isolated from Mallards showed that the HA0 cleavage site lacked basic amino acid residues, which is typical for LPAI viruses. We next determined the genetic relationship between the HA genes of our H7 influenza A viruses isolated from European Mallards and those available from public sequence databases. The phylogenetic tree, based on HA1 amino acid sequences, showed the typical separation of H7 strains in the Eurasian and American genetic lineages. Within the Eurasian H7 HA lineage, the European Mallard influenza A viruses were found in different parts of the tree, clustering closely with influenza A viruses responsible for recent H7 HPAI outbreaks in Europe (Figure 2A). We next generated a DNA maximum-likelihood phylogenetic tree by using prototypic European Mallard influenza A viruses and strains representing each of the H7 HPAI outbreaks that occurred in Europe (H7N1 in Italy
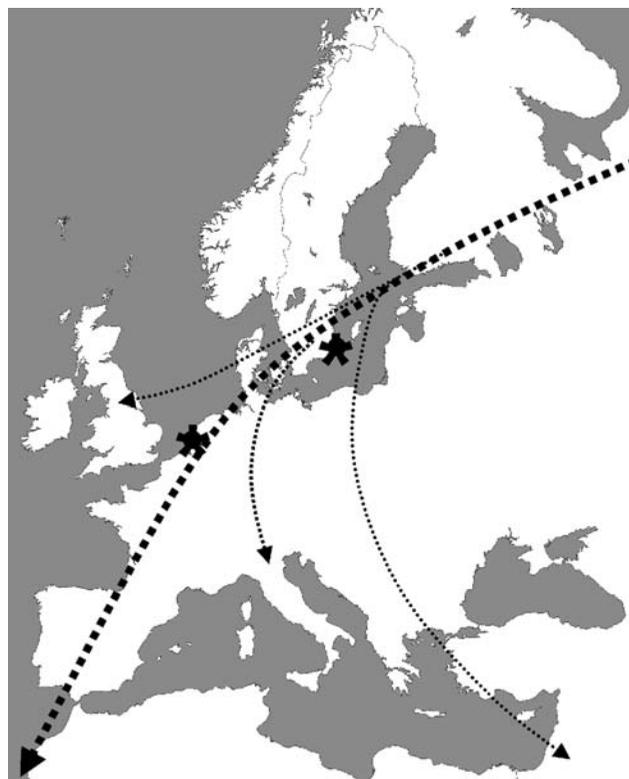
Figure 1. Main fall migration route of wild waterfowl in northern Europe (31). The sample locations Öland (Sweden) and Lekkerkerk and Krimpen a/d Lek (the Netherlands) are marked with asterisks.

2000/2001 and H7N7 in the Netherlands 2003) in the last decade (Figure 2B). This tree showed the cocirculation of 2 genetically distinct lineages of H7 HA in European Mallards, 1 closely related to H7N7 and H7N1 HPAI strains causing outbreaks in the Netherlands (2003) and Italy (2000/2001) and 1 closely related to the H7N7 isolate obtained from a woman with conjunctivitis in the United Kingdom in 1996 (20). The maximum nucleotide/amino acid sequence identity between the Italian H7N1 HPAI virus A/Chicken/Italy/445/99 and the most closely related LPAI virus A/Mallard/Netherlands/12/00 was 98% nt and 98% amino acids (aa). The maximum nucleotide/amino acid identity between the Dutch H7N7 HPAI virus A/Chicken/Netherlands/1/03 and the most closely related LPAI virus A/Mallard/Netherlands/12/00 is 98% nt and 99% aa. The maximum nucleotide/amino acid identity between the LPAI virus A/Mallard/Sweden/56/02 and A/Turkey/Ireland/ PV74/95 (H7N7) was 95% nt, 96% aa; between A/Mallard/Sweden/56/02 and A/England/268/96 (H7N7), it was 96% nt and 97% aa.

We next analyzed the antigenic relatedness of the H7 influenza A viruses obtained from wild Mallards in HI assays with postinfection ferret antisera and hyperimmune

Figure 2. Phylogenetic trees of hemagglutinin H7 sequences. A) Phylogenetic tree based on the amino acid sequence distance matrix for the HA1 open reading frames of all H7 sequences available from public databases. The scale bar represents ≈10% of amino acid changes between close relatives. *Represents the locations of the Mallard influenza A virus isolates. B) DNA maximum likelihood tree for the European highly pathogenic avian influenza viruses and the low pathogenic avian influenza H7 influenza A viruses isolated from migrating Mallards by using A/FPV/Dutch/27 as outgroup. The scale bar represents 10% of nucleotide changes between close relatives.

rabbit antisera. The hyperimmune rabbit antisera were chosen on the basis of their ability to provide a broad response, which would recognize a wide range of strains within 1 subtype, whereas the postinfection ferret antisera were chosen on the basis of high specificity. HI assays showed that the antigenic properties of the H7 influenza A viruses from Mallards were relatively conserved, and that the HI data for the Mallard influenza A viruses did not differ significantly (i.e, up to 4-fold) from those obtained with

strains causing the HPAI outbreak in the Netherlands in 2003 (Table 1). The antigenic analyses therefore confirmed the genetic data, which showed little genetic diversity between the H7 strains isolated from wild Mallards and the strains causing the H7 HPAI outbreaks.

Sequence Analysis, Phylogeny, and Antigenic Characterization

Sequence analyses of the HA ORFs of the 17 H5 influenza A viruses isolated from Mallards showed that the HA0 cleavage site lacked basic amino acid residues, which is typical for LPAI viruses. To determine the genetic relationship between the H5 influenza A virus isolates obtained from wild birds and strains causing recent H5 HPAI outbreaks (H5N2 in Italy 1997), we generated a phylogenetic tree based on the amino acid sequences of the HA1 domain of all H5 influenza A viruses currently available from public sequence databases (Figure 3A). As for H7, this tree showed the 2 clearly distinguishable Eurasian and American genetic lineages. The H5 HA sequences that we obtained from influenza A viruses isolated from European Mallards were closely related to the influenza A virus strains responsible for the H5 HPAI outbreak in Italy in 1997. The H5 HPAI influenza A strains isolated in Southeast Asia beginning in 1997 form a continuous genetic lineage, presumably evolving from a common LPAI wild bird ancestor around 1997 (15). Similarly, we did not detect close relatives of the recent HPAI Asian strains in Mallards in Europe (Figure 3A). The DNA maximum likelihood tree based on the full-length HA nucleotide sequences of the 17 H5 HA genes of Mallard influenza A viruses and those of the Italian H5 HPAI influenza A viruses confirmed the close genetic relationship (Figure 3B). Maximum nucleotide/amino acid identity between the Italian HPAI virus A/Chicken/Italy/312/97 H5N2 and the most closely related LPAI virus, A/Netherlands/3/99 H5N2, is 96% nt identity and 98% aa identity (Figure 3B).

In HI assays with postinfection ferret antisera and hyperimmune rabbit antisera raised against H5 influenza A viruses, the influenza A viruses obtained from wild Mallards were antigenically conserved and did not differ significantly (up to 4-fold) from the prototypic strains used in the HI assay (Table 2). In agreement with the phylogenetic analysis of the current H5N1 HPAI influenza A viruses from Southeast Asia, the antigenic properties of H5N1 influenza virus A/Vietnam/1194/04 differ significantly from those of the LPAI strains isolated from Mallards used in this study, when analyzed with the highly specific postinfection ferret antisera. Hyperimmune rabbit antisera failed to discriminate the antigenic properties of all strains because of the broader antigenic reactivity of these sera.

Table 1. Hemagglutination inhibition assays with postinfection ferret antisera and hyperimmune rabbit antisera raised against H7 influenza A viruses

Virus	A/Eq/Prague/ 1/54*	A/Seal/Mass/ 1/80*	A/Neth/219/ 03†	A/Mallard/Neth/ 12/00†	A/Neth/33/ 03†
A/Equine/Prague/1/54 H7N7	**1:1,280‡**	1:1,280	<1:20	<1:20	<1:20
A/Seal/Massachusetts/1/80 H7N7	1:160	**1:1,280**	<1:20	<1:20	1:20
A/Netherlands/219/03 H7N7	1:160	1:1,280	**1:40**	1:40	1:80
A/Mallard/Netherlands/12/00 H7N3	1:160	1:1,280	1:20	**1:80**	1:40
A/Netherlands/33/03 H7N7	1:320	1:1,280	1:80	1:160	**1:160**
A/Mallard/Sweden/56/02 H7N7	1:640	1:5,120	1:80	1:80	1:160
A/Mallard/Sweden/105/02 H7N7	1:320	1:2,560	1:80	1:80	1:80
A/Mallard/Sweden/85/02 H7N7	1:160	1:1,280	1:40	1:80	1:80

*Hyperimmune rabbit antisera.
†Postinfection ferret antisera.
‡Homologous titers are represented **boldfaced and underlined**.

Discussion

Because HPAI outbreaks in poultry find their origin in LPAI viruses present in waterfowl, influenza A virus surveillance in wild birds could function as an early warning system for HPAI outbreaks and as a means to keep panels of reference reagents, required for diagnostic purposes and vaccine production, up-to-date (39,40). Wild bird surveillance would also be relevant for HPAI viruses that represent pandemic threats. However, limited information on the prevalence of avian influenza A viruses in wild birds in Europe, and on the genetic and antigenic variability of the viruses in this part of the world, has made assessing the value of such surveillance studies difficult. We isolated avian influenza A viruses of subtypes H5 and H7 from Mallards in northern Europe. During a 4-year surveillance period, we isolated influenza A viruses of subtypes H5N2, H5N3, H5N6, H5N9, H7N3, H7N7, and H7N9, among many other influenza A virus isolates. All of these H5 and H7 influenza A virus isolates were obtained from Mallards during fall migration at a Swedish location and at 2 Dutch wintering sites. Using this relatively limited setting, we isolated influenza A viruses that possess H5 and H7 glycoproteins and gene segments closely related to those of influenza A viruses responsible for HPAI outbreaks in Europe, H5N2 in Italy (1997), H7N1 in Italy (1999–2000), and H7N7 in the Netherlands (2003). Thus, we conclude that influenza A virus surveillance in wild birds is useful to keep the panels

of reference reagents up-to-date. Whether surveillance studies could be useful as a sentinel system is uncertain.

We observed minor antigenic and genetic diversity between the HA genes of Mallard influenza A virus isolates and those of HPAI virus strains. This finding implies that the influenza A virus isolates obtained during wild bird surveillance studies may also be prototypic vaccine candidates for human or veterinary use. Limited numbers of prototype vaccine strains, representing both the American and Eurasian genetic lineages of influenza A virus, could be generated to cover a wide range of HPAI strains. Such vaccine seed strains can be produced well ahead of outbreaks in poultry, other animals, or humans. The disadvantage of the minor antigenic differences between the vaccine strain and the epidemic strains will likely be compensated by the immediate availability of the vaccine. An additional advantage of the use of LPAI strains from wild birds as prototype vaccine strains is that they do not contain a basic cleavage site in the HA gene. Before HPAI strains can be used as vaccine candidates, the basic amino acid residues in the HA gene need to be removed by using reverse genetics technology; this would result in an extra modification step, which would consume precious time. Moreover, these vaccine strains can only be generated after an outbreak of HPAI has started.

We suggest that a thorough genetic and antigenic characterization of avian influenza A viruses isolated in the

Table 2. Hemagglutination inhibition assays with postinfection ferret antisera and hyperimmune rabbit antisera raised against H5 influenza A viruses

Virus isolate	A/Tern/SA/61*	A/Tern/SA/61†	A/Dk/HK/205/77*	A/Dk/HK/205/77†	A/HK/156/97*	A/HK/156/97†
A/Tern/South Africa/61 H5N3	**1:640‡**	**1:320**	1:80	1:640	1:80	1:20
A/Duck/Hong Kong/205/77 H5N3	1:1,280	1:640	**1:240**	**1:1,280**	1:160	1:80
A/Hong Kong/156/97 H5N1	1:1,280	1:640	1:320	1:1,280	**1:640**	**1:320**
A/Vietnam/1194/04 H5N1	1:1,280	1:40	1:640	1:80	1:640	<1:20
A/Mallard/Sweden/21/02 H5N2	1:640	1:320	1:160	1:640	1:160	1:20
A/Mallard/Sweden/49/02 H5N9	1:320	1:320	1:40	1:320	1:160	1:40
A/Mallard/Netherlands/3/99 H5N2	1:640	1:1,280	1:160	1:5,120	1:160	1:80
A/Mallard/Sweden/7/02 H5N2	1:1,280	1:640	1:320	1:1,280	1:320	1:40

*Hyperimmune rabbit antisera.
†Postinfection ferret antisera.
‡Homologous titers are represented **boldfaced and underlined**.
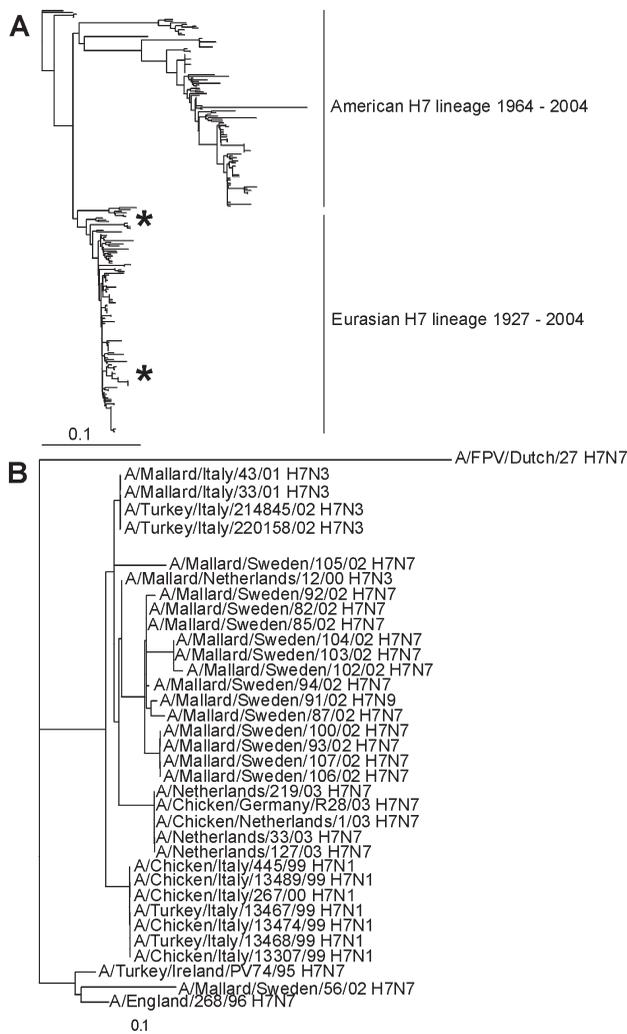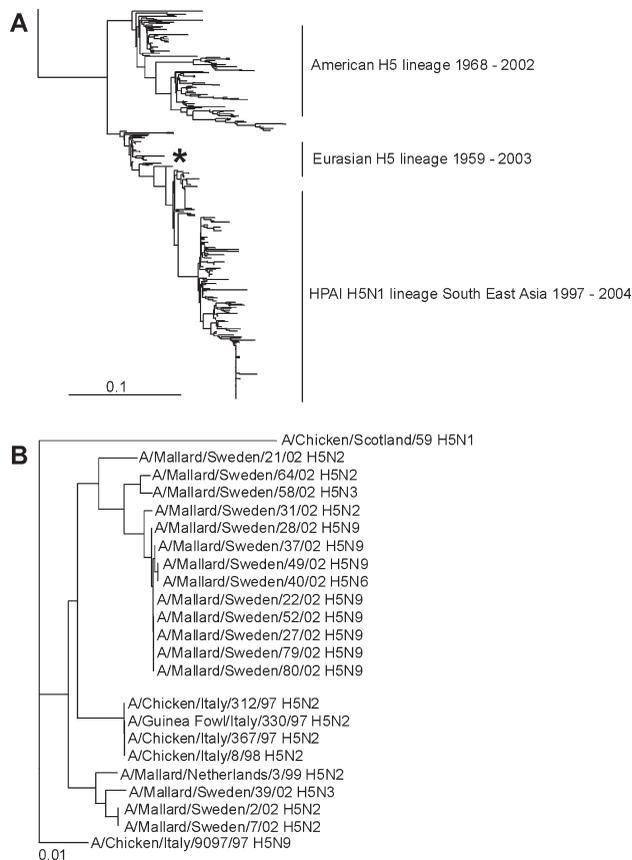
Figure 3. Phylogenetic trees of H5 sequences. A) Phylogenetic tree based on the amino acid sequence distance matrix, representing all H5 amino acid sequences available from public databases. The scale bar represents ≈10% of amino acid changes between close relatives. *Represent location of the H5 influenza A viruses isolated from Mallards. B) DNA maximum likelihood tree for the cluster of European H5 influenza A viruses and the low pathogenic avian influenza H5 influenza A viruses isolated from migrating Mallards by using A/Chicken/Scotland/59 as outgroup. The scale bar represents 1% of nucleotide changes between close relatives.

Americas, Asia, and Europe would be useful to prepare for outbreaks. While this usefulness has been demonstrated in our study with influenza A viruses of the H5 and H7 subtypes, it should be applied also to other influenza A virus strains relevant to animal and public health, in particular, those of subtypes H1, H2, H3, and H9.

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Isolate Removal Methods and Methicillin-resistant *Staphylococcus aureus* Surveillance

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The effect of duplicate isolate removal strategies on *Staphylococcal aureus* susceptibility to oxacillin was compared by using antimicrobial test results for 14,595 isolates from statewide surveillance in Hawaii in 2002. No removal was compared to most resistant and most susceptible methods at 365 days and to the National Committee for Clinical Laboratory Standards (NCCLS) and Cerner algorithms at 3-, 10-, 30-, 90-, and 365-day analysis periods. Overall, no removal produced the lowest estimates of susceptibility. Estimates with either NCCLS or Cerner differed by <2% when the analysis period was the same; with either method, the difference observed between a 90- and a 365-day period was <1%. The effect of duplicate isolate removal was greater for inpatient than outpatient settings. Considering the ease of implementation and comparability of results, we recommend using the first isolate of a given species per patient to calculate susceptibility frequencies for *S. aureus* to oxacillin.

Surveillance of antimicrobial susceptibility is critical for developing strategies to control increasing antimicrobial resistance. Aggregation of institutional antibiograms is commonly proposed as a useful means of monitoring antimicrobial resistance trends in a population (1–3). However, inconsistencies in the methods used to generate antibiogram susceptibility reports, particularly with regard to duplicate isolate removal, make comparing data from different facilities problematic (2,4–6).

To address this situation, in 2002, the National Committee for Clinical Laboratory Standards (NCCLS, currently known as the Clinical and Laboratory Standards Institute) recommended using antimicrobial test results from the first species isolate per patient, per period of data

analysis, to calculate susceptibility frequencies (7). Other approaches currently in use include not removing any isolates, counting only the most susceptible or most resistant isolate from a patient per surveillance period, and applying the Cerner laboratory management system, a widely used software program (4).

Studies comparing the potential effect of using different methods for duplicate isolate removal are limited, i.e., most existing analyses are based on data from a single facility or compared only a few of the many different options for duplicate isolate removal (4–6). We evaluated the effects of 13 distinct duplicate isolate removal strategies on *Staphylococcus aureus* susceptibility to oxacillin by using antimicrobial susceptibility test results from a statewide antimicrobial resistance surveillance system in Hawaii.

Methods and Materials

Data Collection

All available susceptibility data for *S. aureus* isolates identified in Hawaii in 2002 were collected retrospectively from the laboratory information systems of participating facilities and transferred to the State of Hawaii Antimicrobial Resistance Project (SHARP) database. The SHARP system consists of laboratory data from 2 large commercial clinical laboratories and most acute-care hospitals. The 2 commercial laboratories serve most of Hawaii's population (N = 1,211,537) by providing susceptibility testing services for >85% of all nonhospital outpatient settings in Hawaii and performing susceptibility testing for 18 of the 24 acute-care hospitals in the state (8,9). The remaining 6 acute-care hospitals each maintain their own laboratory to perform susceptibility testing for their respective facility. Susceptibility results from 3 of these hospitals were incorporated into the SHARP

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database, yielding a final dataset that encompasses 21 (88%) of Hawaii's 24 acute-care hospitals. A review of antibiograms produced by all laboratories in Hawaii in 2001 indicates that the data sources used in the current analysis capture >90% of all *S. aureus* identified in our state annually.

Laboratories participating in SHARP provide isolate-level data, including the specimen collection date, source (e.g., blood, urine, and cerebrospinal fluid), susceptibility test methods (e.g., Kirby-Bauer), and susceptibility test results. Limited demographic patient information is also included in the record, e.g., date of birth and sex, but detailed clinical histories and patient names are not available. The susceptibility testing method used by all laboratories during the study period was the Vitek automatic system, supported by the Kirby-Bauer disk diffusion method (10). NCCLS criteria were used to interpret inhibitory zone diameters and MIC. Determination of methicillin susceptibility is based on oxacillin susceptibility testing. The breakpoint for oxacillin resistance was MIC ≥ 4 µg/mL or a zone diameter ≤ 10 mm. The breakpoint for intermediate isolates was MIC 2–4 µg/mL or zone diameter 11–12 mm. The breakpoint for susceptible isolates was MIC ≤ 2 µg/mL or zone diameter ≥ 13 mm.

For this analysis, *S. aureus* isolates from inpatients in intensive care units (ICUs), other inpatient settings (non-ICU), and outpatient settings (e.g., physician offices, community health centers, hospital outpatients, and emergency departments) were included. Isolates from patients in long-term care homes and prisons were excluded.

Detection of Duplicate Isolates

Duplicate isolates were identified by using Microsoft Access (Microsoft Corp., Redmond, WA, USA) to sort susceptibility data based on the patient's unique medical record number (MRN), if available, or an assigned patient identifier (APID). When an MRN was not available, the APID was created from the patient's date of birth, sex, reporting laboratory, and identity of the hospital facility or private physician who ordered the culture. The ability of the APID to uniquely identify patients was assessed by generating an APID for the subset of patients who also had MRNs. The corresponding APID was found to be unique for 99% of the records with a unique MRN and assessed to be an acceptable surrogate. The potential effect of using the APID in lieu of the MRN was assessed in a subanalysis that compared the results for records containing an MRN to those from records identified with the APID.

Methods for Duplicate Isolate Removal

Antimicrobial susceptibility frequencies were calculated by using each of the 5 duplicate isolate removal methods described. For the "no removal" method,

susceptibility results for all *S. aureus* isolates in the 2002 database were included in the estimation of the proportion of isolates. For the "most resistant" method, during a 365-day period, irrespective of the number of positive cultures, each patient was counted only once. For any given patient, if a resistant isolate was identified, the first resistant isolate identified was included in the analysis, and all other results, susceptible or resistant, were censored. If no resistant isolates were identified for a patient during the period, the first sensitive isolate was included in the analysis. For the "most susceptible" method, during a 365-day period, irrespective of the number of positive cultures, each patient was counted only once. For any given patient, if a susceptible isolate was identified, the first susceptible isolate identified was included in the analysis, and all other results, susceptible or resistant, were excluded. If no susceptible isolates were identified for a patient during the period, the first resistant isolate was included in the analysis. For the NCCLS method, the susceptibility results for the first *S. aureus* isolate per patient per analysis period, irrespective of body site, antimicrobial susceptibility profile, or other phenotypic characteristics (e.g., biotype), were included in the analysis (7). We applied NCCLS criteria for 5 different surveillance periods: 3, 10, 30, 90, and 365 days. Finally, for the Cerner method, a duplicate isolate was defined as from the same patient, same species, and same NCCLS susceptibility category to an individual antimicrobial agent as an immediately previous isolate (4). For this study, the Cerner method was modified to include surveillance periods commonly used with other duplicate isolate removal methods: 3, 10, 30, 90, and 365 days. Therefore, in our setting, duplicate *S. aureus* isolates were defined by the modified Cerner methods as the same patient and same susceptibility to oxacillin as the immediately previous isolate found during the same analysis period. Any isolate obtained from a given patient during the period of analysis that showed a change in susceptibility from that of the previous isolate was included in calculations of susceptibility.

For each method, the percentage of susceptible isolates was calculated by dividing the number of susceptible isolates by the number of total isolates eligible for the inclusion method in each particular analysis. Tables 1 and 2 show how the various strategies are applied to hypothetical patient isolates and illustrate how the susceptibility

Table 1. Hypothetical data for *Staphylococcus aureus* susceptibility to oxacillin*

Patient	Day						
	1	2	4	11	20	31	100
1	R	S	R	R	S	S	S
2	R	R	R	R	R	R	R
3	S	S	S	S	S	R	R

*R, resistant; S, susceptible.

Table 2. Application of different methods of duplicate isolate removal based on hypothetical data in Table 1*

Method	No. isolates	No. susceptible (%)†
No removal	21	9 (43)
Cerner, 3 d	19	8 (42)
NCCLS, 3 d	18	7 (39)
Cerner, 10 d	15	6 (40)
NCCLS, 10 d	12	4 (33)
Cerner, 30 d	12	5 (42)
NCCLS, 30 d	9	3 (33)
Cerner, 90 d	10	4 (40)
NCCLS, 90 d	6	2 (33)
Cerner, 365 d	7	3 (43)
NCCLS, 365 d	3	1 (33)
Most resistant	3	0
Most susceptible	3	2 (67)

*d, days; NCCLS, National Committee for Clinical Laboratory Standards.
†Susceptibility percentage is calculated as the proportion of the number of susceptible isolates divided by the number of total isolates tested and eligible for inclusion according to the analysis method used.

percentages were calculated for each scenario. Ninety-five percent confidence intervals (CIs) for proportions of susceptibility were calculated by using the binomial method. When susceptibility proportions for specific clinical settings (or institutions) were calculated, only isolates obtained in that particular setting (or institution) were eligible for analysis.

Results

Susceptibility testing results were identified for 14,595 *S. aureus* clinical isolates obtained from 10,892 patients. A total of 3,725 isolates were from 2,749 patients with an associated MRN; 10,870 were from 8,143 patients identified with an APID. For all patients, the isolate-to-patient ratio was 1.3.

Figure 1 depicts the effect of duplicate isolate removal on *S. aureus* susceptibility to oxacillin for all isolates. NCCLS and Cerner methods produced similar estimates of susceptibility for any given analysis period, i.e., the difference between the 2 methods was insignificant. Furthermore, the difference in susceptibility percentage between a 90-day and 365-day period was <1% by either NCCLS or Cerner criteria, which was insignificant. With both Cerner and NCCLS methods, the general trend for estimates of susceptibility increased as the period of analysis lengthened.

No removal resulted in the lowest susceptibility estimate (67%) observed, even lower than that for most resistant (Figure 1). Overall, an inverse relationship was observed between number of isolates included in the analysis and proportion of susceptible isolates (Figure 1). For both Cerner and NCCLS methods, point estimates of susceptibility rose slightly as the period of analysis increased and the number of isolates included in the susceptibility calculations decreased.

The patterns observed for all isolates combined remained unchanged when stratified by different clinical settings, i.e., ICU, non-ICU, and outpatient (Table 3). Within a given clinical setting, the difference in susceptibility frequencies with the 90- and 365-day intervals by either Cerner or NCCLS was <1%.

Differences in the magnitude of the effect of duplicate isolate removal were observed across different clinical settings (Table 3). For example, the effect of removal in the non-ICU and ICU environments was an increase of 8% and 6%, respectively, in the susceptible proportion when comparing 365-day NCCLS results to no removal; this comparison resulted in an increase of 2% in the outpatient setting.

In a subanalysis restricted to the 2,749 patients (3,725 isolates) with an associated MRN, results were highly analogous to those observed for the larger cohort as a whole. Specifically, for each clinical setting, the difference in the susceptibility estimate between NCCLS and Cerner methods was insignificant for any given period of analysis, and the difference in susceptibility percentage between a 90- and 365-day time period was <1% with either NCCLS or Cerner. In addition, the outpatient setting continued to show the least effect of duplicate isolate removal when compared to inpatient settings.

Finally, we examined the effect of each deduplication strategy for inpatients (ICU and non-ICU) at major hospitals. Although the hospitals were of different sizes, and their respective rates of MRSA differed by what was seen with no removal, the effects of deduplication observed for each hospital individually were similar to those observed for the population-based surveillance dataset as a whole. Figure 2 illustrates the results of isolate deduplication from 2 of the hospitals with the largest number of *S. aureus* isolates in 2002.

Discussion

To our knowledge, this report is the first to compare the effect of different deduplication strategies on susceptibility patterns derived from a statewide, population-based antimicrobial resistance surveillance system. The relatively large sample of *S. aureus* isolates in this study was obtained from multiple healthcare settings by a variety of clinical laboratories. Because of the diversity of practice patterns represented in the study, we believe our findings are likely to be applicable to other facilities and agencies conducting antimicrobial resistance surveillance for MRSA.

The major findings from this analysis are the following: 1) NCCLS and modified Cerner methods yield similar results for a given analysis period; 2) with both NCCLS and modified Cerner, the number of total isolates included and the percentage that are MRSA decrease slightly as the

This fact also explains why we observed, for both NCCLS and Cerner, an inverse relationship between the length of the analysis period and the rate of MRSA, i.e., the greater number of isolates included with shorter periods of analysis meant more MRSA isolates relative to MSSA strains. If reculturing patient samples is influenced by prior susceptibility testing results, an institution's MRSA percentages might be determined by the practice patterns of physicians working within the institution. Furthermore, trends in antimicrobial resistance could be obscured if practice patterns changed and reculturing samples from MSRA patients became more or less common.

Duplicate isolate removal facilitates comparing data among institutions and monitoring trends over time. However, at present, no clear consensus has been reached on the definition of duplicate isolates, and duplicate isolates cannot be easily identified with certainty in clinical practice (2,4–6). Our study found that NCCLS and modified Cerner methods yield similar results, and for either a 90-day or a 365-day analysis period, the produced estimates fall between results produced by the most resistant and most susceptible methods. Thus, NCCLS and Cerner might both be considered reasonable approaches. However, the NCCLS method has 1 major advantage: NCCLS is the only method that does not require the infection control practitioner to simultaneously compare susceptibility results for multiple isolates obtained from a given patient during the analysis period. With NCCLS, one simply includes the susceptibility results for the first isolate obtained during the analysis period. This straightforward approach would minimize opportunity for error and result in more consistent implementation of the deduplication process (5).

Regardless of which deduplication strategy is selected, the question remains which analysis period to adopt (5,6,11). A longer surveillance period increases the proba-
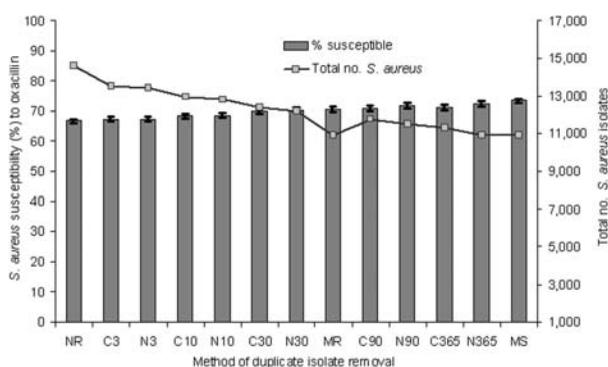

Figure 1. Effect of duplicate isolate removal strategies on the number of *Staphylococcus aureus* isolates and percentage susceptible to oxacillin for all patients in Hawaii, 2002. The 95% confidence interval for the proportion is shown in brackets. NR, no removal; MR, most resistant; MS, most susceptible; N, NCCLS algorithm; C, Cerner algorithm; the number indicates the days in the analysis period.

period of analysis increases; 3) point estimates of the proportion of MRSA produced for a 90- or 365-day analysis period were statistically similar by either NCCLS or Cerner; and 4) the effect of deduplication was greater for inpatient settings compared to outpatient settings.

We also found that no removal produced the highest estimates of MRSA resistance, even higher than most resistant for the same analysis period. While this finding may seem at first paradoxical, it demonstrates the influence that practice patterns may have on reported rates of MRSA and why deduplication is critical. In our setting, cultures of samples from patients with MRSA were obtained more frequently than cultures from those with methicillin-susceptible strains (MSSA) so that, on average, individual patients with MRSA contributed more isolates, which could be included in the estimation of MRSA rates.

Table 3. Effect of duplicate isolate removal on *Staphylococcus aureus* susceptibility to oxacillin, by clinical setting*

	ICU		Non-ICU		Outpatient	
Method	No. isolates	No. susceptible (%, 95% CI)	No. isolates	No. susceptible (%, 95% CI)	No. isolates	No. susceptible (%, 95% CI)
No removal	843	465 (55, 52–59)	3,894	1,971 (51, 49–52)	9,858	7,281 (74, 73–75)
Cerner, 3 d	712	387 (54, 51–58)	3,363	1,705 (51, 49–52)	9,590	7,101 (74, 73–75)
NCCLS, 3 d	708	384 (54, 51–58)	3,328	1,682 (51, 49–52)	9,559	7,078 (74, 73–75)
Cerner, 10 d	629	355 (56, 53–60)	3,090	1,614 (52, 50–54)	9,500	7,045 (74, 73–75)
NCCLS, 10 d	616	352 (57, 53–61)	3,038	1,584 (52, 50–54)	9,461	7,020 (74, 73–75)
Cerner, 30 d	589	345 (59, 55–63)	2,849	1,569 (55, 53–57)	9,280	6,907 (74, 74–75)
NCCLS, 30 d	574	341 (59, 55–63)	2,772	1,534 (55, 53–57)	9,222	6,875 (75, 74–75)
Most resistant	545	317 (58, 54–62)	2,426	1,355 (56, 54–58)	8,427	6,295 (75, 74–76)
Cerner, 90 d	574	339 (59, 55–63)	2,681	1,525 (57, 55–59)	8,905	6,667 (75, 74–76)
NCCLS, 90 d	558	335 (60, 56–64)	2,563	1,480 (58, 56–60)	8,802	6,617 (75, 74–76)
Cerner, 365 d	564	336 (60, 56–64)	2,578	1,485 (58, 56–60)	8,589	6,444 (75, 74–76)
NCCLS, 365 d	545	332 (61, 57–65)	2,426	1,420 (59, 57–60)	8,427	6,368 (76, 75–76)
Most susceptible	545	334 (61, 57–65)	2,426	1,468 (61, 59–62)	8,427	6,433 (76, 75–77)

*ICU, intensive care unit; NCCLS, National Committee for Clinical Laboratory Standards; CI, confidence interval.
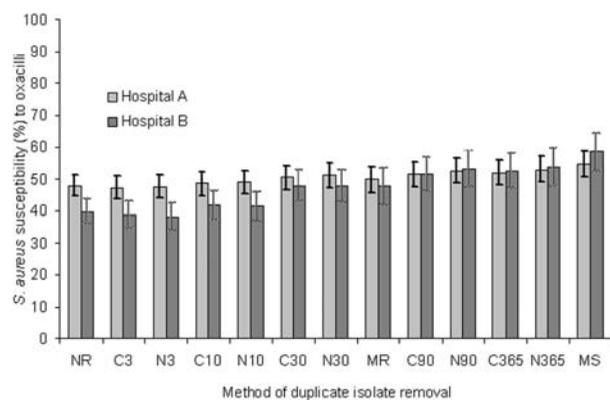
Figure 2. Effect of duplicate isolate removal strategies on the percentage of *S. aureus* isolates susceptible to oxacillin for inpatients at 2 large hospitals in Hawaii, 2002. The 95% confidence interval for the proportion is shown in brackets. NR, no removal; MR, most resistant; MS, most susceptible; N, NCCLS algorithm; C, Cerner algorithm; the number indicates the days in the analysis period.

bility that an isolate representing a truly new resistance event (rather than a duplicate) will be removed (11,12). The purpose of antimicrobial resistance surveillance is to assess temporal trends, evaluate intervention efforts, and ultimately improve clinical outcomes on a population-based level. While the treating clinician will need to consider the susceptibility results for each isolate obtained from the patient, and perhaps promptly change therapy in response to new developments (13,14), population-based recommendations for antimicrobial treatment are not likely to be altered on the basis of 3, 10, or even 30 days of surveillance data. Therefore, adopting either a 90- or 365-day analysis period for MRSA surveillance appears reasonable. At the statewide level in Hawaii, the results seen with the 90- and 365-day NCCLS methods were nearly identical, so either option would be appropriate.

Antibiograms should be individualized for specific clinical areas within an institution (e.g., ICUs) (15,16). In Hawaii, we observed differences in both MRSA rates as well as the magnitude of the effect of deduplication among the ICU, non-ICU, and outpatient settings; outpatient settings had the least effect. The greater effect of deduplication among inpatients may result from both the higher rate of MRSA among hospitalized patients and a greater likelihood of inpatients, especially those with MRSA, to have samples recultured compared to outpatients.

A major limitation of this analysis is that, because of medical confidentiality issues, we did not have patients' names. Since unique identities were determined by using an MRN generated by the treating facility or medical plan, a patient whose sample was cultured in >1 clinical facility during the analysis period might be miscounted as 2

persons. A related concern is that some laboratories did not provide the patient's MRN; for these patients, we had to use other information to generate an APID. While the APID process was not perfect and a small proportion of persons may have been misclassified as nonunique, a sub-analysis that used only records with MRNs produced the same pattern of results as the analysis that used the larger dataset that incorporated the APID. This finding suggests that any misclassifications that resulted from using the APID did not substantially alter the relative effect of the different deduplication strategies we studied.

A second limitation is that we only assessed oxacillin resistance among *S. aureus*, so that conclusions regarding the effect of deduplication on other microorganisms must be made with caution. In addition, our analysis was not stratified by specific anatomic culture site (e.g., blood vs. skin); therefore, the effect of various deduplication strategies on isolates from specific culture sites could not be addressed.

A third limitation is that we did not include deduplication methods that take into account patterns of phenotypic resistance to multiple antimicrobial agents simultaneously (i.e., antibiotypes), as is practiced in some European countries (12). Tracking resistance by antibiotypes may show the actual number of infectious events or the selection of resistance occurring within the surveillance period. While these tasks are important for surveillance in some settings, the main purpose of our study was to evaluate a variety of uncomplicated strategies for generating communitywide susceptibility reports to specifically monitor MRSA trends and guide selection of empiric therapy. Nevertheless, further work is needed to examine the role of antibiotype surveillance in population-based antimicrobial surveillance systems.

We conclude that the NCCLS recommendation of including the first isolate of a given species per patient per analysis period, irrespective of body site, antimicrobial susceptibility profile, or other phenotypic characteristics, yielded results similar to other duplicate isolate removal methods and is straightforward in its implementation. Application of the techniques we examined had the same effect regardless of the institution. To aid our understanding of MRSA in both infection control practice and public health, we urge the widespread adoption of an industry standard. We suggest that adopting the 90- or 365-day NCCLS method would be appropriate, taking into account the goals of surveillance and the resources required.

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Vancomycin and Home Health Care

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The Hospital Infection Control Practices Advisory Committee published guidelines for prudent use of vancomycin to combat increasing resistance to antimicrobial drugs. Studies examining compliance with these guidelines primarily involve hospitalized patients. The growing practice of home use of antimicrobial drugs led to this retrospective cohort study that evaluated parenteral vancomycin use in patients receiving it through a homecare agency. We found that 39.2% of outpatients received vancomycin outside the guidelines, mainly because of prolonged empiric therapy, dosing convenience, and prolonged use after surgery. Patients were more likely to receive vancomycin appropriately if they were >65 years of age, had a history of malignancy, or were discharged from a medical service. In addition, obtaining wound cultures and attempting a microbiologic diagnosis led to more appropriate vancomycin use. Recommendations for prudent vancomycin use are often overlooked when selecting antimicrobial drugs for home infusion. The public health impact of this practice remains unknown.

Vancomycin is an important agent for the treatment of serious infections caused by gram-positive bacteria (1). Over the past 3 decades, its use has steadily increased because of increasing prevalence of β-lactam-resistant nosocomial pathogens, particularly, methicillin-resistant *Staphylococcus aureus* (MRSA) and coagulase-negative staphylococci (CoNS) (2,3). A consequence of this increased use has been the emergence and spread of vancomycin-resistant enterococci, the isolation of *S. aureus* with reduced susceptibility to glycopeptides, and, most recently, reports of infections caused by vancomycin-resistant *S. aureus* (4–6).

Antimicrobial stewardship guidelines have been developed to ensure that vancomycin is used appropriately and retains its viability in the therapeutic armamentarium. The most broadly accepted benchmark was published by the Hospital Infection Control Practices Advisory Committee

(HICPAC) of the Centers for Disease Control and Prevention (7). These guidelines and most efforts to control use of antimicrobial drugs target the hospital setting (8–12). However, the prevalence of drug-resistant pathogens in outpatient and ambulatory settings is increasing, as demonstrated by the prevalence of penicillin-resistant pneumococci and recent emergence of community-onset MRSA (13,14). With an increasing number of patients receiving home infusions of antimicrobial drugs, the appropriateness of choices of drugs for outpatients warrants scrutiny. Guidelines for the administration of outpatient parenteral antibiotic therapy (OPAT) noted this and encouraged adherence to HICPAC guidelines (15).

We conducted a retrospective cohort study of patients discharged from an academic medical center to complete a course of intravenous vancomycin at home. The main objectives were to describe the epidemiology of outpatients receiving vancomycin through a home healthcare agency, determine the appropriateness of outpatient vancomycin prescriptions according to HICPAC guidelines, and examine factors associated with outpatient vancomycin use that conformed to HICPAC guidelines.

Methods

Study Setting and Patient Population

Northwestern Memorial Hospital (NMH) is a 725-bed teaching hospital in Chicago, Illinois. Northwestern Memorial Home Health Care, Inc. (NMHHC), the home healthcare agency affiliated with NMH, receives >200 annual referrals for home infusion of antimicrobial agents.

This study included all inpatients at NMH referred to NMHHC to complete a course of intravenous vancomycin therapy from December 1997 to April 2002. Patients were excluded if they were <16 years of age, admitted to the hospital already receiving vancomycin, discharged to any other facility, or received care from another homecare agency before referral to NMHHC. For patients with

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multiple referrals to NMHHC for vancomycin therapy, only their first treatment episode was included. During this study, although vancomycin use guidelines were published and distributed within NMH, no formal enforcement policy existed within the hospital or homecare setting. The institutional review board of Northwestern University reviewed and approved the study protocol.

Clinical Data

All data were originally collected as part of routine patient care. For this study, clinical data were abstracted retrospectively by review of existing inpatient medical records, home health referral forms, and the inpatient pharmacy database. The data abstractor had no part in the original data collection. The following data were abstracted: demographic information, length of hospital stay, admitting service, insurance status, allergy to β-lactam antimicrobial drugs, level of serum creatinine on the day of discharge, history of end-stage renal disease requiring dialysis, infectious diseases consultation, use of vancomycin in the hospital, reason(s) for vancomycin use, and discharge diagnoses per ICD-9 codes. ICD-9 codes were used to calculate a mean Charlson comorbidity score for each patient (16,17). With 1 exception, the presence of infectious syndromes was determined by review of ICD-9 diagnoses. A diagnosis of bloodstream infection was assigned if multiple positive blood cultures were documented, regardless of coded diagnoses. Because of the retrospective nature of the evaluation, all recorded allergies to β-lactam antimicrobial drugs were considered potentially serious.

Microbiologic Data

The microbiology records spanning the length of the hospitalization for each patient were reviewed. A microbiologic evaluation occurred if cultures were obtained that reasonably corresponded to the infectious diagnosis requiring the use of vancomycin. Record review focused on collection of cultures from blood, other sterile sites, urine, sputum, intravenous catheters or other foreign bodies, and wounds or tissues. Bacterial isolates that were specifically recorded were gram-positive organisms whose treatment might prompt or warrant the use of vancomycin, including methicillin-susceptible *S. aureus*, MRSA, CoNS, streptococci, ampicillin-resistant or -susceptible enterococci, and *Corynebacterium jeikeium*.

Evaluation of Vancomycin Use

HICPAC guidelines served as the basis for determining whether patients received parenteral vancomycin per guidelines or outside guidelines (Table 1). The guidelines pertaining to prophylaxis for endocarditis (1C), surgical procedures (1D and 2A), and low-birthweight infants (2G) did not apply and were disregarded.

In addition, vancomycin use was determined to fall outside HICPAC guidelines for the following situations: 1) treatment of CoNS from superficial wound swabs, or respiratory or urine specimens unless they occurred in the setting of bacteremia; 2) dosing convenience defined as initial treatment with a β-lactam antimicrobial drug during hospitalization with a therapeutic change to vancomycin within 24 h of discharge that was not dictated by culture results or

Table 1. HICPAC guidelines for prudent use of parenteral vancomycin*

1) Situations in which use of vancomycin is appropriate

    A) Treatment of serious infections caused by β-lactam–resistant, gram-positive organisms

    B) Treatment of infections caused by gram-positive microorganisms in patients with serious allergies to β-lactam antimicrobial agents

    C) Prophylaxis, as recommended by the American Heart Association, after certain procedures in patients at high risk for endocarditis

    D) Prophylaxis for major surgical procedures involving implantation of prosthetic materials or devices at institutions that have a high rate of infections caused by MRSA or methicillin-resistant *Staphylococcus epidermidis*

2) Situations in which use of vancomycin should be discouraged

    A) Routine surgical prophylaxis, unless patient has life-threatening allergy to β-lactam antimicrobial drugs

    B) Empiric antimicrobial therapy for febrile neutropenic patient, unless evidence indicates patient has infection caused by gram-positive microorganisms and prevalence of MRSA infections in hospital is substantial

    C) Treatment in response to single blood culture positive for coagulase-negative staphylococci, if other blood cultures taken during same timeframe are negative

    D) Continued empiric use for presumed infections in patients whose cultures are negative for β-lactam-resistant gram-positive microorganisms

    E) Systemic or local (e.g., antimicrobial drug lock therapy)† prophylaxis for infection or colonization of intravascular catheters

    F) Eradication of MRSA colonization

    G) Routine prophylaxis for very-low-birthweight infants

    H) Routine prophylaxis for dialysis patients

    I) Treatment (chosen for dosing convenience) of infections caused by β-lactam–sensitive, gram-positive microorganisms in patients with renal failure

*Summarized from reference 7. HICPAC, Hospital Infection Control Practices Advisory Committee; MRSA, methicillin-resistant *Staphylococcus aureus*.
†Instilling a high concentration of antimicrobial drug to which organism is susceptible into lumen of catheter in attempt to sterilize it.

allergy; 3) prolonged administration of an antimicrobial agent after implantation of prosthetic materials; 4) treatment of cellulitis without identification of a β-lactam-resistant pathogen (additionally, the empiric switch to vancomycin because of slow resolution of cellulitis was considered noncompliant use); and 5) ongoing treatment of infection in a patient with a history of MRSA colonization in the absence of a diagnostic culture. If the use of vancomycin met >1 of these specified criteria, each was included in data collection.

Statistical Analysis

Data were collected on a standardized form and entered onto spreadsheets (Excel 2000, Microsoft Corporation, Redmond, WA, USA). To evaluate predictors for compliance with vancomycin use guidelines, discrete variables were described by percentages and compared by using chi-square or Fisher exact tests as appropriate. Continuous variables were described by means and evaluated by using Student t test. Variables with a p value <0.05 by univariate analysis were evaluated by stepwise logistic regression for inclusion in the final model. SAS version 8.2 for personal computers (SAS Institute Inc., Cary, NC, USA) was used for statistical analysis.

