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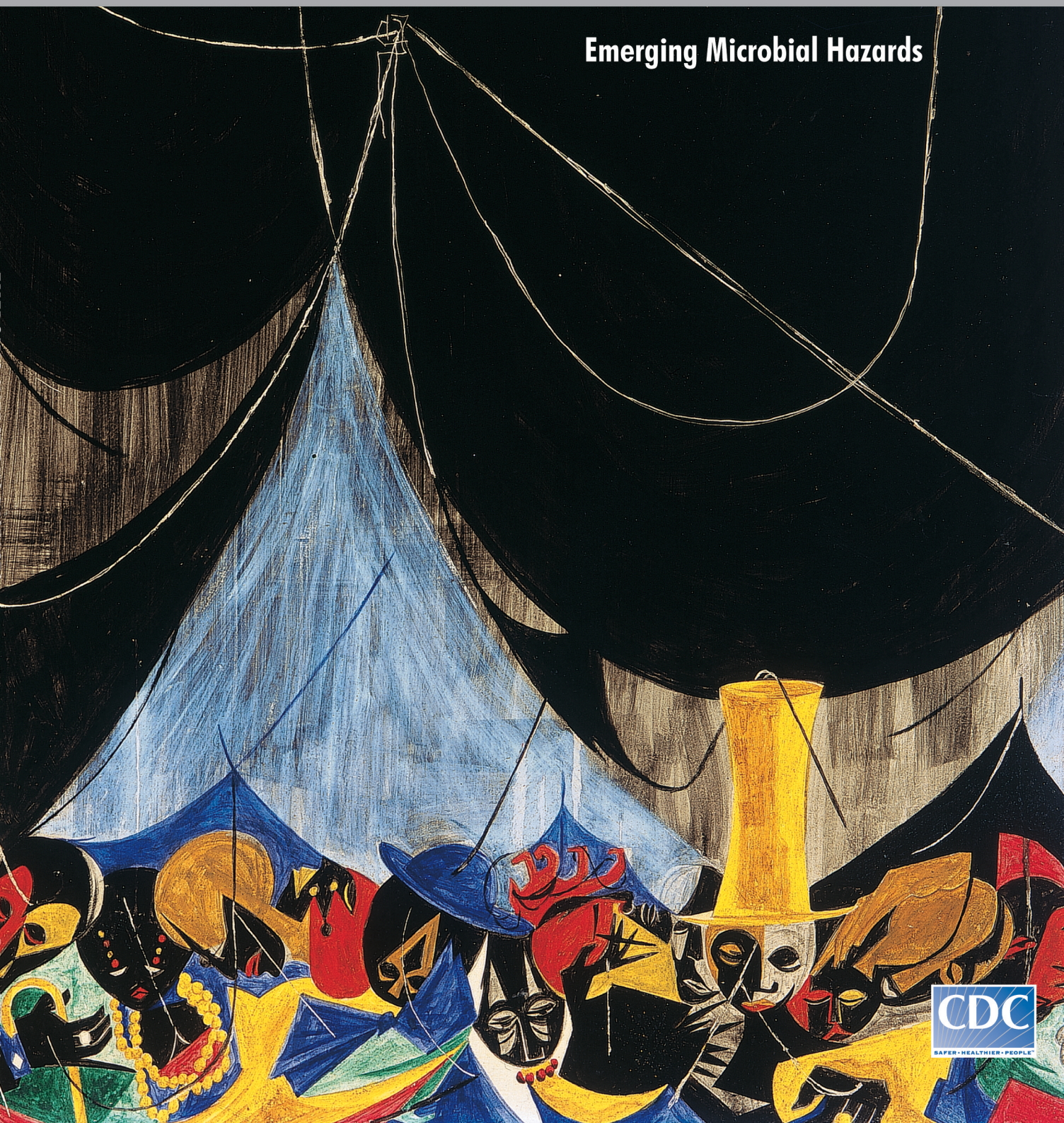

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EMERGING INFECTIOUS DISEASES

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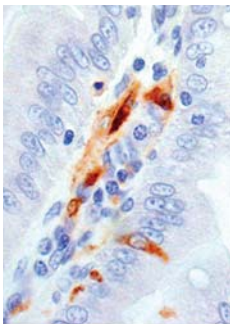
On the Cover

Jacob Lawrence (1917–2000).
Marionettes (1952).
Tempera on panel
(46.4 cm x 62.2 cm).
High Museum of Art,
Atlanta, Georgia, USA.
Purchased with funds from the National Endowment for the Arts and Edith G. and Philip A. Rhodes, 1980.224.

About the Cover p. 962

Perspective

Research



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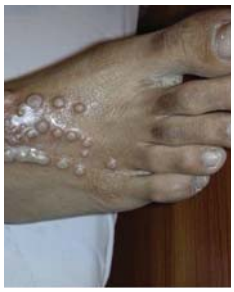

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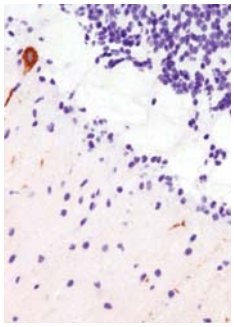
Dispatches



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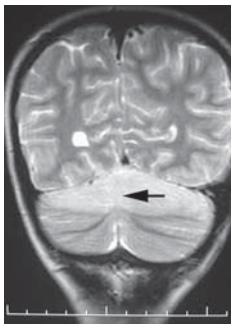

Letters

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Economic Evaluation and Catheter-Related Bloodstream Infections

Kate Halton*† and Nicholas Graves*†

Catheter-related bloodstream infections are a serious problem. Many interventions reduce risk, and some have been evaluated in cost-effectiveness studies. We review the usefulness and quality of these economic studies. Evidence is incomplete, and data required to inform a coherent policy are missing. The cost-effectiveness studies are characterized by a lack of transparency, short time-horizons, and narrow economic perspectives. Data quality is low for some important model parameters. Authors of future economic evaluations should aim to model the complete policy and not just single interventions. They should be rigorous in developing the structure of the economic model, include all relevant economic outcomes, use a systematic approach for selecting data sources for model parameters, and propagate the effect of uncertainty in model parameters on conclusions. This will inform future data collection and improve our understanding of the economics of preventing these infections.

Catheter-related bloodstream infections (CR-BSI) occur at an average rate of 5 per 1,000 catheter days in intensive-care units in the United States (*1*), resulting in 80,000 episodes of CR-BSI per year (*2*). This situation leads to increased patient illness, length of stay, and costs of care (*3*,*4*) and possibly additional deaths (*5*). Empiric evidence (*6*) suggests that >50% of these infections could be prevented. The evidence for the effectiveness of numerous single and multimodule interventions has been reviewed (*2*,*7*), leaving the decision maker with the complex task of selecting the best infection-control programs. This decision should be informed by data on the effectiveness of an intervention as well as an understanding of the cost implications (*8*).

An effective strategy that reduces the risk for CR-BSI will generate health benefits from avoided illness and possibly reduced deaths. At the same time, preventing infections will save costs, and these are offset against cost increases from implementing the strategy. The aggregate of these costs will be either positive (cost-increasing) or negative (cost-saving). An effective program that saves costs must be implemented so as not to waste resources and harm patients at the same time. An effective program that increases costs should be subject to a cost-effectiveness test (e.g., <$50,000 per life year gained) and, if successful, it should be given serious consideration by policymakers. This information can be found in full economic evaluations in which changes to costs and health benefits for a novel strategy are compared with a relevant comparator such as current practice (*8*,*9*). This enables us to identify the course of action that offers optimal returns from our investment of resources.

With the current spending on healthcare in the United States being >15% of the gross domestic product (*10*), the US Food and Drug Administration, as well as the regulatory agencies for the United Kingdom, Australia, and Canada, now require additional programs or therapies to demonstrate cost-effectiveness. The message is clear: new healthcare investments should promote efficiency in resource allocation, not detract from it.

The existing economics literature for CR-BSI includes 2 approaches to full economic evaluation. First are trial-based evaluations in which values for parameters such as costs and health benefits are derived from a single data-collection exercise. Second are modeling studies for which values for these parameters are obtained from a variety of sources and combined in a decision-analytic model. The advantages and disadvantages of each have been discussed (*11*). A major advantage of model-based evaluations is the ability to include long-term cost and death outcomes not observed within the period of a clinical trial. Also, interventions that have not been or cannot be directly compared in a clinical trial can be evaluated side by side in modeling studies. These evaluations allow consideration of all relevant competing infection control interventions and not just

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a single novel strategy compared with existing practice. Finally, model-based evaluations are more generalizable and can be used to evaluate the cost-effectiveness of an intervention in a real-life context not represented by the results of a trial. For these reasons they are the increasingly the preferred approach to the economic evaluation of healthcare interventions (*12*). However, care is needed and only high-quality, appropriately designed and unbiased models should be published and used for policymaking (*11*).

The aims of our study are to summarize the existing literature on model-based economic evaluation of interventions to prevent CR-BSI and then critique this literature, focusing on 2 questions. 1) How useful are the evaluations in terms of how the research questions and findings align with the information needed to make good decisions? 2) What is the quality of the evaluations, in particular, whether the quality of the model structure, the source of parameter data and its incorporation into the model, and the techniques used to evaluate the model are such that the evidence provided is convincing to decision makers? Ultimately, we aim to judge the value of this body of literature in helping us understand the economics of preventing CR-BSI and identify priorities for future research that will lead to a deeper understanding of this topic.

Methods

We reviewed data published between 1990 and November 2005. Searches were conducted in Medline, the Cumulative Index to Nursing and Allied Health Literature, Biologic Abstracts, Academic Search Elite, and Econlit by using the medical subject headings catheterization central venous, costs and cost analysis, and infection; or text keywords catheter and central, cross-referenced with infection, bacteremia, or sepsis, and cost-effective, cost-benefit, or cost-utility. We searched the Centre for Reviews and Dissemination databases (www.york.ac.uk/inst/crd) by using the same subject keywords and limiting the search to economic evaluations. In addition, the reference lists of retrieved articles and review articles in this field of research (*13–16*) were searched to identify published articles that met predefined inclusion and exclusion criteria (Table 1).

To assess the usefulness of the economic evaluations included, summary data for each were extracted by using an audit tool based on the Harvard Cost-Effectiveness Analysis Registry data abstraction forms (*17*). The data extracted included a description of the intervention(s) and population studied, the research question, the structure of the economic model and assumptions used, the data used to inform model parameters, the outcomes considered, and the results and conclusions, including the results of sensitivity analyses. All US dollar figures were adjusted to 2005 prices by using the Bureau of Labor Statistics Consumer Price Index specific to Medical Care (www.bls.gov/cpi), although any common year could have been assumed. When the cost year used for the analysis was not stated, it was assumed to be 1 year before publication. This assumption will not affect evaluation of the analysis.

To assess the quality of the economic evaluations, we used a set of good practice criteria for decision analytic modeling (*18*). Four criteria are used to assess the structure of the model; 6 criteria to assess how data were sourced and incorporated, including approaches to sensitivity analysis; and 1 criterion to judge how the model was evaluated in terms of its own consistency. These 11 criteria were applied as a series of questions that focused on the relevance and coherence of the modeling approach taken in each evaluation, rather than as a prescriptive checklist.

The quality of the data used to inform model parameters was also assessed by using the modified version (*19*) of the potential hierarchies of data sources for economic analyses (*20*). Each component of the decision model was assessed: clinical effect size, baseline clinical data, adverse events, resource use, costs, and utilities. The quality of data sources is ranked from 1 to 6 with the highest quality of evidence ranked 1. Rankings for evidence pertaining to clinical effect size are comparable with the concept of levels of evidence as used in evidence-based medicine (*21*) and Cochrane reviews (*22*). For each article, the highest level of evidence used for each parameter was recorded.

Results

A total of 106 abstracts were identified, and 8 met the inclusion criteria (*23–30*). The reasons for exclusion are shown in the Figure.

Usefulness of Evaluations

Six interventions were evaluated (Table 2); antimicrobial drug–coated catheters were included in 3 separate analyses (*27*,*29*,*30*). One intervention was compared with current practice for all studies, except those of Shorr et al. (*29*) and Ritchey et al. (*28*), who evaluated 3 types of antimicrobial drug–coated catheter and 3 different catheter replacement regimens, respectively. No direct comparisons were made across intervention types, e.g., use of an antiseptic catheter versus introduction of chlorhexidine as a skin preparation, and no evaluations assessed multiple concurrent interventions or bundles. The authors of 6 evaluations (*23*,*24*,*26*,*27*,*29*,*30*) found the intervention to be effective in preventing CR-BSI and cost-saving (Table 3), and the authors of 2 other evaluations (*25*,*28*) generated data to calculate incremental cost-effectiveness ratios.

Sensitivity analysis was performed in addition to baseline analysis in 5 evaluations (*23*,*26*,*27*,*29*,*30*). This provided decision makers with information on the robustness of baseline results to different parameter estimates or characterized the effect of uncertainty in model parameters

Table 1. Inclusion and exclusion criteria for review

Inclusion criteria
Had a full publication or manuscript for review
Conducted a full economic evaluation which valued both costs and benefits of the intervention
Based on a decision-analytic model
Evaluated at least 1 infection-control intervention aimed at reducing incidence of catheter-related bloodstream infection relative to a baseline scenario
Evaluated the intervention with respect to short-term (<21 d), nontunneled, central venous catheters
Based in an adult patient population
Written in English
Exclusion criteria
Cost-analysis studies only
Did not use a comparator
Based on a clinical trial (e.g., randomized controlled trial or pre-post intervention study) or a case study
Did not contain an original analysis (e.g., editorials, reviews)
Contained purely hypothetical data (e.g., methods articles)
Did not provide full details on methods (e.g., letters)
Based in a pediatric patient population
Evaluated interventions aimed at long-term or tunneled or peripherally placed central venous catheters
Evaluated therapeutic or diagnostic interventions, as opposed to preventive interventions

on the results (*23*,*27*,*30*). In 3 cases (*24*,*25*,*28*), sensitivity analysis formed the main body of the evaluation, and decision makers faced multiple sets of results arising from different parameter estimates.

Quality of Economic Evaluations

The extent to which the quality criteria were met for the studies varied from 1/8 for checks on the internal consistency to 8/8 for description of strategies/comparators. This assessment is shown in Table 4.

Model Structure

All authors provided a clear description of the intervention and specified the economic perspective used, which in all cases was that of the hospital or healthcare payer rather than a societal perspective. Only Shorr et al. (*29*) justified their choice of perspective. In 7 evaluations (*23*,*24*,*26*–*30*), a decision tree was used, with a diagram provided in all but 1 report (*26*). In another evaluation (*25*), a regression model was used, and only the formula used for the baseline analysis, not the extension used for sensitivity analysis, was provided. Authors of only 4 evaluations discussed the evidence or expert opinion used to develop the structure of the model (*23*,*27*,*29*,*30*).

Each evaluation used a different representation of the disease pathway in terms of the timing and nature of the relevant clinical events. For example, 1 evaluation modeled colonization as an event preceding CR-BSI (*23*), 4 considered these as mutually exclusive events (*24*,*26*,*27*,*30*), and 3 did not consider colonization (*25*,*28*,*29*). Two models included adverse events specific to the intervention (*28*,*30*), but this was not consistent across studies, with only 1 of the 3 evaluations of antiseptic-impregnated catheters including incidence of hypersensitivity reactions to the catheter (*30*). In 7 evaluations (*23*–*26*,*28*–*30*), only the outcomes that would arise during the period of hospitalization were included. In another evaluation (*27*), the time horizon described the patient's lifetime.

Source and Incorporation of Data

Authors of all evaluations stated the baseline data used in the model along with its source; 5 had information in a table format (*23*,*24*,*27*,*29*,*30*). Most parameter estimates came from the published literature, although 5 evaluations performed their own cost calculations for the intervention (*23*–*26*,*29*) and 1 used original patient trial data for the estimates of daily incidence and relative risk for infectious events (*27*). Seven evaluations (*23*,*25*–*30*) discussed simplifying assumptions and issues of generalizability.

For 6 evaluations (*23*,*26*–*30*), the most important model parameters were identified (Table 5), with the following 3 parameters consistently important: reduction in risk for CR-BSI caused by the intervention, baseline incidence of CR-BSI, and cost of treating a CR-BSI. The ranks of evidence used for these and other model parameters are shown

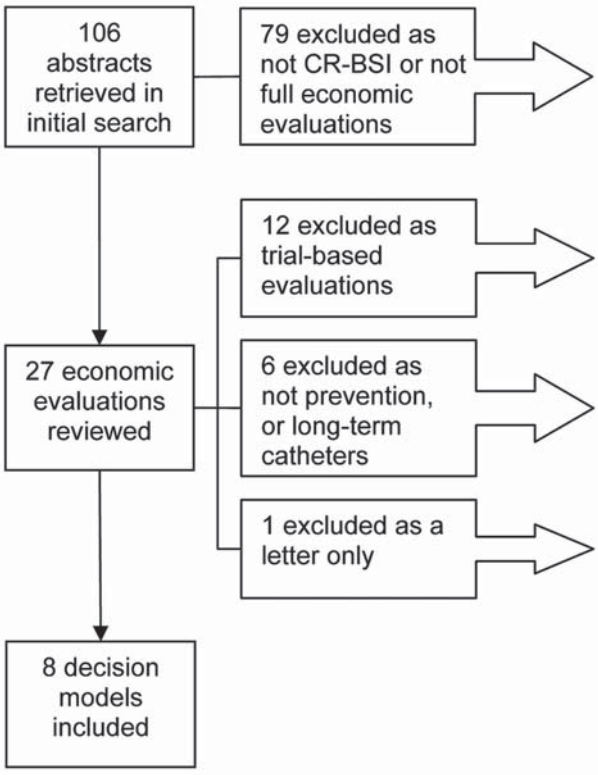

Figure. Reports included in the review. CR-BSI, catheter-related bloodstream infections. The 19 economic evaluations excluded from the review are shown in the online Appendix (available from www.cdc.gov/EID/content/13/6/817-app.htm).

Table 2. Summary of economic evaluations of interventions to prevent CR-BSI included in the review*

Intervention	Comparator	Analysis	Perspective	Sensitivity analysis	Time horizon	Hospitalized patients	Ref.
Antimicrobial catheters							
MR CVC	CHG-SSD CVC	CUA	HC payer	PROB, OW, SC	Patient lifetime	Adults at high risk for CR-BSI likely to require a triple-lumen, noncuffed CVC for ≥3 d	*27*
MR CVC and CHG-SSD CVC	Standard CVC	CEA	HC payer	OW, SC, TH	Duration hospitalized	Critically ill patients requiring a CVC expected to be placed >48 h	*29*
CHG-SSD CVC	Standard CVC	CEA	HC payer	PROB, OW, SC, TH	Duration hospitalized	Patients at high risk for catheter-related infections requiring short-term use (2–10 d) of multilumen CVCs	30
Aseptic technique							
MSB at CVC insertion	Less stringent asepsis	CEA	Hospital	OW, SC	Duration hospitalized	Patients requiring short-term multilumen CVC (specifically, those in ICU, with immunosuppression, or receiving TPN)	*26*
Skin preparation and dressing							
CHG skin prep	PI skin preparation	CEA	Hospital	PROB, OW, SC	Duration hospitalized	Patients requiring either a PVC or CVC (considered separately) for short-term use (<10 d)	*23*
CHG dressing	Standard dressing	CEA†	Hospital	OW, MW, SC	Duration hospitalized	Patients at high risk for catheter-related infections requiring short-term use (2–10 d) of multilumen CVCs	*24*
Total parenteral nutrition							
TPN commercial bags	TPN glass bottles	CMA/CEA	Hospital	MW, TH	Duration hospitalized	Patients receiving TPN through catheter for severe bowel dysfunction secondary to Crohn disease, medical ICU patients, and surgical ICU patients	*25*
Replacement regimen							
Optimal CVC change regimen (10 d, 5 d)	3-d change regimen	CEA	Hospital	OW, MW, TH	Duration catheterized	65-year-old man in ICU with reversible disease process	*28*

*Except for the study in reference 25, which used a regression model, all studies used a decision tree. CR-BSI, catheter-related bloodstream infections; Ref., reference; MR, minocycline and rifampicin; CVC, central venous catheter; CHG-SSD, chlorhexidine gluconate/silver sulfadiazine; CUA, cost-utility analysis; HC, healthcare; PROB, probabilistic sensitivity analysis; OW, one way; SC, scenario; CEA, cost-effectiveness analysis; TH, threshold; MSB, maximal sterile barriers; ICU, intensive-care unit; TPN, total parenteral nutrition; PI, povidone-iodine; CMA, cost-minimization analysis; MW, multi way.
†Crawford et al. (*24*) identified their evaluation as a cost-benefit analysis (CBA) but they conducted a cost-effectiveness analysis with health outcomes multiplied by a dollar value to produce a monetary valuation of health benefits.

in Table 6. The level of evidence used for the effectiveness of the intervention was generally high, and authors of all evaluations provided information on how they selected the data used for this parameter. However, the level of evidence used for the cost and baseline incidence of CR-BSI was generally of lower quality; little detail was given in the reports of the evaluations as to why 1 particular estimate for a parameter was chosen over another. In particular, in all evaluations, reference was made in the introduction or discussion section to relevant information on the cost and deaths attributable to CR-BSI that was not used in the analysis. This explains the wide variation in the source and value of the estimates used for parameters between the evaluations (Table 5).