Results

During the study period, NMHHC received 323 patient referrals for continuation of parenteral vancomycin therapy after hospitalization. The records of 27 patients (8.4%) could not be located and were not included in the study. Thus, the final analysis included 296 patients. Table 2 summarizes the criteria that determined whether vancomycin was prescribed within HICPAC guidelines. One hundred eighty patients (60.8%), 5 of whom met >1 criteria for appropriate use, received vancomycin within guidelines. A total of 118 (65.6%) were treated for infections caused by β-lactam-resistant, gram-positive bacteria. Sixty-seven patients (37.2%) received vancomycin for a reported allergic reaction to β-lactam antimicrobial drugs. Although only the first referral for home vancomycin was analyzed for each patient, 44 (14.9%) were referred multiple times to receive van-

comycin as outpatients (2–8 referrals per patient) during this study.

Of the 296 patients, 116 (39.2%), 8 of whom met >1 criteria, received vancomycin outside HICPAC guidelines. Eighty-four (72.4%) cases were for continued empiric treatment of presumed infections in patients whose cultures were negative or not obtained. This practice was prevalent across all services. Dosing convenience led to the use of the drug in 18 (15.5%) patients, 12 (67%) of whom were admitted to a medical service. In 13 patients (11.2%), home-infusion vancomycin was continued after major surgical procedures involving implanted devices. This practice occurred exclusively in orthopedic and neurosurgery services. Finally, in 9 patients (7.8%), vancomycin was used to treat infection with a CoNS isolate from a single blood culture.

Demographic and clinical characteristics are shown in Table 3. Patients whose use of vancomycin followed guidelines were older than those whose use did not follow guidelines (mean age 53.6 years vs. 48.9 years, p = 0.016). No significant differences were noted in sex or ethnicity, although African-Americans showed a trend toward receiving vancomycin within guidelines (p = 0.054). Appropriate vancomycin use was more likely after a longer mean hospital stay (12.2 days vs. 9.5 days, p = 0.007). No significant differences were noted in the mean Charlson comorbidity score or frequency of diagnosed coexisting medical conditions between the 2 groups with the exception of a history of malignancy (21.7% vs. 10.3%, p = 0.012) among patients who received vancomycin according to guidelines. Insurance status did not differ between groups.

Compliance with HICPAC guidelines varied according to the inpatient prescribing service. Appropriate prescriptions for vancomycin were more likely to be preceded by discharge from a medical service (60.0% vs. 37.9%, p<0.001). This finding was true both for discharges from general medicine and medical subspecialty services, with the exception of hematology/oncology. More episodes of vancomycin infusion outside guidelines followed discharge from a surgical service (59.5% vs. 37.2%, p<0.001), namely, orthopedic and neurosurgery services (35.3% vs. 17.2%,

Table 2. Comparison of HICPAC guidelines with home infusion use of vancomycin in 296 patients*	
	No. (%)
Manner in which vancomycin use met guidelines	180 (60.8)
Treatment of infections with β-lactam–resistant, gram-positive bacteria	118 (65.6)
Treatment of gram-positive infections in patients with allergies to β-lactam agents	67 (37.2)
Manner in which vancomycin use did not meet guidelines	116 (39.2)
Continued empiric vancomycin use in patients with negative or no cultures	84 (72.4)
Use of vancomycin for dosing convenience	18 (15.5)
Prolonged administration of antimicrobial drugs after implantation of prosthetic materials or devices	13 (11.2)
Treatment of a single blood culture showing coagulase-negative staphylococci	9 (7.8)

*Some patients fulfilled >1 criteria. HICPAC, Hospital Infection Control Practices Advisory Committee.

Table 3. Demographic and clinical characteristics of 296 patients referred for home infusions of vancomycin from December 1997 through May 2002*

Characteristic	Use per guidelines, N = 180	Use outside guidelines, N = 116	p value†
Mean age, years (range)	53.6 (19–90)	48.9 (19–86)	0.016
Male, no. (%)	109 (60.6)	76 (65.5)	0.389
Ethnicity, no. (%)			
Caucasian	100 (55.6)	75 (64.7)	0.120
African-American	52 (28.9)	22 (19.0)	0.054
Asian	3 (1.7)	0	0.283
Hispanic	7 (3.9)	2 (1.7)	0.490
Other	18 (10.0)	15 (12.9)	0.434
Mean length of stay, days (range)	12.2 (2–52)	9.5 (2–67)	0.007
Coexisting conditions, no. (%)			
Diabetes mellitus	47 (26.1)	28 (24.1)	0.703
Malignancy	39 (21.7)	12 (10.3)	0.012
Spinal cord injury	28 (15.6)	13 (11.2)	0.290
Decubitus ulcer	31 (17.2)	11 (9.5)	0.063
Acute renal failure	15 (8.3)	6 (5.2)	0.301
ESRD	5 (2.8)	1 (0.9)	0.409
Immunocompromised status	10 (5.6)	8 (6.9)	0.637
HIV	3 (1.7)	1 (0.9)	1.000
Charlson score, mean (range)	1.4 (0–8)	1.1 (0–14)	0.217
Insurance status, no. (%)	176 (97.8)	116 (100)	0.158
Private	121 (67.2)	86 (74.1)	0.205
Medicare	74 (41.1)	36 (31.0)	0.080
Medicaid	48 (26.7)	25 (21.6)	0.319
Discharging service, no. (%)			
Medical	108 (60.0)	44 (37.9)	<0.001
General medicine	66 (36.7)	29 (25.0)	0.036
Medicine subspecialties	29 (16.1)	10 (8.6)	0.063
Hematology/oncology	13 (7.2)	5 (4.3)	0.306
Surgical	67 (37.2)	69 (59.5)	<0.001
General surgery	2 (1.1)	4 (3.5)	0.215
Transplant surgery	5 (2.8)	6 (5.2)	0.350
Vascular surgery	14 (7.8)	7 (6.0)	0.569
Orthopedics/neurosurgery	31 (17.2)	41 (35.3)	<0.001
Other surgical subspecialties	15 (8.3)	11 (9.5)	0.733
Other hospital services	5 (2.8)	3 (2.6)	1.000
Consultation by infectious diseases, no. (%)	85 (47.2)	58 (50.0)	0.641

*ESRD, end-stage renal disease requiring dialysis; immunocompromised status, immunocompromised from causes other than HIV.
†Values <0.05 were considered significant.

p<0.001). Inpatient consultation by an infectious diseases specialist did not affect the appropriateness of home vancomycin prescriptions by managing services (p = 0.641).

The infection diagnoses of patients referred for home infusions of vancomycin are outlined in Table 4. Patients were more likely to receive vancomycin per guidelines in the setting of bloodstream (33.9% vs. 13.8%, p<0.001) and urinary tract infections (20% vs. 11.2%, p = 0.042). The microbiologic investigations undertaken and the organisms identified during hospitalization are delineated in Table 5. Appropriate use of vancomycin was more likely to follow an attempt to make a microbiologic diagnosis (96.1% vs. 77.6%, p<0.001). More blood, urine, and wound cultures were obtained in this group, and the number of cultures obtained was higher when vancomycin was used appropriately.

Results of the multivariate analysis are shown in Table 6. Patients <65 years of age were less likely to receive appropriate vancomycin (odds ratio [OR] 0.50, 95% confidence interval [CI] 0.26–0.94). Appropriate use of vancomycin was more likely to occur after discharge from a medical service rather than a surgical service (OR 2.62, 95% CI 1.53–4.48). Although discharge from a hematology/oncology service was not associated with appropriate use of vancomycin, patients with a history of malignancy were more likely to receive vancomycin within HICPAC guidelines (OR 3.02, 95% CI 1.40–6.53). Obtaining a wound culture was associated with appropriate use of vancomycin (OR 2.08, 95% CI 1.19–3.64). Finally, patients who underwent any microbiologic evaluation were more likely to receive appropriate vancomycin through home care (OR 5.93, 95% CI 2.26–15.54).

Table 4. Infection diagnoses in patients referred for home infusions of vancomycin

Diagnosis*	Use per guidelines, no. (%), N = 180	Use outside guidelines, no. (%), N = 116	p value†
Skin or soft tissue infection	89 (49.4)	51 (44.0)	0.356
Osteomyelitis or septic arthritis	30 (16.7)	25 (21.6)	0.294
Postoperative wound infection	38 (21.1)	19 (16.4)	0.310
Orthopedic device-related infection	5 (2.8)	7 (6.0)	0.227
Central nervous system infection‡	3 (1.7)	6 (5.2)	0.161
Urinary tract infection	36 (20.0)	13 (11.2)	0.042
Pneumonia	9 (5.0)	1 (0.9)	0.095
Bloodstream infection	61 (33.9)	16 (13.8)	<0.001
Vascular device infection	21 (11.7)	10 (8.6)	0.398
Infective endocarditis	8 (4.4)	5 (4.3)	0.956

*Some patients had >1 diagnosis.
†Values <0.05 were considered significant.
‡Includes shunt infections.

Discussion

This study examined a large group of patients referred for home infusions of vancomycin over a 5-year period and applied established guidelines to determine if outpatient use conformed to a widely accepted benchmark. A total of 39.2% of the prescriptions were given outside guidelines. Several authors have applied these HICPAC guidelines to evaluate inpatient use of vancomycin and found the incidence of outside guidelines use to range from 36% to 79% (11,12,18,19). Our study, however, is the first to critically evaluate the appropriateness of vancomycin in the outpatient setting.

The most common reason for outside guidelines use of vancomycin was continuation of empiric therapy in patients without a culture-defining indication. Singer et al. have found similar results in hospitalized patients (12). In contrast, other studies found that the most common reasons for inappropriate inpatient prescriptions for van-

comycin were surgical prophylaxis and failure to modify prescriptions for antimicrobial drugs based on culture results (19,20).

We found that the other reasons for vancomycin use outside guidelines were dosing convenience, prolonged use after surgical procedures, and treatment of CoNS isolated from a single blood culture. Use for dosing convenience is likely underestimated (15.5%) because of the conservative definition used in this retrospective analysis. The incidence of vancomycin use for prolonged periods after implantation of devices and for the treatment of CoNS from a single blood culture was less than the incidence among inpatients (12,19). This incidence may reflect that continuing vancomycin for these indications is more convenient in the inpatient setting and that physicians are likely to reevaluate the true need for outpatient vancomycin in these circumstances.

Table 5. Microbiologic investigations and results for home infusions of vancomycin*

Investigation or result	Use per guidelines, no. (%), N = 180	Use outside guidelines, no. (%), N = 116	p value†
Microbiologic diagnostic attempt	173 (96.1)	90 (77.6)	<0.001
Cultures by site			
Blood	137 (76.1)	74 (63.8)	0.022
Sterile site	25 (13.9)	11 (9.5)	0.258
Urine	96 (53.3)	48 (41.4)	0.045
Sputum	17 (9.4)	6 (5.2)	0.180
Wound	96 (53.3)	45 (38.8)	0.015
Other culture	15 (8.3)	11 (9.5)	0.733
>1 culture	136 (75.6)	65 (56.0)	<0.001
Bacterial isolates			
MRSA	81 (45.0)	2 (1.7)‡	§
Coagulase-negative staphylococci	59 (32.8)	20 (17.2)	
Ampicillin-resistant enterococci	3 (1.7)	0	
Methicillin-susceptible S. aureus	16 (8.9)	19 (16.4)	
Other streptococci and enterococci	52 (28.9)	18 (15.5)	
Corynebacterium jeikeium	2 (1.1)	0	
Culture considered contaminated	20 (11.1)	20 (17.2)	

*MRSA, methicillin-resistant Staphylococcus aureus.
†Values <0.05 were considered significant.
‡The 2 patients outside the guidelines with an MRSA culture included 1 patient with MRSA in the urine but no diagnosis of a urinary tract infection, and 1 with a positive intravascular catheter tip culture but no evidence of infection.
§A p value is not included because infection with these isolates was 1 factor used to determine whether vancomycin was given per guidelines.

Table 6. Factors associated with appropriate use of vancomycin by multivariate analysis using stepwise logistic regression analysis

Variable	Odds ratio (95% CI)*	p value
Attempt at a microbiologic diagnosis	5.93 (2.26–15.54)	0.0003
Discharge from a medical service	2.62 (1.53–4.48)	0.0004
History of a malignancy	3.02 (1.40–6.53)	0.0050
Obtaining a wound culture	2.08 (1.19–3.64)	0.0107
Age <65	0.50 (0.26–.094)	0.0321
*CI, confidence interval.		

Examined data showing the prescribing patterns of physicians demonstrate that patients discharged from a medical service are more likely to receive vancomycin appropriately. Of surgical subspecialists, orthopedic and neurosurgeons were more likely to prescribe vancomycin outside guidelines. These prescribing differences are consistent with the findings of inpatient vancomycin use evaluations (21–24). Although patients with a history of malignancy received vancomycin according to HICPAC guidelines, hematology/oncology was the only medical service not associated with appropriate use. These results suggest that the vancomycin-prescribing practices of certain subspecialists offer the opportunity for education regarding the existence of and rationale for such guidelines and targeted intervention to reduce unnecessary outpatient vancomycin usage (25). Only 6 patients with end-stage renal disease received vancomycin through homecare. Intuitively, one might expect more vancomycin use in this patient population; however, this finding probably reflects that these patients receive vancomycin during hemodialysis and, thus, do not require referral to home health. In contrast to other studies, consultation by infectious diseases physicians did not impact compliance (26–28). This finding warrants further examination to determine if infectious diseases physicians recommend vancomycin for use outside of HICPAC guidelines or if their recommendations are disregarded.

If a microbiologic evaluation was attempted, vancomycin use was more likely to follow guidelines. Obtaining wound cultures was also associated with appropriate use. A thorough microbiologic evaluation aids in clinical decision making. When clinicians have culture and susceptibility results, they are more likely to use vancomycin appropriately, particularly for patients with skin and soft tissue infections.

Patients >65 years of age were more likely to receive vancomycin per guidelines. The reasons for this are unclear but were not impacted by insurance status. This finding probably reflects that patients referred for intravenous antimicrobial drugs through homecare either have insurance that will reimburse for the service or have the ability to pay for the drugs.

This study had several limitations because of its retrospective nature. A substantial number of patients were classified in the compliant group on the basis of a reported allergy to β-lactam drugs. Because we were unable to determine the nature of reported allergies to penicillin, all allergies were assumed to be serious in nature. Thus, this study overestimates appropriate vancomycin use for this purpose. Another limitation of this analysis is the inability to account for the impact of vancomycin courses patients may have received before this study. Finally, this study does not address the financial consideration that influenced the choice of antimicrobial drug. Other investigators have explored this issue and found that the costs of outpatient vancomycin therapy are substantial (29). The patients in this study were preselected to the extent that they were able to receive vancomycin at home.

HICPAC guidelines were developed to promote judicious use of vancomycin in an attempt to curtail the spread of vancomycin-resistant enterococci and forestall the development of S. aureus with reduced susceptibility to glycopeptides. Although these guidelines were initially applied to the inpatient setting, the OPAT guidelines have recommended that they also apply to outpatients receiving vancomycin. Apart from vancomycin, however, the OPAT guidelines lack information regarding choices of antimicrobial drugs for outpatients. In addition, they do not clearly prioritize conscientious use of antimicrobial drugs above other considerations, such as cost and dosing convenience, when choosing outpatient therapy. These issues need to be addressed as the emergence and spread of antimicrobial-resistant gram-positive pathogens in the community continue to increase.

One in 1,000 patients in the United States is estimated to receive outpatient infusion of antimicrobial drugs each year (15). The trend toward increased inpatient acuity and shorter hospital stay will undoubtedly increase this practice. Our study on first-time referrals from 1 tertiary care hospital to its homecare agency represents only a subset of vancomycin use in the community. The propensity for readmissions and repeated referrals of these chronically ill patients must be considered when analyzing the impact of outpatient vancomycin use. In addition, vancomycin administered by other homecare agencies, extended care facilities, outpatient infusion centers, and outpatient dialysis centers all contribute to its burgeoning use outside the hospital. Our study indicates that further investigations into the consequences of this practice on individual persons and the community are warranted. Do the favorable pharmacokinetics and economic attributes of vancomycin that make it attractive for home infusion outweigh the potential consequences of unnecessarily broad-spectrum gram-positive coverage? Further studies are needed to address these issues if we are to understand the dynamics

of resistant pathogens in the community and the overall emergence and spread of antimicrobial resistance.

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Antibacterial Cleaning Products and Drug Resistance

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We examined whether household use of antibacterial cleaning and hygiene products is an emerging risk factor for carriage of antimicrobial drug–resistant bacteria on hands of household members. Households (N = 224) were randomized to use of antibacterial or nonantibacterial cleaning and hygiene products for 1 year. Logistic regression was used to assess the influence of antibacterial product use in homes. Antibacterial product use did not lead to a significant increase in antimicrobial drug resistance after 1 year (odds ratio 1.33, 95% confidence interval 0.74–2.41), nor did it have an effect on bacterial susceptibility to triclosan. However, more extensive and longer term use of triclosan might provide a suitable environment for emergence of resistant species. Further research on this issue is needed.

Concern is growing over the use of household cleaning and hygiene products labeled as antibacterial as a result of laboratory data showing a link between exposure to ingredients in these products, particularly triclosan, and emergence of antimicrobial drug resistance (1–3). This study aimed to determine whether home use of antibacterial cleaning and hygiene products (including use of a handwashing soap containing 0.2% triclosan) or other potential risk factors was associated with carriage of antimicrobial drug–resistant bacteria on household members' hands. We also assessed the association of these antibacterial products with carriage of organisms with reduced susceptibility to triclosan.

Materials and Methods

Study Population

The data for this study were collected as part of a double-masked and randomized home intervention trial (4); participant enrollment began in October 2000, and follow-

up occurred for a 12-month period. The methods and randomization procedures for this study have been reported elsewhere (5). A total of 238 households were recruited at baseline; 224 households completed the entire 1-year follow-up (Figure 1). The study was approved by Columbia University Medical Center Institutional Review Board.

Intervention Methods

Households were supplied with over-the counter, generically repackaged consumer cleaning and personal hygiene products free of charge on a monthly or as-needed basis. Households randomly assigned to use antibacterial products received the following: 1) liquid handwashing soap containing 0.2% triclosan, 2) liquid kitchen spray and liquid all-purpose cleaner for hard surfaces that contained a quaternary ammonium component, and 3) oxygenated bleach laundry detergent. Households randomly assigned to the nonantibacterial group received the same products but without antibacterial ingredients. Both groups received the same nonantibacterial liquid dishwashing detergent and bars of body soap to control for potential use of other products that might contain antibacterial ingredients. Study participants were required to use only assigned home hygiene products and were asked not to change any of their normal hygiene practices. Participants, interviewers, and study coordinators were blinded to brand names and ingredients in all products. Adherence to product treatment group was assessed monthly, and products were weighed during each visit to monitor compliance. Households were immediately dropped from the study if they did not adhere to randomized treatments.

Data Collection

At baseline, and quarterly during the 1-year period, a trained interviewer collected demographic information from the person self-identified as the primary caregiver in the household. The baseline interview determined the type of handwashing soap, hygiene, and cleaning products that were used before randomization into the study (i.e., the

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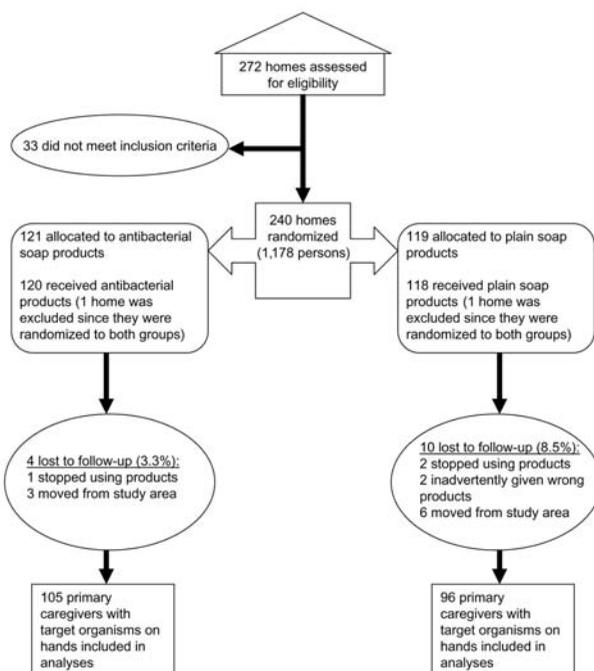
Figure 1. Flow chart for randomized trial. After randomization and loss to follow-up, the remaining study participants who carried target organisms were included in the logistic regression analyses.

brand and whether or not the ingredients were labeled as antibacterial). The baseline and quarterly assessment forms provided information such as the number and age of household members, childcare attendance, symptoms of infectious illnesses (fever, diarrhea, sore throat, vomiting, conjunctivitis, skin boils, runny nose), antimicrobial drug use, chronic diseases, self-rated health, birthplace, travel outside of the United States, and occupation. In addition, reported number of handwashes per day by the primary caregiver and a timed observation of the handwash before hand culturing were gathered.

The hands of the primary caregiver were cultured during the home visit at baseline and at the end of the 12-month period before and after washing with the assigned liquid handwashing product. The trained data collector used a coin flip to choose the test hand, which was then inserted into a sterile polyethylene bag containing 50 mL culture medium (0.075 mol/L phosphate buffer, pH 7.9, containing 0.1% polysorbate 80). The hand was massaged for 1 min through the wall of the bag containing culture medium. Only postwash samples were used in analyses since they were considered to be representative of normal versus transient flora found on hands.

Laboratory Methods

The laboratory methods for this study have been described previously (5,6). The microbiologic analysis and antimicrobial drug–susceptibility testing were conducted at New York Presbyterian Hospital, Columbia University Medical Center, New York. Selective media were used to isolate gram-positive cocci, gram-negative bacteria (GNB), *Staphylococcus aureus*, and enterococci.

Only clinically important bacterial species that were prevalent (species with ≥38 isolates recovered at baseline and end of year combined) on the hands of homemakers were selected for susceptibility analyses (7,8). These included the following GNB: *Acinetobacter baumannii*, *A. lwoffi*, *Enterobacter agglomerans*, *E. cloacae*, *Klebsiella pneumoniae*, and *Pseudomonas fluorescens/putida*; and the following gram-positive staphylococci: *S. aureus*, *S. warneri*, *S. epidermidis*, and *S. capitis*. Therefore, only persons who were carrying at least 1 of these organisms on their hands were included in the final analyses (N = 164 at baseline and N = 201 at year-end). No significant differences were noted between the measured demographic characteristics (Tables 1 and 2 for listing of demographics) among those included in the final analyses versus those excluded (all p>0.10).

Bacterial isolates were tested against a panel of antimicrobial agents by using MicroScan WalkAway 96 SI (Dade Behring, Deerfield, IL, USA). Using the recommendations of the Clinical and Laboratory Standards Institute (formerly NCCLS), we classified antimicrobial drug susceptibility as resistant, intermediate, or susceptible to a particular antimicrobial agent (9). Organisms that tested as either resistant or intermediately resistant to antimicrobial agents were classified as "antibiotic resistant" (10). The selection of antimicrobial agents to be tested for each organism was based on clinical applicability of the antimicrobial drug and consistency with earlier studies that examined a link between triclosan and antimicrobial drug resistance (11–14). GNB were tested against several antimicrobial agents, and staphylococci were tested against oxacillin to indicate methicillin resistance. For analytic purposes, GNB species were classified as resistant if a given isolate was resistant to ≥1 antimicrobial agent(s).

Triclosan susceptibility was examined at Tufts University School of Medicine, Boston, Massachusetts, by using a modified NCCLS agar dilution method (10). Minimum inhibitory concentration (MIC) was defined as the lowest dilution of triclosan that inhibited visible growth. A detailed description of antimicrobial drug and triclosan testing, including controls used and MIC distribution for each organism, has been described previously (6). Since data from the literature regarding triclosan susceptibility testing are sparse and provide no standardized breakpoints (6), we dichotomized triclosan MIC values for each isolate by using the median MIC as a cutoff; low MIC represents less than or equal to the median value and high MIC indicates greater than the median value.

Table 1. Proportion of all study participants with baseline characteristics

Characteristics*	Nonantibacterial groups† (N = 118), %	Antibacterial groups† (N = 120), %
Primary caregiver		
Male primary caregivers	4.2	4.2
Caregivers born outside of United States	94.1	98.3
Caregivers with high CFU counts on hands‡	35.8	39.4
Household		
Antibacterial cleaning and hygiene products used prebaseline	41.5	40.0
Characteristics reported for ≥1 members of the household		
Child in daycare	15.9	17.8
Chronic illness	39.0	37.0
Chronic illness or fair to poor health	61.0	55.8
Symptoms of infection in past 30 days	54.2	54.2
Use of antimicrobial agents in past 30 days§	11.9	11.7
Traveled outside United States in past month	12.8	12.5
Healthcare or daycare occupation	41.0	45.0

*No significant differences in demographic characteristics between persons with or without available cultures or between participants with or without gram-negative bacteria or staphylococci of interest were noted in this study (all p≥0.10).
†No significant differences between the antibacterial and nonantibacterial users in any of the characteristics measured were noted (all p>0.05).
‡Culture information was not available at baseline for 20 study participants. High counts were determined by whether the participant had a CFU above the mean for the entire group.
§Information on use of antimicrobial agents use was only gathered from study participants reporting infectious symptoms. Therefore, all persons reporting no infectious symptoms were coded as having "no reported antibiotic use."

Analytic Methods

First, chi-square and Student *t* tests were used to compare demographic characteristics of antibacterial and nonantibacterial users. Next, chi-square tests were used to compare the overall proportion of antimicrobial drug–resistant isolates found on the hands of the antibacterial and nonantibacterial groups. Finally, multivariate logistic regression analyses were conducted to examine the relationship between antibacterial product use and 2 separate outcome variables: antimicrobial drug resistance (measured by the presence of ≥1 antimicrobial drug–resistant species on the hand) and increased triclosan MICs (measured by the presence of ≥1 species exhibiting a triclosan MIC above the median value).

Each potential covariate (i.e., characteristics of the household and primary caregiver) and our 2 outcome variables were examined in univariate analyses to establish criteria for inclusion in final multivariate models by using a p value <0.05 as the cutoff. Covariates meeting the cutoff criteria were included in multivariate models along with the main effect of the randomized treatment (i.e., antibacterial versus nonantibacterial product use). Analyses were conducted separately for baseline and after 1 year of study participation. Unadjusted and adjusted odds ratios (OR) and 95% confidence intervals (CIs) were generated from logistic regression analyses by using SPSS V.10 (SPSS Inc., Chicago, IL, USA).

Results

GNB and staphylococci were recovered from 164 participants at baseline and 201 participants at year-end. None of the measured demographic and hygiene characteristics differed significantly between the randomized groups (all p≥0.10) (Tables 1 and 2). When comparing isolates from

Table 2. Mean values for baseline or year-end characteristics of study participants

Characteristic	Nonantibacterial group* (N = 118)		Antibacterial group* (N = 120)	
	Mean	SD	Mean	SD
Primary caregiver				
Age (y) of primary caregiver (baseline)	34.6	10.0	33	8.1
No. of daily washes (reported)				
Baseline	13.3	9.8	11.6	7.1
End of year	11.6	6.3	10.3	5.1
Length(s) of handwash (observed)				
Baseline	15.5	9.4	16.4	9.7
End of year	18.7	8.3	18.5	8.3
Household				
Age (y) of all household members combined (baseline)	20.1	4.9	20.0	5.9
No. of children ≤5 y in home (baseline)	1.5	0.6	1.5	0.7
No. of persons in household (baseline)	5.0	1.5	5.0	1.8

*No significant differences were observed between the antibacterial and nonantibacterial product users in any of the characteristics measured (all p>0.05).

the antibacterial users and nonantibacterial users (Figure 2 and online Appendix Figure, available from http://www.cdc.gov/ncidod/EID/vol11no10/04-1276_app.htm), no significant differences in the proportions of resistance were found in all species combined or within single species (all p>0.05).

The odds of carrying ≥1 antimicrobial drug–resistant strain(s) among antibacterial product users and nonusers were not significant at baseline (OR 0.97, 95% CI 0.50–1.89) or after 1 year of antibacterial product use (OR 1.33, 95% CI 0.74–2.41) (Table 3). In addition, the odds of carrying ≥1 organism with high triclosan MIC among antibacterial product users or nonusers were similar at baseline (OR 1.59, 95% CI 0.84–3.01) and at year-end (OR 1.73, 95% CI 0.97–3.09).

Individual and Household Characteristics and Susceptibility

At baseline, primary caregivers with higher than average CFU on their hands were twice as likely to carry antimicrobial drug–resistant organisms (Table 3). A slightly increased risk of carrying antimicrobial drug–resistant organisms occurred among those who washed their hands for a longer duration before the culture sample at baseline (Table 3). However, longer duration of handwashing was not associated with reduced bacterial CFU on hands (OR 1.02, 95% CI 0.99–1.06).

At year-end, both the number of times hands were washed per day and the presence of any household member(s) with a healthcare or daycare occupation were significantly associated with reduced carriage of antimicrobial drug–resistant organisms on hands of the primary caregiver (Table 3). Primary caregivers residing in households with members working in healthcare or daycare were significantly more likely to report above-average number of handwashes per day (OR 3.05, 95% CI 1.71–5.44). None of the other characteristics, such as health conditions or antimicrobial drug use, were significantly associated with carriage at baseline or after 1 year (all p>0.05).

Discussion

This study is the first randomized intervention study to investigate the relationship between antibacterial cleaning and hygiene product use and antimicrobial drug susceptibility of hand microflora within the community setting. Our earlier research, conducted among the same study population described here, showed that use of antibacterial hand soap containing 0.2% triclosan was no more beneficial than plain soap in reducing infectious illness symptoms or bacterial counts on hands of household members (4,5,15). Several avenues of research have contributed to the view that use of products containing triclosan may foster the emergence of antimicrobial drug– or biocide-

resistant organisms. This concern stems from reports that exposure to triclosan can lead to bacterial target mutations conferring cross-resistance to isoniazid and selects for mutants bearing resistance to various antimicrobial agents through expression of multidrug-resistant efflux pumps (12,16). Our findings suggest that household use of antibacterial cleaning and hygiene products for a 1-year period is not a significant risk factor for increasing antimicrobial drug–resistant organisms on the hands of persons in the home.

Few data compare resistance patterns among hand microflora and susceptibility to antibacterial handwashing ingredients. One recent cross-sectional study (17) reported a higher prevalence of decreased susceptibility to triclosan among methicillin-resistant *S. epidermidis* compared to methicillin-sensitive *S. epidermidis* clinical isolates. The findings reported in other cross-sectional studies have mainly examined environmental and clinical isolates of bacteria, and the correlations reported have been inconsistent (11,13,18–20).

Other Factors Associated with Antimicrobial Drug Resistance

Several hygiene-related factors were significantly associated with carriage, regardless of antibacterial product use. Longer handwashes were slightly associated with increased risk for carriage of antimicrobial drug–resistant species at baseline; as reported previously, these findings may be an artifact of sampling technique (5). The culture was taken directly after the handwash; an increased

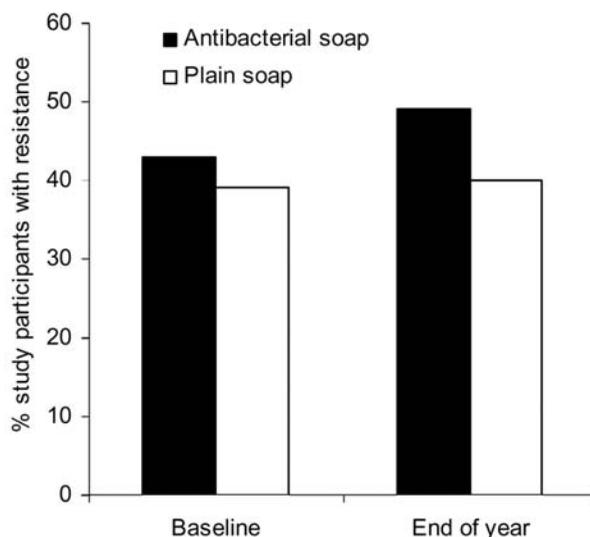Figure 2. Proportion of study participants with ≥1 bacterial species resistant to an antimicrobial agent on their hands. In the group that used antibacterial products, 82 and 105 hand samples were available at baseline and at year-end, respectively. In the group that used nonantibacterial products (i.e., plain soap), 82 and 96 hand samples were available at baseline and at year-end, respectively.

Table 3. Logistic regression models for examining factors associated with carriage of organisms with antimicrobial resistance or increased triclosan MICs*

Outcome 1 (≥1 organism with resistance to antimicrobial agents on hand)	OR	95% CI, p value	aOR†	95% CI, p value
Baseline characteristics (N = 164)				
Antibacterial product use in household‡	1.16	0.62–2.17, 0.63	0.97	0.50–1.89, 0.91
Observed no. of seconds for handwash by primary caregiver	1.05	1.01–1.09, 0.01	1.05	1.01–1.09, 0.01
Above average log total CFU on hands of primary caregiver after handwash	2.06	1.08–3.93, 0.03	1.81	0.93–3.52, 0.08
Reported no. of hands washes per day for primary caregiver	1.01	0.97–1.04, 0.74	–	–
≥1 household members with job in healthcare or daycare	1.28	0.68–2.40, 0.44	–	–
Year-end characteristics (N = 201)				
Antibacterial product use in household	1.44	0.82–2.52, 0.20	1.33	0.74–2.41, 0.34
Observed no. of seconds for handwash by primary caregiver	1.00	0.97–1.04, 0.91	–	–
Above average log total CFU on hands of primary caregiver after handwash	0.62	0.35–1.98, 0.09	–	–
Reported no. of hands washes per day for primary caregiver	0.94	0.89–0.99, 0.04	0.95	0.89–1.01, 0.10
≥1 household members with job in healthcare or daycare	0.51	0.29–0.90, 0.02	0.52	0.29–0.95, 0.04
Outcome 2 (≥1 organism with increased triclosan MIC on hand)				
Baseline (N = 164)				
Antibacterial product use in household‡	1.59	0.84–3.01, 0.16	–	–
Year-end (N = 201)				
Antibacterial product use in household	1.73	0.97–3.09, 0.06	–	–

*OR, odds ratio; CI, confidence interval; aOR, adjusted odds ratio.
†OR adjusted for all variables that were significant in univariate analyses at p<0.05.
‡Prior reported antibacterial product use was controlled for but did not have any effect on the point estimate. Therefore, "group" point estimates reflect use of antibacterial product after randomization.

duration of the wash may have allowed greater dispersal of bacteria into the culture bag.

Primary caregivers residing in households with healthcare or daycare workers had significantly fewer antimicrobial drug–resistant organisms on their hands. This association appears to be influenced by above-average number of handwashes per day by the primary caregiver and indicates that hygiene, regardless of antibacterial ingredients, may reduce household transmission of antimicrobial drug–resistant bacteria.

Limitations for Detecting Changes in Resistance

A factor that might have attenuated the associations found in this study is a higher baseline level of antimicrobial drug resistance in this community. Higher baseline levels would make detecting small changes in susceptibility attributed solely to use of antibacterial cleaning and hygiene products more difficult. Most persons from our study population were from the Dominican Republic, a country that provides over-the-counter access to antimicrobial agents. In an earlier study within this same community, antimicrobial agents were taken by 354 (39%) of 911 persons reporting infectious disease symptoms within the previous 30 days, which suggests high levels of use (21). In addition, this study was conducted for a 1-year period and therefore may not adequately reflect the time-course for development of resistance attributable to use of antibacterial products. Changes in antimicrobial drug resistance during the 1-year period might have been lower than

the level of detection that this study was statistically powered to identify. This study was designed to detect an OR ≥2.11 after 1 year of use, given a power of 80% and a 2-sided α level of 0.05.

Although triclosan susceptibility was examined among various species, we were not able to evaluate potential mechanisms for cross-resistance, such as overexpression of efflux pumps. In addition, when we examined the association between use of antibacterial cleaning and hygiene products and antimicrobial drug resistance, the definition of resistance (≥1 organism[s] with antimicrobial drug resistance) did not allow exploration of the potential association with each separate species or antimicrobial drug tested. However, the purpose of our study was to examine overall trends and shifts in antimicrobial drug resistance attributed to the use of antibacterial cleaning and hygiene products, given that the effects of these products in the community are relatively unexplored.

Conclusion

Currently, no evidence suggests that use of antibacterial soap containing 0.2% triclosan provides a benefit over plain soap in reducing bacterial counts and rate of infectious symptoms in generally healthy persons in the household setting (4,5,15). Our 1-year randomized community intervention study adds to these earlier findings by assessing the potential risks associated with antibacterial product use in the home. The results from our study do not implicate use of antibacterial cleaning and hygiene products as

an influential factor in carriage of antimicrobial drug–resistant bacteria on the hands of household members. Although we did not observe a significant impact on antimicrobial drug resistance during the 1-year period, a longer duration and more extensive use of triclosan might provide a suitable environment for emergence of antimicrobial drug–resistant species in the community setting. Further surveillance for the effect of long-term use of antibacterial cleaning and hygiene products on antimicrobial drug resistance in the community is needed.

At the time of this writing, Dr Aiello was a Robert Wood Johnson Health & Society Scholar in the Department of Epidemiology, Center for Social Epidemiology & Population Health, at the University of Michigan School of Public Health. She is now an Assistant Professor of Epidemiology at that center. Her research interests include antimicrobial drug and antibacterial resistance within the community and clinical setting, multidisciplinary approaches for examining antimicrobial drug resistance, and life-course socioeconomic determinants of infectious diseases.

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Plasmodium falciparum Spatial Analysis, Western Kenya Highlands

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We carried out a population-based study to determine the unbiased, age-specific *Plasmodium falciparum* prevalence, asexual and sexual parasite density, and spatial distribution to establish rates of infection at a site in western Kenya. Three cross-sectional surveys were carried out in western Kenya highlands. Blood samples were taken from 1,388 persons from 6 months to 75 years of age. Parasite prevalence and densities in the population decreased with age and distance from valley bottoms. Children from 1 to 4 years of age had the highest parasite prevalence (38.8%–62.8%); in adults, prevalence declined to 2.9%–24.1%. Malaria prevalence declined by an average of 19% from July to December 2002 across age groups. These observations suggest that parasite transmission is intense at this altitude. Asexual parasite density indicated clustering near major vector breeding habitats. Variability in seasonal prevalence indicates transmission instability and susceptibility to epidemics.

The reemergence of epidemic malaria in the East African highlands (elevation >1,500 m above sea level) is a public health problem (1). Research indicates that the mechanisms leading to epidemic malaria in the highlands are complex and are probably due to the concerted effects of factors such as topography (2), hydrology (3), climate variability (4), land-use/land-cover change (5), and drug resistance (6). Effective disease control calls for a clear understanding of the interaction between these epidemiologic factors.

Perennial malaria transmission in the lowlands has been attributed to high vector densities throughout the year (7). Inhabitants of the basin region of Lake Victoria, western Kenya, experienced up to 300 infective bites per year (8,9). Vector density and transmission intensity in the highlands are much lower than in the lowlands. For example, a transect study from lowland (300 m elevation) to highland

(1,700 m elevation) in the Usambara Mountains in Tanzania found a >1,000-fold reduction in transmission intensity between the holoendemic lowland and the hypoendemic highland plateau (7). At high altitudes in the highlands and on hilltops, where malaria transmission intensity is low, human populations have poorly developed immunity to malaria because exposures are infrequent (10), and persons are vulnerable to severe clinical illness and complications from *Plasmodium* infection (11,12). High risk for severe malaria is seen in persons living in areas with low-to-moderate transmission intensities (13). In such areas, the proportion of asymptomatic persons is usually lower than in high-transmission areas, where *P. falciparum* prevalence and parasite density varies little between seasons (14).

Although malaria incidence has been increasing in the East African highlands (15), the extent and distribution of malaria infections in the asymptomatic human population are largely unknown. Data on malaria in the western Kenya highlands are derived from hospital clinical records (16,17) and do not provide a population-based, epidemiologic profile or information on the unbiased prevalence of malaria in local highland populations. Bias in hospital data can arise from poverty, self-medication, and lack of access to hospitals. Detailed parasitologic data are also not normally available from hospital clinical records. Because data on malaria prevalence in local residents are unavailable, we do not know whether whole populations are susceptible to malaria or the risk is skewed toward children, as is seen in malaria-holoendemic regions. In this study, we investigated malaria parasitologic profiles in a population living in a highland zone in the Kakamega district, western Kenya, where epidemics have been reported (4) to determine age-specific parasitemia prevalence, age-specific parasite densities, and the spatial distribution of infections.

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Materials and Methods

Study Site

The study site is located in Iguhu village (0°17′N, 34° 74′E, elevation 1,450–1,580 m above sea level), Kakamega district, western Kenya, with population ≈11,000. This area experiences 2 rainy seasons and averages 2,000 mm rainfall per year. The long rainy season usually occurs between April and May, with an average monthly rainfall 150–260 mm, while the short rainy season usually occurs between September and October, with an average monthly rainfall 165 mm. Malaria prevalence peaks usually lag 1–2 months after the rain. The mean annual daily temperature is 20.8°C. The area has experienced extensive deforestation and swamp reclamation in recent years as a result of rapid human population growth and the demand for settlement and agricultural land; therefore, only patches of forest remain. Malaria vectors in the area are *Anopheles gambiae* sensu stricto and *A. funestus* (4,18). Maize is the principal subsistence crop, although vegetables are grown on small, irrigated plots in valley bottoms. The area is bisected by the Yala River (Figure 1); most mosquito larval habitats were found on river banks in the bottom of the valley and on the banks of streams during both dry and rainy seasons (18).

Study Design

The working hypothesis was that no difference would be seen in the prevalence among various age groups and that infections would be randomly distributed. The binomial model was used to estimate the confidence interval (CI). Because the prevalence of malaria was not well known in the area, a 50% estimate, which gives the best sample size, was used for the peak-transmission season, and a 25% estimate was used for the low-transmission season. We calculated the 2 sample sizes with a 95% CI and precision level of 5%:

$$n = \frac{z^2(pq)}{d^2}$$

In this equation, *n* is the sample size, *z* is the critical value of the standard normal distribution at the 5% level (1.96), *p* is the malaria prevalence estimate, $q = 1 - p$, and *d* is the precision level. The population size was estimated to be 3,000. The sample size obtained was 341 persons for the peak-transmission season and 263 for the low-transmission season.

Study Population and Data Collection

The study, which involved 1,388 participants, was carried out in 3 cross-sectional surveys undertaken in July 2002 (n = 396), December 2002 (n = 283), and June 2003 (n = 709). Peak malaria transmission occurs 1–2 months after the

rainy season; thus, surveys were conducted 1 month after the rains. The short rains before December are usually not adequate to increase transmission, and this period is considered entomologically dry. Figure 1 shows the spatial distribution of residents examined for *Plasmodium* infections in July 2002. The sample size for each age group during each sampling period is shown in the Table. Adults (≥18 years of age) were recruited into the study upon giving an informed consent; consent for children (<18 years) was provided by the participants and their parents or guardians. Scientific and ethical clearance was given by Kenya Medical Research Institute and State University of New York at Buffalo ethical review boards. Inclusion criteria were provision of informed consent, age >6 months at recruitment, and no reported chronic or acute illness except malaria. Blood samples were collected by the standard finger-prick method, and thick and thin smears were prepared on labeled slides. The thin and thick blood smears were air dried. The thin smears were fixed in methanol and stained in 4% Giemsa for 30 min with the thick smear. Two experienced technicians examined the slides under ×1,000 oil immersion to identify and count the parasite species. Random checks were carried out on the slide counts by independent microscopists to ensure quality control. Parasite density was scored against 200 leukocytes when the slide was positive; otherwise, the whole slide was carefully scanned before being declared negative. Parasite densities were converted to number of parasites per microliter of blood, assuming a leukocyte count of 8,000 cells/μL (19).

We examined the monthly malaria infection dynamics in primary school children, age 6–14, in the study area from June 2002 to June 2003, with the microscopic test. Daily rainfall data were collected by using automated weather stations (HOBO, Onset Computer Corporation, Bourne, MA, USA). These data were offloaded monthly from the weather stations.
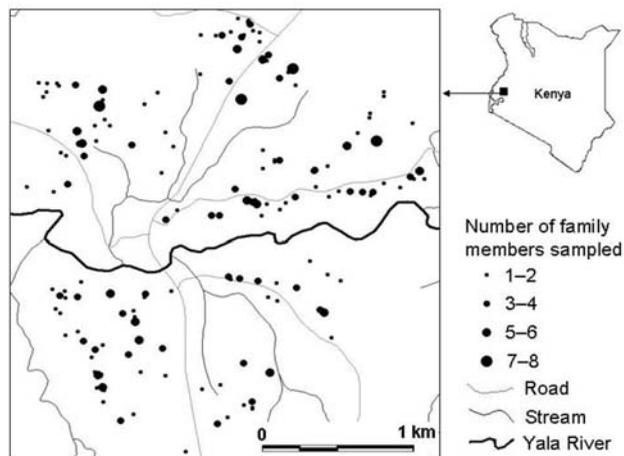

Figure 1. Distribution of residents sampled for *Plasmodium* prevalence, Iguhu village, Kakamega district, western Kenya.

Table. Number of samples by age group and sampling occasion

Age group (y)	July 2002	December 2002	June 2003
<1	19	9	12
1–4	35	54	79
5–9	128	67	230
10–14	129	54	243
15–19	57	31	32
>19	28	68	113
Total	396	283	709

Data Analysis

Most of the homes of the participants in the prevalence survey were georeferenced by using a global positioning system (GPS) unit. A database was created with participants' names, ages, GPS location, and parasite density. Participants' names were coded for confidentiality. The relationship between *P. falciparum* prevalence and parasite density was determined across age, season, and distance to the nearest stream/valley. To examine the effects of age on parasite prevalence and infection density, ages were stratified into 6 age groups: <1, 1–4, 5–9, 10–14, 15–19, and >19 years (20). Age-specific prevalence was determined by expressing positive blood smears as a percentage of all examined blood smears; only positive slides were considered when the geometric mean parasite density was calculated for each age category. The χ^2 test was used to determine differences in prevalence among age groups for each survey. Analysis of variance (ANOVA) with logarithm-transformed parasite density was used to examine the difference among age groups and among surveys in parasite density. Nonspatial statistical analyses were conducted by using the JMP statistical package (SAS Institute Inc, Cary, NC, USA).

To determine the spatial distribution pattern of malaria-infected residents, ArcView 3.3 (Environmental Systems Research Institute, Redlands, CA, USA) was used to create spatial-distribution maps of infected and uninfected participants for the 3 surveys. *P. falciparum* infections were tested for clustering by household with the global spatial statistic, the K function, weighted by parasite density (21–23) by using Point Pattern Analysis software (San Diego State University, San Diego, CA, USA). The global weighted K function, $L(d)$, examines the spatial dependence of malaria infection by household over a wide range of scales of pattern (21). The observed $L(d)$ function values were tested against the null hypothesis that the spatial distribution of all infected residents in the study area was random, by using 1,000 Monte Carlo iterations. Because children and adults showed significantly different prevalence and parasite density, the global spatial cluster analysis was conducted separately for the 0- to 9-year age group, the 10- to 19-year age group, and adults (>19 years old). The local spatial statistic $Gi^*(d)$ (24) was computed to assess clustering of high parasite densities near a partic-

ular transmission source, the Yala River, where most vector breeding sites were found (18), for age groups 0–9 and 10–19 years, but not for adults because sample sizes were too small. To correct for multiple comparisons when using $Gi^*(d)$, significance levels were determined by using Table 3 in reference (25).

Results

Plasmodium Prevalence and *P. falciparum* Density

In addition to *P. falciparum*, we found *P. malariae* and *P. ovale* in our study populations. *P. falciparum* constituted 97.1%, 94.9%, and 94.6% of malaria cases in July and December 2002, and June 2003 surveys, respectively. *P. malariae* constituted 2.9%, 5.1%, and 4.6% of malaria cases, while *P. ovale* was observed only in June 2003 (0.8%). *P. malariae* was often seen in mixed infection with *P. falciparum*. For example, 83.3% of *P. malariae* infections in July 2002 occurred with *P. falciparum*, while 50% of *P. malariae* infections in December 2002, and 81.8% of *P. malariae* infections in June 2003, were accompanied by *P. falciparum*.

P. falciparum prevalence varied significantly among age groups (homogeneity $\chi^2 = 95.82$, df = 5, p<0.001) and
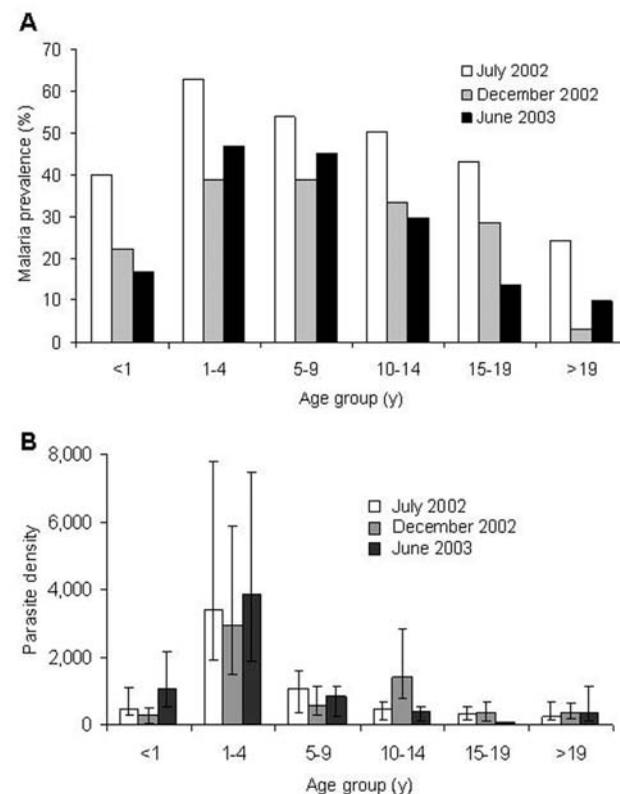

Figure 2. A) *Plasmodium falciparum* trophozoite prevalence. B) Geometric mean of *P. falciparum*–infected erythrocytes per microliter of blood (error bars represent 95% confidence intervals), Iguhu village, Kakamega district, western Kenya.

sampling months (homogeneity χ^2 = 46.81, df = 2, p<0.001; Figure 2A). Malaria prevalence for all age groups ranged from 50.1% in July 2002 to 27.1% in December 2002. Children 1–9 years of age consistently had the highest prevalence (average 47.0%) in the 3 cross-sectional surveys, while adults >19 years of age showed the lowest prevalence (average 9.5%). The mean parasite density did not vary significantly among survey months (ANOVA, F = 0.53, df = 2, 483, p = 0.59) but did vary significantly among age groups (F = 21.17, df = 5, 483, p<0.001; Figure 2B). For example, children 1–4 years of age had the highest parasite density (geometric mean of 3,469.7 infected erythrocytes/µL blood, 95% CI 2,328.0–5,171.3). This amount was >7-fold higher than the average parasite density among all other age groups (477.8 infected erythrocytes/µL, 95% CI 265.6–715.1; Tukey-Kramer HSD [honestly significant difference] test, p<0.001; Figure 2B).

P. falciparum Gametocyte Prevalence and Density

Overall, *P. falciparum* gametocyte prevalence did not differ significantly among the 3 surveys (homogeneity χ^2 = 1.34, df = 2, p = 0.51) or age groups (homogeneity χ^2 = 9.48, df = 5, p = 0.09; Figure 3A). The mean gametocyte prevalence was 2.7% (range 0%–8.3%; Figure 3A). Similarly, gametocyte density did not vary significantly among sampling occasions (ANOVA, F = 0.71, df = 2, 31, p = 0.50) or age groups (F = 0.84, df = 4, 31, P = 0.51; Figure 3B). The geometric mean gametocyte density was 71.7 gametocytes/µL (95% CI 53.8–95.6).

During the study period (June 2002 to June 2003), malaria prevalence showed a general decreasing trend (Figure 4). However, the geometric mean parasite density varied among months. The months with peak parasite density appeared to be 1–2 months behind rainfall peaks.

Spatial Distribution of Parasite Density

The global weighted K function, $L(d)$, was used to examine the spatial dependence of malaria infection by household over an interpoint distance of 100–1,400 m for each of the 3 age groups (0.6–9, 10–19, and >19 years). Figure 5 shows measures of the observed $L(d)$ and the 95% CI plotted for various values of interpoint distance for the July 2002 survey. The spatial distribution of infections is considered evenly dispersed if the observed K function values are below the lower limit of the 95% CI, clustered if above the upper limit, or random if within the 95% CI. The weighted K function indicated that the parasite density distribution pattern was significantly different than expected under complete spatial randomness for age groups 0.6–9 (Figure 5A) and 10–19 years (Figure 5B), but was random in adults (Figure 5C). Spatial clustering for age groups 0.6–9 and 10–19 years occurred at an interpoint
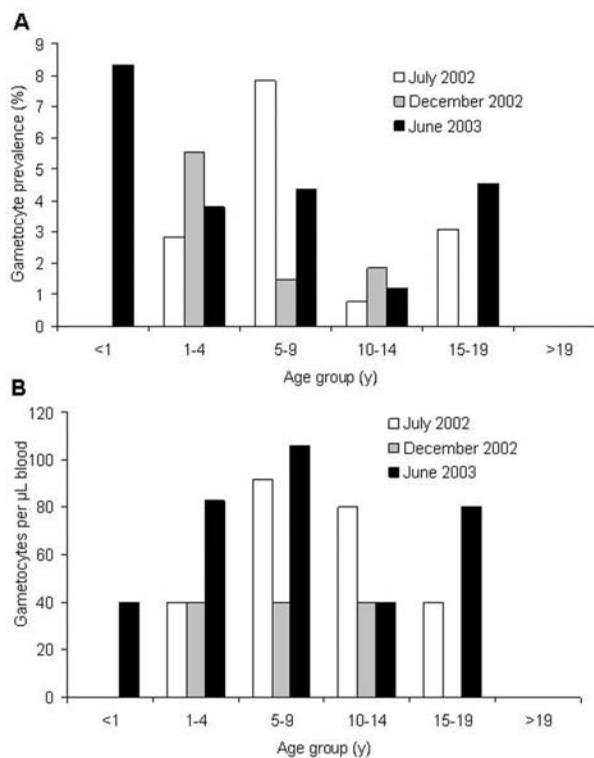
Figure 3. A) *Plasmodium falciparum* gametocyte prevalence. B) Infection densities in different age groups, Iguhu village, Kakamega district, western Kenya.

distance >150 m. This pattern was consistent with 2 other surveys done in December 2002 and June 2003 (data not shown).

Local spatial clustering analysis was performed on parasite densities to determine whether higher infection densities were clustered near the Yala River, where most vector breeding sites existed. Figure 6 shows an example of local spatial clustering for age group 0.6–9 years for the July 2002 survey. We found that parasite densities were positively clustered for children living <500 m from the Yala River and negatively clustered for children residing >1,000 m from the river. That is, children living near the river had significantly higher parasite densities than would be expected in random distribution, whereas those farther away from the river exhibited significantly lower parasite densities. Clustering of parasite densities in age group 10–19 years showed a similar pattern, but fewer households had either positive or negative clustering. Similar results were found for the December 2002 and June 2003 surveys (data not shown). Therefore, the patterns of *P. falciparum* parasite density clustering, as shown in Figures 5 and 6, suggest a relationship between a house's distance from the Yala River and clustering of high parasite densities.
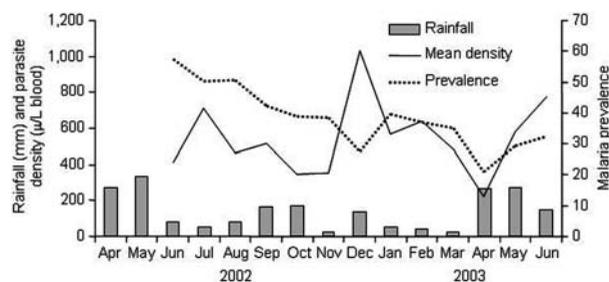
Figure 4. Dynamics of monthly rainfall, monthly (geometric) mean parasite density, and monthly *Plasmodium falciparum* prevalence.

Discussion

In this study, we found that children 1–4 years of age had a 7-fold greater number of parasites in their blood as compared to persons ≥5 years of age, irrespective of season. The rapid fall in parasite density as age increases suggests age-dependent immunity to *P. falciparum* parasites. The low prevalence of infections among adults further supports this notion. However, the highland topography affects drainage and the distribution of mosquito breeding habitats and therefore also affects exposure to infections. For example, 90% of adult mosquitoes in the study area were collected <300 m from valley bottoms (26). Nonuniform exposure patterns to infections as a result of topography and hydrology would lead to similar spatial immunologic profiles. Thus, within the highland human populations, varying states of transmission stability are found.

John et al. carried out an epidemiologic study at an altitude of 2,134 m in the Uasin Gishu district of western Kenya (27). They found no difference in reinfection rates between children and adults, which indicates that malaria transmission could be unstable at this altitude. Whether infections translated into clinical disease at the same rate in children and adults is not clear (28). Hay et al. (17) reported that approximately two thirds of hospital admissions in the Kisii district (elevation 1,500–1,800 m) in the western Kenya highlands were children <15 years of age. Our population-based malaria prevalence data from a village in the Kakamega district (elevation 1,450–1,580 m) indicate that children (1–4 years of age) have the highest malaria prevalence and parasite density, a condition similar to that seen in the holoendemic lowlands. Few records exist of detailed population-based parasitologic and clinical studies of malaria in the highlands of western Kenya, which is why classifying the stability of transmission is difficult. Shililu et al. (3) reported prevalence rates in asymptomatic children from 44% in the dry season to 55.4% in the wet season in Mumias, Kakamega district (≈1,500 m above sea level), western Kenya. During the 1990 malaria epidemic in the Uasin Gishu district in western Kenya (elevation

2,000 m), Some (29) reported a prevalence of 72% in the general population. In the same year, Ayisi et al. (30) observed a mean prevalence of 39.2% in the general population at the Belgut division of the Kericho district (elevation 1,800 m). Our data fall within the range observed by other investigators at similar altitudes. However, we also observed a significant interannual and seasonal variability in malaria prevalence at this highland site. The variation in parasite prevalence between the wet and dry seasons could be explained by differences in vector abundance. For example, the mean monthly anopheline vector abundances increased by 6- to 8-fold in the long rainy season compared to the dry season (26).

The prevalence of *P. falciparum* malaria in school children in the low-altitude region of Lake Victoria basin (elevation ≈1,200 m) adjacent to the highlands reaches >80% (31–34), which is much higher than malaria prevalence in
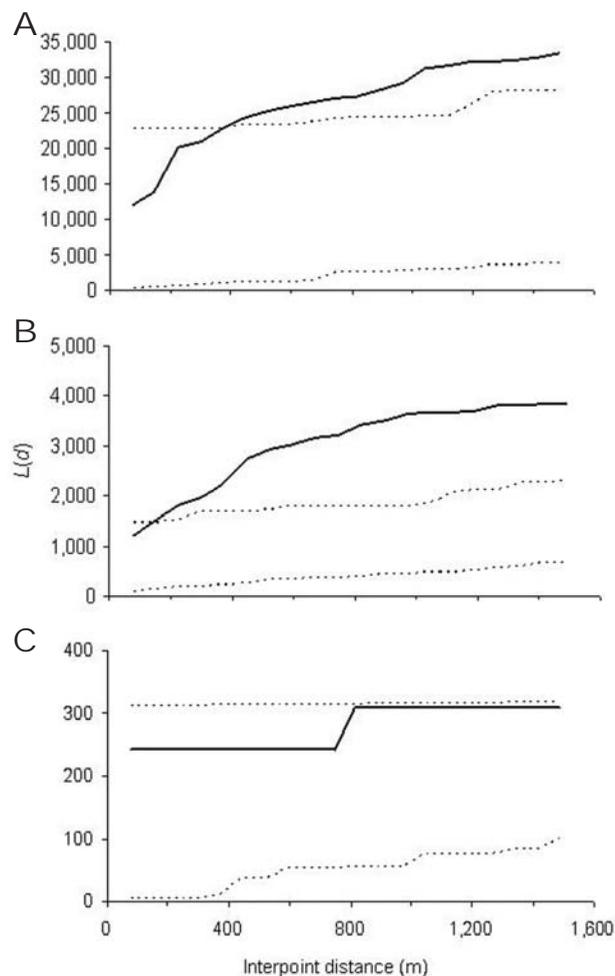

Figure 5. Results of weighted K function analysis on the global spatial clustering of *Plasmodium falciparum* infection intensities in Iguhu for age groups A) 0–9 years, B) 10–19 years, and C) >19 years in the July 2002 survey. The solid line is the observed value of the test statistic *L(d)* at a given distance *d*, and dashed lines indicate 95% confidence intervals.
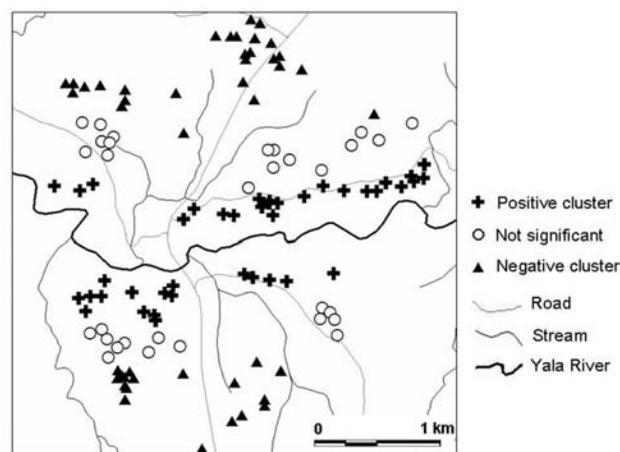
Figure 6. Significant clustering of *Plasmodium falciparum* infection intensities in Iguhu village for children 0–9 years of age.

the highlands. Therefore, in the highlands, a high proportion of residents was susceptible to infection and clinical disease. Under conditions of hypertransmission, outbreaks and epidemics would result because medical facilities would be unable to cope with the number of persons affected. Malaria instability in this area is related to the proportion of susceptible persons and the variability in transmission. Children 1–4 years of age had the highest parasite densities in their blood; after 5 years of age, densities dropped dramatically. The low prevalence of infections in adults suggests that these persons have developed some degree of immunity as a result of lifelong exposure.

The mean parasite density observed in the 1- to 4-years age group at Iguhu was similar to that observed in the <1-year age group in the lowlands (32), which suggests immunity develops more slowly in the highlands. Still, older persons had 7-fold lower parasite loads, which indicates functional, age-dependent immunity.

The age-specific profiles of gametocyte prevalence in the highland site were much lower than those observed in the lowlands; e.g., infants (1–9 months) had a prevalence of 86% in the lowlands, while in the highlands prevalence in this group was 2.9%. Children 5–9 years of age had the highest rate of infection with gametocytes (4.9%) at Iguhu, which is comparatively lower than that reported in the lowland (40%) for the same age group (32). The reservoir of malaria infections in the highlands is much lower than in the lowlands. For example, Ayisi et al. (30) reported a mean gametocyte prevalence of 1.8% in the Kericho district in the western Kenya highlands, while we observed a mean gametocyte rate of 2.8% in the Kakamega district. By contrast, Githeko et al. (32) reported a gametocyte prevalence of 39.1% during the rainy season in the Kisumu district and a rate of 10.8% in the dry season in the Suba

district (35). Similar to observations made in the lowlands (32), the mean gametocyte density in highland residents did not vary significantly among age groups. Compared to what was seen in the lowlands, the proportion of persons carrying gametocytes (the infectious reservoir) in our highland site was 5- to 100-fold less, which suggests a weak transmission system in the highlands.

Spatial analysis of asexual parasite densities indicated clustering in relation to distance from major larval breeding habitats and in relation to age. This phenomenon was not observed in persons living >1,000 m from major breeding sites, which suggests that the rate of parasite transmission may be higher closer to major breeding habitats. This finding is consistent with the spatial distribution of indoor resting vector densities in the area. Our results are consistent with the fact that the risk of malaria is strongly associated with distance from breeding sites (36,37).

This cross-sectional study in a highland site where malaria epidemics have been reported in the past shows that transmission is intense, particularly after the rainy season, but variable with regard to season and distance from major mosquito breeding habitats. The risk of infection is highly variable within the site, and subsequently, the stability of transmission may reflect this variability. A large host population is available for infection before periods of hypertransmission. Although the population has a functional age-dependent immunity to malaria, as indicated by parasite densities, its development is slower than that found in the holoendemic lowlands, which suggests that this population may be more susceptible to malaria infections and more prone to epidemics compared to the lowland populations.

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Botulinum Neurotoxin Detection and Differentiation by Mass Spectrometry

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and David L. Ashley*

Botulinum neurotoxins (BoNTs) are proteases that cleave specific cellular proteins essential for neurotransmitter release. Seven BoNT serotypes (A–G) exist; 4 usually cause human botulism (A, B, E, and F). We developed a rapid, mass spectrometry–based method (Endopep-MS) to detect and differentiate active BoNTs A, B, E, and F. This method uses the highly specific protease activity of the toxins with target peptides specific for each toxin serotype. The product peptides derived from the endopeptidase activities of BoNTs are detected by matrix-assisted laser-desorption ionization time-of-flight mass spectrometry. In buffer, this method can detect toxin equivalents of as little as 0.01 mouse lethal dose $(MLD)_{50}$ and concentrations as low as 0.62 MLD_{50}/mL. A high-performance liquid chromatography–tandem mass spectrometry method for quantifying active toxin, where the amount of toxin can be correlated to the amount of product peptides, is also described.

Botulinum neurotoxins (BoNTs) are the most toxic substances known (1). They are produced under anaerobic conditions by strains of *Clostridium botulinum*, *C. butyricum*, and *C. baratii* (1). Intoxication with 1 of the 7 distinct serotypes of BoNT (A–G) causes botulism. One of 4 serotypes of BoNT (A, B, E, and F) is usually the cause of botulism in humans. BoNTs are zinc metalloproteases that cleave and inactivate specific cellular proteins essential for the release of the neurotransmitter acetylcholine (Figure 1). BoNT-A, -C, and -E cleave SNAP (synaptosomal-associated protein)-25; BoNT-B, -D, -F, and -G cleave synaptobrevin 2 (also called VAMP 2). Of the serotypes, only 1, BoNT-C, cleaves >1 site on a specific protein. In addition to cleaving SNAP-25, BoNT-C also cleaves syntaxin (1).

Current methods for detecting BoNT include a mouse bioassay (2–4) and an enzyme-linked immunosorbent assay (ELISA) (5,6). The mouse bioassay is the accepted standard and is the only widely accepted method for detecting BoNT (2–5). In this assay, mice receiving an intraperitoneal injection containing a sample with more than a minimum lethal dose show symptoms of botulinum intoxication and die (2,4). Many institutional animal care and use committees, including that of the Centers for Disease Control and Prevention, require mice to be euthanized after the onset of severe symptoms. In the mouse bioassay, when the injected dose is high, mice typically develop signs of botulism within 8 hours. At lower doses, mice are affected more slowly; hence, mice are observed for 4 days before a negative result
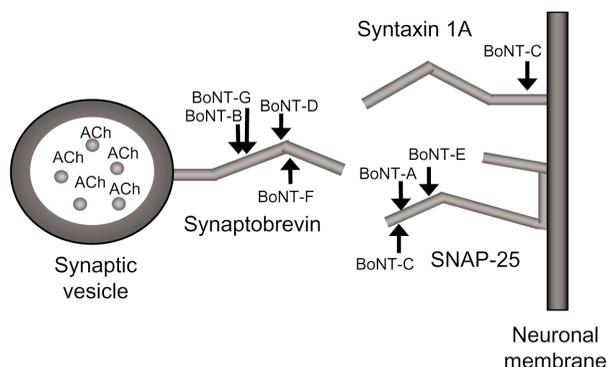
Figure 1. Synaptobrevin on the synaptic vesicle must interact with syntaxin and SNAP (synaptosomal-associated protein)-25 on the neuronal membrane for fusion to occur, which allows the nerve impulse to be delivered across the synaptic junction. The botulinum neurotoxin serotypes cleave the peptide bonds at specific sites on the 3 proteins, as indicated. Cleavage of any 1 of these proteins prevents vesicle membrane docking and nerve impulse transmission.

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is recorded. The mouse bioassay can also be used to differentiate BoNT serotypes (4). Mixtures of neutralizing antibodies are given to mice in conjunction with the sample. Mice receiving the appropriate anti-BoNT serotype antibody are asymptomatic and survive, while mice treated with the other serotype antibodies show symptoms of botulism (4). The mouse bioassay measures active toxin and is sensitive. The absolute amount of toxin detected in the mouse bioassay is not well defined but is thought to be 10–20 pg/mL for BoNT A (7,8). The main disadvantage of the mouse bioassay is that it requires euthanizing many animals. It also requires several days to determine the toxin level and type (4–6). Special animal facilities are also required, personal hazards are associated with injecting animals (5,6), and some clostridia produce nonbotulinal toxins that also kill mice (5).

The ELISA is more rapid than the mouse bioassay, but it is not a functional assay. It recognizes protein antigenic sites and in general is somewhat less sensitive than the mouse bioassay (5,6). The ELISA was validated for detecting BoNT produced in cooked meat medium and tryptone peptone glucose yeast extract (TPGY). The test was designed to detect and differentiate BoNT serotypes A, B, E, and F in a 1-day test and has the sensitivity of ≈10 mouse lethal dose $(MLD)_{50}$/mL (5,6). In a recent study, the ELISA performed well in most laboratories at the 100 MLD_{50}/mL and 10,000 MLD_{50}/mL levels (5). In this study, some cross-reactivity occurred among BoNT cultures and with nonbotulinum cultures (5). At 100 MLD_{50}/mL, a >7% false-negative rate in TPGY was observed; at 10,000 MLD_{50}/mL, a 1.5% false-positive rate for BoNT-A and a 28.6% false-positive rate for BoNT-F occurred (5). The ELISA is currently used primarily as a fast screening technique, and results are verified by the mouse bioassay (5).

Several in vitro assays have been developed to detect the activities of the different BoNT serotypes (7–14). This approach has led to methods that are based on the natural substrates that are cleaved by the BoNTs and use fluorescence to detect toxin activity. These types of assays are >2 orders of magnitude less sensitive than the mouse bioassay and have not been proven for use with environmental, food, or clinical samples. They are also prone to giving false-positive results in samples that contain proteases because the specific site of cleavage cannot easily be determined in a fluorescence-based assay (15). Another method that combines immunoaffinity chromatography with specific antibodies for cleavage products has been successful in detecting BoNT B in some foods at lower detection limits than the mouse bioassay (10).

We introduce here the concept and preliminary data on a new, rapid, mass spectrometry–based, functional method for detecting, differentiating, and quantifying 4 BoNT serotypes. This method is based on both the unusual

endopeptidase activities of these enzymes and specific detection of the unique peptide products by mass spectrometry (Endopep-MS). Because each BoNT serotype has a unique cleavage site on a unique peptide, the mass-specific product peptides detected by mass spectrometry differentiate active BoNT serotypes. Substrate peptides that are specific for each serotype are incubated with BoNT; then serotype-specific product peptides are detected by either matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) or by high-performance liquid chromatography (HPLC)-electrospray ionization-tandem mass spectrometry (HPLC-ESI/MS/MS). Thus, this method combines the biologic specificity of the BoNT enzymatic activity with the unparalleled detection specificity of mass spectrometry.

Methods

Materials

BoNT complex toxins were purchased from Metabiologics (Madison, WI, USA) and were provided at 1 mg/mL total protein in 50 mmol/L sodium citrate buffer, pH 5.5. The toxin activities in MLD_{50}/mg protein were 3.6×10^7 BoNT-A, 1.6×10^7 BoNT-B, 2.8×10^7 BoNT-E, and 5.5×10^7 BoNT-F. All reagents were from Sigma-Aldrich (St. Louis, MO, USA), except where indicated. HPLC-purified peptide substrates were synthesized by Los Alamos National Laboratory (Los Alamos, NM, USA). Figure 2 shows the peptide sequences used to detect and differentiate each BoNT serotype along with the specific cleavage products and their masses.

Peptide Cleavage Reactions

For the Endopep-MS method, BoNT serotypes A, B, E, and F endopeptidase activities were determined in 20-μL volumes of buffer containing 0.05 mol/L Hepes (pH 7.3), 25 mmol/L dithiothreitol (DTT), 20 mmol/L $ZnCl_2$, 1 mg/mL bovine serum albumin (BSA), and the target peptides, at 1 nmol each. Specific BoNT serotype complexes were added at various concentrations and incubated at 37ºC from 2 h to 16 h. Control tubes without BoNT were run at the same time as BoNT cleavage reactions and served as an analytic blank. The analytic sensitivity of the reaction was tested by diluting the toxin in Hepes reaction buffer to 100, 10, 1, 0.1, and 0.01 MLD_{50}/μL. An aliquot (1 μL) of each dilution was added to 19 μL reaction buffer containing specific peptides 1–4.

Multiplexing Reactions

Endopeptidase reactions were multiplexed by adding all 4 peptides (1–4) at 1 nmol each to the reaction buffer described above and incubating for 2 h at 37ºC. Each BoNT serotype was added to separate reaction mixtures.

Figure 2. Substrate peptide sequences, the botulinum neurotoxin (BoNT) serotype predicted cleavage product sequences, and masses of the substrate and product peptides. Peptides for BoNT-A and -E were derived from the human SNAP (synaptoso-mal-associated protein)-25 protein. The substrate peptide for BoNT A, 187-SNKTRIDEANQRATKML-203, was modified to biotin(ε)-KG(K189->R and K201->R)GGK-(ε)Biotin. The BoNT-E substrate sequence was also from human SNAP-25 (156–186). Substrate peptides for BoNT-B and -F are from human synapto-brevin 2; the BoNT-B substrate (3) is from 59–93 in the sequence and the BoNT-F substrate is from 35–74.

Control tubes with no BoNT were always run at the same time to serve as an analytic blank. High levels of BoNT (200 ng/20 µL reaction) were used for each serotype to look for cross-reactivity between any of the BoNT serotypes. No cross-reactivity between the various BoNT serotypes was observed.

Detection Limits in MLD$_{50}$/mL

Larger volume reactions were run to test the sensitivity in mouse LD$_{50}$/mL. BoNT serotype A, B, E, or F complexes ranging from 100 to 0.31 MLD$_{50}$ were spiked in 1 mL of deionized water. A 168-µL aliquot was spiked with a 10× reaction buffer and peptide solution to yield final concentrations identical to those above. The target peptides used for this experiment were the same as above, except minor modification to the BoNT A and B substrate peptides were used since they showed slightly more activity than the previous peptides. These peptides were Biotin-(ε)-KGSNRTRIDEGNQRATR(Nle)LGGK-(ε)-Biotin for BoNT A and LSELDDRADALQAGASQFESSAAK-LKRKYWWKNLK for BoNT B. The final reaction volumes were 200 µL. One set of reactions was allowed to proceed for 4 h, and a second set proceeded for 16 h. The longer reaction times were used to enhance the amount of product peptide produced and, therefore, lower the detection limits. For MALDI-TOF analysis, 2 µL of the 200-µL reaction mixture was mixed with the matrix and analyzed

as discussed in the MALDI-TOF-MS section. For HPLC-ESI/MS/MS analysis, 50 µL of the 200-µL reaction mixture was injected on the instrument.

MALDI-TOF-MS Analysis

Specific cleavage products were detected by mass spectrometry. For all experiments, the reaction mixture, at the incubation times indicated, was added to alpha-cyano-4-hydroxy cinnamic acid (CHCA) at 5 mg/mL in 50% acetonitrile, 0.1% trifluoroacetic acid, and 1 mmol/L ammonium citrate (CHCA matrix), at a ratio of 1:5 or 1:10. This mixture was applied at 0.5 µL per spot to a 192-spot stainless steel MALDI plate (Applied Biosystems, Framingham, MA, USA). Mass spectra of each spot were obtained by scanning 650–4,500 m/z in MS-positive ion reflectron mode on a Model 4700 MALDI-TOF-TOF-MS Proteomics Analyzer (Applied Biosystems). The instrument used a nitrogen laser at 337 nm, and each spectrum was an average of 2,400 laser shots.

HPLC-ESI/MS/MS Analysis

The HPLC-ESI/MS/MS system consisted of an API4000 triple quadrupole mass spectrometer with a TurboIonSpray interface (Applied Biosystems, Toronto, Canada) and a Shimadzu (Kyoto, Japan) liquid chromatograph. We used Luna C18 (Phenomenex, Torrance, CA, USA) columns (150 mm × 1 mm internal diameter, 5-µm particles). Solvents were A: H$_2$O with 1% (vol/vol) formic acid and B: 80:20 acetonitrile:H$_2$O plus 1% (vol/vol) formic acid. Peptides were eluted with a linear gradient of 0% to 80% solvent B in 25 min, at 50 µL/min. A parallel column format was used, giving a cycle time of 34 min. Tandem MS was performed by monitoring precursor to product transitions under individually optimized conditions, typically from the most abundant [M+nH]$^{n+}$ precursor ion to an ammonium ion. For BoNT-A, the N-terminal product 1699.9 m/z, triply charged ion (567.5 m/z) fragmenting to 84 m/z, was monitored. For BoNT-B, the doubly charged ion (880.7 m/z) fragmenting to 84 m/z was monitored. For BoNT-E, the C-terminal product (705.8 m/z), doubly charged ion 353.7 → 84.0 amu, was monitored. For BoNT-F, the triply charged ion (587.6 m/z) fragmenting to 84.1 m/z was monitored. All reaction runs and transitions were monitored for the entire 32-min run time. Isotopically labeled product peptides for BoNT-A (432 m/z → 70 m/z and 368.8 m/z → 70 m/z) were used as an internal standard for accurate quantification.

Results and Discussion

We developed a rapid, sensitive method for detecting and differentiating BoNT serotypes A, B, E, and F. Each BoNT serotype recognizes and cleaves a unique site on either SNAP-25 or VAMP-2 (Figure 1). We synthesized

the specific portions of SNAP-25 and VAMP that are substrates for the 4 BoNT serotypes that commonly cause human botulism (serotypes A, B, E, and F). We used the endopeptidase activity to detect and differentiate the specific BoNT serotype by allowing the BoNT to cleave its specific peptide substrate and detecting the cleavage products by mass spectrometry.

The substrate peptides were designed to be the same as the sequences of those portions of the natural SNAP-25 (for BoNT-A and -E) or VAMP (for BoNT-B and -F) that are recognized and cleaved, except some modifications were made for BoNT-A and -B. For BoNT-A, the peptide from SNAP-25 that includes serine-187 to glycine-206 is required for cleavage at glutamine-196. Schmidt et al. found that replacing lysines 189 and 201 with arginines showed enhanced cleavage by BoNT-A (14). We also found an increase in the amount of BoNT-dependent cleavage products detected in the modified peptides. The portion of VAMP-2 required for cleavage by BoNT-B at glutamine-75 is leucine-59 to lysine-93, and the portion required for cleavage by BoNT-F at glutamine-57 is from alanine-36 to serine-74. The portion of SNAP-25 from isoleucine-156 to aspartic acid-186 is required for cleavage between arginine-180 and isoleucine-181 by BoNT-E. We biotinylated the N and C termini of the substrate peptide for BoNT-A so that the product peptides of interest can be easily purified from complex matrices. After final peptide sequences are determined, we plan to biotinylate all substrate peptides.

The method was multiplexed by combining all 4 substrate peptides for the BoNT serotypes A, B, E, and F into a sample that contained various levels of a single BoNT serotype or no toxin. The expected product peptide masses and the masses of the substrate peptides are shown in Figure 2. The product peptides for each specific BoNT serotype can be easily distinguished by their mass. The online Appendix Figure (http://www.cdc.gov/ncidod/EID/vol11 no10/04-1279_app.htm) shows typical results for each of the reaction mixtures containing the 4 substrate peptides incubated with only the reaction buffer (a blank) or with 1 of the BoNT serotypes. Each of the BoNT serotypes yielded only the expected cleavage products from its respective substrate peptides, indicating that this method can easily detect and differentiate active BoNT serotypes. No cleavage was observed in the reactions that did not contain BoNT. Additionally, even at this relatively high toxin level, no cross-reactivity was seen between the toxin types; only the expected peptide cleavage reactions were observed.

We also tested sensitivity of the method for each single toxin serotype with a single substrate peptide. Since enzymatic reactions tend to be concentration dependent, this testing was accomplished in 2 ways. First, we determined the sensitivity on the basis of the lowest absolute amount

of toxin that could be detected by the Endopep-MS method in a 20-µL reaction; second, we determined the lowest concentration per milliliter that could be detected by this method. The first approach indicates the minimum amount of toxin that needs to be present, and the second indicates the minimum concentration of toxin in a sample. For BoNT serotypes A, B, and F as little as 0.01 MLD_{50} yielded sufficient quantities of product peptides to be clearly detected by MALDI-TOF-MS. This figure is $100\times$ lower in the absolute amount of toxin than that required by the mouse bioassay. For BoNT-E, product peptides could be detected by MALDI-TOF-MS with as little as 0.08 MLD_{50}. The analytic sensitivity of the method was then tested to determine the lowest measurable concentration of the toxin. An aliquot of a 1-mL sample that contained 100 MLD_{50} to 0.31 mouse LD_{50} in water was tested by both the MALDI-TOF-MS and HPLC-ESI/MS/MS methods. Reactions were also allowed to proceed for 4 h and for 16 h. The 4-h reactions for BoNT-A, -B, and -E showed the sensitivity by MALDI-TOF-MS detection of the product peptides of 1.2 mouse LD_{50}/mL for BoNT-A and -B and 6.2 mouse LD_{50}/mL for BoNT-E. After 16-h reactions, the sensitivity was 0.62 mouse LD_{50}/mL for BoNT-A and -B, 0.31 mouse LD_{50}/mL for BoNT-E, and 6.2 mouse LD_{50}/mL for BoNT-F. Using HPLC-ESI/MS/MS to analyze these same low toxin samples, we found that after 16-h incubation, the detection limits were 0.62 mouse LD_{50}/mL for BoNT-A and -B, <0.31 MLD_{50}/mL for BoNT-E, and 0.62 MLD_{50}/mL for BoNT-F. These data indicate that the Endopep-MS method is very sensitive with respect to the amount and concentration of toxin and that methods that can concentrate active toxins into smaller reaction volumes (thus yielding higher concentration) will result in lower limits of detection.

The HPLC-ESI/MS/MS technique to quantitatively detect and differentiate BoNT activities is highly selective. Correct identification of the BoNT product peptides depends on both a retention time match, with respect to standards, and on a chemical-specific fragmentation (a precursor to product ion multiple reaction monitoring [MRM] transition) monitored by tandem MS. To further enhance selectivity, 2 separate MRM transitions can be monitored for each peptide. In addition, our HPLC-ESI/MS/MS technique is very sensitive. Quantification of the BoNT product peptides is achieved by using stable isotope-labeled internal standards that have the same sequence as the native product peptides but are labeled with ^{13}C. Figure 3 shows typical HPLC-ESI/MS/MS chromatograms obtained during the quantification of the activity of BoNT-A, and a standard curve for both of the product peptides. The amount of product peptide was then correlated with the amount of toxin that yielded the product peptides. Standard curves between 10 and 1,000
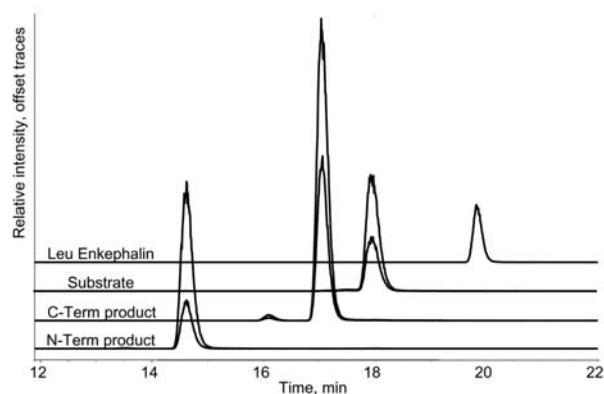
Figure 3. High-performance liquid chromatography-electrospray ionization-tandem mass spectrometry chromatogram showing the botulinum neurotoxin (BoNT)-A substrate and product ions (CT, C-terminal; NT, N-terminal) from a reaction with 25 mouse lethal dose (MLD)$_{50}$ BoNT-A. Each peptide has both a quantification ion (top trace) and a verification ion (lower trace). Isotopically labeled standards are added (traces not shown) as internal standards for quantification. The labeled peptides co-elute with their nonlabeled counterparts and are distinguishable by mass. Leucine enkephalin was included as a secondary reference compound and only 1 ion was monitored.

MLD$_{50}$ were prepared, and individual spiked samples were run as unknown samples to determine the accuracy and precision of the method. The spiked samples were prepared at 16, 32, 65, and 125 MLD$_{50}$; 2 samples were run in duplicate at each spike level giving a total of 4 measurements. The accuracy and precision of these measurements, shown in the Table, were good; relative standard deviations were <2%–5%. This method is the first that can accurately quantify BoNT enzymatic activity. Identical HPLC-ESI/MS/MS strategies can be used to quantify each of the BoNT serotypes.

The Endopep-MS method has many possible applications. Beyond using the Endopep-MS method for identifying the BoNT serotype in a clinical, food, or environmental sample, standardizing BoNT activity in samples used for clinical treatment or research activities may be possible. The standardization of BoNT, both the amount of 150-kDa toxin and the activity of a standard solution, is of great importance in the medical use of BoNT (16). A possible strategy for standardizing BoNT includes correlating the activity obtained by the mouse bioassay to the Endopep-MS method. Additionally, it may be possible to correlate this activity to an absolute amount of toxin that is determined in a similar fashion as was done for apolipoprotein A-1 (17).

Endopep-MS currently has limitations. Mass spectrometry equipment is expensive and requires a high level of technical expertise for optimal operation. The method still needs to be tested in a wide variety of clinical and food samples and may require the use of protease inhibitors and affinity chromatography to partially purify and concentrate the toxin. Also, the Endopep-MS method needs to be validated against the mouse bioassay. The method also has several strengths. It is rapid, and in simple matrices, such as water and buffer, it can obtain similar sensitivities to the mouse in <5 h. Samples can be prepared for the reactions in minutes; incubation times of only 4 h yield sensitivity close to the mouse bioassay, and MALDI-TOF mass spectra can be collected in <1 min. Samples can also be batched so that 50 samples can be analyzed in <6 h. The HPLC-ESI/MS/MS analysis is somewhat slower than the MALDI-TOF-MS, and each sample requires 30 min. However, this process has been automated, and batches of samples can run unattended so that 40 samples a day can be processed and run by HPLC-ESI/MS/MS. The method is well suited for multiplexing and can not only detect but also differentiate toxin type in a single analytic run.

Conclusions

We developed a method based on the unusually specific endopeptidase activity of BoNT that uses highly selective mass spectrometry analysis to detect and differentiate BoNT serotypes A, B, E and F. This method is rapid and sensitive. When the endopeptidase reactions are allowed to proceed for 16 h, it is highly sensitive; 100× more sensitive in absolute amounts of active toxin for BoNT-A, -B, and -F and >10× more sensitive than the mouse bioassay for BoNT-E. Since each BoNT serotype has a unique cleavage site on a substrate peptide, this analysis lends itself well to multiplexing. We multiplexed the analysis and showed that no cross-reactivity occurs within the 4 BoNT serotypes. The Endopep-MS method should be very useful to rapidly detect and differentiate active BoNT in a variety of clinical, food, or environmental samples to quickly establish the serotype(s) and aid in identifying a source during an outbreak. It may also prove useful for standardizing BoNT enzymatic activity for preparations used as clinical treatments or for research activities. This type of approach may also prove useful for detecting other proteolytic toxins.

Table. Accuracy of quantification of BoNT-A*

Spike level (mouse LD$_{50}$)	Measured concentration mean ±SD (N = 4)
16	19.85 ± 1.71
32	33.75 ± 3.24
65	68.05 ± 5.07
125	120.78 ± 2.66

*BoNT, botulinum neurotoxin; LD, lethal dose; SD, standard deviation.

Staff from Battelle Memorial Institute worked under contract at the Centers for Disease Control and Prevention, Atlanta, GA.

Dr Barr is chief of the Biological Mass Spectrometry Laboratory in the National Center for Environmental Health at the Centers for Disease Control and Prevention. His research interests include biologic monitoring, standardization of clinical measurements, and identification and quantification of chemical and biologic warfare agents with mass spectrometry.

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Methicillin-resistant
Staphylococcus aureus,
Western Australia

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Warren B. Grubb,* and Thomas V. Riley‡**

Methicillin-resistant *Staphylococcus aureus* (MRSA)
continues to be a notable cause of hospital-acquired infec-
tions. A statewide screening and control policy was imple-
mented in Western Australia (WA) after an outbreak of
epidemic MRSA in a Perth hospital in 1982. We report on
statutory notifications from1998 to 2002 and review the 20-
year period from 1983 to 2002. The rate of reporting of
community-associated Western Australia MRSA (WAMR-
SA) escalated from 1998 to 2002 but may have peaked in
2001. Several outbreaks were halted, but they resulted in
an increase in reports as a result of screening. A notable
increase in ciprofloxacin resistance during the study period
was observed as a result of more United Kingdom epidem-
ic MRSA (EMRSA) -15 and -16. WA has seen a persistent-
ly low incidence of multidrug-resistant MRSA because of
the screening and decolonization program. Non–multidrug-
resistant, community-associated WAMRSA strains have
not established in WA hospitals.

Recent publications suggest that the epidemiology of
methicillin-resistant *Staphylococcus aureus* (MRSA)
infections is changing, and hospitalization is no longer
necessarily a risk factor (1–3). Reports indicate that com-
munity-associated MRSA infection is now a worldwide
phenomenon. A common theme in these publications is
that the affected populations are usually marginalized,
indigenous peoples, such as American Indians in the mid-
western United States (4,5), Canadian aboriginals (6,7),
and Pacific Islanders in the southwestern Pacific region
(8,9). Aboriginal Australians also appear to be at higher
risk for community-associated MRSA (10). However,
overcrowding or situations where persons are in close

proximity to others, such as in prisons (11) and on sporting
teams (12), may represent the true risk. The increased
prevalence in children (13) may be due to the enhanced
opportunity for exposure at schools and daycare centers.

In the early 1980s, epidemic MRSA (EMRSA) first
appeared on the east coast of Australia; these strains were
often referred to as eastern Australian MRSA (14).
EMRSA were multidrug resistant, and they became
endemic in many large hospitals throughout Australia,
with the exception of Western Australia (WA) (15). The
establishment of EMRSA in WA hospitals has been pre-
vented because of a screening and control program (see
Methods) and the isolation of the state (14,16,17).
However, late in the 1980s, non–multidrug-resistant com-
munity-associated MRSA emerged in WA (14,18,19).
MRSA isolated from patients living in the remote
Kimberley region in the northern part of the state (Figure
1) were phenotypically and genotypically different from
EMRSA and became known as WAMRSA (18,19). Some
WAMRSA have since acquired a multidrug-resistance
plasmid that encodes resistance determinants, including
trimethoprim, tetracycline, and high-level mupirocin
resistance (14,18,19). During the 1990s, WAMRSA spread
to most regions of WA (14,18), and a substantial number of
cases of infection and colonization occurred in metropoli-
tan Perth by 1997 (20).

This retrospective review of statutory MRSA notifica-
tion data was conducted for the period 1998 to 2002. The
aim of the study was to report changes in reporting rates
over time and by location, to describe the distribution by
age and sex of patients, and to document temporal changes
in antimicrobial resistance patterns. The findings were
compared to those in previous publications that covered
MRSA notification data in WA for the period 1983 to 1996
(14,18,20).

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Australian Centre for Pathology & Medical Research, Nedlands,
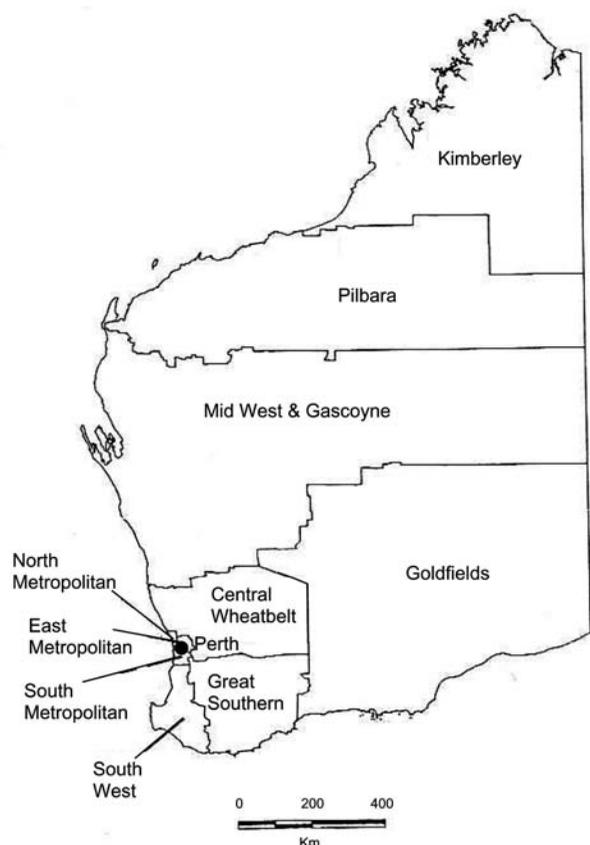Western Australia, Australia

Figure 1. Health regions of Western Australia.

Methods

Background

A statewide screening and control policy was implemented in WA after an outbreak of EMRSA in a Perth hospital in 1982 (16). The policy involved screening all patients who were admitted to hospitals from different states or overseas and all new staff members who had worked outside WA in the previous 12 months (17). After screening, patients infected or colonized with MRSA were isolated and treated; infected or colonized staff members were prohibited from contact with patients until the organism was eradicated. In WA, MRSA infection or colonization has been a reportable condition since 1985. The WA Department of Health electronically flags cases of MRSA, which allows infected persons to be identified and isolated upon admission to any WA public hospital (17).