Model parameters were expressed as probability distributions for only 3 studies (*23*,*27*,*30*), even though this method provided an opportunity to appropriately describe parameter uncertainty. All 3 studies specified the choice of distribution for model parameters and the rationale for this choice. The remaining studies (*24–26*,*28*,*29*) used point estimates and a range for each parameter across which the estimate was varied in sensitivity analyses. Similar to the baseline estimates, no information was given on how ranges used for sensitivity analysis were decided upon, aside from a double-it and half-it approach.

Model Evaluation

All evaluations used deterministic sensitivity analyses by varying parameters across a range of point estimates either 1 at a time (1-way) or concurrently (multiway). Four studies (*25*,*28–30*) reported results of threshold analyses, i.e., the value of each parameter at which the

Table 3. Results of economic evaluations of interventions to prevent CR-BSI*

Intervention	Estimated absolute incremental benefits		Estimated incremental cost	Cost/benefit ratio	Sensitivity analysis	Ref.
	Incidence CR-BSI, %	Mortality incidence, %				
Baseline: CHG-SSD catheter	Variable	Not stated	Not stated			*27*
MR catheter†	–0.7	0.009 QALYs (–0.009, 0.016)	–$83 ($109, –$205)	Cost saving	Robust	
Baseline: standard catheter	3.30	–	$469			*29*
CHG-SSD catheter	–1.94	–	–$222	Cost saving	Robust	
MR catheter	–2.79	–	–$314	Cost saving	Robust	
Baseline: standard catheter	5.20	0.78	$710			*30*
CHG-SSD catheter	–2.20 (–1.2, –3.4)	–0.33 (–0.09, –0.78)	–$262 (–$91, –$522)	Cost saving	Robust	
Baseline: less stringent asepsis	5.30	0.80	$676			*26*
Maximal sterile barriers	–2.49	–0.38	–$274	Cost saving	Robust	
Baseline: Povidone-iodine skin preparation	3.1	0.46	$265			*23*
Chlorhexidine gluconate	–1.6 (–0.6, –2.5)	–0.23 (–0.07, –0.47)	–$134 (–$21, –$286)	Cost saving	Robust	
Baseline: standard dressing	5.00	0.05	$514			*24*‡
Chlorhexidine dressing§	–2.63	–0.03	–$259	Cost saving	Robust	
Baseline: glass TPN bottles	10.0	0.50	Not stated			*25*‡
TPN bags¶	–6.67	–0.33	Not stated	$28,326/life saved	Variable	
Baseline: 5 d	–	0.92	$1,398	Not clear from source what reported cost-effectiveness ratios represented		*28*‡
3 d	–	–0.02	$8		Variable	
10 d	–	–0.13	$63		Variable	

*All estimates have been adjusted to 2005 US dollars. Values in parentheses are 95% confidence intervals. CR-BSI, catheter-related bloodstream infections; mortality, CR-BSI attributable mortality; CHG-SSD, chlorhexidine gluconate/silver sulfadiazine; QALYs, quality-adjusted life year; MR, minocycline and rifampicin; TPN, total parenteral nutrition.
†Refers to results for an 8-d duration of catheterization; intervention was cost-saving for durations >8 d and could not be evaluated for <8 d.
‡Cost year for original analysis not stated; therefore, assumed 1 year before publication.
§Refers to results using baseline conservative assumptions of 5% CR-BSI incidence rate, 1% CR-BSI attributable mortality rate, and $8,000 incremental CR-BSI treatment cost.
¶Refers to results using baseline conservative assumptions of 10% CR-BSI incidence rate, 5% CR-BSI attributable mortality rate, and relative reduction in risk for CR-BSI of 0.33.

conclusions from the analysis would change, and 6 studies (*23*,*24*,*26*,*27*,*29*,*30*) reported results of scenario analyses, i.e., results where all parameters are set to favor each specific intervention in turn (Table 2). The 3 evaluations that characterized parameters as distributions (*23*,*27*,*30*) also used probabilistic sensitivity analysis, which enabled calculation of confidence intervals around their point estimates of incremental costs and benefits.

In the 6 evaluations where the intervention was cost-saving (*23*,*24*,*26*,*27*,*29*,*30*), the conclusions were robust to the sensitivity analyses. In the 2 evaluations where an incremental cost-effectiveness ratio could be calculated (*25*,*28*), different conclusions were drawn in different scenarios (Table 3). Scenario analyses used in 6 evaluations (*23*,*24*,*26*,*27*,*29*,*30*) indicated internal consistency in the models, i.e., they behaved logically and as expected. However, only 1 evaluation (*27*) made an explicit statement on internal consistency about checks performed during the model construction and analysis. Authors of 7 evaluations discussed caveats to their work (*23*–*27*,*29*,*30*).

Discussion

We reviewed existing model-based economic evaluations of interventions to prevent CR-BSI. Given the growing use of economic evidence to inform infection control policy (*13*), the amount of this literature is likely to increase. However, critics have questioned the validity of these evaluations. McConnell et al. (*31*) suggest that "in the absence of evidence-based medicine on the effectiveness of antimicrobial central venous catheters, on the basis of clinically relevant end points, cost-effectiveness studies are an exercise in futility" We would argue that even in this situation the best possible decision still needs to be made (*11*) and that evaluations should be judged not on their ability to predict the precise value of an intervention but on the "ability of a decision model to recommend optimal decisions" (*32*). A decision not to invest in some risk-reducing intervention or program is a decision that leads to economic and clinical outcomes that are either optimal or not optimal. Economic evaluation provides a rational way for the decision maker to rank these outcomes, which in the absence of perfect information, is of more use than producing a single, potentially

Table 4. Assessment of published evaluations and good practice criteria for decision models

Attributes of good practice criteria	No. models meeting criterion, n = 8
Structure	
Perspective specified	8
Description of strategies/comparators	8
Diagram of model/disease pathways	6
Development of model structure and assumptions discussed	4
Data	
Table of model input parameters presented	5
Source of parameters clearly stated	8
Model parameters expressed as distributions	3
Model assumptions discussed	7
Sensitivity analysis performed	8
Key drivers/influential parameters identified	6
Consistency	
Statement about test of internal consistency undertaken	1

misleading, dollar estimate. We critiqued the existing evaluations in terms of their usefulness in providing information relevant to clinical practice. We also assessed the quality of the evaluations and explored the implication that this would have on the information provided to decision makers.

Four interventions were found to be clinically effective and cost-saving: use of antibiotic-coated catheters compared with use of either antiseptic-coated or standard catheters, maximal sterile barrier precautions during catheter insertion compared with less stringent aseptic technique, and use of chlorhexidine gluconate as either a skin preparation or impregnated into the insertion site dressing compared with use of povidone-iodine skin preparation and nonimpregnated dressings. Results of these evaluations are robust to a wide range of parameter estimates and assumptions. Two other interventions showed health benefits and increased costs: use of a 3-day or 10-day catheter replacement regimen rather than replacement every 5 days and use of commercially available plastic bags for delivery of total parenteral nutrition rather than glass bottles. Conclusions about the cost-effectiveness of these interventions changed with use of different parameters and assumptions.

Usefulness of Evaluations

We have data on the cost-effectiveness of only 6 interventions. These interventions were evaluated separately and not compared with each other. Furthermore, many other interventions have been shown to be clinically effective but, there are no data on their cost-effectiveness. This finding is not consistent with current guidelines (*2*), which recommend that "it is logical to use multiple strategies concomitantly." The 100,000 Lives Campaign is also formed on the basis of a group of interventions. The existing economic evidence is therefore incomplete and cannot be used to form a coherent policy for preventing CR-BSI. Infection control practitioners and other decision makers require information on the relative cost-effectiveness of all relevant groups of interventions rather than individual strategies (*8*). A good example of using cost-effectiveness to inform a complete policy is provided by Frazier et al (*33*). They evaluated 21 competing strategies for population-based colorectal cancer screening and included all relevant screening methods and frequencies. This study provides policymakers with complete information in as much as all available choices have been compared.

The failure to specify baseline values (i.e., the value authors believe is most likely) for model parameters is also problematic. Instead of estimating a baseline model and then testing whether the conclusions are robust to high and low values, some authors report all possible results on the basis of all possible values for some parameters. This shifts the responsibility of interpreting the results to the reader. The failure to describe how high and low values were chosen for key parameters (i.e., the double-it and half-it approach) compounds the problem.

Table 5. Variation between economic evaluations in baseline parameter estimates*

Baseline parameters	No. times identified as key parameter	No. different estimates	Minimum estimate	Maximum estimate	Median estimate
Epidemiologic					
Incidence of CR-BSI	6/8	8/8	3.1%	8.0%	5.3%
Effectiveness of the intervention	6/8		Will vary according to intervention		
Attributable mortality	2/7	5/7	5%	15%	14%
Incidence of localized insertion site infection	0/5	4/5	5%	50%	20%
Cost					
Cost of CR-BSI	6/8	6/8	US $2,820	US $13,000	US $10,531
Cost of localized insertion site infection	0/5	3/5	US $195	US $435	US $280
Cost of intervention	2/8		Will vary according to intervention		
Cost of other complications	1/3		Will vary according to complications considered		

*All cost estimates adjusted to 2005 US dollars. Values for parameters are the baseline estimate used in the model (the same patterns of variation were observed with the ranges used for sensitivity analysis). CR-BSI, catheter-related bloodstream infections.

Table 6. Ranks of evidence for parameters used in the decision models*

Evidence ranking	Clinical effectiveness of intervention, n = 8	Baseline incidence CR-BSI, n = 8	Attributable mortality, n = 7	Incidence localized insertion site infection, n = 5	Cost of CR-BSI, n = 8	Cost of intervention, n = 8
High quality						
Rank 1	5	1	–	–	2	–
Rank 2	1	1	1	–	1	7
Medium quality						
Rank 3	–	1	1	–	2	–
Low quality						
Rank 4	1	4	4	4	2	–
Rank 5	–	1	1	1	–	–
Rank 6	–	–	–	–	–	–
Unclear	1	–	–	–	1	1

*CR-BSI, catheter-related bloodstream infections.

Assessing Quality

There was a lack of transparency in the development of model structure. Model structure may have been driven by availability of data rather than careful review of the natural progression of the disease. This could undermine the external consistency of the evaluations as they appear to users. The choice of short-time horizons and narrow economic perspectives inhibits the usefulness of these evaluations by excluding relevant costs and health outcomes from the analysis. The current evidence may represent a blinkered view of the problem and how it should be managed. This situation in turn reduces the extent to which the value of infection control can be compared with other healthcare spending such as cardiac surgery and diabetes prevention.

The quality of data incorporated in the models is highly variable. The authors of 7 studies (*23*,*24*,*26*–*30*) suggest that their results are compromised by an absence of high-quality or precise information, often for key parameters in the model. This finding leads to some skepticism about the results (*31*). Researchers are attempting to provide better estimates of the health and economic outcomes attributable to CR-BSI (*34*). However, a model should not be criticized on the basis of the quality of data used per se. Rather, it should be judged on the techniques used to identify and incorporate the highest quality appropriate and relevant data possible (*35*) for all parameters, not just those relating to effectiveness. Given the lack of information provided by the authors about this process, a more systematic approach to selecting evidence needs to be introduced. Generic tools such as the hierarchy used here (*19*) are useful to judge evidence quality, but this may need to be supplemented with tools such as the hierarchy of quasi-experimental study designs, given the prevalence of the use of these designs in the infection control literature (*36*). Where multiple pieces of relevant information are available, techniques exist for the synthesis of diverse evidence (*37*).

Given the variations in data quality, selecting the best evidence and then propagating the effect of uncertainty in this evidence to the conclusions drawn are important. A good method is probabilistic sensitivity analysis (*38*). This method was used in 3 evaluations (*23*,*27*,*30*). This technique characterizes parameter estimates as distributions rather than discrete values and conducts multiple simulations of the model that draw different parameter values each time from the distributions. This enables the uncertainty around the costs and benefits of a given intervention to be described and the relative contribution to all uncertainty arising from each parameter to be estimated. The next step, which was not conducted for any evaluation, is to estimate the value of collecting more data to inform these parameters (*39*). This step would be particularly relevant to the key parameters identified in this review. The current methods used to derive estimates of costs and deaths attributable to CR-BSI are subject to some bias and may not make intuitive sense to clinicians (*31*). This issue is problematic because these methods are important components in the model, often driving the changes in costs and benefits, and it is likely this finding partly explains why so many interventions appear cost-saving.

This review has some limitations. Despite use of a broad search strategy, we may not have identified all model-based economic evaluations in this area; some evaluations may not have been published or are available only as abstracts. Also, our assessment of the quality of evaluations using the good practice criteria may reflect the way evaluations are reported rather than conducted. In fact, word limits often prevent authors from providing a full description of methods. However, any indication that a criterion was addressed was taken as an evaluation that met that attribute.

Conclusion

We do not have a comprehensive understanding of the economics of preventing CR-BSI. Policymakers and regulatory agencies are unable to recommend the best approach to mitigate risks for CR-BSI in patients in intensive-care units. Those who propose to undertake research in this area would benefit from a careful consideration of this review. Modelers should collaborate and aim to develop a consen-

sus on key issues such as model structure, data sources, and evaluation methods. This activity is promoted by the International Society for Pharmacoeconomics and Outcomes Research and The Cancer Intervention and Surveillance Modeling Network. Ultimately, the best policy for preventing CR-BSI will emerge from an iterative process that includes researchers, clinicians, modelers, and decision makers.

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Levels of Abnormal Prion Protein in Deer and Elk with Chronic Wasting Disease

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Chronic wasting disease (CWD) of deer and elk is a widespread health concern because its potential for cross-species transmission is undetermined. CWD prevalence in wild elk is much lower than its prevalence in wild deer, and whether CWD-infected deer and elk differ in ability to infect other species is unknown. Because lymphoid tissues are important in the pathogenesis of some transmissible spongiform encephalopathies such as sheep scrapie, we investigated whether CWD-affected elk and deer differ in distribution or quantity of disease-associated prion protein (PrPres) in lymphoid tissues. Immunoblot quantification of PrPres from tonsil and retropharyngeal lymph nodes showed much higher levels of PrPres in deer than in elk. This difference correlated with the natural prevalence of CWD in these species and suggested that CWD-infected deer may be more likely than elk to transmit the disease to other cervids and have a greater potential to transmit CWD to noncervids.

Chronic wasting disease (CWD) is an emerging infectious disease first recognized in the 1960s. It is a member of the transmissible spongiform encephalopathy (TSE) disease group that includes sheep scrapie, bovine spongiform encephalopathy (BSE), transmissible mink encephalopathy, and several human diseases, including kuru, Creutzfeldt-Jakob disease (CJD), and variant CJD (vCJD). Several heritable but extremely rare forms of TSE are found, most notably, Gerstmann-Straussler-Schienker syndrome (GSS) and fatal familial insomnia. CWD, like other TSE diseases, is characterized by the accumulation in neural tissues of an abnormal disease-associated prion protein designated PrPres (*1*), PrP^{Sc} (*2*), or PrP^{d} (*3*). Most TSE researchers believe that PrPres is critical in disease pathogenesis, and some evidence suggests that PrPres may itself be the infectious agent (*4*).

As recently as 1997, CWD in the wild appeared to be confined to a few counties of northeast Colorado and southeast Wyoming**.** Since then, new cases have been reported in wild deer from many additional states. The disease has recently emerged in captive elk and deer facilities scattered across the United States and Canada. Whether these new foci of infection resulted from contact with captive cervids or represent established foci of infection discovered by recent surveys is unknown. In disease-endemic areas, the prevalence of CWD in deer is variable but is often >5%. In contrast, the prevalence in wild elk is typically <1% (*5*,*6*). What factors account for different CWD prevalence in the wild between deer and elk are not known.

In some species, the infectious agent and PrPres accumulate in both lymphoid tissues and brain. The extent of lymphoid tissue involvement varies depending on the host and agent involved. In scrapie-infected sheep, lymph nodes and spleen are infected early and are directly involved in the kinetics of disease (*7*–*9*). Lymphoid tissues are likewise important in vCJD (*10*) in humans and also in some TSE mouse models (*11*–*13*). In other TSE diseases such as BSE and sporadic CJD, lymphoid tissues appear to play little or no essential role in disease pathogenesis (*14*,*15*). Knowing the extent of lymphoid tissue involvement in deer and elk might provide clues regarding modes of natural transmission in these species or the potential for transmission to other species.

PrPres in lymphoid tissues of deer (*16*) and elk (*17*) has been primarily detected by using immunohistochemical (IHC) techniques. However, with these techniques, quantification and glycoform analysis of PrPres are not possible. We were interested in determining whether PrPres found in

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[1]Deceased

lymphoid tissues of deer differs from PrPres found in lymphoid tissues of elk in quantity, distribution, or structural features. Immunoblot techniques enabled us to study these questions.

Surveys of CWD-infected deer and elk based on IHC or ELISA analysis of brain or retropharyngeal lymph nodes (RPLNs) have not shown differences between the 2 species that explain why CWD prevalence differs in natural settings (*18*,*19*). In our study, we sought to identify potential differences in biochemical characteristics of PrPres to explain the prevalence differences between the 2 species. We found that lymphoid tissues of CWD-infected deer had much greater quantities of PrPres than were detected in similar samples from elk. Furthermore, we found a wider distribution and higher incidence of positive lymphoid tissues in deer. These differences might account for the disparity in the reported prevalence of CWD in the wild between deer and elk. Our results also support previous observations that suggested CWD surveillance programs based on IHC detection of PrPres in lymphoid tissues alone may not be appropriate for elk (*5*,*18*).

Table 1. PrPres detection by immunoblot in brain and lymphoid tissues of elk and deer*

Species	Brain	Spleen	Tonsil	Lymph node
Elk	10/10	2/10	4/9	5/10
Mule deer	12/12	2/10	9/10	12/12
White-tailed deer	3/3	1/3	2/3	3/3

*PrPres, disease-associated prion protein. Values indicate the number of animals with PrPres detected (numerator) over the number analyzed (denominator). If any PrPres was detected in a given tissue, it was considered positive for the purpose of this table. If even a single lymph node from a given animal was positive, the animal was scored as positive for that tissue. This table does not consider quantitative variation in the amount of PrPres in tissues of elk compared to deer. The denominators vary because not every tissue was available from every animal. Brain, tonsil, and lymph nodes of elk did not differ significantly from mule deer (Fisher exact test).

Materials and Methods

Tissues

Brain, tonsil, spleen, and selected lymph nodes, including RPLNs, prescapular, submandibular, superficial cervical, mesenteric, popliteal, and ileocecal-colic lymph nodes, were obtained from 10 CWD-infected elk and 15 CWD-infected deer (12 mule deer and 3 white-tailed deer). Elk were derived from game farms or research facilities where they became infected by contact with CWD-infected elk, a contaminated environment, or oral inoculation. All of the elk used in this study had definite clinical cases when they were euthanized. The deer used for PrPres quantification all had confirmed clinical cases and were from research facilities where they became infected by contact with infected animals. Three of the mule deer, included in Table 1, were harvested by Colorado Division of Wildlife or Wyoming Department of Game and Fish personnel. Tissues from wild uninfected deer and elk were obtained from Montana Department of Fish, Wildlife and Parks. More than 4,000 wild deer and elk from Montana have been tested for CWD with no positives found.

PrPres Purification

Twenty percent tissue homogenates of brain, tonsil, lymph nodes, or spleen from CWD-infected or uninfected deer and elk were made in 10 mmol/L Tris-HCl, pH 7.4, 5 mmol/L $MgCl_2$ by using either disposable Konex microcentrifuge tubes (Kimble/Kontes, Vineland, NJ, USA) and matched pestles (brain) or an omni tissue homogenizer (tonsil, spleen, and lymph nodes); 75%–90% of the total tissue mass of respective lymph nodes or tonsil was homogenized. Two-hundred–milligram aliquots of the total homogenate were processed further to concentrate PrPres by using ultracentifugation and proteinase K digestion as described (*20*).

Immunoblotting

Protein gel electrophoresis and immunoblotting were done as previously described (*21*,*22*) by using polyclonal antibody R35 (*23*) or monoclonal antibody L-42 (R-Biopharm AG, Darmstadt, Germany). L-42 reacts with PrPres from several species, including deer and elk, and has been well characterized (*24*). Blots were developed by using either an enhanced chemiluminescence (ECL) or enhanced chemifluorescence (ECF) system, according to the manufacturer's instructions (Amersham-Pharmacia, Piscataway, NJ, USA). ECL blots were exposed to film to visualize proteins. ECF blots were scanned by using a STORM fluorescent detection system (Amersham-Pharmacia) as described previously (*25*).

PNGaseF Digestion

Reagents and enzymes for PNGaseF treatment were purchased from New England BioLabs (Beverly, MA, USA). Reaction conditions were as recommended by the manufacturer except that denaturing of 1- to 30-mg tissue equivalents was done in a total volume of 20 μL sodium dodecyl sulfate–polyacrylamide gel electrophoresis sample buffer. Each sample was digested by using 2,500 U PNGaseF and incubated overnight at 37°C. Samples were frozen at –20°C until they were analyzed by immunoblotting.

Results

Quantification of PrPres in Brain, Tonsil, and RPLNs

Tissues from 10 CWD-affected elk and 15 CWD-affected deer were analyzed in this study. All 10 of the elk had advanced clinical CWD when euthanized. The deer

represented various stages of clinical disease. Detailed data showing PrPres glycoform profiles and quantification of PrPres are shown for 5 of the elk and 6 of the deer (Figure 1). Each of the elk brains gave a very strong PrPres signal when 2-mg brain equivalents were analyzed. Deer brain PrPres was more variable, and 20 mg of brain equivalent was analyzed (Figure 1). The relative amount of PrPres in each sample was determined by comparing the PrPres signal to a standard control. The standard control for each blot was RPLN from one of the CWD-infected mule deer in the study (labeled C in each blot, Figure 1). Relative PrPres amounts were determined by using a phosphor-imager and Image Quant software (Storm, Molecular Dynamics, Sunnyvale, CA, USA). The average amount of PrPres in elk brain was consistently higher than the amount in deer brain (Figure 2). The lower amounts of PrPres in deer brain than in elk brain likely reflects the more variable and earlier clinical status of the deer that were analyzed.

Diagnosis of CWD is often based on detection of PrPres in tonsil tissue by using ELISA and IHC analysis. Therefore, we also analyzed tonsil tissue, but because we were interested in quantitative issues we used immunoblot technology.

No PrPres was observed in tonsil from 2 of the elk (Figure 1B), and only a small amount was detected in tonsil from the other 3 elk (Figures 1B, 2B). PrPres in the 3 tonsil specimens that did give a signal averaged 4% of the control's signal. In contrast, tonsil from 5 of the 6 CWD-affected deer gave a strong PrPres signal (Figure 1B), averaging 109% of the reference control's signal. However, the tonsil of the remaining deer (#4, Figures 1B, 2B) gave no PrPres signal on immunoblot.

PrPres quantities in RPLNs from elk were also low. RPLN from 1 elk was negative (#4, Figure 1C), while weak reactions were seen for RPLNs from the other 4 elk at 2%, 2%, 5.1%, and 13% of the control, respectively (Figures 1C, 2C). RPLNs from the deer were much stronger. RPLNs from all 6 deer were positive and ranged from 3.4% to 100% of that of the control (Figures 1C, 2C). One of the deer (#4, Figure 1B, C) had no PrPres detected in tonsil and very little in RPLN, even though the reaction from the brain of this deer was strong. Tonsils and RPLNs from 5 additional CWD-infected elk and 9 additional CWD-infected deer, including 3 white-tailed deer, showed PrPres in amounts similar to those of most elk and deer shown in Figures 1 and 2, but detailed quantification was not carried out on these samples. The combined data for all elk and deer show tonsil and RPLN specimens to be consistently PrPres positive by immunoblot in deer but positive less frequently in elk (Table 1).

We also sought to determine whether all of the nodes from individual deer contain similar levels of PrPres. Considerable variation was observed. In some deer, every node that was tested was PrPres positive, but more frequently only 1 or 2 nodes were positive. Furthermore, the intensity of the PrPres signals varied from node to node. In most deer, RPLN gave the strongest PrPres signal, but in other deer the prescapular or submandibular nodes were best (Figure 3). The mesenteric node was often positive, but generally gave a weak PrPres signal (Figure 3, lane 7). Thus, analysis of a single lymph node other than the RPLN by immunoblot would likely result in some CWD-positive deer being undetected.

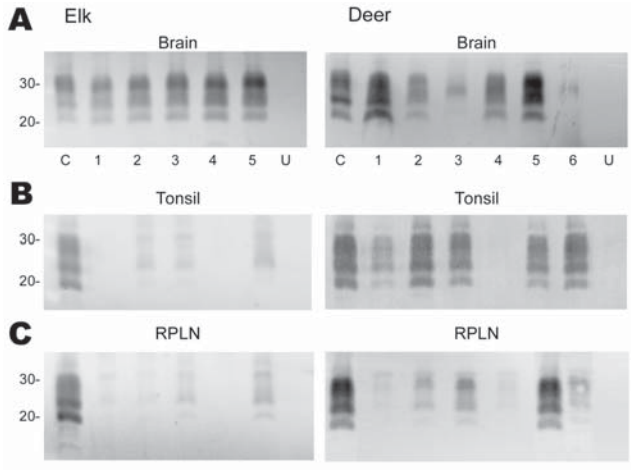

Figure 1. Immunoblot analysis of PrPres from chronic wasting disease (CWD)–affected elk and deer brain, tonsil, and retropharyngeal lymph node (RPLN). Panel A shows the PrPres signal from 2-mg equivalents of elk brain or 20-mg equivalents of deer brain. Individual animals are identified as 1–5 (elk) or 1–6 (deer). C denotes the reference control to which all other samples are compared and consists of 20-mg equivalents of retropharyngeal lymph node (RPLN) from a CWD–affected mule deer. Aliquots of this same control are included on all blots shown in panels B and C. Lanes labeled U in panels A, B, and C contain 20-mg equivalents of the respective tissue from uninfected elk or deer. No PrPres bands were detected when tissues from uninfected deer or elk were analyzed. In panels B and C, 20-mg equivalents of tonsil or RPLN were used. PrPres was obtained as described in Materials and Methods and the blots developed by using antibody L42 at a 0.04 μg/mL dilution and standard enhanced chemifluorescence processing. Approximate molecular weights in kd are indicated on the left side of the panels.

The spleen has been shown to influence disease pathogenesis in both sheep and mouse models of TSE disease (*7*,*12*,*13*,*26*). Therefore, we also sought to quantify the amount of PrPres in elk and deer spleen. However, all of the animals gave very weak or no PrPres signals in spleen (Table 1) (blots not shown). Thus CWD-affected elk and deer differed from scrapie-affected sheep, in which the spleen routinely gives a strong PrPres signal.

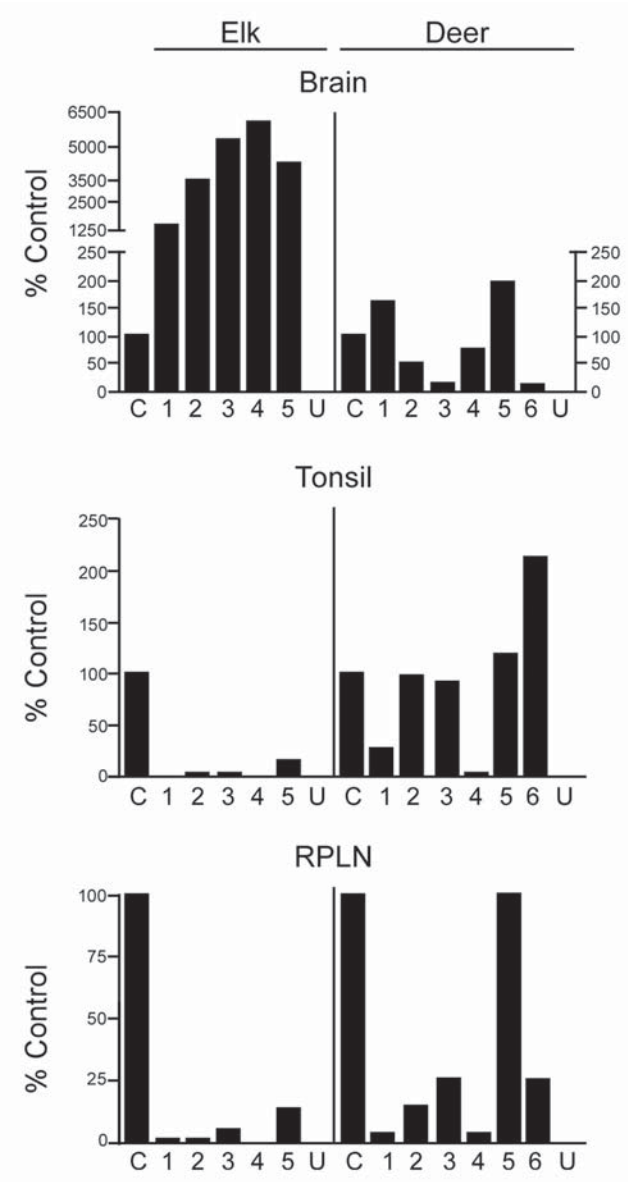

Figure 2. Quantification of disease-associated prion protein (PrPres) in brain, tonsil, and retropharyngeal lymph node (RPLN) from chronic wasting disease–affected elk and deer. The relative amount of PrPres in each lane of Figure 1 is shown relative to a common control described in the Figure 1 legend. A split scale is shown for elk brain because the PrPres signal from each elk brain was strong enough with 2-mg equivalents of tissue to obscure the protein patterns on the gel. Twenty-milligram equivalents were analyzed for all other tissues. The 10-fold difference in the amount of tissue equivalents loaded is accounted for by the split scale, where the result from 2-mg equivalents was multiplied by 10. Data shown are the average of 4 duplicate gels run for each sample. PrPres level in elk brain is significantly different from deer brain ($p<0.001$), elk tonsil is significantly different from deer tonsil ($p = 0.0274$), and elk RPLN is significantly different from deer RPLN ($p = 0.0087$) (Mann-Whitney test). C, reference control; U, uninfected elk or deer.

PrPres Glycoform Patterns in Lymphoid Tissues of CWD-infected Elk and Deer

PrPres glycoform patterns have been used to define TSE strains and have been studied extensively in deer and elk brain (*23*). Therefore, we evaluated the PrPres glycoform patterns of lymphoid tissues of CWD-infected deer and elk to identify profiles that might differentiate deer from elk. The glycoform profile in deer tonsil and lymph node were similar to that of the profile in deer brain (Figure 4). Likewise, there was no convincing difference in the pattern of PrPres found in deer and elk brains (Figure 1). A meaningful comparison of glycoform patterns between elk and deer tonsil and lymph nodes was not possible because none of the elk lymphoid organs gave a sufficiently strong PrPres signal.

Because PrPres band differences can be due both to differing glycosylation and different sites of proteinase cleavage, we treated samples of various tissues with PNGaseF to remove carbohydrates and thus show any differences due to proteolytic cleavages. Such differences in the PrPres structural core might provide evidence for the existence of different CWD strains as seen before in other TSE diseases (*27–29*). However, no differences were detected in PNGaseF-digested PrPres from elk and deer brain (Figure 4). Thus, both glycoform profiles and PNGaseF analysis indicated that PrPres from elk and deer were similar.

Discussion

We found marked differences in the quantity of PrPres in tonsil and lymph nodes of CWD-infected elk versus deer

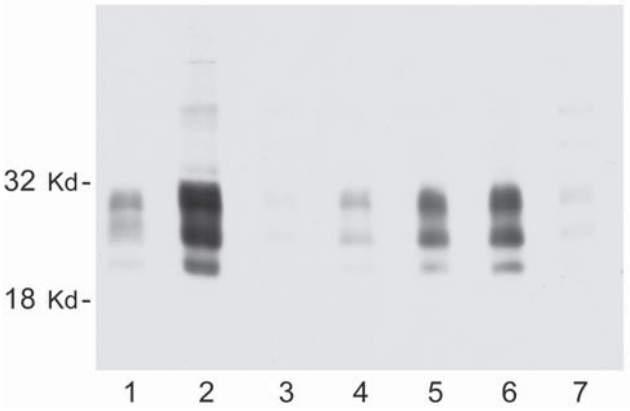

Figure 3. Representative immunoblot showing the relative amount of disease-associated prion protein (PrPres) in brain, tonsil, and selected lymph nodes from a single chronic wasting disease (CWD)–affected mule deer. All lanes were loaded with 10-mg equivalents of tissue (original wet weight basis). Lane 1, brain; lane 2, tonsil; lane 3, popliteal lymph node; lane 4, retropharyngeal lymph node (RPLN); lane 5, prescapular lymph node; lane 6, submandibular lymph node; lane 7, mesenteric lymph node. PrPres bands were visualized by using antibody L42 at 0.04 μg/mL and standard enhanced chemiluminescence processing.

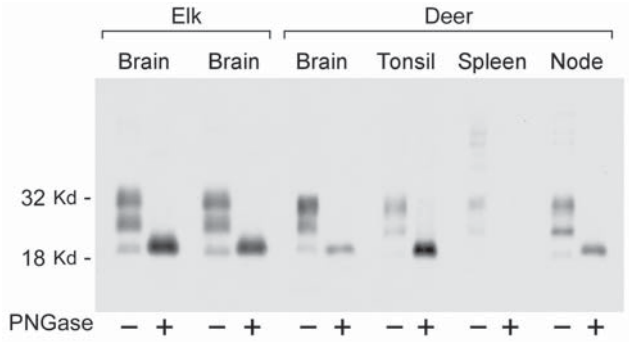

Figure 4. Immunoblot showing disease-associated prion protein from chronic wasting disease–affected elk brain or mule deer brain, tonsil, spleen, and retropharyngeal lymph node before and after treatment with PNGaseF. Alternating lanes show before and after treatment for each tissue. PNGaseF digestion was done as described in Materials and Methods. Ten-milligram equivalents of tissue were used for PNGase F–negative lanes, and 4-mg equivalents of tissue were used for PNGase F–positive lanes. The blot was developed as described in Figure 3.

by using immunoblot analysis. For example, PrPres was detected at high levels in deer, but not in elk, when 20-mg equivalents of tonsil or RPLN were analyzed. In contrast, brains from these deer and elk were all highly positive when the same immunoblot method was used. Both the quantitative PrPres difference (Figure 2) and the differences in the frequency of PrPres detection (Tables 1, 2) between elk brain and lymph nodes were not noted in previous studies in which nonquantitative ELISA or IHC methods were used (*5*,*18*,*19*). In these reports, most elk (85%–94%) had detectable PrPres in both brain and RPLN; however, 6%–15% of the elk had no PrPres in RPLN when brain was positive, which was similar to our immunoblot results. In fact, RPLN from most of the elk in our study were also positive by ELISA or IHC (data not shown). Thus, ELISA and IHC appeared to be more sensitive than immunoblot for PrPres detection and therefore more appropriate methods for diagnosis and surveillance. In contrast, immunoblotting appeared to be more useful for studies requiring quantitation or visualization of PrPres banding patterns.

Our results suggest that fundamental differences may exist in the pathogenesis of CWD between deer and elk. In CWD-infected deer, as with scrapie in sheep, infectivity and PrPres are detectable in lymphoid tissues early after infection, well before they can be detected in brain tissue (*7*,*16*). In deer and sheep, this early lymphoid involvement is considered important in the process of neuroinvasion and the kinetics of disease. After a period of replication in these peripheral sites, the infectious agent moves to the central nervous system. In elk, the low quantity of PrPres in tonsil or lymph nodes suggests that lymphoid infection may not necessarily precede neuroinvasion. Possibly the small amount of PrPres detected in elk tonsil and lymph node may actually originate from the brain. This situation may be similar to that of mink that have mink encephalopathy in which infection of peripheral lymphoid and other tissues is seen only when the animals are in the late stages of disease. It is unclear whether such spread from brain to the periphery is bloodborne or mediated by retrograde transmission through autonomic nerves (*31*).

In the TSE diseases in which lymphoid tissues are substantially involved, i.e., sheep scrapie and CWD in deer, horizontal transmission in natural situations is efficient. In contrast, when peripheral lymphoid tissues are not extensively involved, i.e., BSE in cattle (*14*,*15*), and naturally occurring CWD in elk, horizontal transmission appears to be relatively inefficient (Table 2). Thus, differences in lymph node PrPres levels correlate with differences in the prevalence of CWD in deer and elk in natural settings. This finding might be the result of greater quantities of CWD infectivity released to the environment from lymphoid tissues of deer that have died or been killed. Also because there is widespread distribution of large quantities of PrPres in deer lymphoid tissues, it seems possible that infectivity might also be present in other peripheral tissues such as intestine, kidney, or salivary glands, which could possibly lead to excretion or secretion of infectivity in feces, urine, or saliva. One would also expect brain-associated infectivity to be a source of environmental contamination, and in this regard brain from CWD-infected elk represents as great a risk as CWD-infected brain from deer.

Several other factors might also influence transmission within deer and elk populations. For example, differences in social interaction, the size of typical homeland range,

Table 2. Comparison of PrPres tissue distribution in TSE-affected ruminants*†

Species (condition)	Brain	Spleen	Nodes	Tonsil	Natural transmission
Elk (CWD)	10/10	2/10	5/10	4/9	Low
Mule deer (CWD)	12/12	2/10	12/12	9/10	High
White-tailed deer (CWD)	3/3	1/3	3/3	2/3	High
Sheep‡ (scrapie)	8/8	7/8	6/8	Yes	High
Cattle (BSE)§	6/6	Neg	Neg	Neg	No

*PrPres, disease-associated prion protein; TSE, transmissible spongiform encephalopathy; CWD, chronic wasting disease; BSE, bovine spongiform encephalopathy; Neg, negative.
†Distribution of PrPres or infectivity in peripheral tissues of TSE affected ruminants was compared. Data for deer and elk were determined in the study presented here. Data for sheep are from an earlier publication (*30*) as are the data for cattle (*14,15*)
‡Data from (*30*).
§Data from (*14*).

preferred habitat, population densities, and so forth. The relative contribution of the possible factors is not known.

Although CWD prevalence in elk is low in natural settings, it can be much higher in confinement situations. What differences increase transmission when animals are confined is not known. Apparently, high PrPres levels in lymphoid tissues are not essential for transmission in crowded conditions. However, at least 2 factors might have an additional impact on transmission in captive elk. First, restricting elk to small pastures, sheds, or corrals where infectious material has accumulated over time might facilitate increased transmission. Second, in confined settings, animal-to-animal contact would increase. This might involve exchange of infectivity through saliva, which has been found to be infectious in deer (*32*) and might also be positive in elk, although this remains unproven.

Earlier studies have not shown any evidence for transmission of CWD to humans (*33*–*35*). CWD has been transmitted to cattle by intracerebral but not by oral inoculation (*36*), and no reports have found that co-pasturing of CWD-infected deer or elk with cattle has resulted in transmission. Furthermore, in vitro assays designed to test the susceptibility of humans or cattle to CWD suggested a very low probability of transmission to humans (*37*). Sheep, however, are likely to be more susceptible to CWD. They have been infected by intracerebral inoculation (*38*), and at a molecular level, CWD PrPres was shown to convert sheep PrP to the disease-associated form with relatively high efficiency (*37*). Thus, among livestock, sheep might be a possible target for CWD infection in appropriate situations such as co-pasturing. Also, a CWD agent from putatively infected sheep could have a host range not usually associated with CWD and might cross species barriers more readily than CWD from cervids. Thus, if CWD continues to expand in deer and elk populations, the possibility of transmission to noncervid species will require continued surveillance.