Since the screening and control policy was introduced, the identity of MRSA clinical isolates and those isolated through screening has been confirmed by a reference laboratory by using standard procedures; antimicrobial drug susceptibility was determined by Clinical and Laboratory Standards Institute (formerly NCCLS) methods (21). Until 1997, the reference laboratory was in the Division of Microbiology and Infectious Diseases at the WA Centre for Pathology and Medical Research; thereafter it was in the Royal Perth Hospital/Curtin University Gram Positive Bacteria Typing and Research Unit.

From 1983 to 1997, MRSA was categorized as EMRSA or WAMRSA according to antimicrobial drug resistance patterns based on previous genetic analysis (14). EMRSA strains were resistant to β-lactam antimicrobial drugs, gentamicin, or both erythromycin and tetracycline. Strains resistant to β-lactams only, β-lactams and erythromycin, or tetracycline but not gentamicin were classified as WAMRSA. This approach has several limitations that have been alluded to in previous publications (14,18,20), such as changes in susceptibility pattern as a result of plasmid acquisition. Consequently, a more sophisticated system for differentiating isolates was developed at the Royal Perth Hospital laboratory that detected the introduction into WA of UK EMRSA-15 and Irish-2 strains of MRSA (22).

Data

Information on infection and colonization with MRSA was obtained from the database held by the Communicable Diseases Control Branch of the WA Department of Health. MRSA colonization was determined by screening patients, staff, and contacts by methods as described (17). Isolates recovered on screening and clinical isolates were sent to the Royal Perth Hospital laboratory for characterization by several procedures, including bacteriophage typing, routine antibiogram, urease production, extended antibiogram/resistogram, coagulase gene typing, and pulsed-field gel electrophoresis (23,24). Information collected with isolates included basic case demographics and details pertaining to the organism, including isolation site. In addition, whether the notification was the result of MRSA isolates found in a clinical specimen or from a screening specimen was recorded. Multiple cultures on the same patient were not included unless it had been determined that the patient was clear of MRSA colonization or infection after the process outlined (17).

Crude notification rates for health regions (Figure 1) were calculated with population estimates based on the 2001 census (25). Differences in proportions were compared by using the chi-square test, while changes over time were assessed by using chi square for trend.

Results

From 1998 to 2002, a total of 9,955 notifications of MRSA were made in WA; 1,441 notifications were made in 1998, 1,767 in 1999, 2,102 in 2000, 2,326 in 2001, and 2,319 in 2002. Table 1 shows the numbers of notifications and crude notification rates per 100,000 population of the various health regions of WA. Of the 9,955 notifications,

Table 1. Notifications of MRSA and rates per 100,000 population*

Location	No. notifications (rate)					Total no. MRSA
	1998	1999	2000	2001	2002	
Central Wheatbelt	13 (25.5)	28 (54.9)	34 (66.7)	37 (72.6)	41 (80.4)	153
Goldfields	112 (199.9)	110 (196.3)	83 (148.1)	91 (162.4)	56 (99.9)	452
Great Southern	60 (87.8)	55 (80.5)	43 (62.9)	87 (127.3)	91 (133.2)	336
Kimberley	107 (255.0)	97 (231.1)	140 (333.6)	112 (266.9)	115 (274.0)	571
East Metropolitan	251 (109.2)	348 (151.4)	415 (180.5)	529 (230.1)	446 (194.0)	1,989
North Metropolitan	175 (33.5)	248 (47.5)	324 (62.1)	384 (73.5)	384 (73.5)	1,515
South Metropolitan	508 (86.4)	610 (103.8)	769 (130.8)	745 (126.7)	830 (141.2)	3,462
Mid West & Gascoyne	79 (117.3)	98 (145.5)	116 (172.2)	126 (187.0)	120 (178.1)	539
Pilbara	47 (109.9)	44 (102.9)	47 (109.9)	38 (88.9)	52 (121.6)	228
South West	68 (37.4)	89 (48.9)	78 (42.9)	111 (61.0)	137 (75.3)	483
Other/Unknown	21	40	53	66	47	227
Total	1,441 (77.9)	1,767 (95.5)	2,102 (113.7)	2,326 (125.8)	2,319 (125.4)	9,995

*MRSA; methicillin-resistant *Staphylococcus aureus*.

9,728 gave permanent addresses within WA. The highest notification rates were recorded in the Kimberley region, followed by the East Metropolitan and Goldfields regions. The average yearly notification rate for the whole state during this period was 107.7/100,000 population. Figure 2 shows notifications of WAMRSA and EMRSA in WA from 1983 to 2002. This figure shows a marked increase in WAMRSA from 1991 to 2002 (peak), with a slowing in the notification rate after 2000. EMRSA peaked in 2001 and declined in 2002.

The distribution of MRSA by type is shown in Figure 3. In 1998, 6.4% of MRSA notifications were classified as EMRSA, increasing to 24.4% in 2002. The greatest contributor to EMRSA was UK EMRSA-15, which rose from 55 reports in 1998 to 383 in 2002. UK EMRSA-16 increased substantially from a few notifications in 2000–2001 to 66 notifications in 2002. Irish-2 notifications remained constant early in the 5-year period at ≈40 per year but fell to 29 in 2001 and 18 in 2002. Australian EMRSA was maintained at a variable but relatively low level, except in 2001 when 131 notifications occurred. Overall, 94% of community-associated WAMRSA were classified into 3 clones: ST1-MRSA-IV (55%), ST129-MRSA-IV (30%), and ST5-MRSA-IV (9%). Of the community-associated WAMRSA, 97% were staphylococcal chromosome cassette (SCC) *mec* type IV and 3% were SCC*mec* type V (unpub. data). During this period, Western Samoan Phage Pattern strains were isolated occasionally.

Some of the variability in notifications was related to outbreaks and subsequent contact screenings. Overall, 75% of notifications (7,913) were due to MRSA isolates found in routine clinical specimens; 25% (1,980) were due to a survey (Table 2). The annual proportion of isolates due to screening increased significantly during the 5-year period (from 10% in 1998 to 39% in 2002) (chi square for trend 35.696, p<0.0001). Of the 1,980 MRSA detected in screening specimens, 86.7% were WAMRSA. Of the 9,889 notifications over the 5-year period where the appropriate

information was provided, 93% were due to MRSA isolates found in patient specimens, however, 5% of notifications involved staff, and 2% were from other contacts (Table 3). The number of notifications due to a staff member who had MRSA increased from 73 in 2000 to 173 in 2001.

The distribution of MRSA cases from 1998 to 2002 by age and sex is shown in Figure 4. Notification rates peaked in age groups ≤9 years of age, 20–39 years of age, and 70–89 years of age. Overall, there was a 1:1.1 female-to-male ratio in notifications. In the 20- to 39-year age group, a predominance of female cases was reported; in the 60- to 79-year age group, male notifications predominated. In the >80-year age group, a 61.7% predominance of female notifications was seen; however, this figure was only slightly different than the 64.7% proportion of women in the WA population >80 years of age.

Data were collected on the susceptibility of MRSA isolates to various antimicrobial agents (Table 4). All isolates were susceptible to vancomycin, cotrimoxazole, and clindamycin. Most of the isolates were susceptible to mupirocin. Susceptibility to trimethoprim, tetracycline,
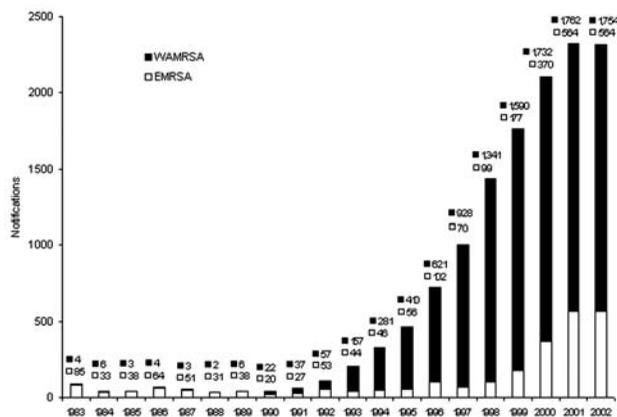

Figure 2. Notifications of methicillin-resistant *Staphylococcus aureus* (MRSA) in Western Australia (WA), 1983–2002, WAMRSA versus epidemic MRSA. Note: Not included are 4 in 2001 and 12 in 2002 of Western Samoan Phage Pattern.
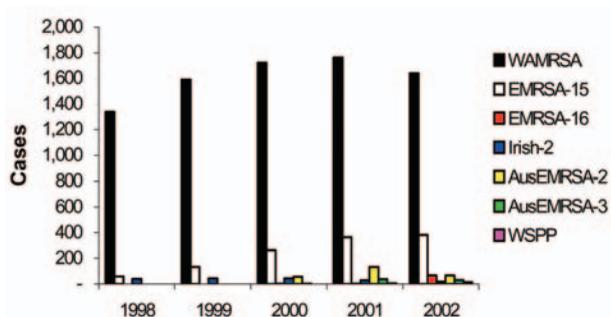
Figure 3. Methicillin-resistant *Staphylococcus aureus* (MRSA) in Western Australia, 1998–2002, by type.

and fusidic acid varied, although these antimicrobial drugs remained reasonably active throughout the data collection period. Approximately 40% of MRSA isolates remained susceptible to erythromycin. The only notable change was a significant increase in the resistance of ciprofloxacin, from 11% in 1998 to 26% in 2002 (chi square for linear trend 8.940, p = 0.002), consistent with the increase in numbers of UK EMRSA-15, UK EMRSA-16, and Irish-2 strains. The proportion of multidrug-resistant strains varied from 5.6% in 1998 to 10.4% in 2001.

Discussion

The increasing prevalence of community-associated MRSA is a global public health concern. In WA, colonization or infection with MRSA has been a reportable condition for >20 years, either voluntarily from 1983 to 1984 or by law since 1985. This time span has afforded a unique opportunity to document 2 important occurrences, 1) preventing EMRSA from becoming established in the hospital system and 2) emerging community-associated MRSA throughout WA.

The epidemiology of MRSA in WA has always differed from that in the rest of Australia because of the "search and destroy" policy (16,26,27) adopted in the early 1980s. In that decade, the proportion of *S. aureus* that was MRSA varied from 10% to 30% in states other than WA, while WA remained at 0.4% (17). After a relatively low number of MRSA notifications in the 1980s in WA, the number increased dramatically in the 1990s. This increase was due

almost exclusively to community-associated WAMRSA. The proportion of WAMRSA notifications after 1989 rose remarkably, increasing from 14% to 94% of total notifications in 1998. An almost exponential trend of MRSA notifications was evident, although a possible reporting bias may have occurred as a result of sporadic outbreaks at various times. As is evident from Figure 2, the epidemic of WAMRSA may have peaked; however, several more years of data are required to verify this. Although MRSA now causes 10% of *S. aureus* bacteremia in WA (28), the proportion is much lower than that seen in other Australian states (29). After 1998, the number of EMRSA notifications in WA started to increase after the introduction of UK EMRSA-15, in particular, and the Irish-2 strains (22).

Significant changes in proportions of WAMRSA isolates that occurred in the Perth metropolitan area were not noticeable until 1991, which suggests spread from the remote Kimberley region in the northern part to the southern half of the state. The Kimberley region has a total population of ≈30,000 in a 25,000-km^2 area. Approximately half the population is indigenous peoples, many of whom live in poor socioeconomic conditions. Infected skin lesions and staphylococcal sepsis occur frequently in this population, and empiric antistaphylococcal therapy is often prescribed (17). Some of the disease spread to the south may be attributed to transporting patients from this region to tertiary hospitals in Perth, particularly Royal Perth Hospital, a major trauma center in the eastern metropolitan area. In addition, a large population of "fly-in/fly-out" workers are employed in the area (14). Finally, traditionally indigenous populations in Australia are highly mobile.

The highest notification rates of MRSA continued to be in the Kimberley region throughout the study period, which suggested continued involvement of the aboriginal population. However, from 1998 to 2002, the second highest notification rate in the state was the East Metropolitan region of Perth. Historically, Royal Perth Hospital has had more MRSA outbreaks than other Perth hospitals (14,18), primarily because of the case-mix at Royal Perth Hospital; consequently, Royal Perth Hospital conducts more screening than other hospitals. Until 1997, WAMRSA had not caused outbreaks when patients who were infected were admitted to hospitals, however, during that year an outbreak of a fusidic acid–resistant WAMRSA occurred at Royal Perth Hospital after a patient from another remote rural location (30) was admitted. From 1983 to 2002, notification rates increased >50- and 70-fold in rural and metropolitan health regions, respectively.

In 1983, the overall rate of notifications in the rural regions was 10/100,000 compared with the metropolitan area rate of 7/100,000 (14). In 2002, notification rates in rural and metropolitan regions were 108 and 104 notifications per 100,000 persons, respectively. In rural regions,

Table 2. Notifications of MRSA from routine and survey specimens*

Year	Routine no. (%)	Survey no. (%)	Total
1998	1,308 (90)	131 (10)	1,439
1999	1,535 (85)	228 (15)	1,763
2000	1,756 (81)	338 (19)	2,094
2001	1,661 (62)	631 (38)	2,292
2002	1,653 (61)	652 (39)	2,305
Total	7,913 (75)	1,980 (25)	9,893

*Data were not recorded for 62 specimens. MRSA, methicillin-resistant *Staphylococcus aureus*.

Table 3. Notifications of MRSA in patients, staff members, and other contacts*

Type	1998	1999	2000	2001	2002	Total (%)
Patients	1,357	1,644	1,986	2,081	2,171	9,239 (93)
Staff	22	71	73	173	110	449 (5)
Other	46	43	36	52	24	201 (2)
Total	1,425	1,758	2,095	2,306	2,305	9,889

*Data were not recorded for 66 specimens; MRSA, methicillin-resistant *Staphylococcus aureus*.

the greatest increase in notification rates since 1983 occurred in the Pilbara, Mid West & Gascoyne, and Great Southern health regions with 56-, 48-, and 45-fold increases, respectively. In the metropolitan regions, the South, East, and North Metropolitan notification rates for 2002 were 50-, 23-, and 8-fold higher, respectively, than those reported in 1983.

From 1998 to 2002, 3 peaks in the age and sex distribution of notification rates were apparent: increases in the ≤9-, 20- to 39-, and 60- to 89-year age groups; male predominance in the 60- to 79-year age group; and female predominance in the 20- to 39-year age group. This female predominance was because the screenees were either nurses on staff or persons who were being screened for potential employment.

Since 1998, notifications as a result of screening increased 4-fold. If the screening tests were removed from the totals, male cases predominated in all age groups. This pattern of age and sex distribution has changed minimally since 1983 (14,20).

From 1998 to 2002, the proportion of MRSA notifications due to screening increased from 10% to 39%. Screening is still a controversial issue. A recent review concluded that screening of at least high-risk patients was necessary to reduce rates of MRSA infections in hospitals; however, further validation, from a variety of different institutions, of the cost-effectiveness of such programs was suggested and would be valuable (31). In WA in 1982, a unified approach to controlling multidrug-resistant MRSA was implemented. The approach was to screen all patients on admission who had been hospitalized or staff members who had worked in a hospital outside WA within the previous 12 months (17). Individual hospitals have varied in their approach to controlling non–multidrug-resistant MRSA, which is now endemic in the WA community, but not WA hospitals.

From 1983 to 1992, the agents for which a major change in susceptibility was observed were tetracycline (an increase from <10% to ≈30%), erythromycin (≈10% to ≈40%), and clindamycin (≈30% to ≈80%) (14). These changes reflected the increasing proportions of non–multidrug-resistant WAMRSA notifications. In 1993, 68% and 65% of WAMRSA were susceptible to tetracycline and erythromycin, respectively (8). The only significant

changes from 1994 to 1997 were that fusidic acid resistance increased from 4.6% to 12.4% (20), and mupirocin resistance decreased from 6.4% to 0.3% after an earlier high of 18% in 1993 (32). Udo et al. first reported high-level mupirocin resistance in WAMRSA strains. This resistance was encoded on a transferable plasmid, which also carried resistance determinants for tetracycline, trimethoprim, and cadmium toxicity (33). Mupirocin was used frequently in the northern part of the state to treat infected skin lesions, which resulted in the emergence, selection, and amplification of a mupirocin-resistant strain of WAMRSA. As a result, guidelines restricting the use of mupirocin were implemented; it was not to be used without laboratory control, its use should not exceed 10 days, and ≥1 month should elapse before further use for the same patient (32). After these measures were implemented, mupirocin resistance fell to levels <1% (32). This low level of resistance has been maintained for the last 5 years.

Resistance to fusidic acid in WAMRSA continues to be a concern. Resistance has gradually risen from 3% of MRSA notifications in 1993 to 5% in 1994, 9% in 1995, and 12% in 1997 (34). From 1998 to 2001, resistance to fusidic acid ranged from 11% to 13% of MRSA, with a slight fall to 8% in 2002. The emergence of fusidic acid resistance in WAMRSA paralleled the decline in mupirocin resistance; some practitioners may have replaced 1 topical antimicrobial drug with another after the guidelines were implemented. The emergence of ciprofloxacin resistance is also of concern. In 1998, 11% of all MRSA isolates were resistant to ciprofloxacin, increasing to 26% in 2002. This change reflects the introduction of UK EMRSA-15, UK EMRSA-16, and Irish-2 into the state (22). Ciprofloxacin has been suggested as a possible agent for MRSA decolonization (35) and has, at times, been recommended for this purpose by the WA Department of Health. This recommendation may need to be reviewed.
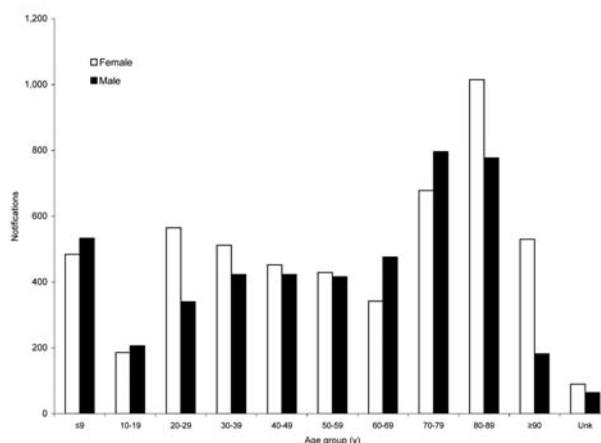

Figure 4. Notifications of methicillin-resistant *Staphylococcus aureus* in Western Australia, 1998–2002, by sex and age group.

Table 4. Antimicrobial susceptibility of MRSA isolates reported in Western Australia (% susceptible)*

Antimicrobial drug	1998 (1,440†)	1999 (1,058†)	2000 (311†)	2001 (2,326†)	2002 (2,316†)
Gentamicin	98	95	95	92	95
Fucidic acid	87	89	87	89	92
Erythromycin	40	43	39	39	40
Mupirocin	99	99	99	99	98
Vancomycin	100	100	100	100	100
Tetracycline	95	94	94	92	94
Rifampicin	99	100	100	99	99
Ciprofloxacin	89	84	76	76	74
Trimethoprim	93	90	93	90	93
Cotrimoxazole	100	100	100	100	100
Chloramphenicol	98	99	99	99	99
Clindamycin	100	100	100	100	100

*MRSA, methicillin-resistant *Staphylococcus aureus*.
†Number of organisms tested.

Endemic persistence of MRSA and the measures that should be undertaken to control or eradicate it from hospitals are likely to remain topical subjects. WA has successfully halted multidrug-resistant MRSA outbreaks. The state has seen a persistently low incidence of multidrug-resistant MRSA because a vigilant screening and decolonization program was implemented. During the last 5 years, growth of non–multidrug-resistant, community-associated WAMRSA has been exponential, and rural and metropolitan rates have apparently stabilized. However, new epidemic strains of MRSA, such as UK EMRSA-15, which were seen initially in 1999, steadily increased in 2000 and 2001. The infection control precautions instituted for patients infected with these strains were the same as those with multidrug-resistant MRSA. We do not know whether this approach will work or whether a similar dramatic rise in prevalence of these strains, as seen in UK hospitals recently, will occur (36).

Ms Dailey is a PhD student who is working on surveillance systems for healthcare-related infections. The work reported in this article was the basis of her MPH dissertation in 2003.

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Atypical Infections in Tsunami Survivors

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After a tsunami hit Asia in December 2004, 2 survivors had severe infections due to multidrug-resistant and atypical bacteria and rare fungi weeks afterwards. Treating these infections is challenging from a clinical and microbiologic point of view.

After a tsunami hit a large part of Southeast Asia in December 2004, >200,000 people died and several hundred thousand were severely injured. First aid was provided in local hospitals under difficult conditions. Most patients had multiple fractures, soft-tissue injuries, and complications from near-drowning events (1,2). Acute complications did not pose diagnostic problems; after emergency situations were resolved, patients were seen in other healthcare facilities, and foreign tourists were repatriated. Two Swiss tourists were treated in Thailand and then transferred to our hospital. Severe infections that were caused by multidrug-resistant bacteria and, subsequently, unusual fungal and mycobacterial infections developed in both of the patients.

The Study

Case 1

A previously healthy, 59-year-old man was treated in Thailand for aspiration pneumonia complicated by multiorgan failure and septic shock, necessitating mechanical ventilation and hemodialysis. *Acinetobacter baumannii* (resistant to all penicillins, cephalosporins, aminoglycosides, and trimethoprim-sulfamethoxazole) and *Escherichia coli* were found in cultures from bronchoalveolar lavage performed 48 h after a near-drowning episode and were caused by massive bronchoaspiration. Both bacteria strains were sensitive to imipenem-cilastatin and ciprofloxacin. Because of persistent fever and cough after 2 weeks of treatment with ciprofloxacin and imipenem-cilastatin, a computed tomographic scan of the chest was performed; an abscess was seen in the left lung. Culture of the abscess yielded an *Acinetobacter* sp. that was resistant to imipenem-cilastatin; drug was changed to ampicillin-sulbactam.

Three weeks later, the patient was transferred to our hospital because he had several pulmonary abscesses and an empyema that required repetitive drainage. A lobectomy of the left necrotic lobe was performed. After 6 weeks of treatment with piperacillin-tazobactam, the patient was discharged from the hospital. One month later, the patient returned to the hospital with back pain with no previous spinal pathology. Paravertebral collection showed spondylodiscitis (T8–T9), caused by *Scedosporium apiospermum*. After surgical drainage, the spine was immobilized with an external corset, and treatment with voriconazole (200 mg IV twice daily) was started. The patient progressed well clinically and had no neurologic complications.

Case 2

A previously healthy, 51-year-old woman with deep cutaneous wounds of the legs, multiple pelvic fractures, and a ruptured bladder lay immobilized in mud for >24 h. At the local hospital, after her hemodynamic status was stabilized, a laparotomy confirmed the bladder injury. Surgical debridement of the patient's wounds was performed. The patient was repatriated and admitted to our hospital on December 31. The soft-tissue wounds were infected with multidrug-resistant bacteria: *A. baumannii* (resistant to all penicillins, cephalosporins, aminoglycosides, fluoroquinolones, and trimethoprim-sulfamethoxazole; sensitive only to colistin); *Stenotrophomonas maltophilia* (sensitive only to piperacillin-tazobactam); and *Achromobacter xylosoxidans* (sensitive to piperacillin-tazobactam, imipenem-cilastatin, ciprofloxacin). Penicillin-resistant *Enterococcus faecium* and *Pseudomonas aeruginosa* were also seen in the cultures. The wounds that were colonized by multidrug-resistant bacteria were treated with aggressive surgical debridement and local instillation of colistin. A computed tomographic scan of the chest was performed because dyspnea developed. The scan showed bilateral infiltration, and pneumonia was confirmed. To minimize further selection pressure by antimicrobial drugs and treat the concomitant pneumonia caused by *Pseudomonas* sp., targeted therapy with piperacillin-tazobactam was administered for 14 days. Pelvic open fractures were in direct contact with urine. The bladder injury was repaired surgically, and urine specimens showed *A. baumannii* (sensitive only to colistin, which was used for bladder irrigation) and *E. faecium* when cultured. Because the pelvic open fractures were in contact with infected urine and stable, orthopedic surgeons decided that bed rest was the treatment of choice. The pelvic fractures healed without sequelae. However, an abscess developed on the patient's thigh 2 weeks after admission. *Nocardia africanum* was cultured from the samples taken

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at the time of intervention. The patient was treated with trimethoprim-sulfamethoxazole for 10 weeks.

On week 8 of hospitalization, persistent fever and progressive alteration of consciousness developed in the patient. A computed tomographic scan showed an intracerebral abscess with hydrocephalus (Figure). The abscess was drained surgically, and a ventricular-peritoneal drain to treat aresorptive hydrocephalus was subsequently put in place. The abscess cultures showed *S. apiospermum*, and voriconazole (4 mg/kg IV twice a day) was started. The clinical course was slow but favorable. Magnetic resonance imaging performed after 3 months of voriconazole treatment showed a reduction in the dimensions of the abscess. Voriconazole treatment was scheduled for 6 months or until resolution of the cerebral lesion.

Three months after the tsunami, the patient still had a residual open wound on the tibial area of the leg. Because of healing difficulties, even with antimicrobial drug treatment, specialized tests were conducted on the wound for a resistant or atypical pathogen. Special cultures for mycobacteria permitted the growth of *Mycobacterium chelonae*, which was sensitive to amikacin and clarithromycin and resistant to imipenem-cilastatin, fluoroquinolones, and trimethoprim-sulfamethoxazole. Magnetic resonance imaging results excluded osteomyelitis. Treatment with clarithromycin was initiated and surgical debridement was accomplished.

Conclusions

These 2 patients had unusual and severe lesions, pathogens that were difficult to treat, and complications that could be encountered in tsunami survivors. The situation constitutes an exceptional event, and several factors put the survivors at risk in the short- and the long-term. In the first days after the event, survivors were likely to have bacterial complications of soft tissue and bone injuries and aspiration pneumonia. Some case reports that were recently published described less frequent infections, such as cutaneous mucormycosis (3) or unusual pathogens, such as *Bacillus pseudomallei* (2), outside the affected region.

To observe multiple infections in the same patient caused by several different multidrug-resistant bacteria is unusual in clinical practice. Treatment is complex, and additionally, using broad spectrum antimicrobial drug therapy in patients with high bacteria count may lead to resistance. In these 2 patients, incorporating optimal antimicrobial drug therapy to treat all isolated germs was very difficult. Aggressive surgical intervention was essential to ensure the efficacy of treatment.

Most of the tsunami survivors who experienced near-drowning events remained in unclean and traumatic conditions without receiving any immediate medical care (4) for several hours. Near-drowning is a rare event; therefore, experience is limited in dealing with the resulting complications (5). Tepid, salty, and brackish water was inhaled and ingested. Patients lay for several hours or days in warm, stagnant water and slush; normally poorly virulent environmental bacteria, fungi, and amoebae found the ideal conditions to colonize in open wounds and bone fractures and disseminate to other body sites.

S. apiospermum is a ubiquitous saprophytic fungus that rarely causes invasive infections in an immunocompetent host. In addition to anatomic barrier alterations, such as burns, trauma, or neurosurgery, near-drowning events promote favorable conditions for elevated numbers of fungal infections. The potent activity of voriconazole against this fungus and its availability, with good penetration of the hematoencephalic barrier, may increase the chance of recovery and survival. Otherwise, the prognosis is poor (6). Both patients reported here have shown good clinical response to treatment.

The second patient had a cutaneous infection with *M. chelonae*, a rapidly growing mycobacteria that is ubiquitous in soil and water worldwide. Generalized infections are seen mainly in immunosuppressed patients; however, isolated cutaneous infections have been reported in immunocompetent patients. The diagnosis may be difficult because of the necessity of obtaining specific mycobacterial cultures. In addition, the treatment may be complex, as *M. chelonae* is among the most resistant mycobacteria, and adaptation of therapy according to sensitivity tests is mandatory (7).

A number of conditions found in tsunami survivors could render infection treatment extremely difficult. These conditions include the large number of relatively rare environmental pathogens that result from particularly traumatic exposure; extensive soft tissue and internal injuries; the

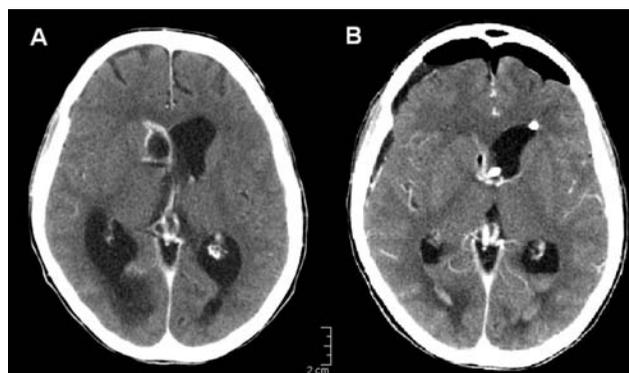Figure. Brain abscess caused by *Scedosporium apiospermum* (patient 2). A) Images from contrast-enhanced computed tomographic scan show a ring-enhancing lesion in the head of the nucleus caudatus (2 × 1.5 cm) bulging in the right lateral ventricle with concomitant aresorptive hydrocephalus. B) Control computed tomographic scan after surgical drainage and placement of ventricle-peritoneal drainage.

possible presence of multidrug-resistant bacteria, atypical bacteria, and fungal infections; and initial treatment administered with limited resources and under difficult emergency conditions. All these factors contribute to the severity of complications and difficulties in treating infections in tsunami survivors. In addition, an optimal infection control policy is required in managing these patients to prevent the spread of imported nosocomial infections (8).

This article aims to raise awareness about the possibility of unusual complications and highly-resistant microorganisms that can lead to extensive illness and death in tsunami survivors. Most clinicians are probably unfamiliar with the pathogens that would be found under these conditions. Therefore, every tsunami survivor should be considered a high-risk patient, even months after the event. The severe and atypical infections they may have pose challenges for diagnosis and treatment, even for experienced infectious disease specialists.

Acknowledgments

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Genetic Characterization of Nipah Virus, Bangladesh, 2004

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Until 2004, identification of Nipah virus (NV)-like outbreaks in Bangladesh was based on serology. We describe the genetic characterization of a new strain of NV isolated during outbreaks in Bangladesh (NV-B) in 2004, which confirms that NV was the etiologic agent responsible for these outbreaks.

Nipah virus (NV) and Hendra virus (HV), the only members of the genus *Henipavirus* within the family *Paramyxoviridae* are different from most other paramyxoviruses because they have a broad host range in vivo and in vitro. Although HV and NV have genetic characteristics and replication strategies similar to those of other paramyxoviruses, the henipaviruses have several unique genetic features (1,2–4).

The first outbreak of NV occurred between late 1998 and early 1999 in peninsular Malaysia and Singapore and was associated with respiratory disease in swine and acute and febrile encephalitis in humans. Direct contact with sick pigs was the primary source of human infection (1). Of 265 human cases, 108 were fatal. Although NV is excreted in respiratory secretions and urine of patients (1,5), a survey of healthcare workers in Malaysia showed no evidence of human-to-human transmission (6). The reservoir of NV is presumed to be fruit bats, primarily of the genus *Pteropus* (7,8), and humans are infected through intermediate hosts such as pigs.

Recently, NV has been established as the cause of fatal, febrile encephalitis in human patients in Bangladesh during the winters of 2001, 2003, and 2004 (9–12). An NV-like virus was identified as the cause of the outbreaks in 2001 and 2003 on the basis of serologic testing (12). Two outbreaks consisting of 48 cases of NV were detected in

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2004 in 2 adjacent districts (30 km apart) of central Bangladesh (Rajbari and Faridpur) with a case-fatality rate of nearly 75%. Because of heightened surveillance, other small clusters and isolated cases (n = 19) were identified during the same period in 7 other districts in central and northwest Bangladesh. Although antibodies to NV were detected in fruit bats from the affected areas in 2004, an intermediate animal host was not identified, which suggests that the virus was transmitted from bats to humans. Human-to-human transmission of NV was also documented during the Faridpur outbreak (10,11). We describe the genetic characteristics of 4 NV isolates from the outbreak in Bangladesh in 2004.

The Study

Virus isolation was performed in the BSL-4 laboratory at the Centers for Disease Control and Prevention in Atlanta. Vero E6 cells were inoculated and observed for characteristic cytopathic effect, syncytium formation (1,5). NV was isolated from 2 oropharyngeal swabs (SPB200401066, SPB200406506), 1 cerebrospinal fluid (SPB200401617), and 1 urine specimen (SPB200405758) from human patients, and isolation was confirmed by reverse transcription polymerase chain reaction (RT-PCR). Two isolates were from Rajbari, and 1 was from Faridpur; the fourth isolate, from the Rajshahi district (100 km from Rajbari), was not linked to the other 2 outbreaks. The complete genomic sequence of the first viral isolate (SPB200401066) from Rajbari was derived and submitted to GenBank (accession no. AY988601) as NV-Bangladesh (NV-B). The sequences of the open reading frame (ORF) coding for nucleoprotein (N) were obtained for the other 3 isolates. The methods used for RT-PCR, sequencing, cDNA cloning, rapid amplification of cDNA ends (RACE), and sequence analysis were previously described (2,3).

The genome of NV-B is 18,252 nt in length, 6 nt longer than NV-Malaysia (NV-M), the prototype strain of NV (SPB199901924). The additional 6 nt map to the 5′ nontranslated region of the fusion protein (F) gene. The length of the NV-B genome is evenly divisible by 6, suggesting that NV-B follows the "rule of six" (3). The gene order and sizes of all the ORFs except V are conserved between NV-B and NV-M (Table, Figure, A). The overall nucleotide homology between the genomes of NV-B and NV-M is 91.8%, but the changes are not uniformly distributed throughout the genome. Nucleotide homologies are higher in the protein coding regions than in the noncoding regions, although the sizes of the nontranslated regions remain highly conserved (Table). The predicted amino acid homologies between the proteins expressed by NV-M and NV-B are all >92% (Table).

Overall, the predicted amino acid homologies of the surface glycoproteins, F and G, of NV-B and NV-M are

Table. Comparison of gene sequences NV-Malaysia and NV-Bangladesh*

Gene	Virus	Open reading frame			5′ nontranslated		3′ nontranslated	
		Length†	% amino acid identity‡	% nucleotide homology‡	Length§	% nucleotide homology‡	Length§	% nucleotide homology‡
N	Malay	532	98.3	94.3	57	100	586	90.8
	Bang	532			57		586	
P	Malay	709	92.0	92.0	105	91.4	469	88.1
	Bang	709			105		469	
V	Malay	52	92.5	95.7				
	Bang	55						
W	Malay	47	100	98.5				
	Bang	47						
C	Malay	166	95.2	97.6				
	Bang	166						
M	Malay	352	98.9	93.4	100	86.0	200	83.5
	Bang	352			100		200	
F	Malay	546	98.4	93.4	284	83.1	412	79.4
	Bang	546			290		412	
G	Malay	602	95.5	93.0	233	75.5	504	80.8
	Bang	602			233		504	
L	Malay	2,244	98.2	93.4	153	82.4	67	80.6
	Bang	2,244			153		67	

*NV, Nipah virus.
†Length in amino acids.
‡Percentage amino acid identity or nucleotide homology after sequences were aligned by using GAP from GCG.
§Length in nucleotides. NV-Malaysia gene lengths and 5′ and 3′ nontranslated sequences and gene lengths were obtained from GenBank accession no. AF212302. The percentage amino acid identity does not take into consideration conserved amino acid changes.

high (Table). In the F protein, the predicted cleavage site, F1 amino-terminal domain, transmembrane domain, and predicted N-glycosylation sites are identical in NV-B and NV-M (2). Four of the 9 predicted amino acid changes occur in the first 11 amino acids (aa) of the precursor of the F protein, F0, which fall within the predicted signal peptide and would be cleaved from the mature protein. Within the G proteins, the predicted transmembrane domains, and the positions of all 17 cysteine residues are conserved between NV-B and NV-M. Of 8 predicted N-linked glycosylation sites in the G protein of NV-M, 6 are conserved in NV-B and in HV (2).

The coding strategy of the P gene is identical in NV-B and NV-M. In these viruses, the P gene contains the C, V, and W ORFs in addition to the P ORF. Like most other paramyxoviruses, the henipaviruses have a conserved AG-rich region that acts as an editing site to facilitate the addition of nontemplated G residues into the transcripts of the P gene. The edited transcripts encode 2 proteins, V and W, which are co-amino-terminal with P but have unique carboxy termini (2). The addition of 1 G residue generates the mRNA for the V protein, and the addition of 2 G residues produces the mRNA for the W protein. Sequence analysis of multiple cDNA clones containing the editing site of NV-B identified edited transcripts that encoded both the V and W proteins (data not shown). The conserved 20-nt region encompassing the editing site is identical in NV-M, HV, and measles virus (MV) (2); however, the editing of site of NV-M (UGGGUAAUUUUUCCCGUGUC) differs from

NV-B (GGGAUAAUUUUUCCCGUGUC) at 2 nt positions (underlined). The functional significance of these 2 substitutions is under investigation. All of the cysteine residues are conserved in the V proteins of NV-B and NV-M; however, the unique portion of the V protein of NV-B is predicted to be 55 aa, 3 aa longer than the V protein of NV-M. The predicted W protein of NV-B is identical in size and sequence to the W protein of NV-M. Recently, aa 100–160 and 230–237 of the V protein of NV-M have been identified as necessary for inhibition of interferon signaling (14). These regions are highly conserved in the V protein of NV-B, which has 4 predicted amino acids substitutions (1 conservative) between positions 100–160 and no predicted substitutions between positions 230–237. In addition, the V protein of NV-B has 3 predicted amino acid substitutions in the CRM1-dependent nuclear export signal that was identified between aa 174–193 in the V protein of NV-M (14). The biologic effects of these amino acid substitutions are under investigation.

The L proteins of NV-B and NV-M had a high level of predicted amino acid conservation (Table). The 6 highly conserved domains of viral polymerases, originally described by Poch et al. (15) and delineated for NV-M by Harcourt et al (3), remain largely unchanged between NV-B and NV-M. Domains 1, 2, and 5 have 1 conservative amino acid change each and domain 3, which is considered the most conserved domain within the L proteins of paramyxoviruses, has 2 aa changes. The 4 motifs identified in domain 3, including the QGDNE motif, which is assumed

to be the active site of the polymerase, are identical between the NV-B and NV-M, as is the predicted nucleotide-binding motif in domain 6.
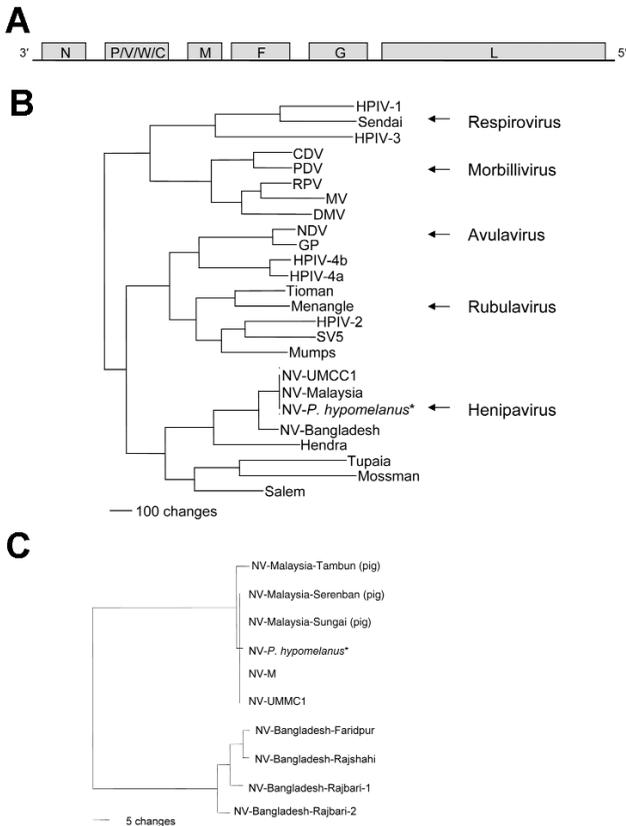
A

B

— 100 changes

C

— 5 changes

Figure. A) Schematic representation of the genome of Nipah virus (NV). Negative-sense genomic RNA is shown in 3′ to 5′ orientation. Open reading frames (ORFs) are indicated by shaded boxes: N, nucleoprotein; P, phosphoprotein; M, matrix protein; F, fusion protein; G, attachment protein; L, polymerase protein. B) Phylogenetic analysis of the N ORFs from members of the subfamily *Paramyxovirinae*. Arrows identify the 5 genera. A phenogram of the N ORFs of members of this subfamily was created by using maximum parsimony analysis with PAUP 4.02 (Sinauer Associates, Sunderland, MA, USA). Abbreviations and accession numbers: HPIV-1, human parainfluenza virus, D01070; Sendai, X00087; HPIV-3, D10025; CDV, canine distemper virus, AF014953; PDV, phocine distemper virus, X75717; RPV, Rinderpest virus, X68311; MV, K01711; DMV, dolphin Morbillivirus, X75961; NDV, Newcastle disease virus, AF064091; GP, goose paramyxovirus, AF473851; HPIV-4b, M32983; HPIV-4a, M32982; Tioman, AF298895; Menangle, AF326114; HPIV-2, M55320; Simian virus 5 (SV5), M81442; Mumps, D86172; NV-UMCC1, AY029767; NV-Malaysia, AF212302; NV-*P. hypomelanus*,* AF376747; NV-Bangladesh; Hendra virus, AF017149; Tupaia paramyxovirus, AF079780; Mossman virus, AY286409; and Salem virus, AF237881. C) The phylogenetic relationship between the N gene sequences of the 4 human NV isolates from the Bangladesh outbreak in 2004 and the N gene sequences from pig and human NV isolates from Malaysia. Accession numbers for the pig isolates of NV (13) are AJ627196, AJ564622, and AJ564621. *NV-*P. hypomelanus* is sequence from a virus isolated from *Pteropus hypomelanus*, the Island Flying Fox (8).

The cis-acting control sequences are highly conserved in the genomes of NV-B and NV-M. As in NV-M, the intergenic sequences in NV-B are GAA, with the exception of the sequence between the G and L genes, UAA, which is unique among the henipaviruses. However, the intergenic sequence between the G and L genes is GAA in the second isolate from Bangladesh. The transcriptional start and stop signals of each gene of NV-B are highly conserved in relation to the other henipaviruses. The 3′ leader sequence of NV-B is identical in length to those of all other paramyxoviruses and has nucleotide changes at positions 14 and 47 compared to NV-M. The 5′ trailer of NV-B is identical in length and sequence to NV-M.

Phylogenetic analysis was used to compare the sequence of the N ORF of NV-B to the sequences of the N ORFs from other members of the subfamily *Paramyxovirinae*. The results confirmed the results of the sequence comparisons, which show that NV-B is most closely related to the henipavirus NV-M, and support the conclusion that NV-B should be regarded as new strain of NV (Figure, B). Phylogenetic analyses conducted with the sequences of the other genes produced similar results (data not shown). The sequences of the N ORFs of 4 NV isolates from Bangladesh share 99.1% nt homology (Figure, C) but exhibited more interstrain nucleotide heterogeneity than the sequences of the human isolates in Malaysia, which were nearly identical (1,8,13). These varying amounts of genetic variability may reflect differences in the mode of transmission of NV in the 2 countries. In Malaysia, molecular evidence suggests that at least 2 introductions of NV into pigs occurred (Figure, C). However, the nearly identical sequences of human and pig isolates from the later phase of the outbreak suggest that only 1 of the variants spread rapidly in pigs and was associated with most human cases (13). In contrast, the sequence heterogeneity observed in Bangladesh may be the result of multiple introductions of NV into humans from different colonies of fruit bats.

Conclusions

This first look at strain variation in NV indicates that viruses circulating in different areas have unique genetic signatures and suggests that these strains may have coevolved within the local natural reservoirs. Until 2004, identification of NV outbreaks in Bangladesh had been based only on serologic testing. The isolation and genetic characterization of NV-B confirm that NV was the etiologic agent responsible for these outbreaks.

Note: After this article was accepted for publication, Nipah virus was isolated from *Pteropus lylei* in Cambodia (16). Phylogenetic analysis of the N gene sequences demonstrated that this virus is more closely related to

Nipah-Malaysia than to Nipah-Bangladesh and represented another lineage of Nipah virus.

Dr Harcourt is a research associate in the Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention. His research interests include viral pathogenesis and emerging viral diseases.

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Diphyllobothriasis, Brazil

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Cases of human diphyllobothriasis have been reported worldwide. Only 1 case in Brazil was diagnosed by our institution from January 1998 to December 2003. By comparison, 18 cases were diagnosed from March 2004 to January 2005. All patients who became infected ate raw fish in sushi or sashimi.

Diphyllobothriasis is an intestinal parasitosis acquired by eating raw or partially cooked fish containing *Diphyllobothrium* spp. plerocercoids. Most persons are asymptomatic, but diarrhea, abdominal pain, or discomfort occurs in $\leq22\%$ of infections. Prolonged or heavy *Diphyllobothrium latum* infection may cause megaloblastic anemia due to parasite-mediated dissociation of the vitamin B_{12}–intrinsic factor complex within the gut lumen, making B_{12} unavailable to the host (1).

Human diphyllobothriasis has been reported in Europe, Asia, North America, and South America. South American has reported cases from Peru, Chile, and Argentina, but not Brazil (2–7). South American diphyllobothriasis is an ancient disease; *D. pacificum* eggs were found in coprolites, 4,000- to 5,000-year-old Chinchorro Chilean mummies (8).

Four recognized species, *D. latum*, *D. pacificum*, *D. klebanovskii*, and *D. nihonkaiense*, infect humans; many species infect fish-eating birds, dogs, foxes, and bears (2,5). Species identification is relevant because *D. pacificum* infects only saltwater fish. *D. latum* infects only freshwater fish or species that spend part of their life in fresh water. Only *D. latum* and *D. pacificum* have been found in humans in South America; other species of *Diphyllobothrium* have been found in freshwater fish from Chile and Argentina (9,10).

The Study

Since diphyllobothriasis was a rare disease in Brazil, 5 cases diagnosed from March to August 2004 were of inter-

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est. At our São Paulo institution, $\approx36,000$ stool specimens are examined for ova and parasites annually. Since 1998, no changes in personnel or protocols used for stool examination have occurred.

A database was searched for the period from January 1998 to December 2003 to determine the number of our patients diagnosed with *Diphyllobothrium* infection. From September 2004 to January 2005, stool specimens of patients who ate raw fish were examined to determine the prevalence of diphyllobothriasis. Patients >15 years of age were asked if they had eaten raw fish in the past 2 months. All patients, except those with *Diphyllobothrium* eggs in their stools, were asked if they had been sick, if they had eaten raw fish, the species of fish eaten, and if they had traveled outside Brazil in the last 5 years. When available, hemoglobin and mean corpuscular volume samples were evaluated to exclude megaloblastic anemia.

Ten eggs were randomly sampled from *Diphyllobothrium* spp.–positive stool specimens from 4 randomly chosen patients; the length and width of the eggs were recorded. Strobilas obtained from 2 specimens were evaluated by scanning electronic microscopic studies (11). Fragments of strobila were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylineosin. Uterine morphology was evaluated in fresh preparations of proglottids.