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West Nile Virus Viremia in Eastern Chipmunks (*Tamias striatus*) Sufficient for Infecting Different Mosquitoes

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In eastern chipmunks (*Tamias striatus*) inoculated intramuscularly with $10^{1.5}$ to $10^{5.7}$ PFU of West Nile virus (WNV), serum titers developed sufficient to infect *Aedes triseriatus* (Say), *Ae. vexans* (Meigen), and *Culex pipiens* (L.). Mean titers (95% confidence interval) of 8 chipmunks were $10^{3.9(3.3–4.5)}$, $10^{6.7(6.4–7.0)}$, and $10^{5.8(4.1–7.5)}$ PFU/mL on days 1–3 postinoculation (p.i.) and $10^{5.8}$ PFU/mL in 1 chipmunk on day 4 p.i. Mean estimated days that WNV titers were $\geq 10^{4.8}$ and $\geq 10^{5.6}$ were 1.7 (1.1–2.3) and 1.4 (1.0–1.6). The longest period of viremia $\geq 10^{4.8}$ PFU/mL was 3–4 days. WNV antigen was detected in the small intestine of 2 chipmunks and the kidneys of 4 chipmunks by immunohistochemistry. WNV also was detected in urine, saliva, and feces of some chipmunks. These data suggest chipmunks might play a role in enzootic WNV cycles and be an amplifying host for mosquitoes that could infect humans.

The role of mammals in the ecology of West Nile virus (WNV) has not been well defined. In many mammals, levels of viremia sufficient to infect mosquito vectors do not develop (*1*). However, mosquito-infective viremia levels do occur in some mammals such as the golden hamster (*Mesocricetus auratus*), in which WNV serum titers can exceed $10^{5.5}$ tissue culture infective dose $(TCID)_{50}$/mL (*2*), and the fox squirrel (*Sciurus niger*), in which a maximum titer of $10^{5.0}$ PFU/mL was reported (*3*). Mosquito-infective WNV serum titers with a mean duration of 2.2 ± 0.6 days also have been demonstrated in the eastern cottontail rabbit (*Sylvilagus floridanus*) (*4*). Minimum estimated infection rates of 12% and 21% occurred in *Culex pipiens* (L.) and *Cx. salinarius* (Coq.) after they fed on cottontail rabbits with WNV serum titers of $10^{4.3}$–$10^{5.0}$ $TCID_{50}$/mL. Infection rates increased to 21% and 25% at WNV titers of $10^{5.0}$–$10^{6.0}$ $TCID_{50}$/mL. The magnitude and duration of WNV viremia levels in cottontail rabbits and fox squirrels, which are peridomestic, raise the question of whether other mammals can serve as WNV-amplifying hosts for mosquitoes in enzootic WNV cycles, with potential for transmission to humans.

This study describes the effect of needle inoculation–induced WNV infection in the eastern chipmunk (*Tamias striatus*). We also demonstrate the potential of chipmunks to serve as a WNV-amplifying host for *Cx. pipiens, Aedes triseriatus* (Say), and *Ae. vexans* (Meigen). These species were selected for the study because they share habitats in common with chipmunks in rural and suburban areas. *Cx. pipiens* is a major WNV enzootic and bridge vector in North America (*5*,*6*). It is ornithophilic but will feed on mammals (*7–9*), especially as it shifts its feeding preference during late summer and early fall (*10*). *Ae. triseriatus* and *Ae. vexans* are competent laboratory vectors of WNV (*6*), and this virus has been isolated from field specimens (*11*,*12*). Both species feed primarily on mammals (*6*) but also will feed on avian species (*9*,*13*,*14*).

Methods

Cells, Media, Diluents, and Solutions

Vero 76 cells were used for virus propagation, plaque assays, and virus isolation. Growth medium (GM) was Dulbecco modified Eagle medium (GIBCO, Invitrogen Corp., Carlsbad, CA, USA) that contained 10% heat-inactivated fetal bovine serum (FBS) and 2.0 mmol/L L-glutamine. Maintenance medium (MM) was GM with 1% FBS. Overlay medium (OM) for virus assay was 1 part MM and 1 part 2% agarose (Difco, Becton, Dickinson and Co.,

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Sparks, MD, USA), prepared in Hanks' balanced salt solution (GIBCO, Invitrogen Corp.). Virus diluent was 1 part MM and 1 part CO_2-independent medium (GIBCO, Invitrogen Corp.) containing 1% FBS. Mosquito grinding diluent (GD) was GM supplemented with 20% FBS. Twenty milligrams of gentamicin sulfate (GentaMax100, Phoenix Pharmaceuticals, Inc., Belmont, CA, USA) was added per 100 mL of all media and diluents. Feeding solution for capillary tubes used for collecting saliva in artificial transmission assays consisted of 5% (w/v) sucrose and 0.5% FBS in 0.15 mmol/L phosphate-buffered saline, pH 7.0.

Virus and Virus Assay

WNV strain IA02-crow was used in all experiments. The virus was isolated from a crow in Iowa in 2002 and passed 4× in Vero 76 cell cultures (*15*). Virus was assayed by the plaque method. Serial 10-fold dilutions of serum were made in cold (≈4°C) virus diluent. Individual 6-well cell culture plates containing confluent Vero 76 cell monolayers were inoculated in duplicate with 0.75 mL of each virus dilution and incubated for 75 min at 37°C in a 5% CO_2 atmosphere. The inoculums were replaced with 3 mL of OM, and plates were maintained at 37°C. Three days later, an additional 3 mL of OM containing 2% stock neutral red solution (Sigma Aldrich, Saint Louis, MO, USA) was added to the original overlays. Plates were maintained at 37°C overnight; plaques were then counted and titers were expressed as PFU/mL.

Experimental Animals

Mature eastern chipmunks were captured with live traps (H.B. Sherman Traps, Tallahassee, FL, USA) in Story County, Iowa, in the fall of 2005 under a state of Iowa permit. Chipmunks were verified to be free of antibodies to WNV by an epitope-blocking ELISA (*16*). Chipmunks were housed in individual cages in the biosafety level 3 animal facility at Iowa State University (ISU) and treated and handled in accordance with guidelines established by the ISU Institutional Animal Care and Use Committee. Chipmunks were anesthetized with ketamine/aceopromazine at 10:1.0 mg/kg for mosquito feeding and blood collection. Chipmunks were bled once from the retroorbital plexus of each eye and once from the heart. Blood was not collected again from the retroorbital plexus of either eye for at least 7 days, or from the heart unless the bleeding was terminal. Two-day-old WNV antibody–free broiler chickens (Hoover's Hatchery, Inc., Rudd, IA, USA) were used to detect virus transmission by mosquitoes that previously fed on infected chipmunks.

Mosquitoes

Ae. triseriatus and Cx. pipiens were collected in Iowa and colonized in 2002. *Ae. vexans* were first-generation mosquitoes collected in central Iowa during the summer of 2004. Mosquitoes were maintained on 10% sucrose (w/v) in controlled conditions (27°C ± 1°C and 80% ± 5% relative humidity) with a 16:8-hour photoperiod. Mosquitoes were deprived of sucrose for 48 hours and water for 24 hours before feeding on chipmunks and chickens or before artificial transmission experiments.

Virus Isolation from Mosquitoes

Mosquitoes were killed by freezing. Whole insects, bodies without legs and wings, and legs alone were triturated individually in 1.5-mL microcentrifuge tubes containing 300 μL of cold (≈4°C) GD. For artificial transmission assays, the contents of individual capillary tubes containing saliva of mosquitoes were deposited into 300 μL of GD and stored at –70°C until assayed. Samples were thawed at 37°C and brought to a final volume of 2 mL with cold MM. Approximately 1.6 mL of each sample was passed through a 0.45-μm filter onto a confluent cell monolayer in a 25-cm^2 cell culture flask. The remaining volume was stored at –70°C. Inoculated flasks were incubated for 1 h at 37°C, and then 5 mL of MM was added. Inoculated cell cultures and controls were observed daily for cytopathic effects (CPE) for up to 7 days postinoculation (p.i.). WNV specificity of CPE was confirmed by the VecTest WNV/SLE antigen assay (Microgenics Corp., Freemont, CA, USA).

Epitope-blocking ELISA

Serum samples were tested for antibodies to flaviviruses by a blocking ELISA as previously described (*16*). The ELISAs were performed by using the WNV-specific monoclonal antibody (MAb) 3.1112G (Chemicon International, Temecula, CA, USA) or the flavivirus-specific MAb 6B6C-1, obtained from the Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, Colorado, USA. The ability of test sera to block binding of the MAbs to WNV antigen was compared to the blocking ability of control sera from chipmunks without antibodies to flaviviruses. Data were expressed as relative percentages and inhibition values; a threshold ≥30% was considered to indicate viral antibodies.

Histopathology and Immunohistochemistry

Tissue samples were fixed in 10% formalin and processed for histologic examination by the Veterinary Diagnostic Laboratory at ISU following standardized protocols. WNV-specific mouse ascites fluid (ATCC Catalog #VR-01267CAF, Manassas, VA, USA) was used as the primary antibody at a dilution of 1:2,000 to detect WNV antigen. A 30-minute staining period was used. Processed tissues, including negative controls, were evaluated blindly by a veterinary pathologist for evidence of microscopic lesions and scoring of the amount of WNV antigen in the tissues.

The amount of WNV antigen in the tissues was given a score of 0 (no staining), + (mild multifocal staining of individual cells), ++ (moderate multifocal staining of cells), or +++ (large amounts of antigen and large numbers of cells staining).

Characterization of WNV Infection of Eastern Chipmunks

Initially, 1 chipmunk was inoculated intramuscularly (i.m.) with $10^{5.7}$ PFU of WNV. Blood was collected 2 days later and found viremic for WNV. Subsequently, 6 chipmunks were divided into 2 equal groups. One group was inoculated i.m. with $10^{3.5}$ PFU of WNV and the other with $10^{1.5}$ PFU. An eighth chipmunk was subsequently inoculated with $10^{2.6}$ PFU. Serum specimens for WNV assay were collected on days 1–4 p.i. and then not again until day 8 p.i. If we encountered difficulty in obtaining blood, we aborted the procedure. Swabs of oral and rectal cavities of chipmunks that were inoculated with WNV were taken on days 1–3 p.i. and assayed for WNV. Swabs of urine also were collected from chipmunks when possible.

Tissues—including the brain, spinal cord, lung, heart, skeletal muscle, liver, kidney, spleen, pancreas, small and large intestine, adrenal glands, and salivary glands—were collected from all chipmunks. These tissues were examined for gross and microscopic lesions and assayed by immunohistochemical tests for WNV antigen. Tissues from 1 chipmunk that had not been inoculated served as negative controls.

Eastern Chipmunks as Source of WNV for Mosquitoes

Groups of up to 15 *Ae. triseriatus*, *Ae. vexans,* and *Cx. pipiens* in the same container were fed on chipmunks between days 1 and 4 p.i. The blood-fed mosquitoes were separated by species, maintained for 14 to 15 days as described previously, and assayed for WNV infection. *Ae. triseriatus* also were assayed for disseminated infections.

Ae. triseriatus transmission of WNV was determined by feeding mosquitoes on 2-day-old chickens or by permitting mosquitoes, from which wings and legs were removed, to feed for 20 minutes from capillary tubes containing feeding solution. Transmission was confirmed by detecting WNV in blood that was collected from chickens ≈48 hours after mosquitoes fed, or in the contents of capillary tubes from which mosquitoes fed.

Results

Characterization of WNV Infection of Eastern Chipmunks

WNV viremia developed in all 8 chipmunks (Figure 1) after they were infected with the virus by needle inoculation. Viremia titers peaked on day 2 p.i. and were generally higher in those chipmunks that were inoculated with higher doses of virus. Peak WNV titers of $10^{7.2}$ and $10^{7.8}$ PFU/mL of serum occurred in 2 chipmunks that were inoculated with $10^{2.6}$ or $10^{3.5}$ PFU of WNV, respectively. The highest titer observed in chipmunks inoculated with $10^{1.5}$ PFU of WNV was $10^{6.5}$ PFU/mL. The mean estimated number of days and 95% confidence intervals that WNV serum titers remained $\geq 10^{4.8}$ PFU/mL and $\geq 10^{5.6}$ PFU/mL were 1.7 (1.1–2.3) and 1.4 (1.0–1.6), respectively. The longest period of time that WNV titers were $\geq 10^{4.8}$ PFU/mL was at least 3 days and occurred in a chipmunk that was inoculated with $10^{3.5}$ PFU of WNV. No WNV was detected in serum specimens from 3 chipmunks that were bled on day 8 p.i.

No WNV was isolated from oral or rectal cavities of infected chipmunks on day 1 p.i., but WNV was isolated on day 2 p.i. from the oral cavities of 4 chipmunks. On day 3 p.i., WNV was isolated from the oral cavities of 4, the rectal cavities of 3, and the urine of 2 chipmunks.

No signs of illness were observed in any of the WNV-infected chipmunks during the first 8 days p.i. Two chipmunks died on days 1 and 2 p.i. during sampling, and a third chipmunk was killed on day 4 p.i. to obtain blood for WNV assay. The first potential signs of WNV infection were observed in the remaining 5 chipmunks between days 9 and 11 p.i. In 3 chipmunks, neurologic symptoms developed, characterized by head tilt and incoordination; the chipmunks were humanely killed. A fourth chipmunk became lethargic and was reluctant to move; it also was humanely killed. A fifth chipmunk had no signs of illness but died unexpectedly on day 27 p.i. WNV-specific antibody was detected in 3 chipmunks that were bled on day 8, 11, or 14 p.i.

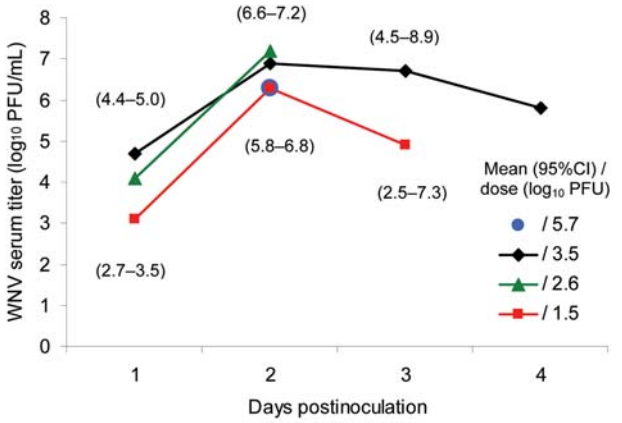

Figure 1. West Nile virus (WNV) viremia profile in 8 eastern chipmunks (*Tamias striatus*) that were inoculated intramuscularly with virus. One chipmunk received $10^{5.7}$ PFU of WNV and was sampled only on day 2 postinoculation (p.i.). Three chipmunks received $10^{3.5}$ PFU of WNV, 1 received $10^{2.6}$ PFU, and 3 received $10^{1.5}$ PFU. The number of chipmunks that received $10^{3.5}$ PFU of WNV decreased to 2 on day 3 p.i. and to 1 on day 4 p.i. The number of chipmunks that received $10^{1.5}$ PFU of WNV was 2 on days 2 and 3 p.i.

No gross lesions were observed in any chipmunk; however, microscopic lesions and/or WNV antigen were observed in tissues of 4 of 8 infected chipmunks (Table 1). Mild to moderate multifocal lymphoplasmacytic meningoencephalitis with gliosis and neuronal necrosis was observed in 2 chipmunks on days 9 and 11 p.i. Mild lymphohistiocytic perivascular cuffing of vessels with moderate to severe multifocal hemorrhage throughout the brain was present in 1 of these 2 chipmunks. WNV antigen also was detected in neurons of both chipmunks. Mild lymphoplasmacytic and histiocytic hepatitis were observed in 3 chipmunks. Viral antigen was also detected in Kupffer cells and macrophages of 2 of these 3 chipmunks. Mild multifocal lymphoplasmacytic interstitial nephritis with mild focal renal tubular necrosis was observed in 2 chipmunks on days 9 and 11 p.i. WNV antigen was present in renal tubular epithelial cells and in the renal arterial walls (Figure 2) of these 2 chipmunks and in 2 others in which no kidney lesions were found. WNV antigen in the absence of lesions was demonstrated in the muscularis mucosa, scattered mucosal epithelial cells, and mononuclear cells in the lamina propria of the small intestine of 2 chipmunks.

Eastern Chipmunks as a Source of WNV for Mosquitoes

The feeding success rates of *Ae. triseriatus*, *Ae. vexan,* and *Cx. pipiens* on viremic chipmunks were 68%± 5%, 27% ± 6%, and 8% ± 4%, respectively. The WNV infection rates of the 3 mosquito species that fed on these chipmunks are summarized in Table 2. Disseminated infections developed in 5 of the 6 WNV-infected *Ae. triseriatus* that fed on chipmunks with WNV serum titers $\geq 10^{5.6}$ PFU/mL. Two (67%) of 3 *Ae. triseriatus* with disseminated infections transmitted WNV in an artificial transmission assay. One *Ae. triseriatus* with a disseminated infection that fed on a 2-day-old chicken did not transmit WNV.

Discussion

In chipmunks infected with WNV, viremia titers developed that were sufficient to infect mosquitoes. This finding is noteworthy because the eastern chipmunk is ubiquitous throughout forested, rural, and suburban areas of southeastern Canada, and in the eastern United States, with the exception of the eastern parts of North and South Carolina, Georgia, and most of Florida. Its western range is described by a boundary extending from northwestern North Dakota to southeastern Louisiana (*17*). Because of its high reproductive potential, the eastern chipmunk frequently becomes a pest around homes, gardens, and public parks. Because of the chipmunk's close association with humans, and its susceptibility to WNV manifested by viremia levels of relatively long duration and high titers (Figure 1), this species, like the cottontail rabbit (*4*), is a potential source of WNV for zoophilic and opportunistic mosquito vectors that have the potential to transmit WNV to humans.

The potential importance of the eastern chipmunk as an amplifying host was demonstrated by the persistence of WNV serum titers $\geq 10^{4.8}$ and $10^{5.6}$ PFU/mL for average periods of 1.7 (1.1–2.3) and 1.4 (1.0–1.6) days, respectively. These levels of viremia were sufficient to infect Iowa strains of *Ae. triseriatus*, *Ae. vexans*, and *Cx. pipiens* that fed on viremic chipmunks (Table 2). Other investigators also have shown that these 3 mosquito species can become infected by the levels of WNV that occur in chipmunks. The regression model developed by Erickson et al. (*15*) to characterize WNV susceptibility of an Iowa strain of *Ae. triseriatus* predicts an infection rate of 26% after mosquitoes feed on a host with a WNV serum titer of $10^{5.6}$ PFU/mL. This infection rate is similar to what we observed in the present study (Table 2). WNV infection and transmission rates of 31% and 12% also have been reported for an eastern strain of *Ae. triseriatus* that fed on chickens with titers of $10^{7.1}$ PFU/mL blood (*6*), a level of viremia that was exceeded in 2 (25%) of the 8 chipmunks infected with WNV in our study. Similarly,

Table 1. Histologic lesions observed in eastern chipmunks (*Tamias striatus*) after intramuscular inoculation with West Nile virus (WNV)

Chipmunk no.	Experiment no.	WNV dose*	Day of death†	Microscopic lesions‡/WNV antigen§¶				
				Brain	Liver	Spleen	Kidney	Small intestine
1	1	5.7	11 (E)	–/–	–/–	–/–	–/–	–/–
2	2	3.5	2 (S)	–/–	–/–	–/–	–/–	–/–
3	2	3.5	11 (E)	++/+++	+/–	–/–	+/+	–/–
4	2	3.5	4 (S)	–/–	+/++	–/+	–/+	–/+
5	2	1.5	27 (U)	–/–	–/–	–/–	–/–	–/–
6	2	1.5	1 (S)	–/–	–/–	–/–	–/–	–/–
7	2	1.5	14 (E)	–/–	–/–	–/–	–/+	–/–
8	3	2.6	9 (E)	++/++	+/++	–/–	+/++	–/++

*Log_{10} PFU of WNV.
†Day of death, day postinoculation that death occurred by euthanasia (E) if a chipmunk had symptoms that prevented it from ambulating to or consuming water or food; S, death associated with sampling; U, cause of death not determined.
‡By histologic examination, lesions were not present (–), mild (+), moderate (++), or severe (+++).
§By histologic examination, WNV antigen was absent (–), mild (+), moderate (++), or extensive (+++).
¶Organs with no detectable lesions or WNV antigen included heart, lung, adrenal and salivary glands, large intestine, pancreas, and urinary bladder.

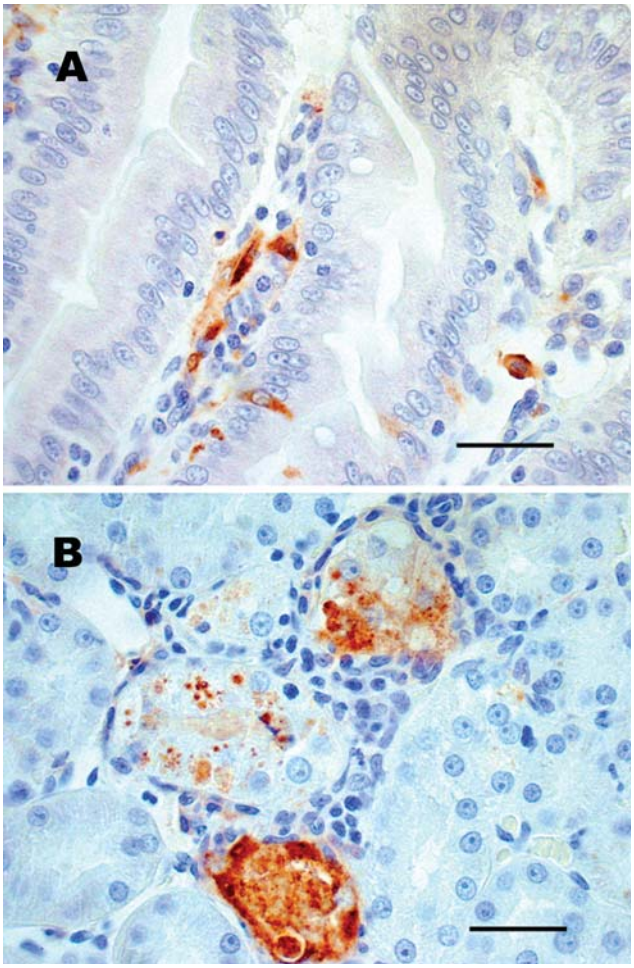

Figure 2. Sections of chipmunk tissues 9 days after intramuscular inoculation with West Nile virus (WNV). A) Lesions were absent, but WNV antigen (brown staining) was demonstrated in scattered epithelial cells and in macrophagelike cells in the lamina propria of the small intestine. B) WNV antigen (brown staining) was demonstrated in necrotic renal tubular epithelial cells. Tissues were stained with hematoxylin, and WNV-specific mouse ascites fluid (ATCC Catalog #VR01267CAF) was used as the primary antibody for immunohistochemical staining. Scale bars = 50 μm.

WNV infection and transmission rates of 44% and 11%, and 32% and 23%, respectively, have been reported for eastern and western strains of *Ae. vexans* that fed on chickens (*6*) or hanging drops of defibrinated blood (*18*) with titers of $10^{7.1}$ PFU/mL. Studies in our laboratory also showed infection and transmission rates of 28% and 9% for *Ae. vexans* that fed on chickens with WNV serum titers of $10^{5.0}$ to $10^{5.4}$ PFU/mL, respectively (S. Tiawsirisup, unpub. data). These same studies demonstrated WNV infection and transmission rates of 43% and 16% for *Cx. pipiens* that fed on chickens with titers ranging from $10^{5.1}$ to $10^{5.4}$ PFU/mL, respectively. WNV infection and transmission rates of 100% and 71%, respectively, also have been reported for a California strain of *Cx. pipiens* that fed on blood with a titer of $10^{7.1}$ PFU/mL (*18*). These observations suggest that all 3 of these species could be involved in a mosquito-chipmunk-mosquito cycle. However, field studies of host preference for *Cx. pipiens* collected throughout mosquito seasons would be necessary for determining the potential role of *Cx. pipiens* in a mosquito-chipmunk-mosquito cycle.

Other zoophilic and opportunistic mosquito species, commonly found in habitats used by chipmunks, which could be infected by feeding on viremic chipmunks include *Ae. trivittatus* (Coq.), *Ae. albopictus* (Skuse), and *Cx. salinarius*. Cumulative infection and transmission rates of 70% and 24%, respectively, have been reported for an Iowa strain of *Ae. trivittatus* that fed on chickens with WNV serum titers of $10^{5.5}$ to $10^{7.0}$ $TCID_{50}$/mL (*19*). Sardelis et al. (*20*) reported WNV infection and dissemination rates up to 53%, and 49% for 3 North American strains of *Ae. albopictus* that fed on chickens with blood titers of $10^{5.7}$ PFU/mL. Infection and estimated transmission rates of 95% and 34% were reported for a Texas strain of *Cx. salinarius* that fed on chickens with WNV blood titers of $10^{6.6}$ PFU/mL (*21*), and a minimum estimated infection rate of 21% occurred in *Cx. salinarius* that fed on cottontail rabbits with WNV serum titers of $10^{4.3}$ to $10^{4.9}$ $TCID_{50}$/mL (*4*).

Four (80%) of the 5 WNV-infected chipmunks that were maintained beyond day 4 p.i. died. The other 3 chipmunks were not maintained beyond day 4 p.i. The deaths of 2 chipmunks on days 1 and 2 p.i. could be attributed to sampling stress or anesthesia, as observed during field surveys; however, WNV infection may have contributed to their deaths because both animals exhibited WNV viremia.

Microscopic lesions observed in the brain, kidney, and liver in some chipmunks were similar to lesions described in related species such as the golden hamster and the fox squirrel (*2*,*3*,*22*). The absence of detectable lesions or WNV antigen in any tissues of chipmunks 2 and 6 on days 1 and 2 p.i. and the absence of brain lesions or WNV antigen in chipmunks 1 and 7 on days 11 and 14 p.i. were not unexpected (Table 1). Xiao et al. (*2*) did not observe any marked histopathologic changes in the kidney, liver, lung, heart or pancreas of hamsters during the first 10 days p.i., although "spotty splenic necrosis" was observed in some hamsters. However, Xiao et al. did observe the beginning of lesions in many areas of the brain on day 5 p.i. These lesions became more numerous throughout the brain by day 6 p.i. but by day 10 p.i. were found mostly in the brain stem. Thus, detectable lesions might have been present earlier in the brain of chipmunks 1 and 7 but diminished to undetectable limits by days 11 and 14 p.i.

The presence of WNV in the urine, saliva, and rectum of some chipmunks raises the question of whether chipmunks can be infected by the fecal-oral route. Oral infection by WNV has been documented in the golden hamster (*23*),

Table 2. Infection rates of *Aedes triseriatus* (Say), *Ae. vexans* (Meigen), and *Culex pipiens* (L.) after feeding on viremic eastern chipmunks (*Tamias striatus*) infected with West Nile virus (WNV)

	Ae. triseriatus		*Ae. vexans*		*Cx. pipiens*	
WNV serum titer*	No. blood meals†	% Positive (no. blood-fed)	No. blood meals	% Positive (no. blood-fed)	No. blood meals	% Positive (no. blood-fed)
3.1–4.4	3	0 (27)	2	0 (10)	2	0 (6)
4.8	2	0 (19)	1	0 (1)	1	0 (1)
5.6–5.8	2	23 (13)	2	0 (4)	1	0 (1)
6.0–6.9	5	0 (14)	3	15 (13)	2	50 (2)
7.2–7.8	2	25 (12)	2	40 (5)	ND‡	

*Log_{10} PFU of WNV.
†No. chipmunks providing blood meals.
‡ND, not done.

the American alligator (*Alligator mississippiensis*) (*24*), the domestic cat (*Felis catus*) (*25*), and some raptors (*26*). Non-mosquito transmission from infected to naive chipmunks might be a mechanism that could contribute to the maintenance of WNV between epidemic periods, particularly if WNV establishes a persistent infection in chipmunks. Recently, Tesh et al. (*27*) described persistent WNV infection in golden hamsters, which shed WNV in urine for up to 247 days p.i. in the presence of serum neutralizing antibody. WNV also has been isolated from kidney tissue of rhesus macaques (*Macaca mulatta*) 5.5 months after infection (*28*). WNV RNA also has been isolated from the spleen, lung, and kidney of birds 6 weeks after experimental infection (*29*). These findings indicate that WNV can persist in some mammals and birds. The presence of WNV antigen in kidney tissue of a chipmunk at 14 days p.i. (Figure 2) in the presence of antibody could indicate that persistent WNV infections also occur in chipmunks. Whether persistent infection occurs and how long it remains are subjects of further study.

Additional study to determine the role of the chipmunk in the ecology of WNV is justified for the following reasons: 1) the high levels of viremia in chipmunks after injection of WNV doses that can be delivered by a variety of mosquitoes (*30*,*31*), 2) the WNV vector competence of several zoophilic and opportunistic mosquitoes that share the same habitats with chipmunks, and 3) the possibility of persistent infection. These studies should include characterizing the profile of the WNV viremia after infection by mosquito bite because the onset and level of viremia can be affected by components in mosquito saliva (*32*). Determining the seasonal seroprevalence of antibodies to WNV in chipmunk populations in WNV-endemic areas and mortality rates after natural infection also would provide an indication of the relative importance of the chipmunk in the ecology of WNV.

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Antimicrobial Drug–Resistant *Escherichia coli* from Humans and Poultry Products, Minnesota and Wisconsin, 2002–2004

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The food supply, including poultry products, may transmit antimicrobial drug–resistant *Escherichia coli* to humans. To assess this hypothesis, 931 geographically and temporally matched *E. coli* isolates from human volunteers (hospital inpatients and healthy vegetarians) and commercial poultry products (conventionally raised or raised without antimicrobial drugs) were tested by PCR for phylogenetic group (A, B1, B2, D) and 60 virulence genes associated with extraintestinal pathogenic *E. coli*. Isolates resistant to trimethoprim-sulfamethoxazole, quinolones, and extended-spectrum cephalosporins (n = 331) were compared with drug-susceptible isolates (n = 600) stratified by source. Phylogenetic and virulence markers of drug-susceptible human isolates differed considerably from those of human and poultry isolates. In contrast, drug-resistant human isolates were similar to poultry isolates, and drug-susceptible and drug-resistant poultry isolates were largely indistinguishable. Many drug-resistant human fecal *E. coli* isolates may originate from poultry, whereas drug-resistant poultry-source *E. coli* isolates likely originate from susceptible poultry-source precursors.

Acquired resistance to first-line antimicrobial agents increasingly complicates the management of extraintestinal infections due to *Escherichia coli*, which are a major source of illness, death, and increased healthcare costs (*1–4*). One suspected source of drug-resistant *E. coli* in humans is use of antimicrobial drugs in agriculture. This use presumably selects for drug-resistant *E. coli*, which may be transmitted to humans through the food supply (*5–7*). Supporting this hypothesis is the high prevalence of antimicrobial drug–resistant *E. coli* in retail meat products, especially poultry (*8–11*), and the similar molecular characteristics of fluoroquinolone-resistant *E. coli* from chicken carcasses and from colonized and infected persons in Barcelona, Spain, in contrast to the marked differences between drug-susceptible and drug-resistant source isolates from humans (*12*).

To further assess the poultry-human connection, we used molecular typing to characterize drug-resistant and drug-susceptible *E. coli* isolates from feces of human volunteers or newly hospitalized patients in Minnesota and Wisconsin and from poultry products sold or processed in the same region. Resistance phenotypes of interest include trimethoprim-sulfamethoxazole (TMP-SMZ), quinolones/fluoroquinolones, and extended-spectrum cephalosporins. These agents are used for treatment of human *E. coli* infections. These drugs (or congeners) are also used in poultry production (e.g., each year in the United States an estimated 1.6 billion broiler eggs or chicks receive ceftiofur [*13*]); *E. coli* isolates resistant to these drugs are found in poultry. We examined, according to phylogenetic group distribution and virulence gene profile, whether drug-resistant human isolates more closely resemble susceptible human isolates, which is consistent with acquisition of resistance within humans, or instead resemble poultry isolates, which is consistent with foodborne transmission of poultry-source organisms to humans. We also examined whether poultry-

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source resistant and susceptible isolates are similar, which is consistent with emergence of resistance on farms under selection from agricultural use of antimicrobial drugs.

Methods

Participants and Bacterial Strains

Human fecal samples were collected from 622 adults newly admitted to local hospitals in 4 rural communities in Minnesota (Willmar) or Wisconsin (Eau Claire, La Crosse, and Marshfield) and from 100 healthy self-identified vegetarians in these and nearby communities (*14*). Hospital patients were recruited from June 2002 through May 2003, vegetarians during the first 6 months of 2004. Fecal samples were collected by study personnel by using rectal swabs (hospital patients) or by the participants (vegetarians). To prevent isolation of hospital-acquired flora, inpatients samples were collected within 36 hours of hospital admission. Guidelines of the authors' institutions regarding use of human subjects were followed in this study. The relevant institutional review boards reviewed and approved the protocol. All participants provided informed consent.

A total of 180 retail poultry products (155 chicken and 25 turkey) were sampled (*14*). Conventional brands were purchased systematically from all food markets in the 4 primary study communities from May 2002 through May 2003, with 40 retail items obtained per community (total 160 items). These represented at least 18 plants in 11 states. Twenty samples with labels indicating that the poultry were raised naturally or without antibiotics were purchased in or near the study communities in August 2004. Additionally, 40 freshly slaughtered chicken carcasses from local farmers who raised chickens naturally or without antibiotics were obtained during plant inspections by the Minnesota Department of Agriculture from September 2003 through August 2004. The latter 2 groups of chickens, designated "no antibiotics," were confirmed to have been raised without antibiotics, based on the product label or by contacting the manufacturer or distributor.

Sample Processing

Human fecal samples were suspended and poultry samples and carcasses were massaged in nutrient broth, which was then incubated overnight at 37°C and stored as aliquots at –80°C in glycerol (*14*). Portions of these frozen stocks were transferred to vancomycin-supplemented (20 mg/L) Luria-Bertani broth. After overnight incubation, these broths were plated directly onto modified Mueller-Hinton (MMH) agar (Amyes medium) (*10*) with and without ciprofloxacin (4 mg/L) and (separately) nalidixic acid (32 mg/L), and were then incubated overnight. Samples of these Luria-Bertani broths containing vancomycin were placed in MMH broths supplemented individually with TMP-SMZ (4 mg/L TMP plus 76 mg/mL SMZ), cefoxitin (10 mg/L and 32 mg/L), and ceftazidime (10 mg/L and 32 mg/L). After overnight incubation, these broths were plated onto MMH agar plates supplemented with the corresponding agent (same concentrations) for overnight incubation. Colonies resembling *E. coli* were identified by using the API-20E System (bioMérieux, Marcy-l'Etoile, France).

Susceptibility Testing

At least 1 *E. coli* colony was randomly selected from each MMH agar plate and tested for disk susceptibility to 24 antimicrobial agents by using Clinical Laboratory Standards Institute (CLSI)–recommended methods, interpretive criteria, and reference strains (*15*). For isolates resistant to TMP-SMZ, nalidixic acid, or ciprofloxacin, the MIC was determined by Etest (AB-Biodisk, Sona, Sweden) according to the manufacturer's directions. Isolates from cefoxitin- and ceftazidime-supplemented plates underwent broth dilution MIC determinations with cefotaxime and ceftazidime regardless of disk test results. Isolates were classified as resistant to TMP-SMZ if the TMP MIC was ≥4 mg/L and the SMZ MIC was ≥76 mg/L, to quinolones if the nalidixic acid MIC was ≥32 mg/L, to fluoroquinolones if the ciprofloxacin MIC was ≥4 mg/L, and to extended-spectrum cephalosporins if the MIC to either cefotaxime or ceftazidime was ≥16 mg/L. The latter threshold corresponds with intermediate susceptibility per CLSI criteria and includes isolates with potentially clinically relevant reduced susceptibility. Because of the small number of isolates within each resistance phenotype, isolates were classified as resistant if they met any of these resistance criteria. Isolates that did not meet any of these resistance criteria were classified as susceptible, even though they may have had reduced susceptibility to other drug classes.

From each sample, 1 colony of each resistance phenotype (TMP-SMZ, quinolones, fluoroquinolones, extended-spectrum cephalosporins) and 1 susceptible isolate, as available, were selected. If multiple isolates from a given sample exhibited similar disk diffusion susceptibility profiles, genomic profiles as generated by using random amplified polymorphic DNA (RAPD) analysis were compared in the same gel (*12*). One representative of each unique RAPD genotype (as determined by visual inspection) was arbitrarily selected for further analysis.

Phylogenetic Analysis and Virulence Genotyping

All isolates were categorized as to major *E. coli* phylogenetic group (A, B1, B2, or D) by a multiplex PCR-based assay (*16*) (Table 1). Genes encoding proven or putative virulence factors of extraintestinal pathogenic *E. coli* (ExPEC) were detected in a sequential fashion. All isolates were screened for 5 ExPEC-defining virulence genes and *hlyD* (hemolysin). Isolates were operationally defined as

Table 1. Bacterial traits by source and antimicrobial drug resistance in 931 *Escherichia coli* isolates from human feces and poultry products, Minnesota and Wisconsin, 2002–2004*

	Prevalence, no. (%)				p value‡		
Trait†	Total (n = 931)	Human, susceptible (n = 460)	Human, resistant (n = 70)	Poultry (n = 401)	HS vs. HR	HS vs. all poultry	HR vs. all poultry
Group A	252 (27)	96 (21)	23 (33)	133 (33)		≤0.001	
Group B1	186 (20)	79 (17)	11 (16)	96 (24)			
Group B2	234 (25)	178 (39)	13 (19)	43 (11)	≤0.001	≤0.001	
Group D	259 (28)	107 (23)	23 (33)	129 (32)		≤0.01	
papA	124 (13)	98 (21)	6 (9)	20 (5)		≤0.001	
papC	163 (18)	100 (22)	10 (14)	53 (13)		≤0.001	
sfa/focDE	69 (7)	65 (14)	2 (3)	2 (0.5)	≤0.01	≤0.001	
afa/draBC	19 (2)	14 (3)	5 (7)	0 (0)		≤0.001	≤0.001
iutA	361 (39)	93 (20)	32 (46)	236 (59)	≤0.001§	≤0.001§	
kpsM II	288 (31)	195 (42)	23 (33)	70 (17)		≤0.001	≤0.01
hlyD	71 (8)	64 (14)	2 (3)	4 (1)	≤0.01	≤0.001	
ExPEC	249 (27)	147 (32)	20 (29)	82 (20)		≤0.001	

*Data are for the total population. Susceptible, susceptible to trimethoprim-sulfamethoxazole, nalidixic acid (quinolones), and ceftriaxone or ceftazidime (extended-spectrum cephalosporins), regardless of other possible drug resistance; resistant, resistant to 1 of the following: trimethoprim-sulfamethoxazole, nalidixic acid (quinolones), and ceftriaxone or ceftazidime (extended-spectrum cephalosporins).
†Groups A, B1, B2, and D, major *E. coli* phylogenetic groups; *papA* and *papC*, P fimbriae structural subunit and assembly; *sfa/focDE*, S and F1C fimbriae; *afa/draBC*, Dr binding adhesins; *iutA*, aerobactin system; *kpsM* II, group 2 capsule; *hlyD*, α-hemolysin; ExPEC, extraintestinal pathogenic *E. coli* defined by presence of ≥2 of *papA* and/or *papC* (counted as 1), *sfa/focDE*, *afa/draBC*, *iutA*, and *kpsM* II.
‡By Fisher exact test. Values are shown only where p≤0.01. HS, susceptible isolates from humans; HR, resistant isolates from humans. Because drug-resistant and drug-susceptible poultry isolates showed only 1 significant difference (for *iutA*), they were combined into an all-poultry group.
§Negative association.

ExPEC if ≥2 of the following were present: *papA* and/or *papC* (P fimbriae structural subunit and assembly), *sfa/focDE* (S and F1C fimbriae), *afa/draBC* (Dr binding adhesins), *iutA* (aerobactin system), and *kpsM* II (group 2 capsule) (*8*). All ExPEC isolates were then tested for 60 ExPEC-associated virulence genes and alleles thereof. Testing was conducted by using 2 independently prepared lysates of each isolate and established PCR-based methods (*12*,*17*). Isolates from various source groups (e.g., hospital volunteers, conventionally raised poultry) were tested in parallel to avoid cohort effects. The virulence score was the number of virulence genes detected adjusted for multiple detection of the *pap*, *sfa/foc*, and *kps* operons (*12*).