The database search found 1 case of diphyllobothriasis. Comparatively, 5 cases were diagnosed from March to August 2004. To determine the prevalence of diphyllobothriasis, we added a standard question about eating raw fish to our admission protocol from September 2004 to January 2005. During this period, we examined fecal specimens of 8,463 patients ≥15 years of age. Among those patients, 623 refused to admit eating raw fish, 5,335 denied eating raw fish, and 2,505 stated they did eat raw fish. Thirteen cases, 5.19/1,000, of diphyllobothriasis were found in the patients who ate raw fish. The infected patients were from 16 to 59 years of age with a mean age of 33 years. None of the patients who denied eating raw fish had *Diphyllobothrium* eggs in their specimens. The most frequently reported symptoms were abdominal discomfort and intermittent diarrhea (83.3%). Twenty-two percent of the patients eliminated the parasite; 16.7% were asymptomatic. Mean corpuscular volume was 81.l–93.9 fL and hemoglobin was 12.2–16.8g/dL in 8 patients. All values were within the normal range. Seven of the 18 patients had not traveled outside Brazil in the last 5 years; 2 of them had never traveled outside Brazil. Eggs observed in stool samples had the characteristic shape observed in *Diphyllobothrium* spp. (Figure, A). The average length was 64–71 μm, and the average width was 48–51 μm.

Of the 4 adult worm specimens (strobila fragments) available for analysis, the largest fragment was 900 mm
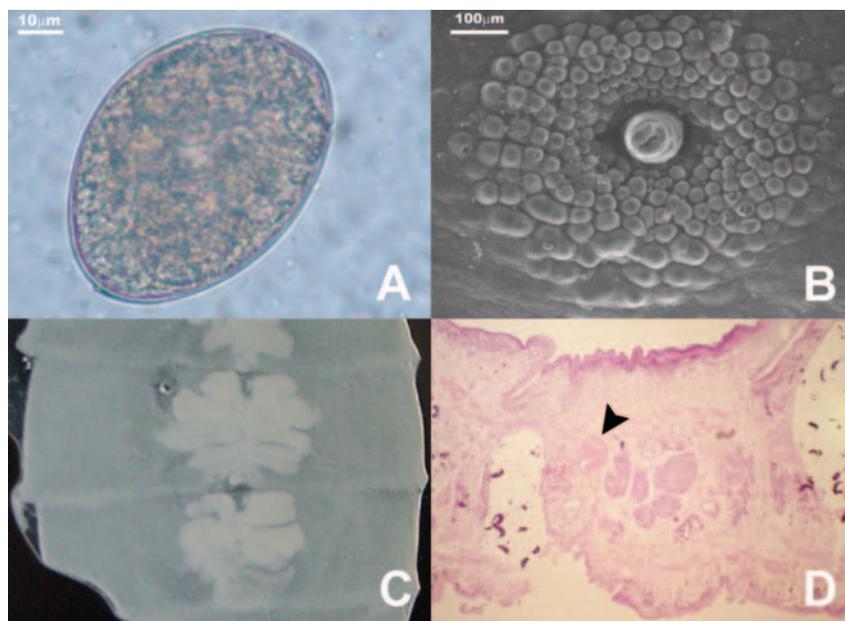
Figure. A) *Diphyllobothrium latum* egg. Note opercular constriction. B) Genital papillae of mature proglottids as seen under scanning electron microscope. C) Uterine loops of gravid proglottids in fresh preparation. D) Sagittal section of the genital pore region stained with hematoxylin-eosin. Note seminal vesicle (arrowhead) situated dorsocaudal to the cirrus sac (magnification ×100).

long by 7 mm wide with an average proglottid length of 2.5 mm. Scanning electronic microscopic studies showed circular and conspicuous genital papillae at the upper third of proglottids (Figure, B). Uteri from gravid proglottids formed a rosette with 4–5 loops (Figure, C). Sagittal sections of adult worms showed the cirrus sac lying horizontally with the seminal vesicle lying dorsocaudal to it. A marked constriction between segments was noted (Figure, D).

The presence of genital papillae showed that the specimens from Brazilian patients were similar to those in published reports for *D. latum* (11). Sagittal sections of the genital pore region of worms in Brazilian patients showed the cirrus sac lying horizontally with the seminal vesicle lying dorsocaudal to it, as described by Dick et al. (12). Our findings exclude *D. pacificum* since uteri from gravid proglottid of worms in Brazilian patients formed a rosette, which was not observed in *D. pacificum*. The largest reported *D. pacificum* eggs (40 µm wide and 60 µm long) (13) were smaller than the smallest eggs (48 µm wide and 64 µm long) in our study. Although egg dimension can not be used as a single criterion for species identification, the values for *D. latum* recorded in our study agree with Andersen et al. (14).

Based on the genital papillae, position of the cirrus sac, shape of the uterus, and egg dimensions, we tentatively identify the specimens as *D. latum* and implicate this species as the source of human infection in São Paulo. All infected patients ate raw fresh Atlantic salmon and some ate a local fish, *Centropomus undecimalis*, in sushi or sashimi. *C. undecimalis* has not been reported as a *D. latum* host, but it is a saltwater fish that spends part of its life in fresh water.

Conclusions

Since the Brazilian climate prevents Atlantic salmon farming, it is imported from Chile, where diphyllobothriasis is endemic in some regions (15). The 18 infections we describe were acquired in Brazil because 2 patients had never traveled outside the country. Imported salmon may be the source of *D. latum* plerocercoids infections; another possibility is that the life cycle of *D. latum* is established in São Paulo coastal waters and rivers. If so, *C. undecimalis*, used in sushi and sashimi, could be a source of infection.

Epidemiologic investigations are being conducted in São Paulo that will identify the source of *D. latum* plerocercoids and help implement educational and sanitary measures and prevent diphyllobothriasis from becoming endemic in Brazil.

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Poultry-handling Practices during Avian Influenza Outbreak, Thailand

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With poultry outbreaks of avian influenza H5N1 continuing in Thailand, preventing human infection remains a priority. We surveyed residents of rural Thailand regarding avian influenza knowledge, attitudes, and practices. Results suggest that public education campaigns have been effective in reaching those at greatest risk, although some high-risk behavior continues.

Since January 2004, Thailand and ≥8 other Southeast Asian countries have experienced outbreaks of avian influenza H5N1 in poultry and >100 million poultry have been culled or died. From January 28, 2004, to February 2, 2005, Cambodia, Thailand, and Vietnam reported 55 human cases, including 42 deaths, to the World Health Organization (1). Twenty-four (44%) of these infections were in children <15 years of age. Although the number of human cases is small compared to poultry cases, human cases continue to occur, usually associated with close contact with sick or dying poultry. Reducing human and poultry contact is a key prevention strategy, and the Thai Ministry of Public Health has pursued an aggressive campaign to educate the Thai population on avian influenza and its prevention. To assess the effectiveness of the campaign, we carried out a survey of knowledge, attitudes, and practices regarding avian influenza in rural Thailand.

The Study

We conducted a community cluster survey in Nakhon Phanom, a province where we have ongoing collaborative projects. A questionnaire was designed to assess knowledge, attitude, and practices before and after the interviewee had heard about avian influenza. To detect a change of ≥15% between results before and after the education campaign, we sampled 200 persons. From a list of villages and their populations, we selected 5 by using a probability proportional to size. The starting house in each village was preselected by randomly selecting 3 numbers between 1 and the total number of households. If no one answered at

the household with the first number, the second then third numbers were tried until a starting house was identified. We interviewed persons who were ≥18 years of age. If >1 adult was home, the interviewer used a pregenerated random number table to determine which person to interview. Once the survey of the starting household was completed, the interviewer followed a written set of detailed instruction to find the next house. Native Thai speakers from the provincial health office were trained and conducted the interviews. Interviewers pilot-tested the survey to assess readability and comprehension, and the questionnaire was translated back into English to confirm accuracy. Interviews were conducted from August 25 to August 31, 2004, between 7:30 a.m. and 6:00 p.m. Data were analyzed by using SPSS 12.0 (SPSS Inc., Chicago, IL, USA).

During the outbreak, the Ministry of Public Health disseminated health messages on avian influenza to the public and healthcare professionals through several different types of media (Appendix Table, available online at http://www.cdc.gov/ncidod/EID/vol11no10/04-1267_app.htm). The ministry established a call-in hotline, and a frequent concern of callers was the safety of eating chicken. Call volume ranged from a minimum of 30 calls per day to a maximum of 200 calls per day. Official television messages were aired only on 3 days in February 2004. In addition, local media coverage was extensive, with daily television, newspaper, and radio reports during the peak outbreak months.

The median age of respondents was 50 years (range 22–87), and 144 (72%) of 200 were women; 122 (61%) had less than a primary school education. The median number of persons per household was 4 (range 1–11), and 110 (55%) had a child <10 years of age living in the household; 148 (74%) reported having poultry in their backyard (Table 1).

All but 4 (98%) persons said they had heard of bird flu, and 179 (91%) of these said they first heard about it on the television. Only 2 (1%) respondents had seen the Ministry of Public Health website on avian influenza. Of the 80 persons who remembered the month they first heard about avian influenza, 51 (64%) heard about it between

Table 1. Presence of poultry in 200 households, Nakhon Phanom, Thailand, 2004

Poultry	No. households (%)	Median no. poultry (range)*
Chickens	101 (51)	10 (1–85)
Fighting cocks	17 (9)	4 (1–40)
Ducks	9 (5)	7 (3–15)
Geese	2 (1)	2 (1–2)
Other†	43 (21)	Not asked
Any poultry‡	148 (74)	10 (1–100)

*Of persons reporting poultry at household.
†Other species and poultry living in backyard owned by others.
‡Some households had >1 type of poultry.

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December 2003 and March 2004, which was during the first big outbreak. Of the people who had heard about avian influenza, 149 (76%) recognized that persons could get the disease from chicken or other poultry.

Overall, knowledge and attitudes about how to protect oneself from diseases from poultry changed significantly after the respondent heard about avian influenza (Table 2). The percentage of adults who thought touching sick or dead poultry with their bare hands was safe decreased from 40% to 14% (p<0.01) and from 23% to 5% for children in their household (p<0.01).

In contrast, practices changed less dramatically. Touching sick or dead poultry with bare hands decreased significantly from 39% to 11% (p<0.01), but the decline was not significant for children in the household (6% to 4%, p = 0.4). Nor did a significant decline occur in the frequency of persons who reported taking dead poultry from their yard and preparing it for consumption (12% to 9%, p = 0.3). Certain practices that did not change significantly were already at somewhat appropriate levels. For example, 77% of persons reported that before they heard about avian influenza, they frequently washed their hands after touching raw poultry.

If persons were required to touch sick or dead poultry, 138 (70%) said they learned they could protect themselves by wearing gloves. Ten (5%) persons thought that wearing a mask was also protective; 172 (86%) persons believed the information they learned about how to protect themselves. When asked how much they changed their actions around poultry and poultry products since hearing about avian influenza, 38 (19%) persons said not at all, 38 (19%) said a little, 62 (32%) said a moderate amount, 47 (24%) said a lot, and 10 (5%) said completely.

Conclusions

In Thailand, public health education campaigns and general media reports about avian influenza appear to have been effective in reaching rural people who are at greatest risk of acquiring the disease through contact with backyard poultry. However, despite widespread knowledge about avian influenza and the effective means of protection, many Thai persons have not changed their behavior. Activities such as taking dead poultry out of the backyard and preparing it for household consumption continue to put persons at increased risk. Given the continued presence of poultry outbreaks and ongoing poultry-to-human transmission, additional efforts are needed to protect humans from infection.

In contrast to the 1997 influenza H5N1 outbreak in Hong Kong, where live poultry markets were the primary source of exposure (2), in Thailand, human cases of avian influenza have largely resulted from contact with sick or dying poultry in the person's backyard (3). A case-control study of the first 12 patients with laboratory-confirmed cases of H5N1 found that contact with dead poultry was a significant risk factor for illness (4). Not only are poultry numerous in Southeast Asia, few birds, except those on large commercial farms, are contained by an enclosure or fence. Culling, which proved highly effective in curtailing the 1997 poultry outbreak in Hong Kong, may be a less effective control strategy in Thailand, where poultry movement is extensive and difficult to control.

Of the 1.2 million chickens and other poultry in Nakhon Phanom, 29% reside on a commercial farm with a quarantine system in place and closed indoor feeding (Nakhon Phanom Provincial Livestock Office, unpub. data). The 5 villages where the survey was conducted contained no large commercial poultry farms.

The province where our survey was conducted has never been officially declared an H5N1-affected area, although poultry die-offs and suspected human cases of avian influenza have been reported. The survey results may not be generalizable to provinces with officially declared "affected areas." However, the improvement in

Table 2. Knowledge, attitudes, and practices before and after* hearing about avian influenza†			
Variable	Before, n (%)	After, n (%)	p value
Knowledge and attitudes			
Thought it was safe to touch sick or dead poultry with bare hands	78 (40)	27 (14)	<0.01
Thought it was safe for children to touch sick or dead poultry with bare hands	45 (23)	9 (5)	<0.01
Thought it was safe to prepare raw poultry and other foods on the same cutting board	98 (50)	73 (37)	0.01
Thought it was safe to eat chicken that was pink in the middle or eggs with a runny yolk	41 (21)	11 (6)	<0.01
Practices			
Touched sick or dead poultry with bare hands	76 (39)	22 (11)	<0.01
Children in household touched sick or dead poultry with bare hands	12 (6)	7 (4)	0.4
Took dead chicken or poultry from yard and prepared it to eat	24 (12)	17 (9)	0.3
Prepared raw poultry and other foods using different cutting boards	64 (33)	83 (42)	0.08
Washed hands with water immediately after preparing raw chicken or poultry	151 (77)	158 (81)	0.3

*Participants were asked to recall the month they first heard about avian influenza and then answer questions recalling their knowledge, attitudes, and practices in the 6 months before versus the 6 months after they heard about it.
†Among the 196 respondents who reported hearing about avian influenza.

knowledge and attitudes seen in our province might be magnified in an affected province where people have been personally affected by chicken and human illness and deaths.

The H5N1 virus has evolved from the strain seen in Hong Kong in 1997 (5). The virus now has an expanded animal range, and although it does not seem well adapted to human-to-human transmission, concerns persist that this adaptation may occur. Although most human cases have been transmitted by poultry, as in the 1997 Hong Kong outbreak, Thailand recently documented limited person-to-person transmission in a family cluster (6–9).

Reducing risk by encouraging behavior change is particularly challenging and can take years. However, change is possible. For example, significant changes in sexual behavior have contributed to a decline in HIV rates in Thailand. Between the 1991 implementation of the 100% condom campaign and 1995, HIV prevalence decreased significantly in Thai military conscripts (10). To prevent avian influenza, changing the behavior with the highest risk, touching sick or dead poultry with bare hands, should be attempted through public education and reinforced through behavioral counseling. This message must reach children because they account for more than half of the cases of avian influenza in Thailand. If complete avoidance of sick or dead poultry is impossible, messages should include information on proper hand protection, such wearing disposable gloves or using a plastic bag, and disposal methods.

This study suggests that public campaigns can be effective at educating rural populations. Renewed efforts are needed to find practical solutions that will induce behavioral change.

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Dr Olsen is an epidemiologist with the International Emerging Infections Program, a collaboration between the Thai Ministry of Public Health and US Centers for Disease Control and Prevention. She leads the program's surveillance and research activities on emerging infectious diseases.

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Anaplasma phagocytophilum in White-tailed Deer

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We examined the reservoir potential of white-tailed deer for *Anaplasma phagocytophilum*. Results suggest that white-tailed deer harbor a variant strain not associated with human infection, but contrary to published reports, white-tailed deer are not a reservoir for strains that cause human disease. These results will affect surveillance studies of vector and reservoir populations.

*A*naplasma phagocytophilum is an obligate intracellular bacterium and the etiologic agent of human granulocytic anaplasmosis (formerly known as human granulocytic ehrlichiosis). From 1999 to 2003, a total of 1,686 cases of human anaplasmosis were reported in the United States, and >95% of these cases occurred in northeastern or upper midwestern states. Transmission within and between reservoir populations in these regions occurs by *Ixodes scapularis* ticks (1,2). Infections occur in humans who have been fed upon by infected nymphal or adult ticks. No evidence shows that *A. phagocytophilum* is transmitted transovarially within the tick population; thus, both infected reservoirs and ticks that can transmit the infection must be available to maintain the agent in nature. Three mammalian species are reservoir competent: the white-footed mouse (*Peromyscus leucopus*), raccoon (*Procyon lotor*), and gray squirrel (*Sciurus carolinensis*), although serologic and molecular evidence has suggested that numerous other small, medium, and large mammals may also be reservoirs (1,3).

Every examined *A. phagocytophilum* sample from a patient with a confirmed case of human granulocytic anaplasmosis from the northeastern or upper midwestern United States has shown identical 16S rRNA sequences. This sequence, referred to as the *A. phagocytophilum* human anaplasmosis (AP-ha) signature sequence, differs by 2 bp from the sequence of the 16S rRNA gene of a variant strain, AP-Variant 1. Recent studies that compared the prevalence of AP-ha to AP-Variant 1 in tick populations showed the variant to be the predominant strain at 2 of 3

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sites and suggest that AP-Variant 1 is common in nature (4,5).

The white-footed mouse serves as a natural reservoir for AP-ha, and laboratory studies have shown that numerous inbred strains of mice (e.g., Balb/C, C3H, DBA/2) are also highly susceptible to infection (1,6,7). In contrast, AP-Variant 1 does not infect the white-footed mouse, DBA/2, and severely immunocompromised (SCID) mice (8). These results suggest that rodents are not a natural reservoir for AP-Variant 1 and that alternative reservoir species exist in nature. Previous reports have identified 3 white-tailed deer (*Odocoileus virginiansus*) from Maryland and 2 white-tailed deer in Wisconsin that harbored an agent with a 16S rRNA gene sequence identical to that of AP-Variant 1 (4,9,10). Several previous studies have also suggested that white-tailed deer are a reservoir for the human agent AP-ha (10–12). These results led to the current study, which was conducted to investigate the relative potential for white-tailed deer to be a reservoir for AP-ha, AP-Variant 1, or both strains. We examined blood samples from white-tailed deer to determine the strains of *A. phagocytophilum* with which these deer were infected and the ticks feeding on these deer to identify strains to which they were exposed.

The Study

I. scapularis ticks were collected from white-tailed deer during controlled hunts at Ridley Creek State Park in Delaware County, Pennsylvania, in December of 2000, 2001, and 2002. Blood samples were also collected from the deer in 2001 and 2002. An unusually high percentage of ticks collected in December 2000 were positive for *A. phagocytophilum* (68 [49.6%] of 137); identification was based on polymerase chain reaction (PCR) amplification of a 546-bp portion of the 16S rRNA gene, as previously described (Table) (9). Further analysis showed a strong correlation between the sex of a tick and the probability of being positive for *A. phagocytophilum*. Most of the positive ticks were females; 62 (84.9%) of the 73 female ticks were positive, compared with 6 (9.4%) of 64 males. DNA sequencing of the PCR products showed that 53 (85.5%) of the 62 PCR-positive females harbored AP-Variant 1, while only 9 (14.5%) of 62 were infected with AP-ha. Blood samples were not collected from white-tailed deer in 2000.

In 2001, both ticks and white-tailed deer blood samples were collected. Similar to the year 2000 tick results, a higher percentage of female ticks (50 [38.8%] of 129) than male ticks (13 [20%] of 65) were positive for *A. phagocytophilum*. Likewise, DNA sequencing showed that more infected female ticks were positive for AP-Variant 1 (74%) than for AP-ha (26%). Of the 38 white-tailed deer blood samples collected in 2001, 11 (28.9%) were positive for

Table. Results of polymerase chain reaction and DNA sequencing of *Anaplasma phagocytophilum* from *Ixodes scapularis* ticks collected from white-tailed deer

Year	No. ticks*	AP-Variant 1, n (%)	AP-ha, n (%)
2000	73 F	53 (72.6)	9 (12.3)
	64 M	4 (6.3)	2 (3.1)
2001	129 F	37 (28.7)	13 (10.1)
	65 M	10 (15.4)	3 (4.6)
2002	4 F	1 (25)	1 (25)
	2 M	0	1 (50)

*All females were either partially or fully engorged.

AP-Variant 1 *A. phagocytophilum*; none of the deer were positive for AP-ha. Most of the AP-Variant 1–positive ticks were obtained from positive white-tailed deer, and AP-Variant 1–positive ticks were collected from 8 of 11 positive deer. Of the 3 positive deer on which AP-Variant 1–positive ticks were not found, 1 deer had only 3 male ticks collected, the second had only 2 males collected, and the third had no ticks. Because adult male ticks do not take a blood meal, we were not surprised that these AP-Variant 1–positive deer had no positive ticks.

The collection of samples in December 2002 resulted in very few ticks (n = 6) because low temperatures inhibited tick activity. PCR amplification and DNA sequencing of *A. phagocytophilum* from female ticks (n = 4) showed that 1 tick was positive for AP-Variant 1 and 1 tick was positive for AP-ha (Table). Only 1 of the 2 males that were collected was positive for *A. phagocytophilum*, which sequencing showed to be AP-ha. Blood samples were collected from 24 white-tailed deer in 2002, and 5 (20.8%) of these were positive for *A. phagocytophilum*. Each of these 5 positive samples was AP-Variant 1.

Conclusions

The differential between the high percentage of AP-Variant 1–positive female ticks (range 25%–72.6%) and the relatively low number of male ticks positive for the variant ($\leq$15.4%), combined with the fact that deer were positive for only the variant, suggest that these partially and fully engorged female ticks were acquiring AP-Variant 1 as they fed on the deer. The presence of AP-ha in both male and female ticks that were feeding on deer showed that these deer were frequently exposed to AP-ha. Despite this demonstrated exposure to AP-ha, infections by AP-ha in white-tailed deer were not found. These results suggest that white-tailed deer are a natural reservoir for the variant, and further, that white-tailed deer are not a reservoir for AP-ha strains. These results also suggest that previous serologic studies that identified white-tailed deer as a reservoir for AP-ha strains were likely the result of the cross-reactivity of the anti-AP-Variant 1 serum from deer with the AP-ha antigens used for detection (10–12). In fact, in a study in which serologic testing showed that 8% of

white-tailed deer in Wisconsin were positive for *A. phagocytophilum*, the only 2 samples that were PCR amplified and sequenced were identical to AP-Variant 1 (10). Strong antigenic cross-reactivity of the AP-ha and AP-Variant 1 strains would not be surprising, considering their 16S rRNA genes are >99% identical.

Seroprevalence studies for infectious agents using animal reservoir and host populations and PCR amplifications from vector species are commonly used to assess the disease risk for humans in a particular region, particularly for viral and bacterial zoonotic agents. Our results show that animal seroprevalence studies for *A. phagocytophilum* must be carefully evaluated to determine whether the agent inducing the immune response is truly infectious in humans. Our results further show that while PCR studies of ticks may identify *A. phagocytophilum*, DNA sequencing of the PCR products is necessary to differentiate AP-ha and AP-Variant 1 and therefore to assess the potential for human infections. These issues were not addressed in earlier studies and likely resulted in overestimation of the prevalence of AP-ha in nature and in the implied risk for human anaplasmosis. Therefore, future studies of host or vector populations must be evaluated and interpreted carefully, with the knowledge that non–disease-causing variant strains may influence results.

While our results suggest that white-tailed deer are a reservoir for AP-Variant 1, additional studies that examine the interaction of AP-Variant 1 with white-tailed deer populations in other parts of the United States are needed to determine if they correspond to our results from Pennsylvania. Differences in AP-Variant 1 strain composition or local white-tailed deer or *I. scapularis* tick populations may alter the interaction of the bacterial agent, vector, and reservoir. AP-ha strains cause a transient, relatively mild febrile illness with no overt signs of disease in immunocompetent mouse species, including the natural reservoir, *Peromyscus leucopus* (13). Inbred laboratory mice infected with AP-ha may remain infected for up to 55 days (6), and previous infections induce an immune response that is only partially protective, since mice may be reinfected (14). We have not determined whether AP-Variant 1 produces any disease manifestation in white-tailed deer, although the high number of positive deer in the current study suggests that persistent infections, reinfections from feeding ticks, or both mechanisms may be involved in maintenance of AP-Variant 1 in white-tailed deer populations.

Diseases Scientific Resources Program for synthesis of oligonucleotides.

Dr Massung is a supervisory research microbiologist in the Viral and Rickettsial Zoonoses Branch, Centers for Disease Control and Prevention. His primary research interests include molecular biology, diagnostic microbiology, and the molecular epidemiology of *Coxiella*, *Anaplasma*, and *Ehrlichia* species.

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etymologia

botulism

[boch′ə-liz-əm]

Food poisoning with neurotoxicity caused by eating food contaminated with *Clostridium botulinum*. From the Latin *botulus*, "sausage," the disease was first recognized in Germany in persons who had eaten tainted sausage and was originally called "sausage poisoning."

Sources: Dorland's illustrated medical dictionary. 30th ed. Philadelphia: Saunders; 2003 and Botulism in Alaska [monograph on the Internet]. [cited 2005 Aug 26]. Available from http://www.epi.hss.state.ak.us/pubs/botulism/bot_03.htm

Melioidosis in New Caledonia

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Recognized melioidosis-endemic areas are widening. In the South Pacific, melioidosis is endemic in New Caledonia, northern Australia, and Papua New Guinea. We report the first 4 documented cases of human melioidosis from New Caledonia. Molecular typing of 2 *Burkholderia pseudomallei* isolates suggests a link to Australian strains.

Melioidosis, a tropical disease endemic in areas of Southeast Asia and northern Australia (1), is caused by *Burkholderia pseudomallei*, an environmental bacterium that lives in soil and surface water (2). This disease is increasingly recognized as an emerging problem because it is more often recognized and identified. Recent new "hot spots" have been reported in Mauritius (3), the Indian subcontinent (4), the Americas (5,6), and the Caribbean (7). In the South Pacific, melioidosis is a rare (8), but likely underdiagnosed, illness.

New Caledonia is located in Oceania, ≈2,000 km northeast of Sydney, Australia. With nearly 220,000 inhabitants, the country is a discrete epidemiologic entity with Pacific Island characteristics and a multicultural (mainly Melanesian, European, and Polynesian) population. The country has 2 provinces on the main island and a third province of smaller islands. Total land area is 18,575 km², slightly smaller than New Jersey. In New Caledonia, 4 cases of melioidosis have been diagnosed in the last 6 years. All were in Melanesians living in the Northern Province, none of whom had traveled abroad, which suggests this area is another discrete focus of endemic melioidosis. The average age of patients was 53 years; 3 had recognized risk factors for melioidosis, and all 4 were heavy kava drinkers.

The Cases

Melioidosis was confirmed for the first time in New Caledonia in February 1999 in a 46-year-old male nurse who worked at the health center of Ouegoa, a village in the Northern Province. He was admitted to the hospital with fever, acute renal insufficiency, pneumonia, and septic shock. Blood culture grew an oxidase-positive, gram-negative rod, which was initially identified as a *Pseudomonas* sp. by the local laboratory but later confirmed as *B. pseudomallei* by the Pasteur Institute when the patient was transferred to the hospital in the capital city, Nouméa. He had been treated for tuberculosis 20 years earlier. He required intubation and ventilation, and treatment was begun with a combination of ceftazidime and amikacin. Ten days after admission, when *B. pseudomallei* infection was confirmed, treatment was changed to the combination of imipenem and trimethoprim/sulfamethoxazole. Numerous cutaneous abscesses cultured *B. pseudomallei*, and he slowly recovered.

The second case came from the same location of Ouegoa but not from the same tribe. The patient was a 43-year-old man with diabetes mellitus and chronic renal failure who required a kidney transplant in 2001. He was taking the immunosuppressant medication tacrolimus when "*B. gladioli*" septicemia was diagnosed in April 2002. Two months later, he was admitted to the hospital with pneumonia, and blood cultures grew *B. pseudomallei*. He made a full recovery after therapy with a combination of ceftazidime and trimethoprim/sulfamethoxazole.

In July 2003, a 58-year-old man, living in Poum, ≈50 km from Ouegoa, was admitted with fever, pneumonia, and renal impairment. This man had tuberculosis in 1993 and was a smoker and alcoholic. Treatment was with amoxicillin/clavulanate for 24 hours followed by a combination of cefotaxime, ofloxacin, and metronidazole for 48 hours. He was transferred to Nouméa with severe hypoxemia from bilateral pneumonia and hepatocellular insufficiency. A gram-negative bacillus was isolated from bronchial secretions. Therapy was changed to a combination of ceftazidime and ciprofloxacin, and he slowly improved. The organism was identified as *B. pseudomallei* 7 days after the patient's transfer to Nouméa. A lung lobectomy was performed after 2 months for persisting pulmonary infection, and *B. pseudomallei* was cultured from the surgical specimen. The patient continued to receive trimethoprim/sulfamethoxazole on a long-term basis.

In June 2004, a 67-year-old woman with a history of diabetes mellitus came to the hospital with abscesses on her thigh, from which *B. pseudomallei* was cultured. She was otherwise healthy and recovered fully after receiving therapy for melioidosis. She lives in Poindimie, about 200 km southeast of Ouegoa.

The bacterium, also called Whitmore bacillus, is an oxidase-positive, gram-negative rod. It is commonly misidentified, sometimes as various species of the *Pseudomonas* genus. The bacterium grows aerobically on most agar media and usually produces clearly visible colonies within 24 hours at 37°C. In our cases, *B. pseudomallei* was identified with API 20NE and API 32GN (API system SA, Lyon, France). Two of the isolates were sent to the Centre

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d'Identification Moléculaire des Bactéries of Pasteur Institute in Paris, where the identity was confirmed by amplifying and sequencing the 16S rRNA gene. These strains were also defined by multilocus sequence typing (MLST) at Imperial College, London, and compared with isolates from Australia and Thailand. Both strains were new sequence types. When a minimum evolution analysis was performed on the concatenated sequences of the 2 strains and compared with Australian and Thai strains on the MLST website (http://bpseudomallei.mlst.net/), the New Caledonian strains clustered well within groups of Australian sequence types (Figure). Furthermore, 1 of the strains was a single-locus variant (i.e., 6 of 7 alleles identical) of a strain from the east coast of Australia. This comparison suggests that New Caledonian *B. pseudomallei* strains are linked to Australian strains (9). New Caledonia is a fragment of the ancient continent of Gondwana and subsequently separated from Australia and New Zealand. The diverse but distinct phylogeny of strains of *B. pseudomallei* in Southeast Asia and Australia may reflect geographic isolation over long periods.

Conclusions

In these cases, a lack of facilities for identifying the bacterium, especially in countries where incidence is unknown, resulted in delays before diagnosis and definitive treatment.
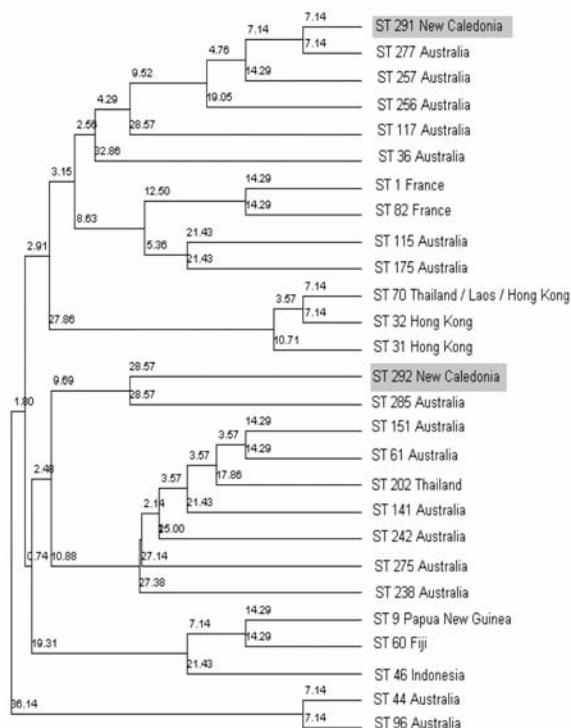

Figure. Phylogenetic tree constructed from the concatenated sequences of the 7 multilocus sequence type loci from *Burkholderia pseudomallei* isolates, illustrating the relationship of the 2 Caledonian strains to Australian and Thai isolates.

The classic resistance to colistin and gentamicin but sensitivity to amoxicillin/clavulanate seen in oxidase-positive, gram-negative rods may facilitate diagnosis.

In New Caledonia, the 4 patients had a variety of symptoms, from pneumonia, with or without septic shock, to cutaneous abscesses. The last case shows that exposure to *B. pseudomallei* does not always result in severe disease. The severity of illness probably depends on a balance between the bacterial strain's virulence, size of the infectious dose, delay before diagnosis, and immune status of the host (2). Risk factors for the patients were similar to those described elsewhere (2,8) and included diabetes, alcohol excess, chronic renal disease, and immunosuppression. Interestingly, all patients were heavy drinkers of kava, an extract of the plant *Piper methysticum*, which is drunk as an alternative to alcohol. Kava may be associated with melioidosis in some aboriginal communities in northern Australia (10). In New Caledonia, as in Australia, this "traditional" consumption is recent. Kava first appeared in New Caledonia ≈15 years ago as an import of Melanesian tradition mainly from Vanuatu. The roots are dried then pounded into powder and exported to New Caledonia and Australia where it is mixed with water to produce a brownish brew consumed for its psychoactive properties. Whether the association of melioidosis with kava consumption is an independent risk and whether it is because of possible ingestion of contaminated brew (11) or because of increased susceptibility of kava drinkers to melioidosis after exposure to *B. pseudomallei* requires further evaluation.

Melioidosis mainly affects persons who have direct contact with wet soil and have an underlying predisposition to infection. In the bush, many Melanesian persons spend a great deal of time outdoors with bare feet or wearing sandals, which increases percutaneous exposure to soil or muddy water during the wet season. New Caledonia often has periodic and heavy rain throughout the year. Four cases in 6 years represent an average annual incidence of 1.81/100,000 in the Northern Province, with 22.4/100,000 in the Ouegoa area, which suggests a high-prevalence focal region of melioidosis.

In conclusion, melioidosis cases have emerged in Melanesian persons, including those with diabetes, in a high-rainfall area in New Caledonia. Sampling of soil and ground water (and kava) for *B. pseudomallei* could be performed in this region to clarify the distribution of the bacterium and increase our understanding of this public health concern.

Acknowledgments

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Oropouche Virus Isolation, Southeast Brazil

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An Oropouche virus strain was isolated from a novel host (*Callithrix sp.*) in Arinos, Minas Gerais State, southeastern Brazil. The virus was identified by complement fixation test and confirmed by reverse transcription–polymerase chain reaction. Phylogenetic analysis identified this strain as a genotype III isolate previously recognized only in Panama.

Oropouche virus (OROV) is one of the most important arthropodborne orthobunyaviruses (*Bunyaviridae*, *Orthobunyavirus*) (1) that infect humans; it causes an acute febrile illness called Oropouche fever (2). OROV was originally reported in Trinidad in 1955, when the prototype virus strain was isolated from the blood of a febrile patient and from a pool of *Coquillettidia venezuelensis* mosquitoes (3). In Brazil, OROV was initially described in 1960, when it was isolated from a sloth (*Bradypus tridactylus*) captured near a forest area during the construction of the Belém-Brasilia highway, and from a pool of *Ochlerotatus serratus* mosquitoes captured nearby (4). More than one-half million persons have been infected with OROV, which makes this virus a public health threat in tropical and subtropical areas of Central and South America (5). OROV genome consists of 3 partite, single-stranded, negative-sense RNAs, named large (L), medium (M), and small (S) RNA. These RNAs are predicted to encode a large protein (L: polymerase activity), viral surface glycoproteins (Gc and Gn), and nonstructural NSM protein, as well as both nucleocapsid (N) and NSS proteins (1). Complete nucleotide sequences have been determined for all 3 RNA segments (6–8), and previous studies of the molecular biology of the N gene (SRNA) of 28 different OROV strains indicated the existence of 3 genotypes, designated I, II, and III (6).

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The Study

A field study was conducted in the Arinos region, Minas Gerais State, within the Grande Sertão Veredas National Park, in February 2000. The park is located 90 km from Arinos city (15°54′S, 46°W) (Figure 1). The Arinos region is part of the epizootic area of sylvatic yellow fever virus transmission, as previously reported (9).

Using a biosafety cabinet class II B-2, we prepared suspensions of viscera (liver, spleen, and kidney) obtained from the monkey and then injected suckling mice by the intracerebral route. Animals were observed daily and were immediately collected when they died or showed signs of disease.

The virus was identified by using the complement fixation test (CF), performed according to a described technique (10). The BeAn 626990 antigen was tested against different mice immune ascitic fluids (MIAFs) prepared for a large number of arboviruses circulating in Brazil.

Molecular studies were conducted to confirm the serologic results. Viral RNA was extracted by using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) technique as described elsewhere, and the entire SRNA segment was amplified, applying a 1-step reverse transcription–polymerase chain reaction (RT-PCR) assay using the

Figure 1. Map of the Arinos region, where the strain BeAN 626998 was isolated from a sylvatic monkey of the genus *Callithrix*.

primers ORO N5 (5′ AAAGAGGATCCAATAATGTCA-GAGTT CATTT 3′), ORO N3 (5′ GTG AAT TCC ACT ATA TGC CAA TTC CGA ATT 3′), ORO 1A (5′ AGTA-GTGTACTCCACTAT 3′) (6), ORONR 271 (5′ CGACTG-GAACTGTGGGAAAT 3′), OROF593 (5′ AAGTCCT-CCGGCAGAGGTAT 3′) and ORO2S (5′ AGT AGT GTG GCT CCA CAT 3′) (6).

Amplicons were direct sequenced in an automated sequencer (ABI 377, Applied Biosystems, Foster City, CA, USA) using the Kit ABI PRISM Dye Terminator (Applied Biosystems) by the dideoxyribonucleotide chain termina-tor method (11). Nucleotide sequence obtained for the strain BeAn 626990 were compared with 44 other OROV N gene nucleotide sequences (AF164531–AF164558; AY704559–AY704568; AY993909–AY993912), and phy-logenetic trees were constructed by using both neighbor-joining (NJ) (12) and maximum parsimony (MP) (13) methods implemented in the Mega 2.1 (14) and PAUP 4.0 software, respectively. Bootstrap analyses with 1,000 replicates were used to place confidence values on group-ings (15).

Mice injected with the strain BeAn 626990 showed signs of disease 48–72 hours after intracerebral injection. By CF, the suspension prepared from the brain of infected mice showed positive reaction with the MIAF prepared for the Brazilian prototype strain of OROV BeAn 19991 (≥32/16). No cross-reactivity was observed with any other MIAF prepared for the most common arboviruses current-ly circulating in the region. The monkey strain was then identified as OROV.

The N gene was found to be 693 nt in length, while the 5′ and 3′ terminal regions showed 290 and 161 nt, respec-tively. After the sequences assembly, accomplished by using the Seq Man program (DNA STAR software pack-age, DNASTAR Inc., Madison, WI, USA), the full-length SRNA was determined (754 nt). The multiple sequence analysis for the entire N gene of the strain BeAn 626990, when Mega 2.1 software was used, showed homology with different OROV strains previously sequenced (92%–100%), as described (6).

Two overlapping open reading frames were determined. One consisted of 693 nt (231 amino acids) and was predict-ed to encode the N protein. The other open reading frame consisted of 273 nt (91 amino acids) and was predicted to encode the NSs proteins. Both coding regions were flanked by 2 noncoding regions, located at the 5′ and 3′ terminals that were 44 nt and 14 nt in length, respectively.

Phylogenetic analysis carried out for 44 OROV N gene sequences (693 nt) with MP and NJ methods resulted in trees with similar topology, even though slightly low boot-strap values had been assigned for the MP consensus tree. The NJ tree suggested that the strain BeAn 626990 was more closely related to those included in genotype III.

Bootstrap support values of 100%, 74%, and 100% were assigned for the genotypes I, II, and III, respectively (Figure 2).

Conclusions

The known OROV epidemic sites are restricted to trop-ical areas of Central and South America, especially those in the Amazon Basin. From 1961 to 1980, in Brazil, OROV was reported in the northern state of Pará, where the most important epidemics occurred in Belém, and other regions of the state; hundred of thousands of persons were affected. From 1980 to 2004, OROV spread to 5 other northern Brazilian states (Amazonas, Amapá, Acre, Rondônia, and Tocantins) and 1 state in the northeast (Maranhão), indicating, in a short period of time, a danger-ous epidemic potential (Table) (5,6).

The first isolation of OROV in the Arinos area in Minas Gerais, Brazil, is of concern because this virus, under favor-able ecologic conditions, can spread, and Oropouche fever may develop in the local people, who are susceptible to
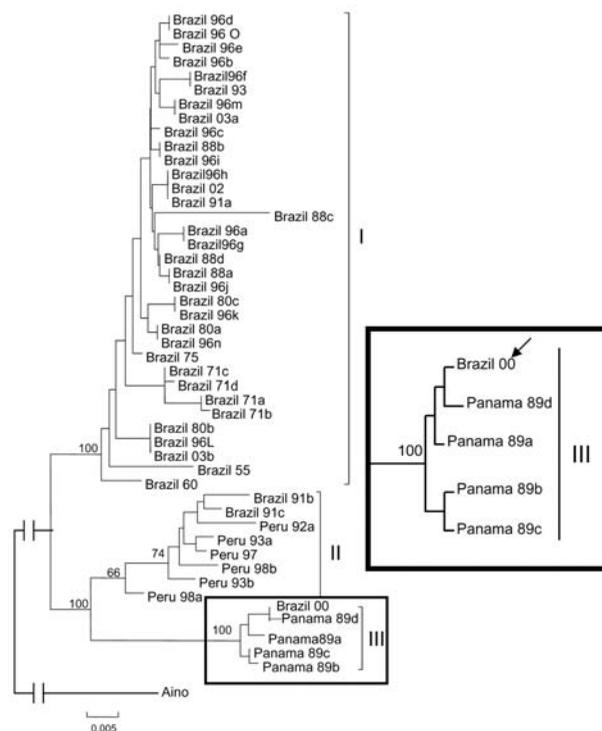
Figure 2. Phylogeny of Oropouche virus (OROV) strains isolated from different sources and periods by using the neighbor-joining and maximum parsimony methods. Bootstrap values were assigned over each internal branch nodes, and highest values were indicated by continuous arrows showing the presence of at least 3 lineages or genotypes (I, II, and III) of OROV. Bootstrap val-ues for the 3 representative genotype clades are placed over each respective branch node. The black arrow indicates the position of the strain BeAn 626990 (Brazil 00) in the tree. The Aino N gene nucleotide sequence was used as an outgroup to root the tree. The scale bar represents 5% nucleotide sequence divergence.

Table. Examples of epidemics and isolates of Oropouche fever virus genetically characterized in Latin American countries, 1955–2004

Years of occurrence	Location	Genotypes
1955	Trinidad	I
	Brazil (Pará State)	
1960	Brazil (Santa Maria*)	I
1961, 1968, 1979–80	Brazil (Belém)	I
1967, 1979-80	Brazil (Bragança region)	
1972	Brazil (Baião)	
1974–75	Brazil (Santarém)	I
1988	Brazil (Tucuruí)	I
1996	Brazil (Oriximiná, Altamira)	I
2003	Brazil (Parauapebas)	I
2004	Brazil (Porto de Moz)	II
1992	Peru (Iquitos)	II
1998	Peru (Madre de Dios)	II
	Brazil (Acre State)	
1996	Brazil (Xapuri)	I
	Brazil (Amazonas State)	
1980–1981	Brazil (Manaus and Barcelos)	I
	Brazil (Maranhão State)	
1988	Brazil (Porto Franco)	I
	Brazil(Rondônia State)	
1991	Brazil (Ariquemes/Ouro Preto)	II
	Brazil (Tocantins State)	
1988	Brazil (Tocantinopolis)	I
2002	Brazil (Paranã)	I
1989	Panama†	III

*Bradypus tridactylus.
†Chame, Chilibre, and San Miguelito.

OROV. In fact, explosive Oropouche fever epidemics have been reported in all of the following areas in Brazil: Maranhão, Tocantins, Amazonas, Acre, Pará, and Rondônia States. Such epidemics have also been reported in Iquitos and Madre de Dios, Peru; and in Bejuco, Panama (5).

Molecular studies (6) recognized 3 genotypes and suggested the distribution for OROV in South and Central America. The authors showed that in Brazil, genotype I, the most widespread in the country, and genotype II, found in Rondônia (a bordering state with Peru), and also in Para state (M.R.T. Nunes, unpub. data) cocirculate. In Peru, only genotype II has been identified. Genotype III has been reported only in Panama, whereas in Trinidad, only genotype I has been isolated (Table).

The genetic data obtained in this study, for the S segment of the strain BeAn 626990 (S: AY 117135), suggest its genetic relationship with other OROV strains. Phylogenetically, the monkey strain was shown to be more closely related to genotype III OROV isolates (Figure 2) and is the first report of genotype III in southeastern Brazil. More importantly, OROV was detected for the first time outside the known epidemiologic area for OROV transmission in South America. Our findings also represent the first report of OROV isolation from a monkey.

The potential impact of OROV in Brazil can be better assessed by viewing it in its demographic context. The southeast is the most populated region in Brazil, comprising urban areas such as the cities of Belo Horizonte, Rio de Janeiro, and São Paulo. The entire region has intense intra- and interregional migration, fostered by improved technology and transportation, which may lead to increases in the dissemination of both human and animal pathogens along these areas. Therefore, further studies on the ecology, epidemiology, and molecular epidemiology of OROV are needed, not only to improve knowledge about the epidemiology of this arbovirus but also to find out if its epidemic area is spreading outside the Amazon region and toward the southeast and other populated regions in Brazil. This situation is of particular concern because Oropouche fever epidemics have been reported in several small villages in the Brazilian Amazon region in 2003 and 2004 (Vasconcelos PFC, unpub. data). As a consequence, the risk of spreading OROV to susceptible areas has increased considerably in Brazil. More research and program development are needed to control this potential epidemic arboviral disease.