Statistical Methods

The unit of analysis was the individual isolate. Comparisons of proportions were tested by using Fisher exact test (2-tailed). Comparisons of virulence scores were tested by using Mann-Whitney U test (2-tailed exact probability). Principal coordinates analysis (PCA), also known as metric multidimensional scaling, is a multivariate statistical technique used to provide a simpler, low-dimensional graphic summary of the similarity between multiple samples (e.g., isolates) across multiple loci (*18*). New axes for plotting the isolates are derived from a data matrix of estimated dissimilarities between isolates. The first 2 principal coordinates, which account for the most variance, are used to plot the data. The distances between points in the plot represent isolate similarity. The dimensions represented by the (statistically uncorrelated) axes have no intrinsic meaning, i.e., they have no units. Using GenAlEx6 (*19*), we applied PCA to the screening dataset (all isolates) and the extended virulence profile dataset (ExPEC isolates) as a way to collapse the multiple variables for simplified among-group comparisons. For each PCA, results for each isolate from the first 2 PCA axes were used in multiple analysis of variance (MANOVA) to test for among-group differences. These values also were plotted to spatially represent the degree of separation or overlap of isolates on the 2-axis plane. For the ExPEC isolates, pairwise similarity relationships according to extended virulence profiles and phylogenetic group were used to construct a dendrogram according to the unweighted pair group method with arithmetic averages (*20*). The criterion for statistical significance throughout was p≤0.01 to account for multiple comparisons.

Results

Isolation of Drug-Resistant and Drug-Susceptible *E. coli*

Selective processing of 942 human fecal and poultry samples yielded 931 unique *E. coli* isolates, which constituted the study population. Of the 931 isolates, 530 (57%) were from human volunteers and 401 (43%) from poultry products. Of the human isolates, 456 (86%) were from hospital patients and 74 (14%) from vegetarians. Of the poultry isolates, 289 (72%) were from conventionally raised retail poultry and 112 (28%) from poultry raised without antibiotics. The median number of unique *E. coli* isolates

per sample was 1 for human fecal samples and 2 for poultry (range 1–4 for both).

Overall, 331 isolates (70 human, 261 poultry) were classified as resistant on the basis of reduced susceptibility to TMP-SMZ, quinolones/fluoroquinolones, and extended-spectrum cephalosporins. The remaining 600 isolates (460 human, 140 poultry) were susceptible to all these drug classes and were classified as susceptible (regardless of other possible drug resistance). The resistant isolates were distributed by resistance phenotype as follows: TMP-SMZ, 154 (47 human, 107 poultry); quinolones, 115 (26 human, 89 poultry); and extended-spectrum cephalosporins, 114 (14 human, 100 poultry). The 7 fluoroquinolone-resistant isolates (5 human, 2 poultry) were analyzed within the quinolone-resistant group.

Phylogenetic Distribution and Prevalence of ExPEC-defining Markers

The initial screening showed the 931 isolates to be fairly evenly distributed among the 4 major *E. coli* phylogenetic groups (20%–28% per group). However, they had various prevalences (2%–39% each) of the screening ExPEC virulence genes (Table 1). Overall, 27% of the isolates qualified as ExPEC by having ≥2 of the 5 ExPEC-defining markers (Table 1).

For enhanced resolution of similarities and differences, the 243 available ExPEC isolates underwent extended virulence genotyping for 60 ExPEC-associated virulence genes. All but 6 of these traits were detected in ≥1 isolate each, with prevalences ranging from 0.4% to 98% (Table 2).

Prevalence Comparisons

Phylogenetic group distribution and virulence gene prevalence differed considerably according to source (human versus poultry) and resistance status. This finding is shown in Table 1 for all 931 isolates (screening virulence genes only) and in Table 2 for the 243 ExPEC isolates (extended virulence profiles). Drug-resistant and drug-susceptible human isolates were separately compared with the combined group of all poultry isolates (i.e., all susceptible and resistant). We analyzed poultry isolates as a single group because the distribution of traits was similar in drug-resistant and susceptible poultry isolates; i.e., only 1 trait (*iutA*) was significantly associated with resistance among poultry isolates.

Consistent differences in phylogenetic and virulence gene distribution were evident between groups (Tables 1, 2). First, drug-susceptible human isolates differed considerably from drug-resistant human isolates. Second, drug-susceptible human isolates differed from poultry isolates. Third, although human drug-resistant isolates and poultry isolates exhibited some differences, these were considerably fewer and less extreme than those between drug-susceptible human isolates and poultry isolates. Similar results were obtained in subgroup analyses when isolates from hospital patient fecal samples were compared separately with isolates from conventionally raised poultry or when isolates from fecal samples from vegetarians were compared separately with isolates from poultry raised without antibiotics.

PCA

PCA was used to concurrently analyze multiple bacterial characteristics. The first PCA was conducted for the total population (n = 931) with the 7 screening virulence genes plus phylogenetic group. According to a 2 × 2 (source × resistance status) MANOVA of the first 2 axes of the PCA (which accounted for 65% of total variance), all 3 independent variables considered (source, resistance status, and interaction term) showed a p value ≤0.001. Accordingly, pairwise comparisons were made between individual source-resistance groups by 1-factor MANOVA. Susceptible human isolates differed ($p<0.001$) from each of the other 3 groups, whereas the other 3 groups differed marginally from each other. The individual axes supported this conclusion. These axes showed more extreme differences between drug-susceptible human isolates and each of the other 3 groups ($p<0.001$ for 5 of 6 comparisons) than among the other groups ($p>0.01$ for 4 of 6 comparisons).

Next, PCA was conducted for the 243 available ExPEC isolates based on all 60 virulence genes plus phylogenetic group. According to an initial 2 × 2 MANOVA of the results from the first 2 PCA axes (which accounted for 57% of total variance), all 3 independent variables (source, resistance status, and interaction term) showed a p value <0.001. Accordingly, pairwise comparisons were made between individual source-resistance groups by 1-factor MANOVA. Susceptible human isolates differed ($p<0.001$) from each of the other 3 groups, whereas the other 3 groups did not differ significantly from each another. In a plot of the (axis 1–axis 2) plane, drug-susceptible poultry isolates, drug-resistant poultry isolates, and drug-resistant human isolates overlapped and were confined largely to the left half of the grid (negative values on axis 1). In contrast, drug-susceptible human isolates, although overlapping somewhat with these groups, were concentrated principally within the right half of the grid (positive values on axis 1) (Figure 1).

Aggregate Virulence Scores

The various source and resistance groups were also compared for aggregate virulence scores (ExPEC isolates only). According to virulence score distribution, drug-susceptible human isolates (higher scores) segregated widely from the other 3 subgroups (lower scores), which were largely superimposed on each another (Figure 2). Because drug-resistant and drug-susceptible poultry isolates had similar virulence scores, they were combined for statistical

analysis. Drug-susceptible human isolates had the highest scores (median 13.0, range 4.25–20.0). Drug-resistant human and poultry isolates had significantly lower scores that did not differ between humans and poultry (median 9.0, range 6.0–15.25, and median 8.75, range 3.75–15.0, respectively; vs. drug-susceptible human isolates, p<0.001). Similar results were obtained when isolates from hospital patient fecal samples were compared separately with the conventionally raised poultry isolates or when isolates from vegetarian fecal samples were compared separately with isolates from poultry raised without antibiotics (data not shown).

Table 2. Bacterial traits by source and antimicrobial drug resistance in 243 extraintestinal pathogenic *Escherichia coli* (ExPEC) isolates from human feces and poultry products, Minnesota and Wisconsin, 2002–2004*

	Prevalence, no. (%)				p value¶		
Trait†‡§	Total (n = 243)	Human, susceptible (n = 144)	Human, resistant (n = 20)	Poultry (n = 79)	HS vs. HR	HS vs. all poultry	HR vs. all poultry
Group A	20 (8)	5 (3)	5 (25)	10 (13)	≤0.01#		
Group B1	7 (3)	0	0	7 (9)		≤0.001#	≤0.001#
Group B2	154 (63)	125 (87)	6 (30)	23 (29)		≤0.001	
Group D	62 (26)	14 (10)	9 (45)	39 (49)		≤0.001#	
papA	117 (48)	97 (67)	7 (35)	13 (16)	≤0.01	≤0.001	
F10 allele	38 (16)	32 (10)	5 (25)	1 (1)		≤0.001	≤0.001
F16 allele	12 (5)	5 (3)	5 (25)	2 (3)	≤0.01#		≤0.01
F48 allele	21 (9)	21 (15)	0	0		≤0.001	
papG III	44 (18)	44 (31)	0	0	≤0.01	≤0.001	
sfa/focDE	62 (26)	61 (42)	1 (5)	0	≤0.001	≤0.001	
sfaS	35 (14)	33 (23)	1 (5)	1 (1)		≤0.001	
focG	13 (5)	12 (8)	1 (5)	0		≤0.01	
afa/draBC	15 (6)	11 (8)	4 (20)	0		≤0.01	≤0.001
iha	52 (22)	38 (26)	16 (80)	0	≤0.001#	≤0.001	≤0.001
hra	108 (44)	67 (47)	2 (10)	39 (49)	≤0.001		≤0.01#
cnf1	54 (22)	51 (35)	2 (10)	1 (1)		≤0.001	
hlyD	67 (28)	67 (28)	2 (10)	2 (3)	≤0.01	≤0.001	
hlyF	73 (30)	28 (19)	1 (5)	44 (57)		≤0.001#	≤0.001#
sat	61 (25)	46 (32)	15 (75)	0 (0)	≤0.001#	≤0.001#	≤0.001#
pic	34 (14)	30 (21)	0	4 (5)		≤0.01	
vat	131 (54)	113 (78)	3 (15)	15 (19)	≤0.001	≤0.001	
astA	48 (20)	7 (5)	1 (5)	40 (51)		≤0.001#	≤0.001#
iutA	162 (67)	67 (47)	18 (90)	77 (97)		≤0.001#	
iroN	118 (49)	78 (54)	3 (15)	37 (47)	≤0.001		≤0.01#
fyuA	199 (82)	138 (96)	17 (85)	44 (56)		≤0.001	
kpsM II	215 (89)	137 (95)	16 (80)	62 (78)		≤0.001	
K5 *kpsM*	35 (14)	28 (19)	4 (20)	3 (4)		≤0.001	
iss	69 (28)	23 (16)	2 (10)	44 (56)		≤0.001#	≤0.001#
usp	144 (59)	127 (88)	6 (30)	11 (14)	≤0.001	≤0.001	
H7 *fliC*	52 (21)	52 (36)	0	0	≤0.001	≤0.001	
ompT	184 (76)	131 (91)	9 (50)	40 (51)	≤0.01	≤0.001	
malX	152 (63)	134 (93)	7 (35)	1 (14)	≤0.001	≤0.001	

*Susceptible, susceptible to trimethoprim-sulfamethoxazole, nalidixic acid (quinolones), and ceftriaxone or ceftazidime (extended-spectrum cephalosporins), regardless of other possible drug resistance; resistant, resistant to ≥1 of the following: trimethoprim-sulfamethoxazole, nalidixic acid (quinolones), and ceftriaxone or ceftazidime (extended-spectrum cephalosporins).

†Traits are shown that showed p≤0.01 for ≥1 comparison each. Groups A, B1, B2, and D, major *E. coli* phylogenetic groups; *papA*, P fimbriae structural subunit with variants F10, F16, and F48; *papG* III, variant P adhesin; *sfa/focDE*, S and F1C fimbriae; *sfaS*, S fimbriae; *focG*, F1C fimbriae; *afa/draBC*, Dr binding adhesins; *iha*, adhesin-siderophore receptor; *hra*, pathogenicity island marker; *cnf1*, cytotoxic necrotizing factor 1; *hlyD*, α-hemolysin; *hlyF*, variant hemolysin; *sat*, secreted autotransporter toxin; *pic*, autotransporter protease; *vat*, vacuolating autotransporter; *astA*, enteroaggregative *E. coli* toxin; *iutA*, aerobactin system; *iroN*, siderophore receptor; *fyuA*, yersiniabactin receptor; *kpsM* II, group 2 capsule; K5 *kpsM*, *kpsM* II variant; *iss*, increased serum survival; *usp*, uropathogenic-specific protein; H7 *fliC*, flagellar variant; *ompT*, outer membrane protease; *malX*, pathogenicity island marker.

‡Traits that did not show p<0.01 but were detected in ≥1 isolate each include the F7–2, F8, F9, F11, F12, F12, F14, and F15 *papA* alleles, *papC* (P fimbriae assembly), *papEF* (P fimbriae tip pilins), *papG* alleles I and II (both internal and flanking sequences), *afaE8* (variant Dr binding adhesin), *gafD* (G fimbriae), F17 fimbriae, *fimH* (type 1 fimbriae), *clpG* (adhesin), *cdtB* (cytolethal distending toxin B), *ireA* (siderophore receptor), *kpsM* III (group 3 capsule), K1 and K2 *kpsM* II variants, *cvaC* (microcin V), *ibeA* (invasion of brain endothelium), and *rfc* (O4 lipopolysaccharide biosynthesis).

§Traits not detected in any isolate include F7–1 and F536 *papA* alleles and K15 *kpsM* II variant.

¶By Fisher exact test. Values are shown only where p≤0.01. HS, susceptible isolates from humans; HR, drug-resistant isolates from humans. Because drug-resistant and drug-susceptible poultry isolates showed no significant differences, they were combined into an all-poultry group.

#Negative association.

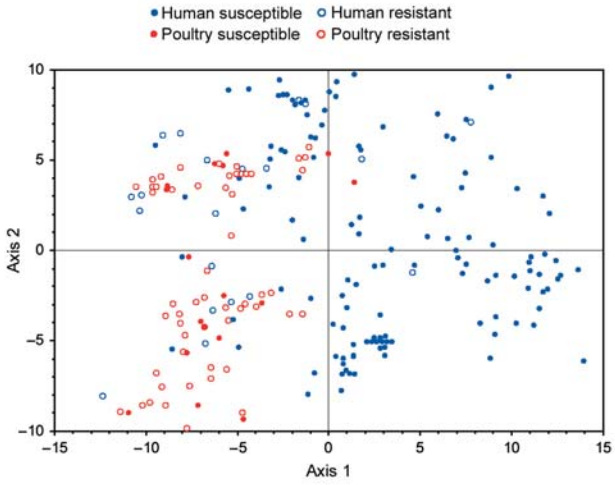

Figure 1. Principal coordinates analysis of distribution of 243 extraintestinal pathogenic *Escherichia coli* isolates from human feces and poultry products, Minnesota and Wisconsin, 2002–2004, on the axis 1–axis 2 plane. Data include extended virulence genotypes (60 traits) and phylogenetic group (A, B1, B2, D). The axes have no units; they reflect the total score for each isolate derived by summing the isolate's partial score for each variable, which is the product of the loading score assigned to the particular variable for a given axis and the isolate's status for that variable. Axis 1 (positive values to right, negative values to left of central vertical line) accounted for 37% of total variance and showed significant differences between susceptible human isolates versus each of the other groups. Axis 2 (positive values above, negative values below central horizontal line) accounted for 20% of total variance and did not show any significant between-group differences. Resistant, resistant to trimethoprim-sulfamethoxazole, nalidixic acid (quinolones), and ceftriaxone or ceftazidime (extended-spectrum cephalosporins). Susceptible, susceptible to all these agents (regardless of other possible drug resistance).

Dendrogram of Extended Virulence Profiles and Phylogenetic Group

Phylogenetic group and extended virulence profiles among the 243 available ExPEC isolates also were used to construct a similarity dendrogram. The dendrogram showed 3 major clusters, each of which contained 2 prominent subclusters (Figure 3). Isolates were distributed by cluster and subcluster according to source and resistance group; that is, drug-susceptible human isolates accounted for almost all of subclusters 1a, 1b, and 2a. In contrast, drug-resistant human isolates were confined largely to subcluster 3a. Poultry isolates, whether resistant or susceptible, were confined almost entirely to subclusters 2b, 3a, and 3b. Thus, compared with drug-susceptible human isolates, drug-resistant human isolates were significantly more likely to occur within a subcluster, or major cluster, that also contained poultry isolates ($p<0.001$ for each comparison).

The possible effects of nonindependence among multiple isolates acquired from the same sample were assessed by limiting the analysis to a single isolate per sample, keeping a drug-susceptible isolate (if available) and randomly selecting among multiple drug-resistant isolates where required. This resulted in reduced sample sizes of 681 (total population) and 226 (ExPEC population). The analysis results closely mirrored the pattern of significant findings obtained in the full samples.

Discussion

In this study, we analyzed the phylogenetic distribution and virulence genotypes of drug-susceptible and drug-resistant *E. coli* isolates from human volunteers and poultry products in Minnesota and Wisconsin. We found that drug-resistant human isolates, although overlapping somewhat with drug-susceptible human isolates, were more similar overall to poultry isolates than to drug-susceptible human isolates. In contrast, drug-susceptible human isolates differed from poultry isolates. This relationship was observed consistently with diverse analytical approaches and various stratifications of the population. It suggests that many of the drug-resistant human isolates were more likely to have originated in poultry (or a similar nonhuman reservoir) and to have been acquired by humans when these isolates were already drug resistant, than to have emerged de novo in humans by conversion of drug-susceptible human isolates to drug-resistant isolates.

We also found that, regardless of analytical approach and population analyzed, resistant and susceptible poultry isolates were highly similar. This suggests that the resistant poultry isolates likely derived from antimicrobial drug–

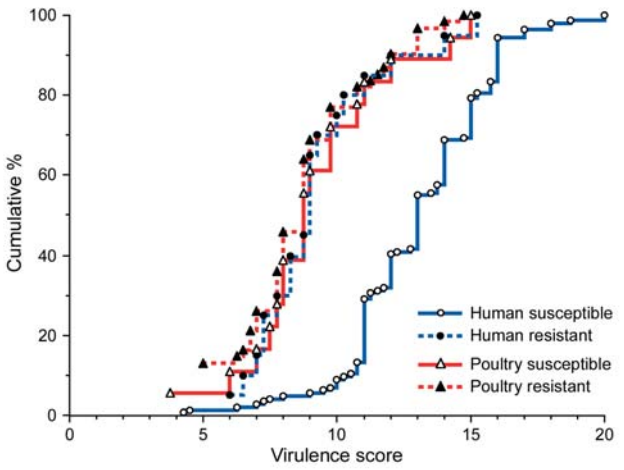

Figure 2. Distribution of virulence factor scores by source and resistance status among 243 extraintestinal pathogenic *Escherichia coli* isolates from human feces and poultry products, Minnesota and Wisconsin, 2002–2004. Resistant, resistant to trimethoprim-sulfamethoxazole, nalidixic acid (quinolones), and ceftriaxone or ceftazidime (extended-spectrum cephalosporins). Susceptible, susceptible to all these agents (regardless of other possible resistances). The virulence scores of the susceptible human isolates are an average of ≈4 points greater than those of the resistant human isolates or poultry isolates.

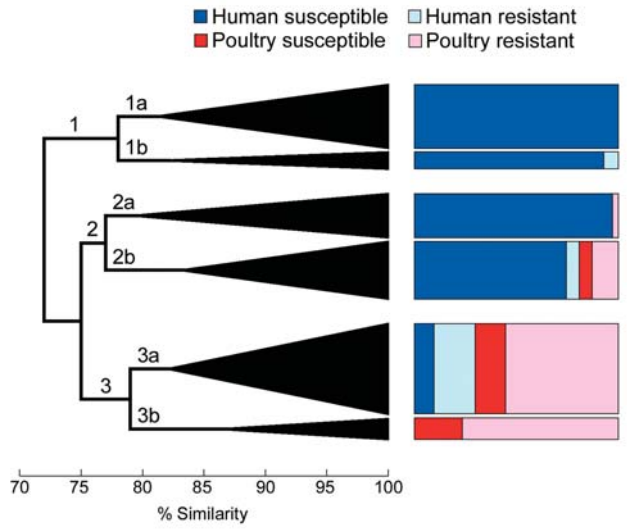

Figure 3. Dendrogram based on extended virulence profiles of 243 extraintestinal pathogenic *Escherichia coli* isolates from human feces and poultry products, Minnesota and Wisconsin, 2002–2004. The dendrogram (shown here in simplified form) was constructed by using the unweighted pair group method with arithmetic averages based on pairwise similarity relationships according to the aggregate presence or absence of 60 individual virulence genes plus phylogenetic group (A, B1, B2, D). Triangles indicate arborizing subclusters. Major clusters 1, 2, and 3, and subclusters 1a, 1b, 2a, 2b, 3a, and 3b are indicated. Colored boxes to right of dendrogram show the distribution (by source group) of constituent members of each subcluster. Resistant, resistant to trimethoprim-sulfamethoxazole, nalidixic acid (quinolones), and ceftriaxone or ceftazidime (extended-spectrum cephalosporins). Susceptible, susceptible to all these agents.

susceptible, poultry-source *E. coli* by conversion to resistance. This most plausibly would occur within the avian fecal flora under selection pressure from on-farm use of antimicrobial drugs.