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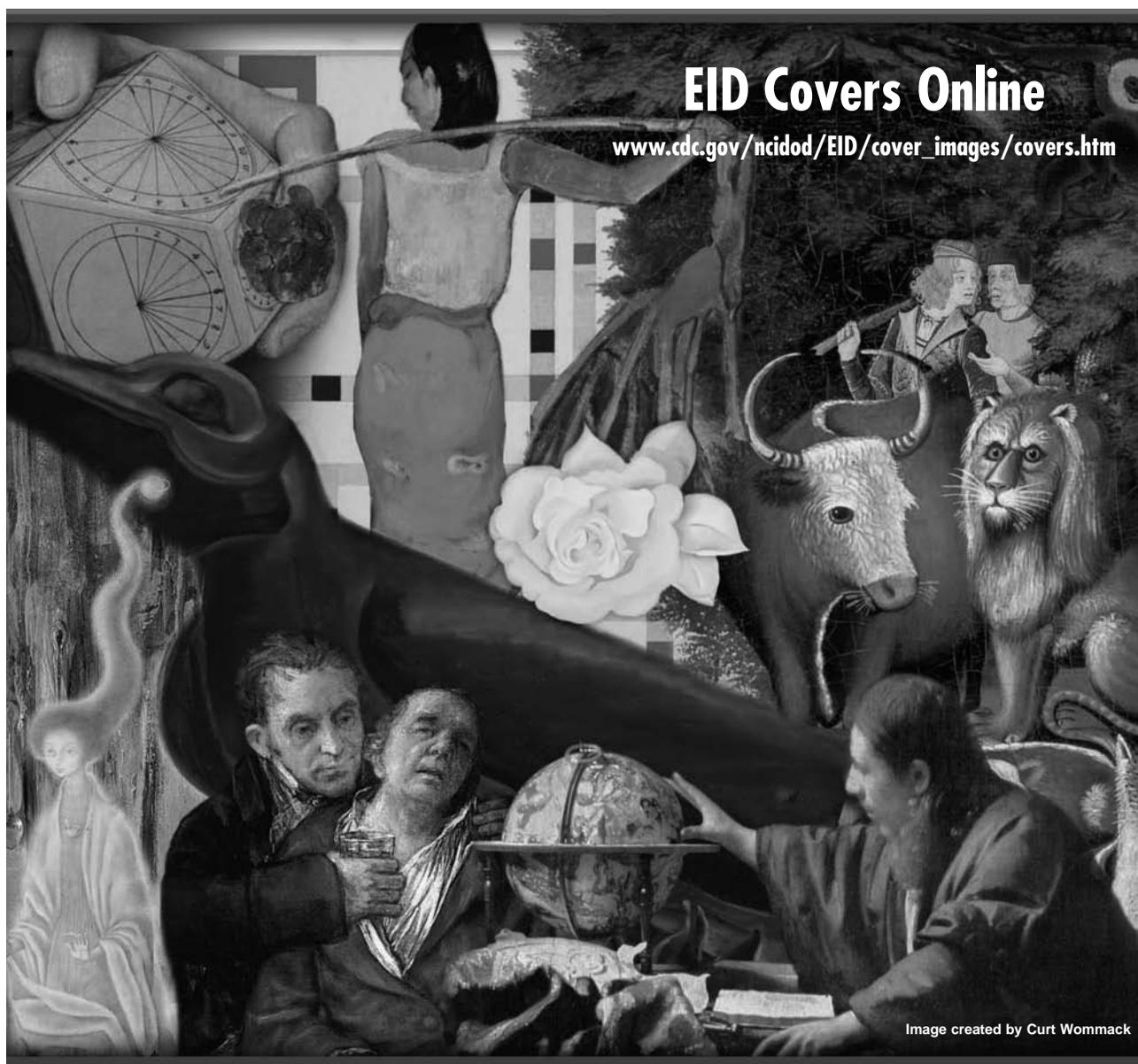
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Drug-resistant *Escherichia coli,* Rural Idaho

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Stool carriage of drug-resistant *Escherichia coli* in home-living residents of a rural community was examined. Carriage of nalidixic acid–resistant *E. coli* was associated with recent use of antimicrobial agents in the household. Household clustering of drug-resistant *E. coli* was observed. Most carriers of drug-resistant *E. coli* lacked conventional risk factors.

A cquisition of drug-resistant *Escherichia coli* may be influenced by food, exposure to flora of contacts, and use of antimicrobial agents (1–3). Few community studies have explored the contribution of these mechanisms to dissemination of drug-resistant *E. coli* in healthy persons (4–6). We examined epidemiologic factors associated with colonization by drug-resistant *E. coli* in home-living volunteers who were not recruited through healthcare settings (7,8). Resistance to trimethoprim/sulfamethoxazole (TMP/SMZ), nalidixic acid (NA), and extended-spectrum cephalosporins (ESCs) was examined (9,10).

The Study

From March to May 2002, a convenience sample of household volunteers was recruited from 1 rural community in Idaho. Consenting adults and parents of children completed an exposure questionnaire. The questionnaire assessed dietary history and livestock contact during the previous month, and other exposures, including antimicrobial drug use and travel outside the United States during the past 6 months. The study was reviewed and approved by the Western Institutional Review Board (Olympia, WA, USA).

Information on antimicrobial drug prescriptions filled by community pharmacies in the preceding year was obtained (beginning March 2001). Pharmacy-documented antimicrobial drug prescriptions were compared with self-reported use. The definition of antimicrobial drug use was either pharmacy documentation of an antimicrobial drug

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prescription or self-reported use of a named antimicrobial agent obtained from a plausible nonpharmacy (e.g., free sample from a doctor's office) or out-of-area source, with dates of use. Recent antimicrobial drug use was defined as use ≤30 days before collection of stool swabs.

Study participants were instructed to use a CultureSwab (Becton Dickinson, Franklin Lakes, NJ, USA) to collect fecal material. All samples were refrigerated and transported to the Idaho State Bureau of Laboratories (state laboratory) in Boise, Idaho. At the state laboratory, samples were streaked across 3 MacConkey agar plates, each containing 1 screening antimicrobial agent (16 mg/L TMP/SMZ, 16 mg/L NA, or 2 mg/L cefotaxime). One phenotypically distinct colony type per plate was further analyzed.

Putative *E. coli* colonies were confirmed by using the Microscan system (Dade Behring Inc., Deerfield, IL, USA). Susceptibility was assessed by MIC using broth microdilution (Microscan) for cefpodoxime, ceftazidime, ceftriaxone, and TMP/SMZ and the Etest (AB-BIODISK, Solna, Sweden) for NA. Manufacturer-specified procedures and reference strains were used, along with Clinical and Laboratory Standards (CSLI) (formerly National Committee for Clinical Laboratory Standards [NCCLS]) guidelines. The CSLI/NCCLS criteria were used to classify isolates as resistant to TMP/SMZ, NA, or ESC. Resistance to ESC was defined as resistance to ceftriaxone (MIC ≥64 µg/mL), ceftazidime (MIC ≥32 µg/mL), or cefpodoxime (MIC ≥8 µg/mL) (11). A sample was resistant if at least 1 *E. coli* isolate from that sample exhibited the corresponding resistance phenotype.

The primary endpoints were intestinal carriage of *E. coli* resistant to the 3 targeted antimicrobial drug classes. Carriage of NA-resistant and TMP/SMZ-resistant *E. coli* were examined separately by comparing carriers and noncarriers of NA-resistant and TMP/SMZ-resistant *E. coli*. Regression models were constructed in which study participants were divided into 3 mutually exclusive groups: carriers of NA-resistant *E. coli* (either TMP/SMZ resistant or susceptible), carriers of TMP/SMZ-resistant/NA-susceptible *E. coli*, and persons who did not carry either resistance (reference group). Crude and adjusted odds ratios were estimated by using generalized estimating equations to account for household-level clustering. Statistical significance was defined as a p value ≤0.05. Analyses were performed with Stata version 8.0 (Stata Corporation, College Station, TX, USA).

Stool swabs were received from 517 study participants representing 167 households (Table 1). The prevalence of intestinal carriage of *E. coli* resistant to NA was 3%, to TMP/SMZ 11%, and to ESCs 1%. All 6 ESC-resistant isolates were found so based on their resistance to cefpodoxime. The ceftazidime MIC was in the susceptible range for 5 of these isolates (≤4 µg/mL for 2 and 8 µg/mL for 3)

Table 1. Characteristics of 517 study participants collected from questionnaires and pharmacy data

Characteristic	No. (%)
Demographic variables	
Male	259 (50)
Income <$35,000/y	339 (66)
Race/ethnicity (n = 497)	
White, non-Hispanic	442 (89)
Hispanic/Latino	30 (6)
American Indian/Alaska Native	9 (2)
Asian	6 (1)
African American	5 (1)
North Hawaiian/Pacific Islander	5 (1)
Age, y	
≤6	163 (32)
7–21	103 (20)
22–50	192 (37)
>50	59 (11)
Travel/daycare factors	
Child in daycare/preschool	88 (17)
Travel out of United States in past year	22 (4)
Dietary factors	
Did not eat hamburger in past month	28 (6)
Ate <1 time/wk	48 (9)
Ate 1–2 times/wk	252 (50)
Ate > 2 times/wk	181 (36)
Did not eat chicken in past month	22 (4)
Ate <1 time/wk	59 (28)
Ate 1–2 times/wk	286 (56)
Ate >2 times/wk	147 (29)
Animal exposure	
Live on farm with livestock	5 (1)
Livestock in past month	21 (4)
Cattle	21 (4)
Horses	20 (4)
Sheep	4 (1)
Swine	3 (1)
Poultry	3 (1)
Goats	1 (0)
Household size	
<3 (referent)	76 (46)
3–4	53 (32)
>4	38 (23)

and intermediate for 1 isolate (16 μg/mL). The isolate with intermediate susceptibility to ceftazidime also showed intermediate resistance to ceftriaxone (32 μg/mL).

Use of antimicrobial agents was associated with carriage of NA-resistant but not TMP/SMZ-resistant *E. coli*; 6 (16%) of 37 study participants who used antimicrobial agents within 30 days of culture carried NA-resistant *E. coli*, compared with 10 (2%) of 480 participants who did not use antimicrobial agents. However, significance was lost after accounting for household clustering (p = 0.13). Carriage of TMP/SMX-resistant *E. coli* was similar in persons with and without recent use of antimicrobial agents; 5 (14%) of 37 study participants with recent use carried TMP/SMZ-resistant *E. coli* compared with 50 (10%) of 480 persons without recent use (p = 0.84).

A similar pattern was seen for recent use of antimicrobial agents in the household. Overall, 92 (18%) persons

resided in a household in which at least 1 member recently used antimicrobial agents. Of these, 11 (12%) of 92 carried NA-resistant *E. coli*, compared with 5 (1%) of 425 in households without recent use. In contrast, the prevalence of carriage of TMP/SMX-resistant *E. coli* was similar in persons with and without recent household use of antimicrobial agents. When we accounted for household clustering, recent use of antimicrobial agents in the household was associated with 9.2-fold increased odds for carriage of NA-resistant *E. coli* (p<0.001). Additionally, the presence of another household member with NA-resistant *E. coli* was associated with 8.8-fold increased odds for NA-resistant *E. coli* carriage (p<0.001), and the presence of another household member with TMP/SMZ-resistant *E. coli* was associated with 2.7-fold increased odds for TMP/SMZ-resistant *E. coli* carriage (p<0.001). Carriage of NA-resistant or TMP/SMZ-resistant *E. coli* was not associated with age, sex, livestock exposure, dietary history, contact with the healthcare system, or travel outside the United States (Table 2). Approximately 94% of persons in the study ate chicken or ground beef in the previous month (Table 1); 14 of 17 persons who did not eat beef or chicken in the previous month were children ≤5 years of age.

The 6 study participants who carried ESC-resistant *E. coli* belonged to 6 separate households. None had used antimicrobial agents within 30 days of culture and only 1 had household use of antimicrobial agents within 30 days. No other epidemiologic or demographic factors distinguished this group. The small number of persons with carriage of ESC-resistant *E. coli* precluded further statistical analysis of this endpoint.

Of the 517 participants, 34% self reported use of an antimicrobial agent during the previous 6 months (Table 3). Of these, 67% had pharmacy documentation of at least 1 antimicrobial agent prescription. However, 22% of the 339 persons who reported not using antimicrobial agents had pharmacy documentation of at least 1 prescription. Of the 178 persons who reported use of ≥1 antimicrobial agent, 108 (61%) provided the name of the agent. However, the specific drug named matched the drug listed in the pharmacy records for only 29% of the persons. Thirteen persons reported receiving an antimicrobial agent from a nonpharmacy source. Six of the 13 purchased antimicrobial agents in Mexico, 4 received a drug sample from their healthcare provider, and 1 person each received the antimicrobial agent from a dairy, another family member, or a leftover prescription.

Conclusions

Carriage of *E. coli* resistant to TMP/SMZ was more common than carriage of *E. coli* resistant to NA or ESC. There was striking evidence of household clustering of resistance, consistent with either spread of organisms

between persons in close contact or common source acquisition, such as through shared contaminated food (8,12). Most carriers of drug-resistant *E. coli* did not have exposures previously associated with antimicrobial drug resistance such as travel, contact with the healthcare system, or chronic illness (13–15).

NA resistance was associated with recent use of antimicrobial agents in the household. Use of antimicrobial agents may have enhanced acquisition of exogenous NA-resistant *E. coli*; alternatively, for persons who had recently taken fluoroquinolones, NA resistance may have emerged during therapy.

Overall, 36% of households had at least 1 member who had received antimicrobial drug treatment within the previous 6 months, illustrating the magnitude of antimicrobial drug selection pressure operating in a community. Self reporting of antimicrobial drug use may be a useful mark-

er of exposure to these drugs when pharmacy records are not available. However, the accuracy with respect to specific drugs was poor.

This study did not convincingly support or refute the hypothesis that contact with contaminated meat contributes to gastrointestinal carriage of drug-resistant *E. coli*. Only a small number of persons reported not eating meat, and those persons lived in households where other members ate meat. Therefore, persons not exposed to meat were not adequately sampled.

The limitations of the study should be acknowledged. Random recruitment of volunteers from the community was not feasible. Since only a single stool specimen was obtained, the duration of carriage of drug-resistant *E. coli* or the timing of its onset in relation to specific exposures could not be determined. The use of thymidine-containing media (MacConkey agar) may have diminished the

Table 2. Comparison of 517 study participants with and without carriage of antimicrobial drug-resistant *Escherichia coli*, by questionnaire responses and pharmacy data*

Characteristics	Noncarriers (n = 452), no. (%)	TMP/SMZ resistant (n = 49)		NA resistant (n = 16)	
		No. (%)	OR (95% CI)	No. (%)	OR (95% CI)
Demographic variables					
Male	228 (50)	24 (49)	1.0 (0.6–1.8)	9 (56)	1.1 (0.5–2.4)
Income <$35,000/y	69 (13)	10 (20)	1.9 (0.8–4.7)	1 (6)	0.8 (0.1–6.7)
High school education or less	149 (33)	18 (37)	1.3 (0.6–2.9)	3 (19)	0.4 (0.1–2.2)
Hispanic ethnicity	24 (5)	6 (12)	2.6 (0.7– 9.3)	0 (0)	–
Age, y					
≤6	144 (32)	16 (33)	Ref	3 (19)	Ref
7–17	71 (16)	5 (10)	1.3 (0.6–2.8)	2 (13)	1.5 (0.7–3.0)
18–50	189 (42)	22 (45)	0.8 (0.4–1.5)	6 (38)	1.3 (0.4–3.7)
>50	48 (11)	6 (12)	0.9 (0.3–2.6)	5 (31)	1.9 (0.3–13.6)
Travel/daycare factors					
Child in daycare/preschool	79 (18)	9 (18)	1.0 (0.5–2.2)	0 (0)	–
Traveled out of United States	17 (4)	1 (2)	0.4 (0.1–1.7)	4 (25)	6.0 (0.9–39.9)
Dietary factors					
Ate hamburger, times/week					
1–2	219 (49)	23 (47)	0.6 (0.3–1.2)	10 (63)	0.7 (0.2–2.0)
>2	164 (37)	14 (29)	0.6 (0.2–1.3)	4 (25)	0.5 (0.2–1.3)
Ate chicken, times/week					
1–2	254 (56)	23 (47)	0.6 (0.3–1.2)	8 (50)	0.8 0.2–2.6
>2	127 (28)	14 (29)	0.6 (0.3–1.5)	7 (44)	1.3 (0.4–4.1)
Household cook	177 (39)	21 (44)	1.0 (0.6–1.7)	7 (44)	0.8 (0.4–1.4)
Primary grocery shopper	169 (37)	20 (41)	1.0 (0.6–1.6)	8 (50)	1.1 (0.6–1.9)
Healthcare/antimicrobial drug use					
Ambulatory visit in past 6 mo	236 (52)	22 (45)	0.8 (0.4–1.3)	9 (56)	1.1 (0.5–2.3)
Diabetic	9 (2)	3 (6)	2.9 (0.7–11.7)	1 (6)	1.5 (0.0–117.4)
Hospitalized in past 6 mo	27 (6)	3 (6)	1.0 (0.3–2.5)	4 (25)	3.4 (0.5–22.4)
Antimicrobial drug use in past 30 d	209 (46)	22 (45)	0.7 (0.2–2.7)	13 (81)	2.6 (0.7–9.7)
Animal exposure in past month					
Livestock	32 (7)	2 (4)	0.5 (0.1–2.1)	1 (6)	1.6 (0.5–4.9)
Cattle	19 (4)	2 (4)	0.8 (0.2–3.4)	0 (0)	–
Horses	18 (4)	1 (2)	0.5 (0.1–4.4)	1 (6)	2.3 (0.7–9.6)
Household size†					
<3	43 (34)	10 (29)	Ref	3 (33)	Ref
3–4	48 (38)	11 (31)	0.7 (0.3–1.6)	3 (33)	1.1 (0.2–5.5)
>4	35 (28)	14 (40)	0.4 (0.2–1.1)	3 (33)	0.4 (0.1–3.4)
Household antimicrobial drug use in past 30 d	21 (17)	5 (14)	0.6 (0.2–1.9)	4 (44)	8.4 (2.4–29.2)

*TMP/SMZ, trimethoprim/sulfamethoxazole; NA, nalidixic acid; OR, odds ratio; CI, confidence interval; Ref, referent.
†n = 126 for noncarriers; n = 35 for TMP/SMZ resistant; n = 9 for NA resistant.

Table 3. Healthcare/antimicrobial use in 517 study participants collected from questionnaires and pharmacy data*

Characteristic	No. (%)
Ambulatory visit in past 6 mo	266 (52)
Diabetic	13 (3)
Antimicrobial use in past month	37 (7)
No. outpatient visits (past 6 mo)	
0	251 (49)
1	125 (49)
2	59 (23)
3	29 (11)
4	14 (5)
≥5	32 (12)
No. hospitalizations (past 6 mo)	
0	483 (93)
1	31 (6)
2	2 (0)
3	1 (0)
No. courses of antimicrobial agents in the past year	
0	333 (64)
1	91 (18)
2	39 (8)
3	19 (4)
4	16 (3)
≥5	19 (4)
Antimicrobial classes, no. with ≥1 course	
TMP/SMZ	
Past month	2 (0.4)
Past year	10 (2)
Fluoroquinolones	
Past month	7 (1)
Past year	17 (3)
Cephalosporins	
Past month	4 (1)
Past year	37 (7)
Penicillin	
Past month	18 (4)
Past year	107 (21)
Macrolide	
Past month	3 (1)
Past year	24 (5)

*TMP/SMZ, trimethoprim/sulfamethoxazole.

activity of TMP/SMZ, thereby reducing the sensitivity of the screening for TMP/SMZ resistance.

In conclusion, most home-living residents who carried drug-resistant *E. coli* lacked conventional risk factors. Household-level antimicrobial drug use was associated with carriage of NA-resistant but not TMP/SMZ-resistant *E. coli*. The role of the food supply in promoting dissemination of drug-resistant *E. coli* in human populations warrants more detailed study.

Dr Hannah is a research professor in the Department of Community and Environmental Health, College of Health Sciences, Boise State University, Boise, Idaho. Her research interests include antimicrobial resistance, evidence-based medicine, using data to improve medical quality, and the intersection between animal and human health.

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Canine Leishmaniasis, Italy

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We report the results of a survey to determine the prevalence of canine leishmaniasis and the presence of sand flies in northwestern Italy, where autochthonous foci of canine leishmaniasis have not been reported. Active foci of canine leishmaniasis were identified, which suggests that the disease is now also endemic in continental climate areas.

V isceral leishmaniasis due to *Leishmania infantum* is a zoonotic disease transmitted in Mediterranean areas by sand flies belonging to the genus *Phlebotomus*. Dogs are the main reservoir of infection, and in some disease-endemic areas seroprevalence of canine leishmaniasis is >30% (1). Recently, the geographic distribution of canine leishmaniasis has spread (2), and new foci of disease have been reported in countries such as the United States (3). Global warming is a possible cause of spread of the disease to cooler areas (4), and the increased movement of infected animals from areas where the disease has traditionally been endemic can, together with the spread of sand fly vectors, facilitate this process. In Europe, both canine leishmaniasis and human visceral leishmaniasis are endemic in Mediterranean areas characterized by a dry, hot summer and mild winter temperatures (4). However, foci of canine leishmaniasis have never been reported in continental climate regions, which are characterized by large seasonal temperature changes between hot summer and cold winters. Until recently, stable endemic foci of both human visceral leishmaniosis and canine leishmaniasis have been present only in southern, central, and insular regions in Italy. However, new foci of canine leishmaniasis and the presence of competent sand fly vectors have also been reported in northern regions of the country, where autochthonous cases had not been reported previously (5,6).

The Study

A serologic survey for canine leishmaniasis was conducted in 3 areas of the Piedmont to study the spread of

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canine leishmaniasis in northwestern Italy and to establish if newly identified foci were stable. Study areas included hill zones close to Turin, Casale, and Ivrea, which are all characterized by a continental climate. Autochthonous cases of canine leishmaniasis had been increasingly reported by practitioners in these areas during the late 1990s, and a focus was recently identified in Turin (6). The survey was also extended to the neighboring Aosta Valley, a mountain region where autochthonous cases of canine leishmaniasis have not been reported or suspected. An entomologic survey was also conducted during 2 consecutive years (2000–2001) in the above areas to assess the presence of *Leishmania* vectors and to study their density and their seasonal dynamics. Sand flies were absent in both the Piedmont and Aosta Valley in a survey conducted 30 years previously (7).

Blood samples were collected from 913 asymptomatic resident dogs (that had never traveled to traditionally leishmaniasis-endemic areas) during the winter and spring months from January 1999 to March 2001. The tested dogs included 313 dogs from the surroundings of Turin (45.4ºN, 7.70ºE), 176 from Casale (45.8ºN, 8.26ºE) 155 from Ivrea (45.28ºN, 7.52ºE), and 269 from the Aosta Valley (45.4ºN, 7.20ºE). Serum samples were tested with the indirect fluorescent antibody test (IFAT), as previously described (8). Titers $\geq$1:160 were considered positive, values $\leq$1:40 were considered negative, and a value of 1:80 was considered doubtful (9). Prevalence values in the 5 study areas were compared by a chi-square test (EpiInfo, version 6.0, Centers for Disease Control and Prevention, Atlanta, GA, USA), and differences were considered significant when p was $\leq$0.05.

For the entomologic survey, 518 collecting sites containing a variety of sand fly diurnal resting sites (animal shelters, houses, and scarp wall cracks) were selected throughout the Piedmont and Aosta Valley regions: 146 in Ivrea, 89 around Casale, 194 near Turin, and 89 in Aosta. The presence or absence of *Leishmania* vectors was assessed once per collecting site from the second half of June to the first half of August, when major sand fly densities are expected (6). In addition, to evaluate sand fly seasonal dynamics, weekly collections were carried out from the end of April to the end of October 2001 in a few representative sites: 8 in Turin, 8 in Ivrea, and 6 in Aosta. Sand fly specimens were captured by sticky traps made from 20 × 20-cm, castor-oiled paper (10); a minimum of 10 sticky traps were used in each collecting site.

Sand flies were identified to the species level (11). Dogs with unambiguously positive serology (IFAT titers $\geq$1:160) were detected in all 4 examined areas. Seroprevalence did not differ significantly in Turin (4.5%), Ivrea (5.8%), and Casale (3.9%) but was significantly lower in Aosta (0.4%) (chi square = 25.6, p = 0.00004). In

Turin, Ivrea, and Casale, infection was observed in resident autochthonous dogs in both 2000 and 2001, and in these areas the observed seroprevalences were higher than the 2.5% threshold usually associated with steadily established canine leishmaniasis foci (12). *Leishmania* amastigotes were also observed in lymph node smears from resident ill dogs from all 4 areas (E. Ferroglio, unpub. data).

Of 518 stations examined, 113 (21.8%) were positive for *Phlebotomus perniciosus* (Figure), the main vector of leishmaniasis in Italy (13), while *P. neglectus*, another recognized vector of canine leishmaniasis (14), was found only in Ivrea (Table). The percentage of positive stations did not significantly differ in the 4 areas.

In 2001, *P. perniciosus* was first captured in Turin on May 11 and during the last week of May in the remaining 2 areas. The last captures occurred in Aosta during the last 10 days of September and in Turin and Ivrea during the first 10 days of October. Peak numbers of *P. perniciosus* were observed in Turin (10.6 individuals/m^2 sticky traps) and Ivrea (7.3/m^2) during the last 10 days of July. Peak density in Aosta was lower (2.5/m^2) and slightly earlier (first 10 days of July).

Conclusions

Our data indicate that canine leishmaniasis is now endemic in at least 3 different areas of the Piedmont (Turin, Ivrea, and Casale), where seroprevalence in resident dogs is 3.9%–5.8%. A possible unstable focus has been identified in the Aosta Valley. Of 518 stations examined, 113 (21.8%) were positive for *P. perniciosus* (Table). The percentage of positive stations did not significantly differ in the 4 areas. In the Aosta Valley, 25 (28.0%) of 89 stations examined were positive for *P. perniciosus*. In this mountain area, sand flies have not been reported previously, and 23 sticky trap capture stations monitored in the late 1960s were all negative (7). Colonization of these areas may have occurred either spontaneously, from Mediterranean coastal areas, or following the increased movement of people towards Mediterranean areas, where phlebotomine sand flies are abundant. The seasonal presence of sand flies extends from the second half of May to September. These results may be usefully exploited to define the risk period for canine (and human) leishmaniasis transmission in northern Italy. Recently, a preliminary

Figure. Traditionally endemic canine leishmaniosis (canine leishmaniasis) areas (slash marks) and new foci in continental climate areas of northwestern Italy (shaded areas).

survey conducted in northwestern Italy showed *L. infantum* infection in the resident human population (15), and polymerase chain reaction–restriction fragment length polymorphism analysis of the human and canine strains provided evidence of the circulation of *L. infantum* between dogs and humans in this area (E. Ferroglio et al., unpub data). Our findings are evidence that canine leishmaniasis is currently expanding in continental climate areas of northwestern Italy, far from the recognized disease-endemic areas along the Mediterranean coasts (Figure). Based on similarities in climate and major landscape features with our study area, spread of canine leishmaniasis to other regions of central Europe can be foreseen in the near future.

Table. Sand flies collected in 4 study areas, 2000–2001				
Study area	*Phlebotomus perniciosus*, no. (% positive stations)	*P. neglectus*, no. (% positive stations)	*P. mascittii*, no. (% positive stations)	*Sergentomya minuta*, no. (% positive stations)
Ivrea	28 (12)	37 (3.3)	2 (1.4)	1,162 (43.3)
Casale	39 (40.4)	0	1 (1.1)	1,708 (34.7)
Aosta	136 (28)	0	3 (3.4)	1,265 (41)
Turin	225 (20.8)	0	1 (0.4)	857 (79.2)
Total	428 (23.2)	37 (1.0)	7 (1.8)	4,992 (36.0)

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Myocarditis Outbreak among Adults, Illinois, 2003

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Sarah Reagan,* Chris Paddock,*
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An outbreak of myocarditis occurred among adults in Illinois in 2003. Diagnostic testing of myocardial tissues from 3 patients and comprehensive tests for enterovirus and adenovirus of other specimens from patients were inconclusive. Appropriate specimen collection from patients with idiopathic cardiomyopathy and further enhancement of diagnostic techniques are needed.

Acute myocarditis is characterized by inflammatory infiltrates of the myocardium. Disease has been attributed to multiple infectious and noninfectious causes, but viruses, particularly the enteroviruses group B coxsackievirus and echoviruses, are believed to be the most common agents of infection in the United States (1). An infectious cause of myocarditis is usually suspected when unexplained heart failure or arrhythmia occurs in a person with a systemic febrile illness or upper respiratory tract infection. Acute myocarditis is typically sporadic, although clusters have been reported during outbreaks of viral disease (2,3). Most cases are idiopathic without a known cause (1). Myocardial biopsy specimens used for pathologic examination, the conventional standard for diagnosis (4,5), have been considered difficult to collect in nonfatal cases. Viruses are infrequently cultured from tissue specimens, although viral nucleic acid identification by polymerase chain reaction (PCR) assays on myocardium has recently enhanced viral detection (6–8). Viral serologic tests and PCR assays of blood, stool, urine, and nasopharyngeal specimens are adjunctive techniques for diagnosing myocarditis that have not been validated.

On March 21, 2003, the Kane County Health Department was notified about 6 cases of presumptive myocarditis and 1 case of pericarditis that occurred in patients hospitalized in Kane County, Illinois, within a 2-week period from February 26 to March 10. Five case-patients were <50 years of age, 1 of whom died within 24 hours of hospitalization. Five of the 6 case-patients were hospitalized at hospital A. Illinois Department of Public Health (IDPH) and Kane County Health Department initiated an investigation to identify additional cases and determine the cause of illness.

The Study

On March 22, IDPH distributed a notice describing the cluster of myocarditis cases to local health departments and healthcare providers in Illinois and requested urgent reporting of similar cases. At hospital A, where most of the initial cases were diagnosed, active surveillance was instituted for patients with a clinical syndrome consistent with myocarditis or pericarditis or an upper respiratory tract illness with profound fatigue or disproportionate shortness of breath of ≥2 weeks' duration. For patients with suspected cases, a testing protocol was implemented, which included a 2-dimensional echocardiogram; electrocardiogram; chest radiograph; measure of serum cardiac enzymes; complete blood count; nasopharyngeal, stool, and urine samples for enterovirus assays; and acute- and convalescent-phase serologic testing for enterovirus.

A review of all records for patients with discharge diagnoses of myocarditis or cardiomyopathy at all 5 hospitals in Kane County from October 1, 2002, through March 31, 2003, was conducted to find unreported cases of myocarditis. Persons with ischemic, alcoholic, postpartum, or chronic cardiomyopathy were excluded. To determine the background number of myocarditis cases for all patients <50 years of age in Kane County, a database search of medical records during the preceding 2-year period (October 1, 2000 to September 30, 2002) at all 5 hospitals was performed by principal International Classification of Diseases, 9th revision (ICD-9) discharge diagnosis codes (Appendix).

A case of myocarditis was defined as 1) a person with myocarditis diagnosed by electrocardiogram, echocardiogram, or cardiac catheterization, which indicates the presence of unexplained arrhythmia or decreased ejection fraction without apparent cause or 2) myocardial inflammatory infiltrates on tissue pathologic examination by using the Dallas criteria (9) or 3) viral isolation or nucleic acid identification in myocardial tissue specimens in persons living in northern Illinois from October 1, 2002, through May 30, 2003.

Medical records of patients were reviewed, and physicians who treated case-patients were interviewed when available. Information was collected about patient demographics; antecedent illness; underlying medical condition; exposure to toxins, pets, or ill persons; recent travel; and smallpox vaccination history.

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The results of echocardiograms and routine specialized laboratory tests, including enterovirus complement-fixation serologic screening, conducted by physicians who evaluated patients at hospitals, were recorded. Nasopharyngeal, urine, and stool specimens from patients were cultured for enterovirus at the IDPH laboratory. Any available serum and myocardial tissue specimens from patients were tested at the California Department of Health Services Viral and Rickettsial Disease Laboratory by using real-time PCR nucleic acid amplification (Amersham Eclipse, Piscataway, NJ, USA) and immunoglobulin M (IgM) enzyme immunoassay for detecting enterovirus and adenovirus (10,11).

Pathology reports on autopsy specimens from patients with fatal cases and myocardial biopsy specimens from patients with nonfatal cases were reviewed. Formalin-fixed, paraffin-embedded tissue from the autopsy of 1 available patients was submitted to the Centers for Disease Control and Prevention (CDC) Unexplained Deaths and Critical Illnesses (UNEX) Laboratory for Gram and calcium staining, enteroviral 5′ noncoding region gene PCR assay, and immunohistochemical staining to detect enterovirus, cytomegalovirus, influenza A, influenza B, and hantavirus.

Sixteen cases, 1 of which (that of patient 8) was recognized through retrospective medical record review, were identified. All patients were hospitalized and admitted between January 28 through April 7 (Figure 1), and 13 patients (81%) were adults <50 years of age. Six (38%) of the 16 patients were hospitalized at hospital A during January through March. For comparison, the number of diagnoses of myocarditis in patients <50 years of age (16 patients) from October 1, 2000, to September 30, 2002, was <1 per month.

The median age for patients was 38 years (range 20–70 years). Among the 16 case-patients, 4 (25%) were residents of Kane County, 8 (50%) were from 5 counties bordering Kane County, and 4 (25%) were from 4 other counties in northern Illinois (Figure 2).

Thirteen case-patients (81%) had an acute, viral-like illness within 1 month before onset of myocarditis. Two female patients, 26 and 39 years of age, had ventricular fibrillation that required an automatic implantable cardioverter defibrillator (AICD) and recovered. There were 2 deaths (see online Table 1, available at http://www.cdc.gov/ncidod/EID/vol11no10/04-1152.htm#table1).

No common exposures could be identified among the patients. None of the patients had recently been vaccinated for smallpox.

Information on acute serologic testing for group B coxsackievirus performed at hospitals was known for 5 patients. Two patients (patients 11 and 14) had elevated antibody titers to group B coxsackievirus. Patient 14 had a
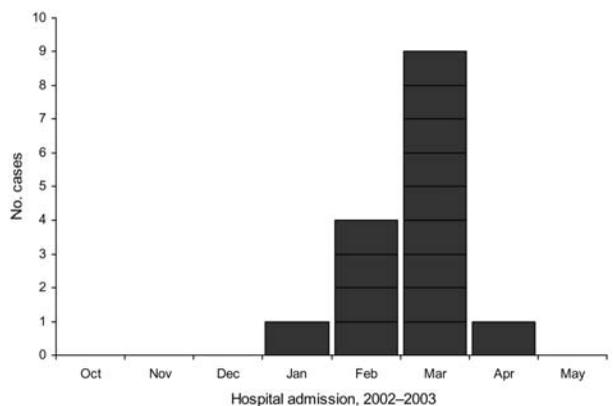

Figure 1. Reported myocarditis case-patients by month of hospital admission, northern Illinois, 2003. (N = 15 because the exact date of admission to hospital was unknown for 1 patient.).

convalescent-phase serum specimen collected for group B coxsackievirus antibody testing that had a 2-fold greater titer than the acute-phase sample. Acute serologic testing for echovirus was performed for 2 patients; results were positive for patient 14 and negative for patient 13. Patient 14 also had an elevated acute-phase influenza B antibody titer but a negative convalescent-phase antibody titer. Patient 12 had no change in acute- and convalescent-phase–positive titers for group B coxsackievirus (online Table 2, available at http://www.cdc.gov/ncidod/EID/vol11no10/04-1152.htm#table2).

IDPH laboratory cultured nasopharyngeal (n = 5), urine (n = 6), stool (n = 6), and myocardial tissue (n = 1) specimens from 9 patients for enterovirus viral isolation. All cultures were negative. Among specimens (serum samples from 11 patients and myocardial tissue from 2 patients) tested for enterovirus and adenovirus by PCR and enzyme immunoassay, all were negative (online Table 2).

For the 2 patients with fatal cases, the primary autopsy diagnosis was acute myocarditis. Autopsy tissue specimens from the 1 case-patient submitted to CDC were negative for viral agents (patient 1).

Conclusions

An outbreak of myocarditis of unknown cause occurred among adults in Kane County (population 400,000) and adjacent areas during winter and early spring 2003. Surveillance for myocarditis cases was initiated throughout Illinois in March and April, although clustering of cases was only evident in and limited to Kane County and surrounding communities. The reporting of myocarditis cases from other counties likely reflected baseline rates of idiopathic myocarditis in those populations that only came to the attention of public health officials through enhanced surveillance.
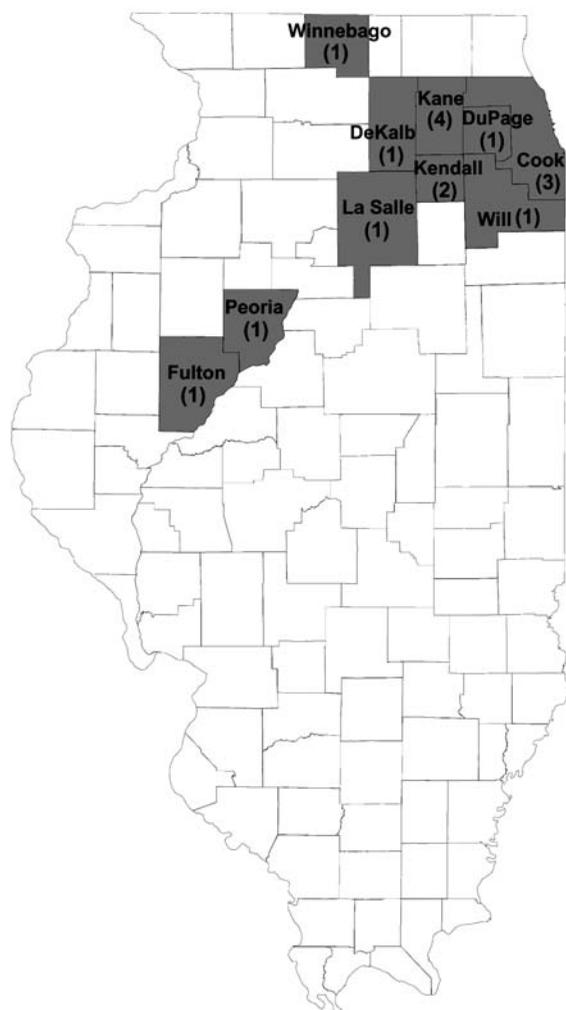
Figure 2. County of residence of reported myocarditis case-patients (N = 16), northern Illinois, 2003.

No common exposures were identified among case-patients. The outbreak occurred within the same period that adverse events of myopericarditis were being reported after smallpox vaccinations among military and healthcare personnel in the United States, including Illinois (12); however, no patients in this outbreak had recently been vaccinated against smallpox. Most illnesses were preceded by a prodrome that suggested the outbreak was viral in origin. Substantial illness and death occurred in these reported cases. All reported patients were hospitalized, 2 required AICD devices, and 2 deaths occurred, a reminder of the severe sequelae associated with this illness.

Despite extensive laboratory testing on submitted specimens, no specific agent was identified. Cross-reactivity of group B coxsackievirus serology with several agents was apparent from initial laboratory tests performed at the hospitals. These results were insufficient to support a specific cause of illness. Tissue specimens from only 3 of the 16 patients were available for testing, which was a major laboratory limitation in the investigation, particularly for detecting viral nucleic acid by PCR assays. The inability to implicate a responsible agent is a common outcome of myocarditis outbreak investigations (1,13).

A better understanding of myocarditis through enhanced diagnostic and therapeutic strategies, increased awareness of possible clusters of illness, and rapid reporting of clusters to public health departments will help improve prevention of future outbreaks. Recent biopsy-based studies suggest that a proportion of life-threatening myocarditis or idiopathic cardiomyopathy in otherwise healthy adults may arise from enteroviral and cytomegalovirus infections (14,15). Research is needed to assess the effect of potential antiviral treatment on illness and death in this patient population. In addition to encouraging appropriate viral testing of acute- and convalescent-phase serologic specimens, further study is required to examine the usefulness of endomyocardial tissue collection for advanced molecular analyses in patients with unexplained cardiomyopathy.

Appendix

Selected Codes for Myocarditis and Pericarditis from the International Classification of Diseases, 9th Revision, Clinical Modification (ICD-9-CM).

Myocarditis: 422.90, 422.92, 422.93, 422.99

Other primary cardiomyopathies: 42.4

Acknowledgments

Dr Huhn completed an infectious diseases fellowship at Rush University Medical Center, Chicago, Illinois, in June 2005. From 2002 to 2004, he was an Epidemic Intelligence Service officer with CDC. His research interests include emerging infectious diseases, tropical medicine, infection control, and HIV.

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The Worriers' Guild
Philip F. Deaver

Today there is a meeting of the
Worriers' Guild,
and I'll be there.
The problems of Earth are
    to be discussed
    at length
    end to end
    for five days
    end to end
    with 1100 countries represented
    all with an equal voice
    some wearing turbans and smocks
    and all the men will speak
    and the women
    with or without notes
    in 38 languages
    and nine different species of logic.
Outside in the autumn
    the squirrels will be
    chattering and scampering
    directionless throughout the town
    because
they aren't organized yet.

Gastroenteritis Outbreak in British Troops, Iraq

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Hazel Appleton,‡ Chris I. Gallimore,‡
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Gastroenteritis affected many British military personnel during the war in Iraq. In the first month, 1,340 cases were seen; 73% of patients required hospital admission and 36% were hospital staff. In a survey of 500 hospital staff, 76% reported gastroenteritis, which was more likely in clinical workers. Investigations showed only caliciviruses.

Infectious diseases are a frequent problem in military campaigns and usually cause more casualties than battlefield injuries (1). Even when they do not have a high death rate, the illness caused may still diminish operational effectiveness. Military deployments are often affected by gastroenteritis (2,3), in particular viral gastroenteritis (4). Norovirus gastroenteritis affected troops during both the Gulf War in 1991 (5) and the Afghanistan campaign in 2002 (6). Caliciviruses (which include both noroviruses and sapoviruses) are well adapted to cause such outbreaks because of their high infectivity, multiple routes of transmission, presymptomatic and postsymptomatic viral shedding, high survivability in the environment, resistance to disinfectants, and poor long-term immunity after infection. The effects and economic costs of these infections in hospital facilities have recently been assessed (7).

British troops invaded Iraq on March 21, 2003, and lived in minimal hygiene facilities for several weeks. During the first week of the conflict, they ate individual ration packs only, but centralized catering and locally produced fresh rations (including salads and fruit) were introduced during the second week. A 200-bed British military field hospital was established in Iraq on March 26 for 2 months. During this time 26,000 British troops were in the area, and ≈50% were close enough to visit the hospital with illnesses such as gastroenteritis. A large outbreak of gastroenteritis affected many British units, including the staff of the field hospital. We reviewed the outbreak in patients from local units and assessed its effect on the field hospital.

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The Study

Data from hospital records were collected from March 28, 2003, when the first gastroenteritis cases were observed, until April 29, 2003, when the number of gastroenteritis cases decreased to <20 per day. A subsequent retrospective survey of hospital staff was carried out from April 19 to May 3, 2003, when the number of cases began to decrease. The survey used a face-to-face structured questionnaire designed to study possible risk factors and outcomes associated with gastroenteritis. Gastroenteritis was defined as having ≥2 of the following: diarrhea, abdominal pain, nausea, vomiting, and either myalgia, headache, or subjective fever. The presence of any of the last 3 symptoms was counted as 1 symptom overall.

Because of the situation and number of cases, conducting fecal investigations for all patients was not possible. Instead, investigations were targeted at the most severe or atypical cases after clinical assessment on daily postadmission ward rounds. Fecal samples were collected within 24 hours of admission. Ova, cysts, and parasites were sought by microscopy with standard wet-preparation techniques and staining with iodine where indicated. Enteropathic bacteria, including *Campylobacter* spp., *Salmonella* spp., *Shigella* spp., and *Escherichia coli* O157, were detected by standard microbiologic culture techniques. We tested for *Vibrio* spp. where clinically indicated. Eight refrigerated fecal samples were sent to the United Kingdom reference laboratory for enteric viruses (Centre for Infections, Health Protection Agency), where enteropathic viruses were sought by electron microscopy and reverse transcription–polymerase chain reaction (RT-PCR) (8). These studies were reviewed and approved by the field hospital ethics committee.

Statistical analysis of results was carried out by using SPSS version 12 software (SPSS Inc., Chicago, IL, USA). Unadjusted odds ratios (ORs) were derived from 2 × 2 tables. Unadjusted ORs for age groups were determined by using a chi-square test for linear trend. Adjusted ORs (AORs) were estimated by logistic regression analysis by using a backwards elimination technique. Variables with the lowest significance were sequentially removed until the best fit model was achieved.

During the first month of the conflict, 2,065 patients were seen at the hospital, of whom 1,466 (71%) were managed by internal medicine physicians. Among these, 1,340 (91%) had gastroenteritis; 975 (73%) required admission. The epidemic distribution of gastroenteritis patients who came to the hospital is shown in Figure 1. Admission rates were initially 93% but gradually decreased to 13% during the study period. Mean length of stay in the hospital was 1.65 days, which resulted in 1,608 bed-days occupied, and bed occupancy rates reached >90%. Hospital staff accounted for 36% of all gastroenteritis patients seen.
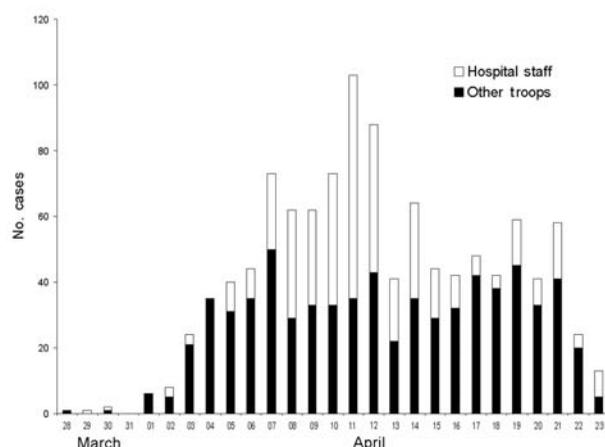
Figure 1. Gastroenteritis patients who came to the field hospital in March and April 2003.

Medical units and armored units appeared to be most affected.

Five hundred (77%) of 648 hospital staff were surveyed when the number of cases began to decrease. Among these, 382 (76%) had gastroenteritis, of whom 202 (53%) had diarrhea that persisted for >48 hours longer than their other symptoms (range 2–14 days). Overall, 292 (60%) of the hospital staff surveyed visited the hospital for medical care, and 192 (39%) were admitted because of gastroenteritis. Those affected required a mean (SD) of 2.1 (1.7) days off work; 796 work days were lost in the 77% of staff surveyed.

Among the 382 hospital staff with gastroenteritis, symptoms are shown in Table 1, and risk factors for gastroenteritis compared with unaffected staff are shown in Table 2. Gastroenteritis was more likely in clinical staff (AOR 1.98, 95% confidence interval [CI] 1.28–3.01) and women (AOR 1.63, 95% CI 1.00–2.65), but no association was found between gastroenteritis and age, blood group, or reported contamination of living areas by vomit, feces, or both.

Fecal microscopy and culture were conducted for 69 patients during this 2-month deployment. No parasites or bacterial pathogens were found during the first month when the initial outbreak occurred. Only 2 fecal samples (collected during week 2 of the outbreak) arrived at the UK reference laboratory. Both samples showed caliciviruses by electron microscopy and had the classic appearance usually associated with sapoviruses rather than noroviruses. They could not be identified by RT-PCRs for noroviruses, sapoviruses, or astroviruses. However, using norovirus primers that amplified contiguous regions of open reading frames (ORFs) 1 and 2 (9), we detected a norovirus-specific amplicon in 1 sample. Subsequently, the norovirus was identified by cDNA sequencing as an unusual strain with 96% identity to NV/Idaho Falls/378/1996/US (GenBank accession no. AY054299) and designated NV/Shaibah/2003/IQ (Figure 2). This strain does not group within any of the 15 previously identified norovirus genotypes (10) but is similar to novel strains identified in US military personnel during this same period (11).