Our findings closely resemble those of a recent study of ciprofloxacin-resistant *E. coli* from humans and chickens in the late 1990s in Barcelona, Spain (*12*). These data indicate that these relationships remain valid and are applicable in the United States, to additional resistance phenotypes (specifically quinolones, TMP-SMZ, and extended-spectrum cephalosporins), and to retail poultry products (*12*). Moreover, similar results were obtained with retail poultry products and poultry carcasses from processing plants. This implies that drug-resistant poultry-source *E. coli* isolates originate in the birds, rather than being introduced from some exogenous reservoir later during the packaging and distribution process. This in turn suggests that on-farm practices, including use of antimicrobial agents for growth promotion, metaphylaxis, and therapy (*21*,*22*), may influence characteristics of *E. coli* that contaminate retail poultry products and, seemingly, are then transmitted to humans (*7*).

The greater overall similarity of drug-resistant human isolates to poultry isolates than to drug-susceptible human isolates applied not only to the hospital patient isolates compared with isolates from conventionally raised poultry, but also to the isolates from vegetarians compared with isolates from poultry raised with no antibiotics. This was surprising because the vegetarians ostensibly did not consume poultry and, therefore, should not have been directly exposed to poultry-source *E. coli*. However, this seeming paradox is consistent with the difficulty in confirming poultry consumption (along with most other individual-level exposures) as an epidemiologic risk factor for colonization with drug-resistant *E. coli* isolates among community-dwelling persons ([*23*]; J.R. Johnson, unpub. data). Assuming that the drug-resistant human isolates were derived from poultry, occurrence of poultry-source *E. coli* in both vegetarians and persons with conventional diets suggests that poultry-source drug-resistant *E. coli* may spread extensively through the human population without requiring individual exposure to poultry products. This suggestion would be consistent with evidence that household-level risk factors may be more predictive of colonization with drug-resistant *E. coli* than individual-level risk factors, and that household members often share *E. coli* clones with each another (*23*–*25*). The mechanisms for such diffusion, and methods to block the entry of such strains into the human population and their subsequent spread, need to be defined.

The virulence potential for humans of the present drug-resistant human and poultry *E. coli* isolates, which is related to their direct threat to human health, is unknown. Predictions regarding virulence potential await molecular comparisons with human clinical isolates (*9*,*10*,*12*) and experimental virulence assessment in vivo (*26*,*27*). Nonetheless, the abundance of ExPEC-associated virulence genes in some of these strains is of concern because it suggests a high likelihood of virulence. This would augment any health threat these strains may pose as passive vehicles for drug-resistance genes (*6*,*7*).

Potential limitations of this study warrant comment. Because we did not examine alternative sources for drug-resistant human isolates, we cannot exclude the possibility that other foods (*28*) or nonfood reservoirs (*29*) might yield even closer similarities to drug-resistant human isolates. Whether persons in the study consumed poultry products from the same lots or suppliers as those sampled is not known. Because the study was conducted in Minnesota and Wisconsin in mostly rural communities and with newly hospitalized patients and nonhospitalized vegetarians, generalizability of the results is unknown. We combined several resistance phenotypes because of low frequencies, which may have obscured differences. We also did not assess other molecular characteristics of strains, e.g., pulsed-field gel electrophoresis profiles (*12*), sequence types (*30*),

and resistance elements (*28*). Use of multiple comparisons increased the likelihood of spurious associations (which we addressed by specifying a strict criterion for statistical significance), whereas the small sample size in certain subgroups reduced power for finding true associations.

Strengths of the study include substantial overall sample size, standardized concurrent processing of fecal and poultry samples, close matching of human and poultry samples, extensive molecular typing using virulence-relevant markers, and use of multiple analytical modalities. Additionally, we examined clinically relevant resistance phenotypes.

In summary, our findings suggest that in a contemporary US-based population, many human-source drug-resistant fecal *E. coli* isolates more likely originated in poultry than in humans, whereas drug-resistant poultry isolates likely derive from drug-susceptible poultry isolates. Our data extend this paradigm to clinically relevant agents other than fluoroquinolones, heighten concerns regarding the potential human health risk for antimicrobial drug use in poultry production, and suggest that avoidance of poultry consumption may not reliably provide personal protection.

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Molecular Characteristics and Epidemiology of Meningococcal Carriage, Burkina Faso, 2003

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To describe *Neisseria meningitidis* strains in the African meningitis belt in 2003, we obtained 2,389 oropharyngeal swabs at 5 monthly visits a representative population sample (age range 4–29 years) in Bobo-Dioulasso, Burkina Faso. A total of 152 carriage isolates were grouped, serotyped, and genotyped. Most isolates were NG:NT:NST sequence type (ST) 192 (63% of all *N. meningitidis*), followed by W135:2a:P1.5,2 of ST-11 (16%) and NG:15:P1.6 of ST-198 (12%). We also found ST-2881 (W135:NT:P1.5,2), ST-751 (X:NT:P1.5), and ST-4375 (Y:14:P1.5,2) but not serogroups A or C. Estimated average duration of carriage was 30 days (95% confidence interval 24–36 days). In the context of endemic group W135 and meningococcal A disease, we found substantial diversity in strains carried, including all strains currently involved in meningitis in this population, except for serogroup A. These findings show the need for large samples and a longitudinal design for *N. meningitidis* serogroup A carriage studies.

In the African meningitis belt, serogroup A of *Neisseria meningitidis* (NmA) is the most frequent cause of bacterial meningitis. Since an epidemic in 1987, these invasive NmA strains have been identified as belonging to clonal complex sequence type (ST)–5 (*1*). From 1980 through 2000, meningococcal serogroup W135 (NmW135) was found in Africa only occasionally (*2*) and never as an epidemic strain. At the end of the 2001 epidemic season in Burkina Faso and Niger, however, similar proportions of cases caused by NmW135 and NmA were found (*3*). During a large epidemic in Burkina Faso in 2002, phenotype W135:2a:P1.5,2 was the predominant strain; this strain belonged to the ST-11 clonal complex (*4*), as did NmW135 strains found in an outbreak among Hajj pilgrims in 2000. Since early 2003, NmW135 has gradually decreased, and in 2005 and 2006 NmA again predominated, with some NmW135 outbreaks in Uganda, Sudan, and Kenya (*5*,*6*).

Most published carriage studies on sub-Saharan Africa were conducted after outbreaks, with transversal design or with nonsystematic specimen collection. These studies often found a predominance of the outbreak strain. Our longitudinal study describes meningococcal phenotypes and genotypes circulating in an urban Burkina Faso population 1 year after an *N. meningitidis* W135 epidemic, their dynamics during a nonepidemic meningitis season, and the carriage prevalence of disease-causing strains in the healthy population.

Methods

Recruitment and Swab Collection

Methods, population characteristics, and 4-month carriage prevalence by serogroup have been previously reported (*7*). The study was reviewed and approved by the Ethics Committee of Centre Muraz, Bobo-Dioulasso, Burkina Faso, and the Comité de Vigilance of Pasteur Institute, Paris. Briefly, after written informed consent was obtained from study participants or their guardians (for persons <18 years of age), a random sample of the healthy residents of urban Bobo-Dioulasso, Burkina Faso, were examined at 5 clinic visits from February 3 to June 7, 2003. The sampling design required that 1 participant 4–14 years of age and 1 participant 15–29 years of age were included from each

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selected compound (community of households). At all 5 visits, swabs were taken from the posterior pharyngeal wall through the mouth by using cotton-tipped sterile swabs, which were streaked immediately onto plates containing selective medium. The plates were stored immediately in an atmosphere of 5% CO_2 at room temperature for a maximum of 2 hours until incubation at 37°C.

Microbiologic Analyses

N. meningitidis strains from incubated plates were isolated and identified by using established bacteriologic methods, following recommendations of the World Health Organization when applicable (*8*). *N. lactamica* isolates were also cultured and identified. Confirmation and genogroup prediction of *N. meningitidis* isolates was conducted on the basis of PCR testing as previously described (*9*,*10*). All groupable *N. meningitidis* isolates and a subset of nongroupable isolates were further tested with immune serum for serogroup confirmation.

Serotypes and serosubtypes were determined by using monoclonal antibody kits obtained from the National Institute of Public Health and the Environment (Bilthoven, the Netherlands) by the whole-cell enzyme immunoassay technique, as previously described (*11*). Chromosomal DNA restriction patterns were analyzed by pulsed-field gel electrophoresis (PFGE). Whole chromosome DNA macrorestriction fragments generated by digestion with *Spe*I endonuclease were separated by PFGE as previously described (*12*). DNA fragments were separated by using a Chef-DR II system (Bio-Rad Laboratories, Hercules, CA, USA). PFGE fingerprint patterns were compared by using the criteria of Tenover et al. (*13*).

Multilocus sequence typing (MLST) was performed on a subset of 53 isolates chosen to represent different PFGE variants of different serogroups (*14*). Fragments from 7 housekeeping genes (*abc*Z, *adk*, *aro*E, *fum*C, *gdh*, *pdh*C, and *pgm*) were used for typing, as given on the *Neisseria* MLST website (http://pubmlst.org/neisseria/). After DNA preparation and amplification by PCR, each locus sequence was analyzed on an ABI Prism 3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed by using Vector NTI suite software (InforMax, Bethesda, MD, USA). The sequences were compared with existing alleles on the *Neisseria* MLST website for determination of allele numbers, STs, and clonal complexes of the isolate. After internal validation tests, strains that had the same PFGE profile were considered to belong to the same ST, and thus were defined as having the same ST (Figures 1, 2).

Estimation of Carriage Duration

Mean duration of carriage and 95% confidence intervals (CIs) were calculated by the truncated observations method described by de Wals and Bouckaert (*15*). We assumed that isolates with identical phenotypes and genotypes collected from a person at consecutive visits, and only those, indicated an ongoing carriage event.

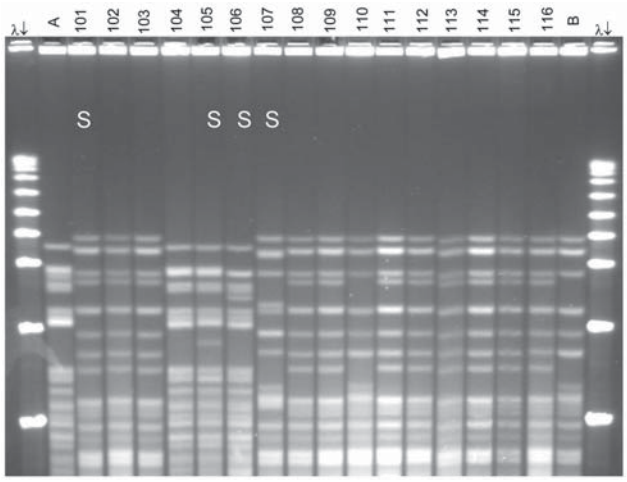

Figure 1. Pulsed-field gel electrophoresis (PFGE) analysis of chromosomal DNA from pharyngeal meningococcus isolates (stained with ethidium bromide). Whole chromosome DNA macrorestriction fragments were generated by digestion with endonuclease *Spe*I. Shown are examples of sequence type (ST) prediction by PFGE in carried meningococci and diversity among STs. S, isolates tested by multilocus sequence typing (MLST). Lanes λ (arrows), PFGE marker I (Boehringer Mannheim, Mannheim, Germany); lane A, ST-2881, meningitis case isolate, Niger 2003; lanes 101, 102, and 103, ST-11, W135:2a:P1.5,2; lanes 104 and 105, ST-2881, W135:NT:P1.5,2; lane 106, ST-4151, W135:NT:P1.5,2; lane 107, ST-11_{2000}, W135:NT:P1.5,2; lanes 108–116, ST-11, W135:2a:P1.5,2; lane B, meningitis case isolate, ST-11, Niger 2003. Isolates 101 and 107 were identified as ST-11 by MLST. Isolates 102, 103, 108, 109, and 111–116 are indistinguishable from isolate 101 and are therefore considered ST-11. The 19 ST-11 isolates had 4 different PFGE patterns, of which 3 are represented by isolates 101, 107, and 110. The pattern of isolate 107 is indistinguishable from the 2000 Hajj epidemic strain (not shown).

Results

A total of 488 persons were included in the study; ≥96% were seen at each respective visit. Eighteen percent of the population carried a meningococcus at least once during the study. All genogroupable isolates could be serogrouped. The 152 meningococcal isolates were attributed to serogroups NmW135 (n = 28), NmX (n = 5), NmY (n = 3), and nongroupable, autoagglutinable, or polyagglutinable Nm (n = 116). No NmA, NmB, or NmC were found (Table 1).

Among the 151 meningococcal isolates submitted for serotyping, most could not be serotyped or serosubtyped with existing antibodies (n = 98, 65%). Serotype 2a:P1.5,2 (n = 17, 11%) and 15:P1.6 (n = 16, 11%) were the most frequently found serotypes, followed by NT:P1.5,2

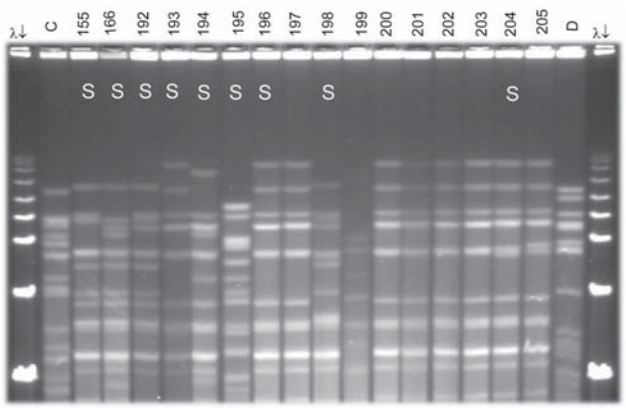

Figure 2. Pulsed-field gel electrophoresis (PFGE) analysis of chromosomal DNA from pharyngeal meningococcus isolates (stained with ethidium bromide). Whole chromosome DNA macrorestriction fragments were generated by digestion with endonuclease *Spe*I. Shown are examples of sequence type (ST) prediction by PFGE in carried meningococci and diversity among STs types. S, isolates tested by multilocus sequence typing (MLST). Lanes λ (arrows) PFGE marker I (Boehringer Mannheim, Mannheim, Germany); lane C, ST-2881, meningitis case isolate, Niger 2003; lanes 155, 166, and 192–194, ST-192, NG:NT:NST; lane 195, ST-198, NG:15:P1.6; lanes 196–198, ST-192, NG:NT:NST; lane 199, isolate unrelated to the presented study; lanes 200–205, ST-192, NG:NT:NST; lane D, meningitis case isolate, ST-11, Niger 2003. Isolates 192, 193, 194, 196, 198, and 204 were identified as ST-192 by MLST. Isolates 197, 200, 201, 202, and 203 are indistinguishable from isolate 196 and are thus considered ST-192. ST-192 isolates from this study had 10 different PFGE patterns, of which 6 are represented by isolates 155, 166, 192, 194, 196, and 204.

(n = 11, 7%), NT:P1.5 (n = 6, 4%), and 14:P1.5,2 (n = 3, 2%) (Figure 3, Table 1).

Among the 151 isolates analyzed by MLST or PFGE, most were ST-192 (n = 96, 63%), followed by ST-11 (n = 19, 13%), ST-198 (n = 13, 9%), and ST-2881 (n = 8, 6%, including 1 strain with the single locus variant ST-4151) (Table 1). Other STs represented <5% of the carriage strains and included ST-4426 (in the clonal complex ST-198), ST-751 (including the single locus variant ST-4376), ST-4375 (in the clonal complex ST-23), and ST-4377.

Serogroup W135 was mostly found in combination with serotype 2a:P1.5,2 and genotype ST-11. Serogroup X was found with NT:P1.5, ST-751, and serogroup Y was found with 14:P1.5,2, ST-4375 (Table 1).

Diversity among STs

The 19 isolates belonging to ST-11 showed moderate diversity in restriction patterns (Figure 1). Three (16%) isolates found in February 2003, among them 2 isolates with phenotype W135:NT:P1.5,2, were indistinguishable from the 2000 Hajj outbreak strain, and 1 (5%) isolate each showed 1- and 2-band differences from the 2000 Hajj strain. The other 14 ST-11 isolates (74%), among them an isolate with phenotype NG:2a:P1.5,2, showed a 6-band difference from the 2000 Hajj strain.

The 8 ST-2881 isolates belonged to 1 clone with a 0- to 2-band difference between them. Although most of the ST-2881 isolates belonged to group W135, their PFGE patterns were unrelated to ST-11 isolates in group W135; they were closely related to ST-2881 invasive strains of serogroup W135 found in 2003 in Niger (*16*).

Among the 96 ST-192 isolates, 86 had interpretable results by PFGE, which showed considerable diversity in restriction patterns (Figure 2). Of these isolates, 41 (48%) were indistinguishable from each other and 10 (12%), among them the isolate with phenotype W135:NT:NST, were closely related with 1- to 3-band differences. Thirty-one isolates (36%) were possibly related to the central

Table 1. Characterization of 152 meningococcal isolates, Bobo-Dioulasso, Burkina Faso, 2003*

Sequence type (ST)	No. isolates (% ST)	No. tested by MLST	Phenotype	No. (%) ST isolates with phenotype
ST-192	96 (63)	40	NG:NT:NST	95 (63)
			W135:NT:NST	1 (1)
ST-11	19 (13)	5	W135:2a:P1.5,2	16 (11)
			W135:NT:P1.5,2	2 (1)
			NG:2a:P1.5,2	1 (1)
ST-198	13 (9)	3	NG:15:P1.6	12 (8)
			W135:15:P1.6	1 (1)
ST-4426 (clonal complex ST-198)	2 (1)	2	NG:15:P1.6	2 (1)
ST-2881	8 (5)	2	W135:NT:P1.5,2	8 (5)
ST-4151 (single locus variant of ST-2881)	1 (1)	1	W135:NT:P1.5,2	1 (1)
ST-751	5 (3)	3	X:NT:P1.5	5 (3)
ST-4376 (single locus variant of ST-751)	1 (1)	1	NG:NT:P1.5	1 (1)
ST-4375 (clonal complex ST-23)	3 (2)	1	Y:14:P1.5,2	3 (2)
ST-2049	1 (1)	1	NG:15:P1.6	1 (1)
ST-4377	2 (1)	2	NG:NT:NST	2 (1)
Not tested	1 (1)	–	NG: Not determined	1 (1)
Total	152 (100)	61		152 (100)

*MLST, multilocus sequence typing. NG includes nongroupable, autoagglutinable, and polyagglutinable strains.

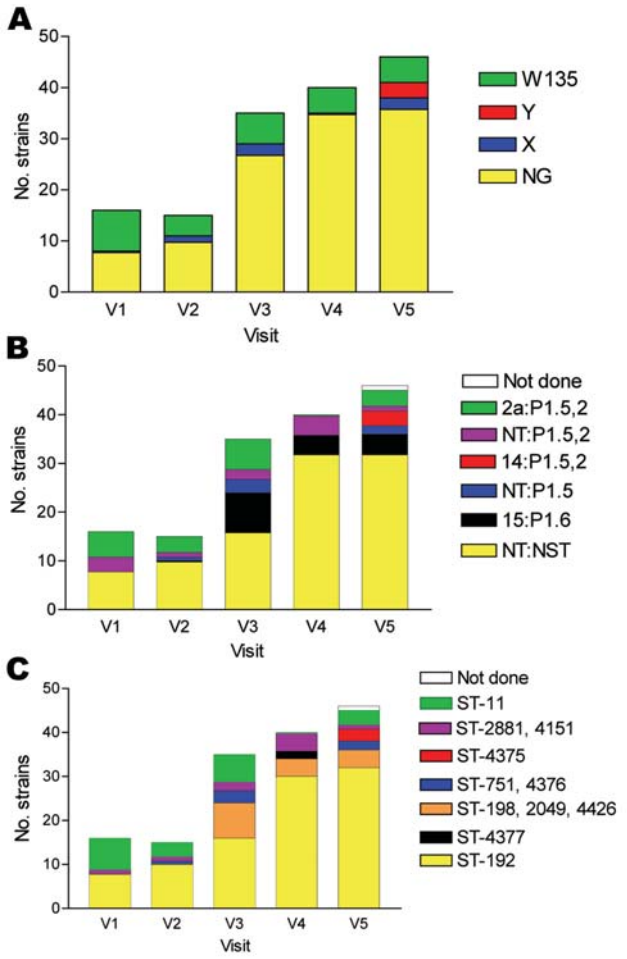

Figure 3. Serogroups (A), serotypes (B), and sequence types (C) of 152 meningococci carried by 488 persons obtained at 5 monthly study visits from February through June 2003, Bobo-Dioulasso, Burkina Faso. NG, nongroupable; ST, sequence type.

clone (4- to 6-band difference), and 4 (5%) were unrelated (≥7-band difference).