Conclusions

This study was conducted in a war zone by clinicians in a busy military field hospital. Surveying hospital cases inevitably underestimates the true scale of the problem faced by military units in Iraq. The fact that 71% of patients were seen by internal medicine physicians is typical of the disease-to-trauma ratio that occurs in military conflicts (1) and overseas training exercises (12).

Gastroenteritis was the major cause of illness in hospital cases during this operation and probably diminished the operational effectiveness of those units affected. Admission rates were initially high because of exhaustion, dehydration, and poor living conditions during the first month of the conflict. Viral gastroenteritis is readily transmitted in healthcare settings (7) and confined living spaces, which may explain why medical and armored units were most affected. Environmental health measures planned for this deployment had high standards, but they were not fully implemented in time to prevent this outbreak, which coincided with the delivery of fresh rations.

The survey of hospital staff indicates the scale of the problem faced by the field hospital, but this may also be an underestimate since cases were still occurring while data were collected. Since gastroenteritis was more common in clinical workers, infection control at the hospital may have been inadequate. Hygiene standards for the hospital were also high (13), but they were not fully implemented in time to prevent the outbreak among hospital staff.

The wide epidemic distribution suggests a prolonged period of disease transmission, rather than a single point source outbreak. Although this outbreak fulfills the criteria of Kaplan et al., which suggest that a calicivirus was responsible (14), data were insufficient to determine the cause. However, a similar outbreak on a Royal Navy hospital ship occurred during this period when locally produced fresh salad was taken on board; several enteric viruses were subsequently identified (8).

Table 1. Symptoms in 382 hospital staff with gastroenteritis	
Symptom	No. (%)
Diarrhea	341 (89)
Abdominal pain	323 (85)
Nausea	304 (80)
Vomiting	215 (56)
Fever (subjective)	212 (55)
Myalgia	210 (55)
Headache	209 (55)

Table 2. Analysis of risk factors for gastroenteritis in 500 hospital staff*

Risk factor	GE, no. (%) (n = 382)	No GE, no. (%) (n = 118)	OR (95% CI)	p value	AOR (95% CI)
Clinical worker	208 (54)	43 (36)	2.09 (1.36–3.19)	<0.01	1.98 (1.28–3.01)
Female	153 (40)	35 (30)	1.58 (1.02–2.47)	<0.05	1.63 (1.00–2.65)
Contaminated accommodation	138 (36)	53 (45)	0.69 (0.46–1.05)	0.09	–
Age, y					
18–29	126 (33)	36 (31)	1.00 (Referent)	–	–
30–39	155 (41)	51 (43)	0.87 (0.52–1.45)	0.57	–
>39	101 (26)	31 (26)	0.93 (0.52–1.67)	0.80	–
Blood group					
O	181 (47)	54 (46)	1.07 (0.69–1.65)	0.76	–
A	147 (38)	53 (45)	0.77 (0.49–1.19)	0.21	–
B	36 (9)	7 (6)	1.65 (0.68–4.19)	0.24	–
AB	18 (5)	4 (3)	1.41 (0.44–5.03)	0.54	–

*GE, gastroenteritis; OR odds ratio; CI, confidence interval; AOR, adjusted odds ratio after logistic regression analysis.

This outbreak in Iraq would have had an even greater effect if it had been caused by a virulent species of *Salmonella* or *Shigella*, which are common foodborne pathogens in this region. We believe introducing fresh rations during a military operation, before adequate health and infection control measures were fully implemented, was inadvisable. In contrast, Royal Marines based on the Al-Faw Peninsula were not supplied with fresh rations dur-

ing this period, and no outbreaks of gastroenteritis were reported (S. Bree, pers. comm.). More rigorous, prospective studies are required to understand the epidemiology and effect of gastroenteritis in deployed military personnel (15).

We recommend that improvements be made in the implementation of environmental health and infection control measures during operational deployments. Military units should avoid fresh rations during military operations until adequate hygiene measures have been fully implemented and inspected. Further studies and changes to working practices are required to prevent and control similar outbreaks in the future.
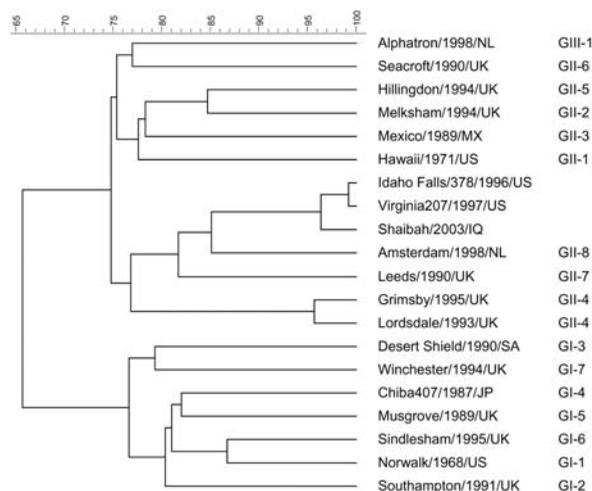
Acknowledgments

Figure 2. Dendrogram of the 5′ end of open reading frame 2 of noroviruses, including the Shaibah strain. Scale at the top shows percent relatedness between different strains. GenBank strains are Alphatron/1998/NL (AF195847), Seacroft/1990/UK (AJ277620), Hillingdon/1994/UK (AJ277607), Melksham/1995/UK (X81879), Mexico/1989/MX (U22498), Hawaii/1972/US (U07611), Idaho Falls/378/1996/US (AY054299), Virginia207/1997/US (AY038599), Amsterdam/1998/NL (AF195848), Leeds/1990/UK (AJ277608), Grimsby/1995/UK (AJ004864), Lordsdale/1995/UK (X86557), Desert Shield/1990/SA (U04468), Winchester/1995/UK (AJ277609), Chiba407/1987/JP (AB022679), Musgrove/1989/UK (AJ277614), Sindlesham/1995/UK (AJ277615), Norwalk/1969/US (M87661), and Southampton/1991/UK (L07418). The sequence of the Shaibah/2003/IQ strain can be obtained from the Enteric Virus Unit, Virus Reference Department, Centre for Infections, Health Protection Agency (christopher.gallimore@hpa.org.uk).

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Detecting Biological Warfare Agents

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We developed a fiber-optic, microsphere-based, high-density array composed of 18 species-specific probe microsensors to identify biological warfare agents. We simultaneously identified multiple biological warfare agents in environmental samples by looking at specific probe responses after hybridization and response patterns of the multiplexed array.

Accurately detecting and identifying biological warfare agents (BWAs) is the focal point for countering bioterrorism. Current methods used to identify BWAs are primarily focused on a single target agent (1–10). In contrast, the fiber-optic, microsphere-based, multiplexed arrays described in this paper can rapidly screen and simultaneously identify multiple potential BWAs, enabling an efficient response to a bioterrorism attack. Such multiagent analysis is difficult because it is usually complicated by interferences between assays and the large number of BWA probes.

We developed a platform to simultaneously detect BWAs with an array composed of DNA probe-functionalized microspheres that are randomly distributed onto microwells generated on the end of an etched coherent optical fiber bundle (11,12) that contains 6,000–50,000 individual fibers. Methods are described more fully in the Appendix, available online at http://www.cdc.gov/ncidod/EID/vol11no10/05-0269_app.htm. A multiplexed array was fabricated by distributing up to 18 different microsphere sensors into the optical fiber array with species-specific 50-mer DNA probes corresponding to 6 BWAs of interest (*Bacillus anthracis*, *Yersinia pestis*, *Francisella tularensis*, *Brucella melitensis*, *Clostridium botulinum*, and vaccinia virus) and 1 BWA simulant, *Bacillus thuringiensis kurstaki*, with each organism represented by at least 2 probe sequences (online Appendix Table 1). All probe sequences were designed with software by Illumina, Inc. (San Diego, CA, USA), and the specificity of the probe sequences was checked against GenBank by using BLAST to have minimal cross-hybridization potential. Employing multiple probes for each BWA reduces the potential for both false-negative and false-positive results in environmental samples. The inherent redundancy of each probe type on the array, because each

microsphere type has many replicates, provides additional confidence in detection and enhances the signal-to-noise ratio. Because the assembly process for the arrays is random, each bead type is encoded with an identifying optical bar code (11,12) created by entrapping dyes in various combinations within microspheres, to differentiate microsphere probe types from one another.

To ensure sensitive detection of BWAs with high accuracy, the performance of this multiplexed, high-density array was assessed by examining the detection limits and specificity of probes with Cy3-labeled synthetic targets (the synthetic target is a 50-mer oligonucleotide complementary to the probe). We modified our previous protocol of microsensor preparation (12) by using an increased concentration of cyanuric chloride solution in the probe activation step to achieve a more complete and efficient coupling of DNA probes to microspheres. The improved microsensor preparation enabled a detection limit as low as 10 fmol/L (in 50 μL volume) within 30 min hybridization time (data not shown). While the probes were selected to avoid the formation of secondary structures, such structures were not completely eliminated and probably affected the hybridization efficiency, resulting in higher detection limits for some probes on the array. To evaluate probe specificity, arrays containing beads with only a single probe type were exposed sequentially to the synthetic complementary target as well as to all noncomplementary targets. Cross-reactions were observed between probes from the same organism such as BA1/BA2, BA5/BA6, BTK1/BTK2, YP1/YP2, and FT1/FT2 (probes are abbreviated according to the organism from which they were derived: BA, *B. anthracis*; YP, *Y. pestis*; FT, *F. tularensis*; BM, *Brucella melitensis*, CB; *C. botulinum*; VA, vaccinia virus; and BTK, *Bacillus thuringiensis kurstaki*; details in online Appendix Table 2) because some of the sequences for each probe pair overlapped, as probes were designed to be specific to the same target gene of the BWA. While in some applications cross-reactions are undesirable, in the present context the cross-reactions between probes from the same BWA confirmed each BWA in the sample. In addition, some minor cross-reactions between probe types from different BWAs were observed because of sequence similarity in spite of the careful probe design for each BWA (online Appendix Table 2). These cross-reactions could be advantageous, since they generated a unique response pattern across all the probes on the multiplexed array for each target BWA and provided additional information to identify BWAs. As a result, any BWA can be simultaneously identified with this multiplexed array by looking at the high signals resulting from the specific response of each individual species-specific probe as well as the response pattern across the array.

To sensitively detect target BWAs, polymerase chain reaction (PCR) was used as an amplification step before

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hybridization on the multiplexed array platform. Amplification was essential, especially when BWAs were present at trace levels in environmental samples (see below). Primer pairs that corresponded to each of the target sequences were designed specifically for each BWA (online Appendix Table 1). Each primer pair amplified the respective BWA sequence from an autoclaved bacterial culture (online Appendix Table 3) during asymmetric PCR. Figure 1 shows the detection of PCR products derived from each individual primer pair based on single bead–type arrays. The variation of the probe responses to PCR products can be explained by the different concentrations of the autoclaved bacterial cultures used as templates in PCR, the varying efficiency of primer pairs, or the potential of PCR products to form secondary structures. While carefully designed and optimized to have PCR products with a shorter length (147–201 bp), some of the amplified PCR fragments might form secondary structures (e.g., BA5). The formation of secondary structures makes the DNA fragments that were complementary to the array probe sequences inaccessible for hybridization. For these targets, the hybridization efficiency was affected, giving low hybridization responses to PCR products. In addition, the position of the target sequences within the PCR amplicons could affect hybridization efficiency. Oligonucleotide probes complementary to the middle part or the 5′ position of PCR products showed lower hybridization efficiencies than did target sequences close to the 3′ positions. This observation was likely related to steric hindrance that led to less accessibility of PCR products to the probe sequences on the array.

To assess the limit of detection with real bacterial samples, 10-fold serial dilutions were made from the autoclaved cultures of each BWA (online Appendix Table 3) and used for PCR followed by array hybridization (summarized in the Table). Specificity tests of primer pairs were performed by applying each primer pair to other nontarget bacterial cultures individually, and no nonspecific amplifications were observed. To investigate the ability to simultaneously detect BWAs, multiplex PCR was used to analyze mixed bacterial cultures that contained multiple BWAs, e.g., *B. anthracis* mixed with *B. thuringiensis kurstaki* and *Y. pestis* mixed with *F. tularensis*, in varying ratios. We prepared 2 primer pools for the 7 BWAs of interest, and each pool contained 1 primer pair specific for each BWA. Figure 2 shows the amplification of BWAs in each mixed culture sample using primer pools I and II. The amplified PCR products were subjected to multiplexed array detection with each BWA represented by at least 2 probe types. The online Appendix Figure shows the results of simultaneous detection of BWAs in different mixed culture samples after amplification with primer pool I (online Appendix Figure, panel A) and pool II (online Appendix
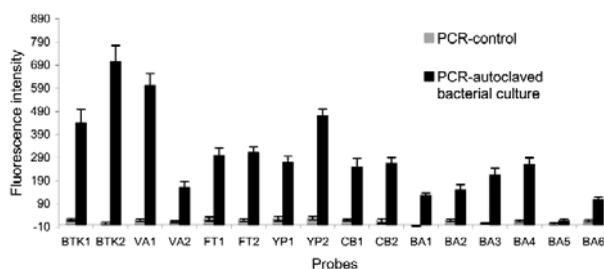

Figure 1. Array detection of single polymerase chain reaction (PCR) products from autoclaved bacterial cultures of the 6 microorganisms listed in online Appendix Table 3 by using single bead–type arrays. PCR and array hybridization conditions are described in the Appendix. Single bead–type arrays were fabricated containing ≈100 replicates of each microsphere probe. The standard deviation (SD) of background images is 15 (N = 3), and the detection limit is 45, defined as 3 × SD.

Figure, panel B). For the mixtures of *B. anthracis* and *B. thuringiensis kurstaki*, both probes BTK1 and BTK2 gave positive signals, as did probes BA1, BA2, and BA4, which indicates the presence of *B. anthracis* and *B. thuringiensis kurstaki*. Since the primer pair specific for *B. anthracis* in primer pool I and pool II corresponded to the BA2 and BA4 sequences, respectively, no positive signals were observed from the other BA probes (BA3, BA5, and BA6); no cross-reactions were seen between them except for probes BA1 and BA2 (online Appendix Table 2). For mixtures of *Y. pestis* and *F. tularensis*, differentiating 2 bands (Figure 2B, lane 9 and lane 10) on the gel is difficult because the product size is similar; however, positive signals from the respective probes after hybridization showed these 2 BWAs in the mixed culture samples (online Appendix Figure, panel B). Array detection in conjunction with multiplex PCR is advantageous in that it not only simultaneously identifies target BWAs but also minimizes the possibility of misinterpreting PCR results because it adds an additional level of specificity. With gel analysis only, PCR results are usually complicated because of nonspecific amplification during multiplex PCR with environmental samples.

Since real environmental samples will likely involve complex matrices with many potential interferents, the performance of the multiplexed array was further assessed

Table. Detection limits of real samples determined with polymerase chain reaction followed by array detection

Organism	DNA copies/mL
Bacillus anthracis	10^3
B. thuringiensis kurstaki	10^2
Vaccinia virus	10^2
Yersinia pestis	10^2
Francisella tularensis	10^3
Clostridium botulinum	10^3

with wastewater spiked with individual autoclaved bacterial cultures of BWAs at various volumetric dilution factors (1:3 and 1:10) (online Appendix Table 3). Both primer pools were applied to all spiked wastewater samples for multiplex PCR. The probes on the multiplexed array gave positive responses only to the amplified PCR products of interest, without responding to any nonspecifically amplified products. Online Appendix Figure, panel C gives an example of array detection of the amplified BWAs from spiked wastewater samples (1:10 dilution) with primer pool I. BWAs seeded into wastewater at various dilution factors were all identified successfully within 30 minutes of hybridization.
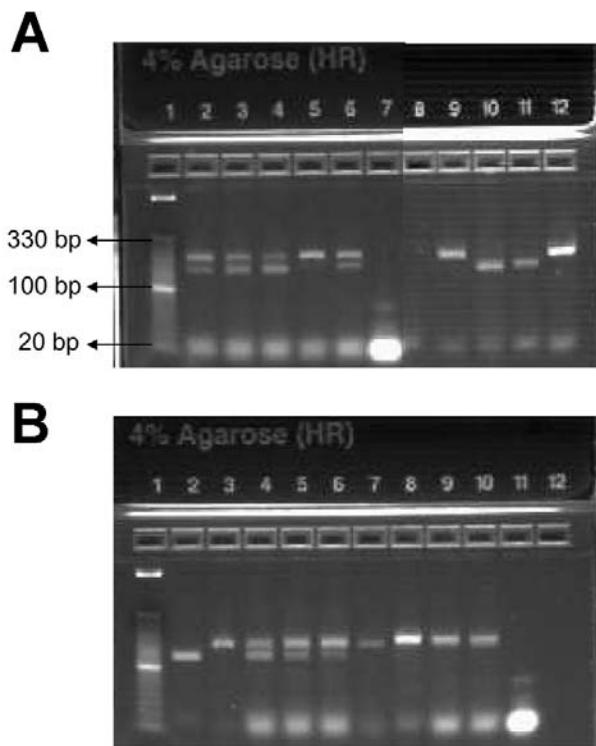

Figure 2. Gel analysis of multiplex polymerase chain reaction (PCR) with mixed autoclaved bacterial culture samples using primer pool I (A) and II (B). A) Lane 1, DNA ladder; lane 2, *Bacillus anthracis* (BA) + *B. thuringiensis kurstaki* (BTK), 1:1; lane 3, BA + BTK, 1:5; lane 4, BA + BTK, 1:9; lane 5, *Yersinia pestis* (YP) + *Francisella tularensis* (FT), 1:1; lane 6, YP + FT, 1:9; lane 7, negative control (no template); lane 8, BA-negative control (no template); lane 9, BA-positive control; lane 10, BTK-positive control; lane 11, FT-positive control; lane 12, YP-positive control. B) Lane 1, DNA ladder; lane 2, BA-positive control; lane 3, BTK-positive control; lane 4, BA + BTK, 1:1; lane 5, BA + BTK, 1:5; lane 6, BA + BTK, 1:9; lane 7, YP-positive control; lane 9, YP + FT, 1:1; lane 10, YP + FT, 1:9; lane 11, negative control (no template); lane 12, blank well. Primer pool I contained 7 primer pairs, BA2, BTK1, FT1, YP1, CB1, VA1, and BM1. Primer pool II contained BA4, BTK2, FT2, YP2, CB2, VA2, and BM2. Each primer pool has 1 primer pair for each biological warfare agent of interest. A positive control was run in single PCR with the corresponding primer pair. See Appendix for the conditions of multiplex PCR.

In summary, this fiber-optic, microsphere-based array platform provides fast, sensitive, and simultaneous identification of BWAs with high accuracy. The high density of this array format can accommodate additional probe types while still maintaining a high redundancy for each probe type on the array. Additional probe types could be added to the array without affecting the performance of the existing microspheres. The ability to expand the probe types in the array opens up the opportunity to incorporate a large number of potential BWAs when their genome sequences become available. As a result, the multiplicity of arrays could be increased by incorporating an even broader class of potential BWAs and other pathogens, leading to a universal array for all pathogenic agents of interest.

Ms Song is a graduate student in chemistry at Tufts University. Her research interests include applications of DNA microarrays and microarray-based detection of biological warfare agents and other infectious biological agents.

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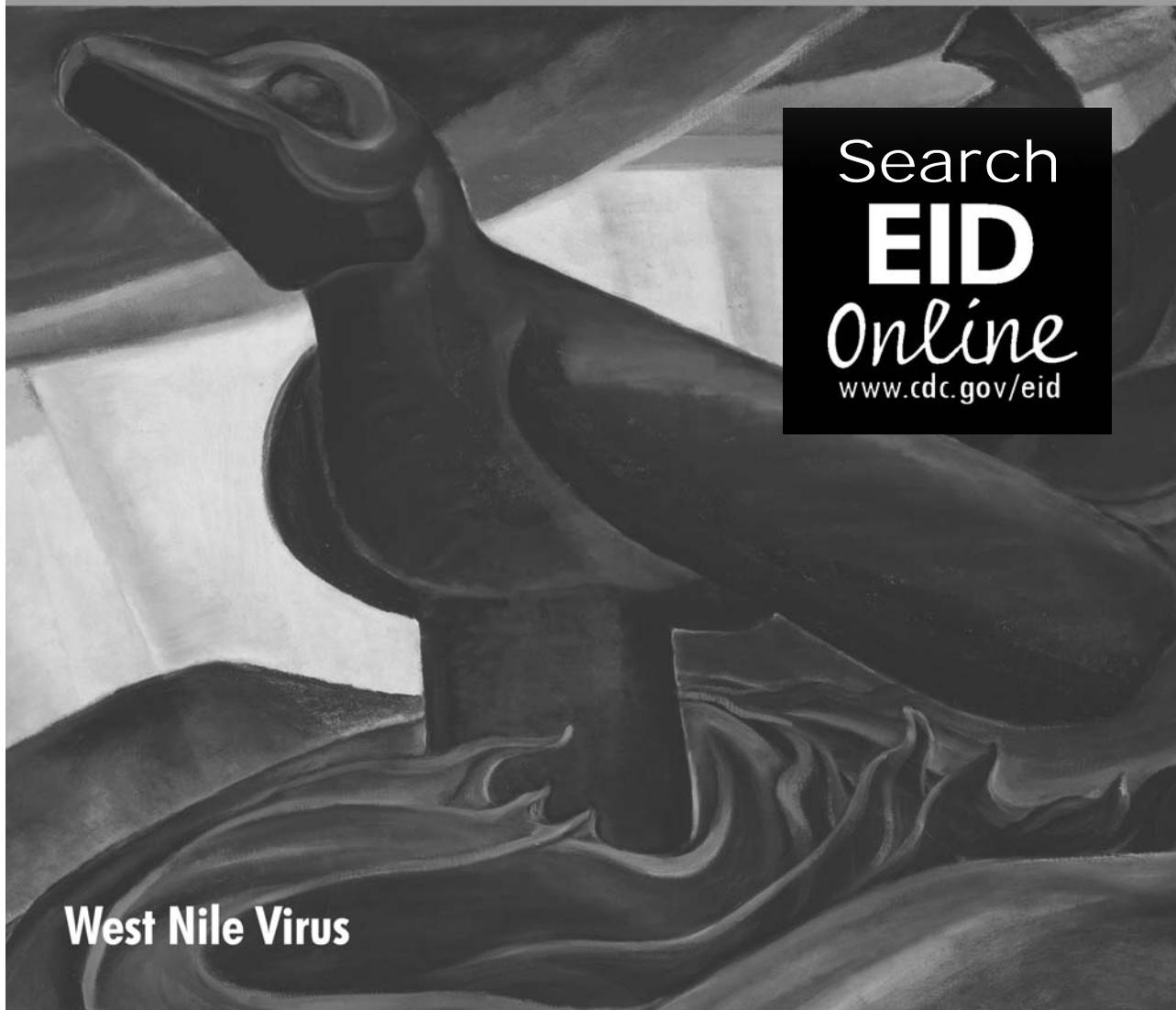
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West Nile Virus

Rapid West Nile Virus Antigen Detection

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We compared the VecTest WNV antigen assay with standard methods of West Nile virus (WNV) detection in swabs from American Crows (*Corvus brachyrhynchos*) and House Sparrows (*Passer domesticus*). The VecTest detected WNV more frequently than the plaque assay and was comparable to a TaqMan reverse transcription–polymerase chain reaction.

Dead bird surveillance is an effective way to monitor the presence and spread of West Nile virus (WNV) in North America (1), and assays to detect infectious WNV virions, antigen, and RNA in tissues from infected birds are reliable techniques (2–4). More than 28,000 bird carcasses were tested for WNV in the United States from 1999 to 2002 (5). Processing and testing these carcasses require a substantial commitment of resources from federal, state, and local health departments. Simplifying diagnostic procedures by implementing rapid antigen-capture assays would permit increased specimen processing and, ultimately, improved surveillance.

Cloacal and oral (nasopharyngeal) swabs from dead corvids (crows and jays) are reliable sources of WNV RNA and infectious virions (6). Three field evaluations of an antigen detection assay applied to corvid carcasses collected shortly after death found that oral swabs were more sensitive than cloacal swabs for detecting WNV antigen, and that sensitivity of the VecTest WNV antigen assay (Medical Analysis Systems, Camarillo, CA, USA) applied to oral swabs was >80% for American Crows, lower for other corvids, and variable for a variety of other species (7–9). Several questions remain unanswered regarding the usefulness of swab specimens for WNV surveillance. How long after death of a bird can WNV be detected in swab specimens? Can swabs from noncorvid birds be used to detect WNV? Can reverse transcription–polymerase chain reaction (RT-PCR) or VecTest detect WNV in oral swab samples that have remained dry and at room temperature?

To address these questions, we compared the VecTest WNV antigen assay with standard methods of virus detec-

tion from oral and cloacal swabs taken 1–4 days postmortem from experimentally infected American Crows (*Corvus brachyrhynchos*) and House Sparrows (*Passer domesticus*). The VecTest, which was originally developed for mosquito pools as a simple, 1-step wicking assay available in a kit, requires no specialized equipment, storage conditions, or highly trained personnel and provides results in 15 minutes (10,11).

The Study

Oral and cloacal swab samples were collected daily (for 4 days) from carcasses of crows and sparrows that had been experimentally infected with either the NY99-4132 strain (30 crows and 6 sparrows) or the Kenyan KN-3829 strain (1 crow and 5 sparrows) of WNV. Carcasses were stored at ambient temperature ($\approx 20°C$) during this period. The samples were collected with standard, cotton-tipped applicators by inserting them into the cloaca or oral cavity and then placing them directly into 1 mL VecTest grinding solution A, a physiologic buffer similar to phosphate-buffered saline. Samples were subsequently frozen at –70°C until tested by a variety of methods for detecting WNV. Some oral swabs from infected crows were left at room temperature without diluent for 24 or 48 hours before testing. Negative control swab samples were collected from 25 live, healthy, uninfected crows.

All swab specimens collected from crow carcasses were positive by the TaqMan RT-PCR method, using 2 sets of WNV-specific primers (2). Several TaqMan RT-PCR–negative swabs for the sparrows were also negative by the other assays; these were disregarded in summarizing the results. Results of VecTest and Vero plaque assay (11) of the RT-PCR–positive swab specimens are shown as sensitivities (using RT-PCR as the standard for detecting WNV RNA) in Table 1. A logistic regression model accounting for anticipated correlation induced by multiple and repeated observations on each bird was used to compare sensitivities for each day postmortem, with significance determined using $\alpha = 0.05$ (12). For crows evaluated 1 day postmortem, no significant difference between swab types (oral versus cloacal) (p = 0.63) and no significant difference between the 2 assays (p = 0.10) were detected. At 2 days postmortem, the effect due to swab type was not significant (p = 0.07), but a significant difference was seen in the sensitivities of the 2 assays (p = 0.004), excluding the non-significant effect of swab type from the logistic regression model. At 3 days postmortem, both swab type and assay differences were significant (p<0.01), with oral swabs more likely to yield a positive finding (compared with cloacal swabs) and VecTest more sensitive than plaque assay. For sparrows, no significant differences were seen between the sensitivities of the VecTest and plaque assay for either swab type on any of the 3 days (McNemar test).

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Table 1. Sensitivities of the VecTest and plaque assay in detecting West Nile virus in American Crows and House Sparrows, by swab type and day postmortem*

Species	Day postmortem	Assay	Swab type	
			Oral, % positive (no. tested)	Cloacal, % positive (no. tested)
American Crow	1	VecTest	90 (30)	90 (29)
		Plaque	83 (30)	69 (29)
	2	VecTest	93 (30)	100 (29)
		Plaque	90 (30)	55 (29)
	3	VecTest	97 (30)	83 (30)
		Plaque	83 (30)	13 (30)
	4	VecTest	100 (5)	80 (5)
		Plaque	80 (5)	0 (5)
House Sparrow	1	VecTest	70 (10)	67 (9)
		Plaque	90 (10)	56 (9)
	2	VecTest	75 (8)	100 (2)
		Plaque	75 (8)	50 (2)
	3	VecTest	83 (6)	NA
		Plaque	83 (6)	NA

*NA, not available (valid sampling was not possible given the physical state of the bird).

VecTest detected WNV in 90% of 22 crow oral swabs that were tested after remaining dry and at room temperature for 24 hours and in 70% of 13 crow oral swabs assayed after 48 hours. By comparison, TaqMan RT-PCR detected WNV in 86% and 70% of these oral swabs, respectively, at the same time points.

Over the 4-day sampling period, geometric mean viral titers in crow oral swabs, determined by Vero cell plaque assay, decreased from $10^{3.6}$ to $10^{2.2}$ PFU/swab (Table 2). In contrast, the geometric mean viral titer in crow cloacal swabs decreased from $10^{3.0}$ PFU/swab at 1 day postmortem to undetectable by 4 days postmortem. RNA levels, as detected by the TaqMan assay, also decreased over time.

Conclusions

VecTest has the potential to simplify dead bird surveillance for WNV by reducing required resources such as specialized equipment and costly reagent kits needed to achieve a rapid and accurate result. With appropriate biosafety measures, the assay can be conducted in the field, or in centralized regional laboratories, obviating the need for expensive shipping of bird carcasses to remote reference laboratories.

One objective of our study was to determine whether oral or cloacal swabs were preferable for WNV testing of dead birds. To answer this question, several criteria were evaluated, including the ability of 3 different assays to detect WNV, the feasibility of collecting specimens postmortem, and postmortem duration of WNV positivity. TaqMan RT-PCR detected WNV RNA and antigen in similar proportions in all cloacal and oral specimens collected from crows. However, virus isolation by Vero plaque assay was more successful when oral swabs were tested. Virus appears to be more rapidly inactivated in the cloaca compared with the oral cavity. This phenomenon was consistent for both sparrows and crows.

Fewer postmortem swab samples were available from sparrows compared with those from crows because fewer sparrow carcasses were available (sparrows are less susceptible to fatal WNV infection than crows) (13). Collecting cloacal swabs from the smaller sparrows was also more difficult after 1 day postmortem because they tended to desiccate quickly. RT-PCR detected WNV RNA in sparrows from 24/24 oral swabs, but only 11/13 cloacal swabs. Antigen was detected by VecTest from 18/24 oral and 8/13 cloacal swabs. Infectious virus was detected by plaque

Table 2. Log geometric mean titer (SD) of West Nile virus PFU equivalents (TaqMan RT-PCR) and PFU (plaque assay) for American Crows and House Sparrows, by swab type and day postmortem*

Species	Swab type	Assay	Day postmortem			
			1	2	3	4
American Crow	Oral	TaqMan	4.8 (1.1)	4.7 (0.6)	4.3 (0.8)	3.5 (1.5)
		Plaque	3.6 (1.9)	3.2 (1.5)	2.4 (1.6)	2.2 (2.0)
	Cloacal	TaqMan	5.2 (1.2)	5.5 (1.3)	4.6 (1.9)	3.5 (0.7)
		Plaque	3.0 (2.4)	1.4 (1.5)	0.3 (0.7)	0.0 (0.0)
House Sparrow	Oral	TaqMan	4.3 (1.4)	5.0 (1.7)	4.6 (1.7)	NA
		Plaque	3.8 (2.0)	3.0 (1.9)	2.9 (2.5)	NA
	Cloacal	TaqMan	3.8 (1.6)	4.3 (3.3)	NA	NA
		Plaque	1.9 (2.9)	0.7 (0.9)	NA	NA

*RT-PCR, reverse transcription–polymerase chain reaction; NA, not available (valid sampling was not possible given the physical state of the bird).

assay in 20/24 oral swabs but in only 6/13 cloacal swabs. Virus titers and RNA concentrations in the carcasses decayed over the 4-day period of observation and this decay was most pronounced in the cloacal swabs. Thus, oral swabs were more effective than cloacal swabs to detect WNV in both crows and sparrows.

VecTest consistently detected WNV antigen in a greater proportion of samples than Vero plaque assay detected virions. Thus, although the detection of infectious virus was inconsistent, carcasses contained sufficient quantity of viral components, both RNA and protein, to permit detection for ≥4 days after death. In a natural setting, carcasses most likely would decay more rapidly than in these experiments, given exposure to temperature fluctuations, microbial attack, and predation. Guidelines for WNV surveillance recommend sampling carcasses <24 hours old (14). These results suggest that older carcasses may have detectable WNV RNA and antigen that still are readily detectable with the TaqMan and VecTest assays. Thus, carcasses should be tested regardless of age, as long as they are not in a condition where sampling is impossible. In addition, swabs collected in the field can be stored at room temperature in empty cryovials for up to 48 hours and then reliably assayed for WNV antigen by VecTest.

Detecting WNV from sparrow carcasses demonstrates that swabs are useful to test species other than corvids. House sparrows, like corvids, are passerine birds that develop high levels of WNV in blood and tissues (13). Stone et al. showed that the VecTest had a sensitivity of 76% in detecting WNV in oral swabs of field-collected carcasses of house sparrows (9).

In summary, oral swabs are more useful than cloacal swabs for obtaining a reliable result with the diagnostic assays described in our study. Moreover, swabs from non-corvid birds may also be effectively assayed for WNV. Our findings suggest that large numbers of dead corvids of any age, and possibly other passerine birds, could be screened by cautiously collecting dry oral swabs in the field, storing them properly, and then testing them within 48 hours by rapid antigen detection assay or RT-PCR.

Acknowledgments

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Saving Lives through Global Safe Water

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Unsafe water is a global public health threat, placing persons at risk for a host of diarrheal and other diseases as well as chemical intoxication. Unsanitary water has particularly devastating effects on young children in the developing world. Each year, >2 million persons, mostly children <5 years of age, die of diarrheal disease (1,2). For children in this age group, diarrheal disease accounted for 17% of all deaths from 2000 to 2003 (3), ranking third among causes of death, after neonatal causes and acute respiratory infections. Severe, prolonged diarrheal disease can also lead to malnutrition and impaired physical and cognitive development (4). Nearly 90% of diarrhea-related deaths have been attributed to unsafe or inadequate water supplies and sanitation (5)—conditions affecting a large part of the world's population. An estimated 1.1 billion persons (one sixth of the world's population) lack access to clean water and 2.6 billion to adequate sanitation (5).

At the 2000 United Nations (UN) Millennium Summit, member states adopted a set of 8 goals and related targets and indicators aimed at helping end human poverty and its ramifications (6). Among these Millennium Development Goals is a call to halve by the year 2015 the proportion of persons without sustainable access to safe drinking water and basic sanitation. Although some progress has been made, much remains to be done. Toward this end, in March 2005, the UN launched the "International Decade for Action: Water for Life 2005–2015." A UN Millennium Project Task Force has identified 5 guiding principles and 10 actions needed to intensify efforts to meet the targets (7,8). Building on lessons learned from the previous International Drinking Water and Sanitation Decade during the 1980s will also be an important part of this process. Success in reaching these targets will help achieve the other 7 Millennium Development Goals, increase workforce productivity, and substantially reduce the amount of time that women and children spend collecting and storing water, which will free them to pursue other productive and educational activities. Moreover, reaching these goals will

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be an important step toward breaking the cycle of poverty and disease.

A collaborative, interdisciplinary effort to ensure global access to safe water, basic sanitation, and improved hygiene is the foundation for ending this cycle. In addition to mobilizing political will among national leaders and heads of international agencies, this effort will require sustained involvement and commitment from a broad range of public and private-sector organizations, such as CARE and Procter & Gamble, which have long been involved in efforts to provide safe water in developing countries. Other corporations joining these efforts include The Coca-Cola Company and Starbucks, the latter through its recent purchase of Ethos Water, whose profits have supported safe water projects in India and several African countries. Identifying the specific roles and responsibilities of the many organizations and agencies with missions to improve access to safe water and sanitation will be critical to the success of this effort. The World Health Organization–sponsored International Network for the Promotion of Safe Household Water Treatment and Storage, a global collaboration of UN and bilateral agencies, nongovernmental organizations, research institutions, and the private sector (8), could serve as a model for improving coordination of international efforts in this area.

Innovative approaches toward improving water, sanitation, and hygiene must be implemented and evaluated. A number of studies conducted in a variety of geographic settings have shown that interventions such as point-of-use disinfection of water and educational efforts to improve personal hygiene help reduce disease prevalence (9). These studies also highlight the importance of tailoring such interventions to local situations. For example, a recent study in an area in rural western Kenya that had turbid source water found that household use of a flocculant-disinfectant preparation helped reduce the prevalence of diarrhea in children <2 years of age (10). Studies in refugee camps in Africa (11) and urban slums in Asia (12) have documented that handwashing with soap reduced the prevalence of diarrhea in all age groups (11) and lowered

the incidence of diarrhea and pneumonia in children <5 years of age (12). The reduced incidence of pneumonia found in the second study is noteworthy and warrants further study. Although interventions for improving sanitation have lagged behind those for water, promising advances have been made, especially in the development of ecologic sanitation systems. Recent experiences with the tsunami in Asia and Hurricane Katrina on the US Gulf Coast are grim reminders of the need to address water and sanitation urgently following natural disasters.

The United States currently ranks last among the 22 member countries of the Development Assistance Committee of the Organization for Economic Cooperation and Development in net official development assistance provided to developing countries, when such assistance is measured as a percentage of gross national income (13). Today's political and social climate presents an important opportunity to improve this situation. As Barry Bloom, Dean of the Harvard School of Public Health, has written, "The United States should be investing efforts and funds to strengthen the health structures in countries around the world. This investment would protect our country and every other against global epidemics, save million of lives, and change the US image from one of self interest to one of human interest" (14).

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Melioidosis in Tsunami Survivors

To the Editor: A tsunami devastated coastal areas of the Indian Ocean rim in December 2004. Of the affected countries, more than half of the ≈300,000 deaths occurred in the Aceh Province of Indonesia, close to the epicenter of the earthquake near northern Sumatra. Infrastructure, including medical and laboratory facilities, in this region was severely damaged. Of >1,000,000 survivors, >500,000 likely were injured. Most injuries were from trauma, but a substantial number were caused by aspiration of, or immersion in, saltwater that may have been contaminated by soil, sewage, or other environmental sources.

Melioidosis, caused by the saprophytic gram-negative bacillus *Burkholderia pseudomallei*, is endemic in Southeast Asia and northern Australia. Most cases have been found in northeastern Thailand, Singapore, and northern Australia. Melioidosis has been reported only sporadically from Indonesia and mainly in returning travelers (1–3).

In the context of acute medical relief efforts to the town of Banda Aceh, we report on 10 patients with pneumonia, including 4 patients with culture-confirmed melioidosis, after their immersion in contaminated saltwater during the tsunami. Clinical and laboratory services were reestablished on January 3, 2005, at Fakinah Hospital and on January 13, 2005, at Zainoel Abidin Hospital by the relief teams. Patients were identified opportunistically; details of treatment and outcome were reviewed retrospectively. Cultures were taken when clinically indicated and when specimens were available. Sputum cultures were plated onto horse blood agar, cystine lactose electrolyte-deficient agar, *Haemophilus* agar, and colistin/nalidixic acid blood agar incubated in a candle jar at 35°C for ≤3 days. Colonies suspicious for *B. pseudoma-*

llei were subcultured to *B. cepacia*–selective media (Oxoid, Adelaide, South Australia, Australia). Blood cultures and screening cultures of throat and rectum specimens were not performed routinely. Isolates of *B. pseudomallei* and characteristic antimicrobial drug susceptibilities were confirmed with API20NE (bioMérieux, Marcy l'Etoile, France).

From January 3 to January 28, 2005, a total of 10 cases of postimmersion pneumonia were identified. All patients were <18 years of age and previously well; 6 were male. No cases were epidemiologically linked to others. The patients were treated 10–35 days after the tsunami. Eight had bilateral alveolar opacities on chest radiograph; 3 also had empyema. Clinical, radiologic, and microbiologic details are summarized in the online Table (Available from http://www.cdc.gov/ncidod/EID/vol 11no10/05-0740.htm#table).

The sputum cultures of 4 patients were positive for *B. pseudomallei*. Except posttsunami exposure, none had risk factors for melioidosis, including diabetes, renal failure, or thalassemia. Other co-isolated organisms included *Pseudomonas aeruginosa* and *Klebsiella* sp.; 2 patients who did not have cultures taken had cavitatory lung disease. All patients with melioidosis were treated with meropenem, and all but 1 clinically improved in the hospital.

This is the second report of melioidosis from within Indonesia (1) and the second published report of melioidosis after the tsunami disaster (4). Cases from this event were included in a preliminary communication (5). However, exported cases of melioidosis from Indonesia, as well as the neighboring countries of Singapore and Malaysia, have been reported previously (2,3), a likely indication that this infection is underrecognized in Indonesia. Melioidosis has also been reported in tsunami survivors from Sri Lanka (4) and Thailand.

A striking feature of this event is the lack of predisposing factors and the young age of the patients. This feature likely represents the unique mode of acquisition and magnitude of the infecting inoculum, as well as the vulnerability of children to near drowning after flooding. One third of the patients had empyema, which reflects both the severity of pulmonary disease and delay in receiving medical care. Undoubtedly, a substantial selection bias occurred by including only patients who sought hospital treatment, and this is suggested by the low death rate. Melioidosis, acquired after near drowning, has been associated with a short incubation period and severe disease. However, patients who had melioidosis before medical aid arrived in the region would likely have died. Patients with longer incubation periods also may have acquired melioidosis through contaminated wounds with subsequent hematogenous dissemination.

Medical services to the region were by no means comprehensive during this time. Many other patients with postimmersion pneumonia and melioidosis may have been overlooked, both during the study (for persons who were not treated or if cultures were not taken) and afterwards; the incubation period of melioidosis may be ≤62 years. A further limitation is the lack of denominator data because no reliable records were kept on hospital admissions, and the exact number of survivors is not yet known. Conflicting data are found on the accuracy of the API20NE test kit used to identify bacteria in this study (6–9), but we believe that the clinical features and microbiologic findings suggest melioidosis.

This report confirms that *B. pseudomallei* exists in the Aceh Province of Indonesia and that melioidosis and gram-negative pneumonia may complicate saltwater immersion in this region. After near drowning incidents, melioidosis is characterized

by severe pulmonary disease, including pleural effusions. Clinicians worldwide should be mindful that melioidosis in tsunami survivors may appear many years after exposure.

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Chytrid Fungus in Europe

To the Editor: Amphibian species are declining at an alarming rate on a global scale (1). One of the major reasons for these declines is chytridiomycosis, caused by the chytridiomycete fungus, *Batrachochytrium dendrobatidis* (1,2). This pathogen of amphibians has recently emerged globally (2,3) and has caused mass die-offs and extensive species declines on 4 continents (1,3); knowledge of its distribution and effects on amphibian populations remains poor. In Europe, little is known about *B. dendrobatidis* distribution, which is disturbing when one considers that at least 3 European amphibian species are undergoing chytrid-associated die-offs that will likely lead to local extinction (4,5) (J. Bosch et al., unpub. data).

We screened 1,664 current and archived samples of wild amphibians collected in Europe from 1994 to 2004 by researchers using amphibians as study organisms. *B. dendrobatidis* infects the skin of adult amphibians and the mouthparts of anuran larvae; samples included toe clippings and skin samples from adults and mouthparts of tadpoles. Our sampling was opportunistic, including both caudates and anurans. We screened all samples for chytrid fungus with quantitative real-time polymerase chain reaction (PCR) of the ITS-1/5.8S ribosomal DNA region of *B. dendrobatidis* (6), including appropriate positive and negative controls. We confirmed real-time PCR positives by amplifying a subset of these positives with a second *B. dendrobatidis*–specific PCR with a nested reaction developed from the *ctsyn1* locus (3). To confirm that detection with real-time PCR indicated a viable chytrid infection, when actual tissue samples were available, we examined a generous subset using histologic features for typical signals of pathogenic *B. dendrobatidis* infection. Specifically, we found intracellular zoospore-carrying sporangia within the stratum corneum and stratum granulosum of toe and skin samples. We also compared real-time PCR amplification profiles of suspected positives to those generated from samples from animals involved in chytrid-driven die-offs and found these results to be comparable. Furthermore, attempts to isolate the fungus from dead animals were successful when animals were obtained in a suitable condition for this purpose (see below).

Our survey found *B. dendrobatidis* in amphibians in 5 European countries, Spain, Portugal, Italy, Switzerland, and Great Britain. Previously, chytrid infection has been

reported in wild amphibians only in Spain, Germany, and Italy (4,5,7,8). We detected chytrid fungus in 20 of 28 amphibian species examined, representing 9 different genera, 5 anuran, and 4 caudate, in 6 families. We found signs of chytrid in archived samples from as early as 1998. The number of infections per country we found were Austria 0/24, Croatia 0/8, Czech Republic 0/18, Italy 2/101, France 0/60, Germany 0/51, Greece 0/88, Portugal 1/25, Slovenia 0/29, Spain 108/345, Sweden 0/197, Switzerland 63/252, and United Kingdom 2/466. Infection prevalence was exceptionally high in Spain and Switzerland. In Spain, ongoing chytridiomycosis-driven declines of midwife toads (*Alytes obstetricans*) and salamanders (*Salamandra salamandra*) have been documented since 1997 (4) and 1999 (5), respectively, and confirmed with scanning electron microscopy, histologic examination, and molecular detection methods (4,5). Common toads have been suffering apparently minor chytrid-related die-offs in Spain for several years, but mass die-offs were observed in 2004 (5) (J. Bosch et al., unpub. data). No chytrid-related die-offs have been reported in Switzerland. Furthermore, the infected animals from Switzerland were all adults in good breeding condition, many of which reproduced successfully in behavioral and ecologic experiments. Real-time PCR amplification profiles for the Swiss samples were quantitatively equivalent to those generated from samples of *A. obstetricans* collected during mass die-off events in Spain; from these latter samples, we successfully isolated viable *B. dendrobatidis* cultures from 2 geographically distinct areas. In Great Britain, we found chytrid in 2 of 14 introduced North American bullfrogs (*Rana catesbeiana*) caught in 2004 but did not find it in wild-captured native British species. Examination by microscope and electron micro-

scope of 180 native British amphibians from 1992 to 1996 did not find chytrid infection (A.A. Cunningham, unpub.data). The ability of the North American bullfrog to act as a vector for chytrid range expansion has been hypothesized (9,10). Our data may indicate that bullfrogs can fulfill this role in Great Britain and other areas; we have found the molecular signal of chytrid infection from introduced North American bullfrogs collected on 3 separate continents (T.W.J. Garner et al., unpub. data).

This survey shows that *B. dendrobatidis* is widely and irregularly distributed in Europe and infects a broad range of amphibian species. Furthermore, because of the opportunistic nature of our sampling strategy, our results certainly underestimate the overall prevalence of *B. dendrobatidis* in Europe. These findings are surprising considering that chytrid-related die-offs have been infrequently described in Europe. This may be because *B. dendrobatidis* has only recently and rapidly expanded its range into Europe (3), and the consequences are only now being detected in wild amphibian populations; because the expression of chytridiomycosis is environmentally limited (11); or because European amphibians exhibit highly variable levels of resistance to chytrid infection. Notwithstanding, our knowledge of the epidemiology of *B. dendrobatidis* is insufficient to effectively manage wildlife and conduct disease abatement. As data regarding the distribution of chytrid fungus accumulate and the ecologic requirements for disease persistence and transmission are identified (11), management of the pathogen can become more predictive. Basic management practices, such as restricting transportation of potential carriers and restricting pet trading and reintroduction projects, coupled with field monitoring, must be improved to prevent further global emergence of this pathogen. Our

results also show that asymptomatic amphibians must be included in any broad-scale epidemiologic screening for this emergent pathogen.

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Vibrio metschnikovii Pneumonia

To the Editor: *Vibrio metschnikovii* is a gram-negative, oxidase-negative bacillus. This species was isolated in 1981 from blood culture of an 82-year-old diabetic woman with cholecystitis (1). It was previously isolated from river water, clams, oysters (2), fish (3), and birds that died of a choleralike illness (4). We report isolation of *V. metschnikovii* in bronchial aspirate from a patient with pneumonia.

A 63-year-old man was admitted to the intensive care unit (ICU) of A. Calmette hospital in Lille, France, for acute respiratory failure related to community-acquired pneumonia. The patient had a history of chronic obstructive pulmonary disease with a forced expiratory volume of 820 mL in 1 s (32% of predictive value); he was treated with oral salmeterol, terbutaline, and prednisolone (40 mg/day). He was HIV negative. On ICU admission, he had the following values: respiratory rate 30/min, temperature 39°C, pulse rate 140/min, blood pressure 140/90 mm Hg, Glasgow coma score 15, leukocyte count 13.7×10^9/L, hemoglobin level 10.2 g/dL, procalcitonin level 22 ng/mL, and C-reactive protein level 73 mg/L. A chest radiograph showed diffuse bilateral infiltrates. Analysis of arterial blood gases with 6L of oxygen/min showed respiratory acidosis: pH 7.30, pCO_2 59 mm Hg, pO_2 78 mm Hg, HCO_3 23 mmol/L, O_2 saturation 94%. Other laboratory test results were normal. After noninvasive ventilation failed, the patient was immediately intubated and mechanically ventilated.

Blood cultures and bronchial aspirate samples were obtained before initiating treatment with antimicrobial drugs. The patient was treated with amoxicillin/clavulanic acid and ciprofloxacin. Blood cultures showed negative results. Microscopic examination of the bronchial aspirate showed no squamous epithelial cells, a few gram-negative bacilli, leukocytes, and many ciliated bronchial cells. The presence of ciliated cells was the best indicator that secretions originated from the lower respiratory tract. A urinary antigenic test result for *Legionella* spp. was negative. Quantitative culture of the bronchial aspirate on bromocresol purple agar, blood agar (grown in an atmosphere of 5% CO_2), and chocolate agar plates yielded *V. metschnikovii* (10^7 CFU/mL) and nonhemolytic streptococci (10^5 CFU/mL) as the oropharyngeal flora. These streptococci (gram-positive, catalase-negative) were not considered to be the pathogenic agent.

The strain of *V. metschnikovii* isolated was a gram-negative, curved rod. This facultative aeroanaerobic bacillus formed opaque colonies (diameter 3 mm) on blood agar in 24 h and showed complete hemolysis. It was catalase positive, oxidase negative, and did not reduce nitrate to nitrite. This strain was identified as *V. metschnikovii* with an ID GBN Vitek 2 card (bioMérieux, Marcy l'Etoile, France) (acceptable T identification index 0.22). Confirmation was done with a Microseq 500 16S ribosomal DNA bacterial kit (PE Applied Biosystems, Foster City, CA, USA). A 475-bp fragment was sequenced in an automated sequencer (360 ABI Prism, PE Applied Biosystems). The fragment was compared with National Center for Biotechnology Information (Bethesda, MD, USA) GenBank entries and showed 99% homology with *V. metschnikovii* (GenBank accession no. X74712.1). In vitro susceptibility testing with the AST-N032 Vitek 2 card (bioMérieux) showed that the organism was resistant to ampicillin, ticarcillin, piperacillin, and aminoglycosides.

The patient was successfully extubated. He was transferred to a pneumology ward on day 9 and discharged on day 15. Antimicrobial treatment was stopped on day 10.

Most nonhuman strains of *V. metschnikovii* are usually found in aquatic habitats (e.g., lakes and marine waters). Human clinical infections with this bacterium are rare; however, cases of epidemic diarrhea caused by *V. metschnikovii* have been reported (5,6). Contamination by water or fish was not always demonstrated in these cases, but an orofecal source is possible. In coproculture, this microorganism is probably not diagnosed: it was initially identified as normal aerobic flora because it was oxidase negative.

The first case of septicemia with *V. metschnikovii* was reported in 1981 in a patient with peritonitis and an inflamed gallbladder (1). Three other patients with similar septicemia, all >70 years of age, were described (7,8); 2 had polymicrobial results in blood cultures. *V. metschnikovii* was also found in a mucocutaneous site (wound infection) after saphenectomy in swab samples of the wound site (9).

The patient in our study denied contact with lake or sea water, and he had not eaten any seafood. He was a retired carpenter without contact with domestic or wild animals and did not recall an episode of diarrhea before his hospitalization. The source of contamination that caused his acute respiratory failure was not identified.

Miyake et al. showed that *V. metschnikovii* produces a cytolysin with hemolytic properties (10). This finding might explain the invasive process of this bacterium, which resulted in pulmonary lesions in a patient with respiratory deficiency. As far as we know, this is the first case of pneumonia caused by *V. metschnikovii.*

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Lassa Fever, Nigeria, 2003 and 2004

To the Editor: Suspected outbreaks of Lassa fever have been reported in the northern part of Edo, Nigeria, including Ekpoma, Igarra, and Ibilo, in 2001 and between November 2003 and March 2004 (1,2). To confirm Lassa fever activity in this area, serum samples were collected at the Specialist Teaching Hospital in Irrua (ISTH) from September 2003 to January 2004. Approximately 16,000 patients are seen each year at ISTH, and ≈80% of them have febrile illness. Serum specimens were taken from patients with febrile illness (n = 31), healthy contact persons (n = 17), and healthy hospital staff (n = 12). The samples were analyzed by Lassa virus–specific reverse-transcriptase polymerase chain reaction (RT-PCR) at the University of Lagos. Aliquots of specimens were sent to the Bernhard-Nocht Institute (BNI in Hamburg, Germany) for confirmatory PCR analysis, serologic testing, and virus isolation. The PCR used at both facilities was based on primers 80F2 and 36E2 that targeted the glycoprotein precursor (GPC) gene (3), although the protocols were slightly different. At BNI, virus RNA was purified by QIAamp viral RNA kit (Qiagen, Hilden, Germany), and RT-PCR was performed with Superscript II RT/Platinum Taq polymerase 1-step reagents (Invitrogen, Karlsruhe, Germany). This PCR assay has a 95% detection limit of 2,500 copies/mL (4). At the University of Lagos, virus RNA purification and RT-PCR were performed with diatomaceous silica and Brilliant single-step RT-PCR kit (Stratagene, Heidelberg, Germany), respectively. Serologic testing for Lassa virus–specific immunoglobulin G (IgG) and IgM was performed by indirect immunofluorescence assay

(IFA) by using Vero cells infected with Lassa virus strain Josiah. Virus was isolated in the biosafety level 4 laboratory at BNI with Vero cells. Results of the tests are summarized in the Table.

Acute Lassa virus infection, as shown by a positive PCR result, was diagnosed at the University of Lagos in 1 patient. This result was independently confirmed at BNI, and 2 additional samples tested positive by PCR. The PCR signals were weak, which suggests that discrepancies between laboratories stem from higher sensitivity of the assay used at BNI. Presence of a low IgM titer in the absence of IgG in 2 of the PCR-positive samples is also consistent with an acute infection. Two of the Lassa virus–positive persons (04-02 and 04-10) had febrile illness that indicated symptomatic Lassa fever, while 1 (04-04) had been classified as an asympto-

matic contact at the time of sampling. Retrospective investigation showed no evidence of illness in this person before or after sampling. Sequencing the diagnostic PCR fragments (300 nucleotides [nt] of GPC gene) from the 3 patients indicated infections by closely related strains. The sequence of patient 04-10 (GenBank accession no. DQ010031) differed by 4% from those of patients 04-02 and 04-04, while the latter sequences were identical (GenBank accession no. DQ010030). The facts that patients 04-02 and 04-04 were sisters who lived in the same house, that their samples were taken on the same day (January 28, 2004), and that the sequences were identical suggest a common source of infection or an infection chain. The detection of an asymptomatic or mild Lassa virus infection in the contact person agrees with population-based studies in

Sierra Leone that show only 9%–26% of all Lassa virus infections are associated with fever (5).

In an additional 10 samples, IgM with or without IgG was detected, primarily in patients with febrile illness. IgG in the absence of IgM was detected in 1 contact and 4 healthcare workers. All serologic IFA findings were confirmed with μ-capture and IgG enzyme-linked immunosorbent assays developed at BNI. Virus isolation was attempted with all samples that tested positive by PCR or IgM IFA. Lassa virus was isolated from 1 PCR-positive serum (04-10). The strain was designated Nig04-010. To characterize Lassa virus circulating in north Edo, phylogenetic analysis was performed. In addition to the GPC sequences of the diagnostic PCR fragments, part of the L gene of Nig04-010 was amplified and sequenced (780 nt, GenBank accession no. AY693637). Phylogenetic analysis of these sequences showed that the virus circulating around Irrua belongs to phylogenetic lineage II, which comprises Lassa virus strains from the southeastern part of Nigeria (6). Thus, genotype and geographic origin of the viruses characterized here correspond.

These data provide evidence for Lassa fever activity in north Edo. Approximately 6% of febrile patients tested had PCR-confirmed Lassa fever, which extrapolates to hundreds of patients with Lassa fever per year, when one considers the number of patients with febrile illness seen at ISTH. As shown here and elsewhere, PCR is a useful tool to diagnose Lassa virus infection (3,7), a prerequisite for effective ribavirin treatment (8). First steps have been made to establish molecular diagnostics for Lassa virus at the University of Lagos. Further efforts are necessary to improve the laboratory infrastructure in the country.

Table. Lassa virus–specific findings in 60 serum samples from Irrua Specialist Teaching Hospital, Edo, Nigeria*

Patient	RT-PCR†	IgM titer‡	IgG titer‡
Patients with fever (n = 31)			
04-10	Positive§	–	–
04-02	Positive	1:40	–
04-51	–	1:160	–
04-34	–	1:40	–
04-03	–	1:>20,480	1:20,480
03-05	–	1:320	1:20,480
03-01	–	1:160	1:10,240
04-08	–	1:80	1:20,480
04-33	–	1:20	1:640
04-52	–	1:160	1:40
04-53	–	1:40	1:40
Contact persons (n = 17)			
04-04	Positive	1:20	–
03-04	–	1:160	1:>20,480
04-11	–	–	1:80
Hospital staff (n = 12)			
04-31	–	–	1:80
04-32	–	–	1:80
04-17	–	–	1:80
04-20	–	–	1:20

*Data not shown for patients whose samples were negative in all tests. RT-PCR, reverse-transcriptase polymerase chain reaction; Ig, immunoglobulin; –, negative result.
†RT-PCR targeting the Lassa virus glycoprotein gene (4). PCR products were detected in ethidium bromide–stained gel and sequenced (GenBank accession nos. DQ010030 and DQ010031 for 04-02 and 04-10, respectively).
‡Immunofluorescence assay used cells infected with Lassa virus strain Josiah. Findings were confirmed with μ-capture and IgG enzyme-linked immunosorbent assays (data not shown).
§Lassa virus was isolated in cell culture (strain Nig04-010), and part of the L gene was sequenced (GenBank accession no. AY693637).

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Methicillin-resistant *Staphylococcus aureus* Skin Infections

To the Editor: Moran et al. write, "In areas with a high prevalence of CA-MRSA [community acquired methicillin-resistant *Staphylococcus aureus*], empiric treatment for skin and soft tissue infections (SSTIs) with β-lactam agents such as cephalexin may no longer be appropriate. Oral agents such as clindamycin or trimethoprim/sulfamethoxazole and rifampin should be considered in CA-MRSA" (1). However, some studies have had different results. Lee et al. reported that 31 (84%) of 37 Texas children with CA-MRSA SSTIs showed clinical improvement after incision and drainage, even though they received an "ineffective" antimicrobial agent that was not changed after the susceptibility results became available (2). These researchers also reviewed some reports with similar experience in the United States and further suggested that incision and drainage without adjunctive antimicrobial therapy were effective in immunocompetent children for CA-MRSA SSTIs <5 cm in diameter.

Several studies on Taiwanese children with CA-MRSA SSTIs agree with the viewpoint of Lee et al. Chen and colleagues reported that 22 (63%) of 35 episodes of CA-MRSA superficial soft tissue infections in children were cured by nonsusceptible antimicrobial therapy, regardless of surgical intervention (3). In a study by Wang et al., oxacillin, with or without incision and drainage, was effective in 16 (89%) of 18 children with CA-MRSA SSTIs, even in a case with high-level oxacillin resistance (MIC≥8 μg/mL) (4). Fang et al. also reported that 16 (55%) of 29 children with CA-MRSA SSTIs were eventually cured with therapy to which their infections were not susceptible (5). With these experiences and concerns about the growing problem of bacterial resistance, we suggest that incision and drainage, with or without adjunctive antimicrobial therapy, are adequate to treat noninvasive CA-MRSA SSTIs in immunocompetent children and that oxacillin or first-generation cephalosporins are still effective and sufficient under such conditions. Vancomycin and other agents that are effective against MRSA isolates should be reserved for invasive CA-MRSA infections or for immunocompromised patients. Although Moran's study was focused on adults, not on children as these studies were, we believe these suggestions are also appropriate when applied to CA-MRSA SSTIs in adults.

Finally, the antibiogram of CA-MRSA isolates may vary from country to country. In Taiwan, CA-MRSA isolates are also resistant to multiple antimicrobial agents; 71.4%, 91.4%, and 41.2% are resistant to clindamycin, erythromycin, and chloramphenicol, respectively (4). Trimethoprim/sulfamethoxazole is more effective against CA-MRSA isolates than

other first-line antimicrobial agents: the resistance rate is 0%–65.7% (4,5). Therefore, clindamycin and trimethoprim/sulfamethoxazole may be not adequate empiric antimicrobial agents for SSTIs in Taiwan or other areas with a high prevalence of CA-MRSA.

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In response: Dr Ma makes an excellent point about the limitations of study data on antimicrobial drug treatment of skin abscesses (1). All of the patients described in our study (2) required antimicrobial drug therapy, and most were admitted to the hospital. However, we did not mean to imply that all skin abscesses require antimicrobial drug treatment. Our own practice is to give antimicrobial drug therapy only when a skin abscess is associated with definite surrounding cellulitis, systemic signs, or both. Although various criteria have been published, in practice this is a judgment call, and we suspect that physicians vary considerably in use of antimicrobial agents for skin infections.

Because most cellulitis associated with skin abscess will improve with adequate drainage, designing a study that will find a difference in outcome attributable to the antimicrobial drug is difficult. More studies are needed to determine whether antimicrobial agents with in vitro activity against methicillin-resistant *Staphylococcus aureus* (MRSA) are more clinically effective than those lacking such activity. Perhaps these studies should focus on those infections for which antimicrobial agents would be expected to have the greatest impact (e.g., infected wounds with cellulitis), rather than abscesses that can be expected to improve with incision and drainage alone.

When the decision is made to use an antimicrobial agent, it is difficult to justify choosing one to which the infecting organism will likely be resistant. Because MRSA is now the most common cause of skin infections at our institution, we choose agents with activity against the MRSA strains in our community. We do not believe that choosing an antimicrobial agent to which the infecting organism is susceptible is more likely to contribute to the general problem of antimicrobial drug resistance.

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Angiostrongyliasis, Mainland China

To the Editor: The first case of angiostrongyliasis caused by *Angiostrongylus cantonensis* in mainland China was reported in 1984; only 3 cases were reported between then and 1996 (1). Recently, however, cases of angiostrongyliasis have increased rapidly because of its natural focus and a change in human dietary patterns. For example, snails have become a popular food in many regions of this country. Nearly 100 cases of angiostrongyliasis have been reported in mainland China, including 2 outbreaks (2,3).

From 1994 to 2003, 84 cases of angiostrongyliasis were documented in mainland China. Of all the cases, 29 were reported individually, and 55 were reported from the 2 outbreaks that occurred in Zhejiang and Fujian. Sixty-three of the 84 patients had eaten raw or undercooked snails, 5 had eaten raw crabs, 1 swallowed tadpoles, and several pediatric patients had close contact with snails. Some researchers believe that the larvae of *A. cantonensis* can be released from mollusks into slime fluid and contam-

inate produce and other objects as they crawl. However, Liang et al. (4) reported finding no larvae in body fluid washed from 23 *Achatina fulica* that were infected with *A. cantonensis*. Therefore, whether slime fluid plays a role in human infection remains unclear.

The clinical symptoms of the patients from the 2 outbreaks of angiostrongyliasis that occurred in China are shown in the Table. Eosinophilia is a typical characteristic of eosinophilic meningitis or meningoencephalitis caused by *A. cantonensis*. In our study, eosinophilia was detected in 79 of 84 patients. Therefore, clinical patients with eosinophilic meningitis or meningoencephalitis with eosinophilia should be presumptively considered to be infected with *A. cantonensis* and parasitologic or serologic tests should be performed. The parasitologic detection

rate of *A. cantonensis* infection in humans is low; because this parasite is found in the human central nervous system (CNS) and the tiny larvae often stick to meninges or nerve root, a false-negative result is often shown when cerebrospinal fluid is examined. In the 84 cases reported in this article, worms were isolated from only 8 patients (9.5%), whereas 64 cases were diagnosed as angiostrongyliasis by immunologic methods. The most common immunologic methods used to diagnosis angiostrongyliasis in this country are indirect fluorescent antibody test, immunoenzymatic staining technique, and enzyme-linked immunosorbent assay. All antigens used in these methods were prepared from whole adult worms. Reports indicated that serologic cross-reaction occurred between trichinosis and angiostrongyliasis when whole-worm lysate was used as the antigen (5). In

addition, whole-worm antigens cannot discriminate between new and previous infection, or monitor the efficacy of the treatment. Therefore, finding potential diagnostic antigens will be essential to solving this problem and applying recombinant antigens may achieve this goal. A cDNA library from the larvae of *A. cantonensis* was constructed and screened with acute infection sera, and a diagnostic antigen that can detect early infection of *A. cantonensis* (3 weeks) was identified (6).

Some researchers considered that anthelminthics, such as albendazole, ivermectin, mebendazole, and pyrantel, did not affect *A. cantonensis* but noted that the death of worms in the CNS might exacerbate neurologic symptoms (7). However, many studies in mainland China showed that anthelminthics can relieve symptoms and reduce the duration of disease. For example, Wang et al. (8) reported that albendazole could relieve the symptoms of angiostrongyliasis and suggested that it can be used to treat the disease. Lin et al. (3) also reported that in 8 patients who were treated with 20 mg/kg albendazole for 9 days, the symptoms and signs of acute angiostrongyliasis were rapidly relieved in 3–6 days. All of these patients had recovered by 10 days after treatment, and no side effects were observed.

Angiostrongyliasis is an emerging foodborne public health problem in mainland China. However, most clinicians are not familiar with this disease and little is known about the prevalence of *A. cantonensis* in China. Thus, more studies should be conducted on the biology, epidemiology, and clinical characteristics of angiostrongyliasis, and more effective diagnostic methods and treatments for *A. cantonensis* should be developed.

Table. Clinical characteristics of patients with angiostrongyliasis in 2 outbreaks in China*

Characteristics	Outbreak 1 (%)	Outbreak 2 (%)
Patients, no.	47	8
Male patients, no. (%)	24 (51)	8 (100)
Age, y		
Median	32	12
Range	6–58	11–13
Incubation, d		
Median	10.35	6
Range	1–27	5–7
Symptoms†, no. (%)		
Headache	44 (93.6)	8 (100)
Nuchal rigidity or neck pain	0	6 (75.0)
Fatigue	7 (14.9)	7 (87.5)
Vomiting	9 (19.1)	8 (100)
Paresthesias	30 (63.8)	3 (37.5)
Muscle pain	43 (91.5)	8 (100)
Fever	27 (57.4)	3 (37.5)
Cough	4 (8.5)	0
Somnolence	4 (8.5)	7 (87.5)
Skin eruption	10 (21.3)	0
Skin itch	13 (27.7)	0
Laboratory detection No. positive/no. examined (%)		
Eosiniphils in cerebrospinal fluid	23/25 (92)	8/8 (100)
Eosinophils in blood	23/25 (92)	8/8 (100)
Serologic diagnosis	21/25 (84)	NT
Pathogenic test	ND	1/8‡ (12.5)

*NT, not tested; ND, not detected.
†No patients had visual disturbance or photophobia, hyperesthesias, muscle weakness, or diarrhea.
‡Two larvae were found in cerebrospinal fluid.

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Methicillin-resistant *Staphylococcus aureus* Necrotizing Pneumonia

To the Editor: Methicillin-resistant *Staphylococcus aureus* (MRSA) strains account for >40% of all hospital-acquired *S. aureus* infections in Italy (1). Although cases of community-acquired MRSA (CA-MRSA) infections have been reported in recent years (2), these isolates have not been characterized for Panton-Valentine leukocidin (PVL) (3); therefore, the presence of isolates with the typical characteristics of CA-MRSA (4) in Italy remains unknown.

At the beginning of April 2005, a 37-year-old woman was admitted to the University Hospital Policlinico in Rome because of fever, cough, and headache. Her medical history was unremarkable. She was a teacher in a school for foreign students in Rome, smoked 3 cigarettes per day for 15 years, and reported no recent travel abroad. Her 5-year-old daughter had influenzalike symptoms in the previous week. At hospital admission, her temperature was 39°C, heart rate was 108 beats/min, respiratory rate was 32 breaths/min, and blood pressure was 105/70 mmHg. Arterial blood gas analysis showed mild hypoxemia and hypocapnia (PaO_2 73 mm Hg and $PaCO_2$ 34 mm Hg on room air). Leukocyte count was 24,360 cells/μL (81% polymorphonuclear cells), and platelet count was 506,000/μL. Chest radiograph showed infiltrates in the right upper and lower lobes and left lower lobe. Empiric treatment with clarithromycin and ceftriaxone was started, but the patient's clinical conditions did not improve. Culture of sputum samples obtained at admission yielded growth of MRSA. Computed tomographic scan showed multiple lung cavitary lesions, indi-cating necrotizing pneumonia. On day 3 of admission, antimicrobial drug therapy was changed to linezolid (600 mg 3 times a day). Fever resolved, and the patient's condition rapidly improved. The patient was discharged after 14 days of linezolid treatment. At discharge, leukocyte count was 6,040 cell/μL (58% polymorphonuclear cells), and arterial blood gas analysis showed PaO_2 of 88 mm Hg.

The MRSA isolate from sputum was susceptible to all the non–β-lactam antimicrobial drugs tested, including erythromycin, clindamycin, ciprofloxacin, tetracycline, kanamycin, and fusidic acid. With established molecular methods, the isolate was found to harbor *SCCmec* type IV (5); *lukS* and *lukF*, the genes coding for the 2 subunits of the PVL toxin; and *hlg*, the γ-hemolysin gene (3). The genetic background of the isolate was determined by multilocus sequence typing (MLST) (6) and sequence typing of the tandem repeat region of protein A gene (*spa* typing) (7). Results showed that the isolate belonged to ST30 according to the MLST database (http://saureus.mlst.net), and *spa* typing, analyzed by the Ridom Staphtype software (http://www.ridom.de), indicated a novel *spa* type, to which type 755 was assigned. ST30, 1 of 6 clones more commonly associated with PVL-positive CA-MRSA (4), is designated also the southwest Pacific (SWP) clone, because of the area in which it circulates. Recently, the SWP clone has caused CA-MRSA infections in northern European countries (England, Scotland, the Netherlands, Sweden, and Latvia) (8,9). Molecular analysis suggests that the SWP clone has evolved from a methicillin-susceptible clone of *S. aureus*, termed phage type 80/81, that was pandemic in the 1950s and considered to be unusually virulent and transmissible (8). In fact, strains belonging to phage type 80/81 carry the PVL gene and

appear to have subsequently acquired methicillin resistance through horizontal transfer of *SCCmec* type IV. The *spa* type of the Italian isolate comprises 7 nucleotide repeats, indicated by XJ4AKAOM in the alphabetical code. This repeat sequence differs from that of the classical SWP clone, indicated by XKAKAOMQ (8), by only 1 bp in the second repeat and loss of the last Q repeat. In spite of these differences, the *spa* type is in substantial agreement with the MLST result and indicates that the Italian isolate is either a descendent or a local variant of the SWP clone. The most common clone of CA-MRSA described in Europe is ST80, *spa* type 44. CA-MRSA belonging to ST80 tend to be more antimicrobial drug resistant than isolates belonging to other clones (4). Resistance to fusidic acid, typical of ST80, has been proposed as a marker for CA-MRSA in Europe (10). In light of our finding, we cannot rely on resistance to fusidic acid to screen for PVL-producing CA-MRSA in our country.

To our knowledge, this is the first report from Italy of necrotizing pneumonia caused by PVL-positive CA-MRSA. The presentation was typically that of a severe pneumonia that occurred in a previously healthy, young adult with no risk factors for MRSA acquisition, as described in other cases (11). This is also the first report of a SWP clone isolate in southern Europe; if the strain is circulating in Italy or is occasionally imported from the SWP area, whether our patient acquired it through contact with a foreign contact remains unknown.

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West Nile Virus Infection and Conjunctival Exposure

To the Editor: Corvids (crows, blue jays, magpies, and their relatives) are particularly susceptible to West Nile virus (WNV) (1). Birds are useful indicators of the spread of WNV (1), and Canada has implemented WNV surveillance strategies that use these species as sentinels.

Direct acquisition of WNV through percutaneous injuries has been reported in 2 laboratory circumstances, involving a blue jay and a mouse (2). We describe a conjunctival exposure to WNV that occurred in the field and probably resulted in infection in the exposed person.

As part of the local WNV bird surveillance activities in 2003, an animal control officer collected sick and dead corvids at the Canadian Forces Base, Suffield, Alberta. He had a protective suit on, but he wore no mask or face shield. While killing an injured crow (*Corvus brachyrhynchos*), the officer struck the struggling bird on a nearby horizontal pipe gate, which resulted in fracture of the skull, causing brain tis-

sue and cerebrospinal fluid to spray onto his head, face, neck, and right shoulder. Body fluids and brain material of the bird entered his eyes, but not his mouth; he had no known open lesions on the exposed area. His co-workers immediately wiped off visible material, and a few hours later he showered.

The dead crow was sent for analysis to the Fish and Wildlife Division, Government of Alberta, where laboratory tests using the VecTest assay (Medical Analysis Systems, Inc., Camarillo, CA, USA), indicated that the crow was positive for WNV antigen. This test has been validated for detecting viral antigen in oropharyngeal and cloacal swabs in crows (3).

Seven days after exposure, the animal control officer became unwell and sought medical assistance. His symptoms included headaches, dizziness, spiking fevers, and sweats; on examination, mild otitis was noted, but he did not display meningismus or other neurologic signs. A whole blood sample with EDTA and a serum sample were collected, together with a throat swab for viral culture to exclude a possible enteroviral infection, as part of a standardized provincial protocol for investigating suspected WNV infections in Alberta. Betahistine dihydrochloride was prescribed for the dizziness and a cephalosporin for otitis. A cerebrospinal fluid sample was not collected, since his clinical signs did not suggest neurologic involvement.

At the Provincial Laboratory, WNV RNA was detected in the plasma by nucleic acid sequence-based amplification, with primers described by Lanciotti and Kerst (4), which was confirmed by the Artus RealArt RT-PCR assay (artus biotech USA Inc, San Francisco, CA, USA) in a Roche LightCycler. The serum sample, collected at the same time as the plasma sample, was negative for immuno-

globulin M (IgM) antibody by enzyme immunoassay using 2 kits (Panbio, Windsor, Queensland, Australia; and Focus Technologies, Cypress, CA, USA). Fourteen days after exposure, a convalescent-phase serum sample showed IgM antibody to WNV in both kits; the plasma sample was negative for viral RNA. Hemagglutination inhibition assay on the acute- and convalescent-phase serum samples, collected 7 days apart, showed rising titers, from <1:10 on the acute-phase serum, to 1:40 for dengue virus (serotypes 1–4), 1:40 for St. Louis encephalitis virus, and 1:80 for WNV on the convalescent-phase serum. Preliminary data from our laboratory indicate that in ≈40% of cases of acute West Nile fever, the acute-phase plasma sample shows viral RNA before IgM antibody develops, after which viral RNA is no longer detectable (J. Fox, unpub. data). Two weeks after culture was initiated for virus isolation, the throat swab was negative for enteroviruses.

The patient's severe fever, sweats, headaches, anorexia, fatigue, and diminished concentration and memory continued. His symptoms peaked 2 weeks after the initial exposure. Three months later, his symptoms of fatigue, dizziness, headaches, and poor memory were severe enough to prevent him from returning to fulltime work. Eight months after exposure, he continues to have fatigue and headaches.

We believe this is the first reported case of apparent conjunctival transmission of WNV in an occupational setting. As the officer spent considerable time outdoors in areas where WNV transmission was relatively high in 2003 and repeatedly handled infected birds, we cannot eliminate the possibility of a mosquito bite or other percutaneous route of transmission. However, the nature of the exposure and the time to symptom development strongly suggest that infection

occurred after conjunctival exposure. Persons who dispatch sick wildlife are encouraged to use appropriate, humane methods and should take precautions against exposure to tissues and body fluids.

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Revenge of the Microbes: How Bacterial Resistance Is Undermining the Antibiotic Miracle

Abigail A. Salyers
and Dixie D. Whitt

American Society for Microbiology Press, Washington, DC, 2005
ISBN 1-55581-298-8
Pages: 186, Price: US $29.95

Professional journals these days brim with new developments in the field of antimicrobial resistance, and scarcely a week goes by without a flurry of new reports on "super bugs" in popular media. Given the unrelenting blitz of information, that 2 self-proclaimed "fusty old pedants" could produce a fresh perspective in the ongoing arms race between man and microbe is all the more noteworthy.

Although their traditional milieu is microbiology textbooks, Salyers and Whitt have provided a concise yet readable history of the rise of resistant organisms, as well as the social and economic effect of "these indomitable little critters." The history, from the first hints of penicillin resistance to the recent rise of vancomycin resistance, is as insightful as it is entertaining.

Lay readers will get a digestible dose of the basic science often missing from the mass media. And professionals will find the kind of incisive analysis—and even a touch of humor—that is often missing from scientific journals. Both audiences will find eminently compact descriptions of the major mechanisms that enable bacteria to develop and pass on resistant traits, the hurdles that pharmaceutical companies face in developing new antimicrobial drugs, the dilemmas doctors and patients face in

finding better ways to use drugs, and a thoughtful appraisal of possible future trends.

In contrast to prophecies of an approaching "post-antibiotic era," the authors' own "realistic vision of the future" is far from apocalyptic. Still, they worry that increasing numbers of treatment failures like those occurring in hospitals and community settings will erode confidence in the health-care system. Some diseases, they believe, will remain treatable, some new drugs will emerge, and bacteria, with 3 billion years of evolution on their side, will continue to adapt. So perhaps, they suggest, "the best we can hope for is détente, a running standoff between science and the bugs' remarkable ability to adapt to their changing environment."

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The Microbe-Host Interface in Respiratory Tract Infections

Jan L.L. Kimpen and
Octavio Ramilo, editors

Horizon Biosciences,
Norfolk, UK, 2005
ISBN 0-8493-3646-5
Pages: 323, Price US $139.05

How thoroughly can 1 book address 2 complex aspects of the host-agent-environment triad, especially

for a topic as broad as respiratory tract infections? Every author of an infectious disease topic assumes this task, at least implicitly. For example, the clinical aspects of adenovirus infection are hard to discuss without also highlighting the host factors that lead to greater susceptibility. The value of a book dedicated to the host-pathogen interaction depends on the book's ability to focus explicitly and narrowly on this relationship as the main topic.

Common to all 13 chapters of this first edition is the subject matter expertise of the authors. In addition to their thorough treatment of each subject, extensive referencing shows clearly the authors' command of current and past literature (in some instances, more space is devoted to references than to text). Beyond these common features, different chapters address particular facets of the host-agent relationship. Several chapters treat the host itself as the key subject, for example, the chapter on genetic background. Others place greater emphasis on the features of the microbes themselves, such as their pathogenicity and mechanisms for evading the host immune system. Still other chapters dissect and analyze every aspect of the complex relationship between host and agent, successfully making this interaction the central topic. The chapter on the pathogenesis of bacterial respiratory tract infections is a particularly strong example. Finally, some chapters look at the host-microbe interface over a period longer than the time of acute infection. For example, the chapter on atypical bacteria summarizes the evidence for a causal relationship between infection with *Mycoplasma pneumoniae* and the subsequent development of asthma.

If the authors' expertise is the primary strength of the book, the lack of organization and focus is its principal weakness. Most infectious disease textbooks adopt a pyramidal structure,

beginning with foundational concepts, such as clinical syndromes, followed by specifics, such as the clinical presentation and treatment of individual pathogens. No such analogous structure is apparent in this book. Although the book begins with a discussion of genetics and the hygiene hypothesis, it quickly digresses into issues less relevant to the main point of the book, such as new diagnostic tests. A clear structure would also help ensure that all major topics are included. For example, many respiratory tract infections have a bimodal age distribution with the greatest incidence in the very young and the very old. However, this book largely omits any discussion of host-microbe interactions among the elderly. Similarly absent is a description of how pandemic influenza viruses emerge and evade the host immune system. Simply put, structure would unify what could otherwise be considered a series of well-done monographs.

Most readers who want to understand the host-agent interplay in respiratory infections might find that a general infectious disease text meets their needs. However, others who need more depth in selected topics should search the table of contents before adding this book to their library.

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Infections of Leisure, 3rd ed.

David Schlossberg, editor

ASM Press, Washington, DC, 2004
ISBN: 1-55581-299-6
Pages: 444; Price: US $59.95

If you have ever thought about spending more time away from work, here is a book that could help change your mind. Infections of Leisure provides a detailed survey of the infective hazards associated with a wide range of human leisure activities and pursuits, from lazing on a beach to relaxing in a spa, dining out, or simply staying home and doing the gardening.

Now in its third edition, this book covers infections linked to salt and freshwater activities, camping and the outdoors, gardening, contact with animals, eating, foreign travel, sports, sexually transmitted diseases, body piercing, tattooing, and trekking to high altitude. The menu of topics is somewhat eclectic, and the balance between them is sometimes uneven, e.g., 30 pages on diseases associated with "Man's Worst Friend" (the rat), but only 20 pages on overseas travel. The result is nonetheless fascinatingly readable, even for the armchair practitioner.

On the subject of rats, I was intrigued to discover that 40,000 human rat bites are reported annually, and that *Rattenbisskrankheit*, or rat-bite fever in its various forms, has been noted clinically for >2,000 years. Bacterial zoonoses from domestic pets include salmonellosis from illegally kept turtles (i.e., those

measuring <4 inches long). Both of these conditions have been the subject of recent case reports in the MMWR (1,2), confirming the continuing topicality of the book's contents.

There is much to whet the appetite of any connoisseur of bizarrely named syndromes, from "toxic sock" syndrome (pitted keratolysis caused by *Corynebacterium* in athletes) to "hot-foot" syndrome (plantar *Pseudomonas* folliculitis associated with abrasive swimming pool floors). But anyone looking for up-to-date information about more common conditions, from leptospirosis to Lyme disease, will find plenty of clear, concise, well-referenced material, contributed by experts in each field.

Leisure is a precious commodity, and this book remains a useful resource for anyone interested in knowing more about the pathogens that conspire against our pursuit of it, from the mundane to the truly outlandish.

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Instructions for Infectious Disease Authors

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.

Yellow Jack—How Yellow Fever Ravaged America and Walter Reed Discovered Its Deadly Secrets

Yellow Jack is a compelling and thorough narrative of one of the most interesting chapters in medical history. The book, which is highly readable even to those who may think they know this story, is based on a series of articles written by one of the authors, John R. Pierce, for Stripe, a publication of the Walter Reed Army Medical Center. Dr Pierce was a colonel in the Army Medical Corps and recently retired after 30 years of active duty service.

The first 5 chapters describe the introduction of yellow fever in North America before 1900. Of particular interest is the chapter detailing events of the 1793 outbreak in Philadelphia, and the efforts of Benjamin Rush to treat patients and determine the specific cause. Chapter 6 compares the roles played by Carlos Juan Finlay and George Miller Sternberg before and during the work of the US Army Yellow Fever Board. Dr Finlay was a Cuban physician who had theorized that mosquitoes transmitted the yellow fever virus, while Sternberg, a US Army physician, claimed to have discovered a bacterium that was the etiologic agent of yellow fever.

Most of the remaining 10 chapters primarily discuss the work of the US Army Yellow Fever Board, led by Major Walter Reed. Yellow fever had ravaged North America for >200 years, bringing death and economic ruin to several major cities. Major Reed designed a series of simple experiments, using human volunteers, which clearly showed that yellow fever was transmitted only by the bite of infected mosquitoes and not by contaminated items or "poison air." Although the story is familiar to some, the authors present an exciting narrative with details not available elsewhere in the literature. The periodic use of quotations from letters and original sources is most welcome.

The book includes a useful Notes section and an extensive bibliography. Additionally, 12 pages of photos and illustrations are provided, some of which are not as clear as one might wish. Overall, however, this book is a valuable addition to the literature on medical history. It will have broad appeal to scientists and nonscientists alike because of the nature of the story, the magnitude of the problem that was solved, and the easy-to-read writing style of the authors. I recommend it highly.

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Correction: Vol. 11, No. 4

In "*Staphylococcus aureus* Bacteremia, Australia," by Peter Collignon et al., an error occurred. On page 556, in Table 1, in line 10 of column 6, the value indicating total bloodstream infections over study period (all orgs) in Hospital E should be 1,296.

The corrected table appears in the online article at http://www.cdc.gov/ncidod/EID/vol11no04/04-0772.htm#table1

We regret any confusion this error may have caused.

EID Online
www.cdc.gov/eid

Katsushika Hokusai (1760–1849). Thirty-six Views of Mt. Fuji: The Great Wave off Kanagawa (1830–2) (detail). Color and ink on paper (25.7 cm × 37.9 cm). Honolulu Academy of Arts, Hawaii, USA, Gift of James A. Michener, 1991 (13,675)

Of Tidal Waves and Human Frailty

Polyxeni Potter*

From the age of six I had a penchant for copying the form of things, and from about fifty, my pictures were frequently published; but until the age of seventy, nothing that I drew was worthy of notice. At seventy-three years, I was somewhat able to fathom the growth of plants and trees; and the structure of birds, animals, insects and fish. Thus when I reached eighty years, I hope to have made increasing progress, and at ninety to see further into the underlying principles of things, so that at one hundred years I will have achieved a divine state in my art, and at one hundred and ten, every dot and every stroke will be as though alive.

From Hokusai's autobiography, written in 1835, at age 75

"The old man mad about painting" was how Katsushika Hokusai signed some of his work in his later years (1). Passion for art defined his life. And on his deathbed, at age 89, he bemoaned, "If only Heaven will give me just another ten years… just another five more years, then I could become a real painter" (1).

Hokusai was born in Edo, present-day Tokyo. He showed early interest in art and was apprenticed to

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Katsukawa Shunshō, master painter and printmaker, to paint *ukiyo-e*, "images of the floating world," a style focused on everyday activities and their fleeting nature. He painted the transient lives of actors in Edo's theater district, then moved on to study other art styles and become famous for his illustrations of poetry and popular novels. He drew from diverse artistic traditions, among them Chinese and Western art, which was then beginning to appear in Japan. Versatile and prolific, he left thousands of works, signed in more than 30 artistic names. He created a series of sketchbooks as instruction to those who wanted to draw in his style. The series was called Hokusai manga, a term he coined (2).

In a traditional society of Confucian values and rigid regimentation, Hokusai was bohemian. Eccentric, rebellious, and temperamental, he cared nothing about convention and was reputed to move each time the notorious clutter and disorder of his home became unbearable. Legend has it that when invited once to paint maple leaves floating on the Tatsuta River, he drew a few blue lines and then repeatedly imprinted atop the scroll chicken's feet he had dipped into red color. When his contemporaries drew the shoguns and samurai, he portrayed the common people,

and when he painted landscapes, it was strictly from his own point of view (3).

Even though Hokusai's work did not receive full appreciation in Japan, it gained high status and respect abroad. The Great Wave (on this month's cover) became a global icon, as recognizable and revered as Leonardo da Vinci's Mona Lisa or Vincent van Gogh's Sunflowers. Hokusai prints were collected by Claude Monet, Edgar Degas, Mary Cassatt, and many others, who were influenced by them.

Hokusai reached the peak of his creativity in his seventies, when he began work on his thirty-six views of Mount Fuji (3,776 m), Japan's summit and spiritual epicenter. These images, like much of his mature work, reflect familiarity with such European trends as innovative pigments and the telescope. Fascinated by Western design principles, he integrated them with Japanese technique, not only in landscape paintings but also with flowers and birds, which he showed in horizontal close-ups and cut-outs as if seen by a telescope. His imaginative efforts captured the essence rather than the likeness of what he painted and created an altogether novel effect, which appeared Japanese to outsiders and Western to the Japanese.

The Great Wave is Hokusai's most celebrated work. Although renowned nature scenes featured often in Japanese art, the landscape as *ukiyo-e* theme did not gain prominence until after views of Mount Fuji prints became popular. The Great Wave inspired other artistic works, as diverse as Rainer Maria Rilke's poem Der Berg (The Mountain) and Claude Debussy's symphonic masterpiece La Mer (The Sea), whose full score featured The Great Wave on its first edition at the request of the composer (4).

This refined woodblock print epitomizes the artist's skills. Although meticulously structured, it appears effortless, its flair equaled only by the purity of its composition. Undulating lines are fine, at times almost invisible, the colors deliberate and intense. The viewer is guided through the perilous ebb, past the boats to the landmark mountain. The wave is menacing and ghostly, hardened by thick skeletal lines, softened by bubbles of mist, sparkling and voluminous. An eerie feeling is punctuated by the pale sky and frosty white of breaking waves and mountain peak.

The scene could not be more *ukiyo-e*: three light boats carrying fish to market on a work day. But on this day, the sea is in charge, a monstrous wave commanding the foreground, cresting high above the horizon, dwarfing majestic Mt. Fuji now a bump in the fluid scene. Like leaves tossed to sea, the boats tumble, their tiny occupants crouched in fear, clinging to the sides, unable to face the wave and its claws of foam curling toward them.

In The Great Wave, Hokusai captured the uneasy sentiments of a nation surrounded and defined by water, as well as the deeper, primal, human terror of the sea. Enchanting but treacherous, water lures and repels. Seeking livelihood, fortune, adventure, or just solace in its calm, humans ride the waves, risking capricious tempests, settling in precarious coastal regions frequently battered and overpowered by the sea. When the earth moves or climate and other elements stir the waters, environmental markers shift, boats and settlements crumble, and humans perish. In the aftermath comes infectious disease, originating in the disruption and lingering for lack of hygienic conditions and adequate medical care.

Hokusai's fishermen typify human plight against overwhelming force. Their posture embodies the horror of imminent physical harm and death. Fear and anxiety about the long-term consequences of environmental catastrophe are left to survivors and public health workers, who face, along with the loss of infrastructure, compromised sanitation, contamination of water supplies, secondary wound infections, unsafe food, increased poverty, and compounded disease.

The formidable challenge of water-related illness and death persists, from the Indian Ocean to the Gulf of Mexico—despite global prevention and control efforts. Like the fishermen caught in Hokusai's wave, unable to confront the culprit, we cling to a lifeline: managing the physical trauma and addressing resultant infections and complications.

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"Hope" is the thing with feathers/That perches in the soul,
And sings the tune—without the words,/And never stops at all...

Emily Dickinson

EMERGING INFECTIOUS DISEASES

Upcoming Issue

Look in the November issue for the following topics:

Emergence of Toscana Virus in Europe

Highly Pathogenic Avian Influenza H5N1, Thailand, 2004

Cervical Human Papillomavirus Screening

Cryptococcus gattii in AIDS Patients, Southern California

Toscana Virus, Spain

Detection of Tickborne Pathogens, Western Siberia

Rift Valley Fever in Small Ruminants, Senegal, 2003

Neutralizing Antibody Response and SARS

Respiratory Infections during SARS Outbreak, Hong Kong, 2003

Smallpox and the Australian Public

Evaluation of West Nile Virus Education Campaign

West Nile Virus Epidemic, Israel, 2000

Vibrio cholerae Pathogenic Clones

Methicillin-resistant *Staphylococcus aureus* in Taiwan

Salmonella Paratyphi A Increase, Asia

Stigmatization of AIDS and SARS

**Complete list of articles in the November issue at
http://www.cdc.gov/ncidod/eid/upcoming.htm**

Upcoming Infectious Disease Activities

October 4–5, 2005
Intensive Update Course in
Clinical Tropical Medicine and
Travelers' Health
Immediately preceding
the IDSA 42nd Annual Meeting
Contact: 847-480-9592 or
astmh@astmh.org
http://www.astmh.org

October 13, 2005
Launch of Clean Care is Safer Care,
Global Patient Safety Challenge
World Health Organization,
Geneva, Switzerland
http://www.who.int/patientsafety/
challenge/en/

November 12–14, 2005
6th International Conference on
Typhoid Fever and Other
Salmonelloses
Guilin, China
Contact: tandongmei112@
yahoo.com.cn or yyyjin@126.com

November 13–18, 2005
Fourth MIM Pan-African Malaria
Conference
Yaoundé, Cameroon
http://www.mim.su.se/conference2005

December 5–9, 2005
National Viral Hepatitis Prevention
Conference
Hyatt Regency Hotel on Capitol Hill
Washington, DC, USA
http://www.nvhpc.com

December 10, 2005
2005 Pre-Meeting Course: Immune
Regulation: Parasites and Chronic
Infectious Diseases
Hilton Washington Hotel and Towers
Washington, DC, USA
Contact: 847-480-9592 or
astmh@astmh.org
http://www.astmh.org

December 10, 2005
2005 Clinical Pre-Meeting Course:
Anti-Malaria Chemoprophylaxis
Hilton Washington Hotel and Towers
Washington, DC, USA
Contact: 847-480-9592 or
astmh@astmh.org
http://www.astmh.org

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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 is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles see below and visit http://www.cdc.gov/eid/ncidod/ EID/instruct.htm.

Emerging Infectious Diseases is published in English. To expedite publication, we post articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (http://www.cdc.gov/eid/ncidod/EID/trans.htm).

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 email address). Include separate word counts for abstract and text.

Keywords. Include up to 10 keywords; use terms listed in Medical Subject Headings Index Medicus.

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje. org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text.

Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

Tables 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. Complete provided Authors Checklist. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid).

Types of Articles

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

Synopses. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch of first author—both authors if only 2. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research Studies. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary, and a brief biographical sketch of first author—both authors if only 2. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and brief biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and a brief biographical sketch of first author—both authors if only 2. Dispatches are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no figures or tables.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. All letters should contain material not previously published and include a word count.

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

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted on the journal Web page only, depending on the event date.)

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only. Summaries, which should contain 500–1,000 words, should focus on content rather than process and may provide illustrations, references, and links to full reports of conference activities.