Carriage Dynamics

The gradual increase in carriage point prevalence of any Nm from 3.5% in February to 9.9% in May–June was caused by an increase in ST-192 with a phenotype of NG:NT:NST (Figure 3). The number of isolates of this genotype and phenotype at each of the 5 visits was 7, 8, 15, 26, and 27, respectively.

Among the 84 persons who carried meningococci during ≥1 visit, half (n = 42) were carriers at only 1 visit and half at multiple visits. Of the 42 persons with meningococcal carriage at several visits, 21 always had the same strain (Table 2) and 19 had different strains (Table 3). The latter group carried ≤3 different strains during the 4 months of observation, and 4 persons had the same strain on multiple occasions but with interruption. Twenty-seven persons (32% of all carriers) had the same strain ≥2 times at subsequent visits. The mean duration of carriage for all serogroups was estimated as 30 days (95% CI 24–36 days). Estimated mean carriage duration was 20 days (95% CI 15–23 days) for NmW135 and 34 days (95% CI 27–42 days) for nongroupable strains. The estimated mean duration of overall Nm carriage increased with the increasing prevalence of nongroupable strains toward the end of the meningitis season: 21 days (95% CI 18–24 days) during February–April compared with 35 days (95% CI 28–43 days) during April–June.

In addition to the 152 meningococci, 103 *N. lactamica* were isolated. Prevalence of *N. lactamica* was highest in 4 to 8-year-old children and increased gradually from 5.5% (95% CI 2.1%–13.2%) at the first visit to 16.1% (95% CI 10.9%–23.1%) at the last visit. For persons 9–18 years of age, prevalence of *N. lactamica* carriage varied from 2% to 4% over the 5 visits; carriage for adults was ≥1.5%.

Discussion

This longitudinal carriage study in a healthy young population in the African meningitis belt describes the diversity of carried meningococcal serogroups, serotypes, and genotypes during a nonepidemic meningitis season. Eleven STs and 4 serogroup categories (including nongroupable strains) were identified. Parallel culture- and PCR-based meningitis surveillance in this population during 2003 showed a high incidence of endemic meningococcal disease (annual rate = 77/100,000 among persons <5 years of age and 5/100,000 among persons >14 years of age). During February–April 2003, 9 cases of NmA meningitis and 28 cases of NmW135 meningitis were found in urban Bobo-Dioulasso (435,000 inhabitants), as well as sporadic cases caused by serogroup X and nongroupable meningococci (Table 4) (*5*,*17*).

Despite frequent serogroup A disease, no serogroup A meningococcal carriage was found. NmA was likely circulating at low levels during our study but not found because of low transmission density or short duration of NmA carriage, together with sample size limitation. Our study thus provides evidence for low prevalence of serogroup A carriage in nonepidemic conditions, which is similar to results of a study in Nigerian schoolchildren (*18*). This finding is useful for assessment of group A conjugate meningococcal vaccines by carriage studies. To show a reduction in NmA carriage prevalence after vaccination, as was recently reported from the United Kingdom for group C conjugate vaccine (*19*), studies need to include several thousand persons to achieve appropriate statistical power.

Apart from NmA, all phenotypes and genotypes isolated from meningococcal meningitis cases in this population from 2000 through 2005 (Table 5) were represented

Table 2. Analysis of 21 persons carrying the same meningococcal strain at 5 monthly visits, Bobo-Dioulasso, Burkina Faso, 2003

Person	Age, y	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5
1	4	W135: ST-2881		W135: ST-2881		
2	6			NG:ST-192	NG:ST-192	NG:ST-192
3	7	NG:ST-192			NG:ST-192	NG:ST-192
4	8	NG:ST-192			NG:ST-192	
5	10			NG:ST-192	NG:ST-192	NG:ST-192
6	11				NG:ST-192	NG:ST-192
7	11				NG:ST-192	NG:ST-192
8	12			NG:ST-192	NG:ST-192	NG:ST-192
9	14			NG:ST-192	NG:ST-192	NG:ST-192
10	14		NG:ST-192		NG:ST-192	
11	14			NG:ST-192	NG:ST-192	NG:ST-192
12	15		NG:ST-192			NG:ST-192
13	16				NG:ST-198	NG:ST-198
14	17				NG:ST-192	NG:ST-192
15	17			NG:ST-192	NG:ST-192	
16	18			NG:ST-192	NG:ST-192	NG:ST-192
17	19		NG:ST-192	NG:ST-192		NG:ST-192
18	20	NG:ST-192			NG:ST-192	NG:ST-192
19	20				NG:ST-192	NG:ST-192
20	22		NG:ST-192			NG:ST-192
21	26		W135: ST-11			W135: ST-11

in this 4-month carriage study of 488 persons. This finding supports the use of carriage studies in nonepidemic conditions for surveillance of meningococcal strains of specific serogroups. For surveillance of new genotypes expressing a group A capsule, however, disease surveillance will be more appropriate. For example, ST-2859, a new genotype that expresses group A capsule, has become a major meningitis agent in Bobo-Dioulasso since 2002 (*17*). Our carriage study did not detect this development. In addition, results from localized carriage studies should not be generalized to West Africa and the African meningitis belt as a whole because only 4 of 7 serogroups and 5 of 15 genotypes found in meningococcal meningitis cases in the region during 2000–2005 were represented in our carriage study.

During bacterial meningitis surveillance in the Bobo-Dioulasso population in 2004, we observed 2 invasive strains whose genotypes had been associated with different serogroups and serotypes in our carriage study 1 year earlier (Table 5). ST-11, which is usually associated with phenotype W135:2a:P1.5,2 in invasive strains, had phenotype Y:14:P1.5,2 (seen in ST-4375 carriage strains), and ST-4375, which is usually associated with phenotype Y:14:P1.5,2, had phenotype W135:NT:P1.5,2 (seen in ST-11 carriage strains). These findings could be evidence for a

Table 3. Analysis of 19 persons carrying >1 meningococcal strain at 5 monthly visits, Bobo-Dioulasso, Burkina Faso, 2003

Person	Age, y	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5
22	4			NG: ST-198	NG:ST-192	NG:ST-192
23	5	W135: ST-11			NG:ST-192	NG:ST-192
24	8				W135: ST-2881	NG:ST-4426
25	8			X: ST-751	NG:ST-192	X: ST-751
26	9		NG:ST-192	W135: ST-11		
27	11			W135: ST-11	NG:ST-192	W135: ST-2881
28	13			NG: ST-198	NG:ST-192	NG:ST-192
29	14			NG: ST-198	NG:ST-192	
30	16				W135: ST-2881	Y:ST-4375
31	18		W135: ST-11	NG:ST-2049	NG: ST-198	NG: ST-198
32	18			NG:ST-11	W135: ST-2881	NG:ST-192
33	18		NG:ST-192	NG: ST-198	NG:ST-192	
34	20			NG: ST-198	NG: ST-192	NG: ST-192
35	21	W135: ST-11		NG:ST-192	W135: ST-11	
36	22		X: ST-751	NG: ST-198	NG:ST-192	X: ST-751
37	22			X: ST-751	W135: ST-2881	
38	23			W135: ST-11	NG:ST-4377	NG:ST-192
39	26		NG:ST-192		NG:ST-4377	
40	26				NG: ST-192	NG: ST-4426

Table 4. Meningococci isolated during surveillance of acute bacterial meningitis, Bobo-Dioulasso, Burkina Faso, February–April 2003

Sequence type (ST)	Phenotype	No. cases
ST-11	W135:2a:P1.5,2	28
ST-2859 (ST-5 complex)	A:4:P1.9	9
ST-751	X:NT:P1.5	1
ST-192	NG:NT:NST	2

capsular and serotype switch between co-colonizing meningococci, as described by Swartley et al. (*23*). However, that report described only gene conversion for capsule expression, not for outer membrane protein (PorB) expression. The potential capacity of meningococci to exchange capsular plus subcapsular genes needs to be further evaluated.

Our study and previous studies of meningococci in sub-Saharan Africa have shown a similar number of different serogroup categories, including nonserogroupable strains (*18*,*24–26*). However, assessing whether the genetic diversity we found is a new phenomenon is difficult because most studies do not report genotypes of all isolates. Five ST strains expressed group W135 capsule in this population that was followed up over a 4-month period. This variation has not been reported for other meningococcal serogroups in sub-Saharan Africa but is consistent with results of a report on increasing genetic diversity of W135-encapsulated strains in France since the Hajj-associated outbreak in 2000 (*27*). In contrast to NmW135, NmA has a relatively low genetic diversity, with only 6 genotypes found to express the A capsule over the past 30 years ([*1*]; http://pubmlst.org/neisseria). This difference between the 2 serogroups suggests that NmW135 may not replace NmA as the major epidemic agent in the future. Nevertheless, the easy adoption of a W135 capsule by various genotype stains, in combination with infrequent immune induction by NmW135 carriage (*7*), may cause regular NmW135 outbreaks to occur.

Nongroupable strains were predominant in our study and other carriage studies during nonepidemic conditions in Burkina Faso, Ghana, Europe, and the United States (*20,24,28–31*). In our study, nongroupable and nontypeable isolates were predominantly ST-192, which represented 63% of all carried meningococci. Data from the *Neisseria* MLST website indicate that ST-192 isolates were present in The Gambia and Niger in the 1990s, but no published data are available on the dimension of prevalence of this strain in these or other countries. This strain deserves closer observation because in Bobo-Dioulasso during 2003 and 2004, 3 persons were found with disease caused by nongroupable ST-192 isolates. Unencapsulated strains rarely cause invasive disease and usually only among complement-deficient persons (*32*). This may have occurred in the 3 patients, whose complement status was not determined. However, the isolates from Bobo-Dioulasso also showed

Table 5. Overview of meningococci reported from meningitis patients in West Africa and the African meningitis belt, 2000–2005*

Sequence type (ST)	Phenotype	Place and time of meningococcal disease cases
ST-11	W135:2a:P1.5,2	Sporadic in Cameroon, Senegal, Burkina Faso, Central African Republic, Chad, Niger, and Ghana since 2003; epidemic and major seasonal agent in Burkina Faso during 2002–2004 (including Bobo-Dioulasso)
	W135:NT:P1.5,2	Sporadic in Bobo-Dioulasso, 2004
	W135:2a:P1.2, W135:NT:P1.2	Sporadic in Niger, 2003
	Y:14:P1.5,2	Sporadic in Bobo-Dioulasso, 2004
ST-1966 (ST-11 complex)	W135:2a:P1.5,2	Sporadic in Burkina Faso
ST-2881	W135:NT:P1.5,2	Sporadic in Benin since 2003; major seasonal agent in Niger during 2003
ST-5	A:4:P1.9, A:21:P1.9	Major seasonal agent in Niger, Senegal, and Burkina Faso during 2000–2001
ST-7 (ST-5 complex)	A:4:P1.9, A:21:P1.9	Major seasonal agent and epidemic in Cameroon, Chad, Niger, Senegal, Benin, Burkina Faso, Ethiopia, and Nigeria
ST-2859 (ST-5 complex)	A:4:P1.9, A:21:P1.9	Major seasonal agent and epidemic in Burkina Faso since 2003 (including Bobo-Dioulasso)
ST-751	X:NT:P1.5, X:NT:P1.5,2	Sporadic in Burkina Faso (including Bobo-Dioulasso), Niger, and Ghana
ST-181	X:NT:P1.5	Sporadic in Niger
ST-2880	Y:14:P1.5,2	Sporadic in Niger
ST-4375 (ST-23 complex)	Polyagglutinable:14:P1.5,2	Sporadic in Bobo-Dioulasso, 2004
	W135:NT:P1.5,2	Sporadic in Bobo-Dioulasso, 2004
ST-23 (ST-23 complex)	Y:14:NST	Sporadic in Senegal
ST-32, ST-2496 (ST-32 complex), ST-291 (ST-41/44 complex)	C:4:P1.16, B:4:P1.16, B:4:P1.7,16, B:4:P1.9	Sporadic in Cameroon
ST-192	NG:NT:NST	Sporadic in Bobo-Dioulasso, 2003 and 2004

*Data were obtained from references *1,5,16,17,20–22*, and the *Neisseria* multilocus sequencing typing website (http://pubmlst.org/neisseria).

enhanced capacity to escape human immune defenses (*33*), which would enable these isolates to cause invasive disease in immunocompetent persons.

In our study, carriage of the NG:NT ST-192 strain increased from the early phase of the meningitis season to just past its end, as did carriage of *N. lactamica* in children. This finding may be an annual phenomenon that is associated with a decreasing meningitis incidence by late April (Figure 4), which would be caused by a reduced risk for infection or disease by virulent meningococci, given the increased carriage prevalence of nongroupable meningococci (*34*). However, our data were from a small sample and only 1 population during 1 meningitis season. A more systematic evaluation by longitudinal carriage studies in several African sites is needed to further explore this hypothesis.

Carriage during this study was dynamic and short-lived compared with other studies in Europe (*15*,*30*,*35*). This finding emphasizes the need for a large sample size in cross-sectional carriage studies and the need for multiple assessment points over short intervals for studies on carriage association with risk factors or immune status. Estimated carriage duration was longer for less virulent nongroupable strains than for NmW135. This could be due to a more accentuated immune response to carriage of encapsulated strains, although serologic evaluation during the same study suggested that the immune response to NmW135 carriage does not occur frequently (*7*).

In the context of hyperendemic NmW135 and NmA disease in Bobo-Dioulasso in 2003, we found a large diversity of phenotypes and genotypes in carried Nm strains (including all strains, except for serogroup A) that caused meningococcal meningitis in this population. NmW135 showed substantial prevalence and high genetic diversity. These features distinguish this serogroup from NmA and indicate that, in combination with poor immune induction by carriage, this serogroup may be a potential epidemic agent. The absence of NmA during this nonepidemic meningitis season and the pronounced dynamics of meningococcal carriage emphasize the need for large samples and a longitudinal design for most carriage studies. By decreasing the risk for infection with a virulent clone, expansion of a nonvirulent clone in carriage toward the end of the meningitis season may be 1 of the mechanisms causing a seasonal decrease in the incidence of meningococcal disease.

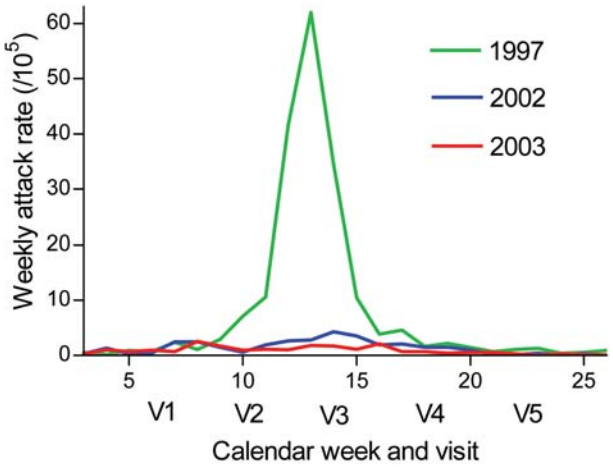

Figure 4. Weekly incidence rates of reported meningitis in the Bobo-Dioulasso region from January through June of 1997, 2002, and 2003. V1, February 3–15 (n = 488); V2, February 25–March 15 (n = 480); V3, March 25–April 12 (n = 465); V4, April 22–May 10 (n = 463); V5, May 27–June 7 (n = 470).

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Incidence and Cost of Rotavirus Hospitalizations in Denmark

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In anticipation of licensure and introduction of rotavirus vaccine into the western market, we used modeling of national hospital registry data to determine the incidence and direct medical costs of annual rotavirus-associated admissions over >11 years in Denmark. Diarrhea-associated hospitalizations coded as nonspecified viral or presumed infectious have demonstrated a marked winter peak similar to that of rotavirus-associated hospitalizations, which suggests that the registered rotavirus-coded admissions are grossly underestimated. We therefore obtained more realistic estimates by 2 different models, which indicated ≈2.4 and ≈2.5 (for children <5 years of age) and ≈4.9 and ≈5.3 (for children <2 years of age) rotavirus-associated admissions per 1,000 children per year, respectively. These admissions amount to associated direct medical costs of US $1.7–1.8 million per year. Using 2 simple models to analyze readily available hospital discharge data resulted in more consistent and reliable estimates.

Rotavirus is the main cause of acute, severe, dehydrating diarrhea in infants and children throughout the world (*1*). Rotavirus disease incidence is similar worldwide, regardless of infrastructure and other levels of development (*2*), which suggests that traditional diarrheal disease control measures, such as safe water and improved hygienic standards, are inadequate. In industrialized counties, hospitalizations are often the most costly events associated with rotavirus disease and often constitute a major expense for national health budgets (*3*).

A recent outbreak of rotavirus diarrhea in a daycare center in Denmark demonstrated that even small outbreaks of rotavirus in childcare facilities can be associated with substantial expense on a personal and a public scale due to parental loss of work (*4*). A major strategy for control of rotavirus disease is prevention through vaccination. With 2 new rotavirus vaccine candidates almost ready for marketing (*5*), valid and updated data on rotavirus disease extent and circulating rotavirus strains are essential for several purposes: to address the need for disease prevention, to generate reliable data for vaccine cost-benefit/effectiveness assessments, and to establish a platform for disease surveillance to monitor the effectiveness of a future vaccine program.

Because the immediate focus for development of rotavirus vaccines has mainly been prevention of associated deaths in Asia and Africa, valid data exist to some extent from prospective disease surveillance studies in these regions. However, despite the considerable problems associated with hospitalization, including nosocomial transmission of rotavirus disease (*6,7*) and the cost of parental loss of work (*8*), few data exist regarding the epidemiologic features of rotavirus infection in industrialized countries.

For this study we used Danish National Patient Registry (NPR) data for all hospital admissions in Denmark since 1977 to address the epidemiology and cost of rotavirus hospitalizations in Denmark. On the basis of findings from other studies of rotavirus disease (*9,10*), we anticipated an underreporting of rotavirus among patients hospitalized with diarrhea. This underreporting is due to various factors, the most important of which seems to be that rotavirus testing is routinely conducted in only a few settings because the same therapy, regardless of test results, is prescribed: symptomatic treatment with fluid replacement. Therefore, testing is used mostly for differential diagnostic purposes or to establish a diagnosis during outbreaks or for immunocompromised persons for whom rapid intervention against other diarrhea agents (bacteria, parasites) is crucial. Also, rotavirus laboratory results often are available only after the average diarrhea patient has already been discharged, so these results rarely get recorded in the patient's medical file.

The issue of underreporting is supported by a study in a major county hospital in Copenhagen, Denmark. The findings suggest that among children 3–36 months of age,

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rotavirus was responsible for ≈60% of all admissions due to diarrhea from December 1998 through May 1999 (*11*). In Denmark, rotavirus-associated hospitalizations occur with a marked seasonality, from January to June, and peak in March and April. So far, no other diarrhea-associated microbial agent with a similar seasonal pattern has been identified. We took advantage of the unique seasonality of rotavirus to obtain a more realistic estimate of rotavirus incidence among hospitalized children. We used modeling of the 11 years of registry data as well as indirect estimates from similar rotavirus disease burden studies. Finally, we used these estimates to assess the extent of severe rotavirus disease in Denmark and the associated direct medical costs.

Materials and Methods

Data Sources

The NPR contains information on hospitalizations for all reasons except psychiatric in Denmark since January 1977, including outpatient treatments since 1995. Information on date of admission, date of discharge, diagnoses, surgical procedures, and personal identification number is recorded for every hospitalized patient. Diagnoses were classified according to the World Health Organization International Classification of Diseases, version 10 (ICD-10) (*12*). To identify specific rotavirus infections, we extracted ICD-10 diagnosis DA080. To compute data on diarrhea, we extracted ICD-10 diagnoses DA000 to DA099 (Table).

Data Analyses

From the NPR we extracted information for all Danish children <5 years of age who were hospitalized during January 1994–July 2005 and had diarrheal disease (ICD-10 codes DA000–DA099) as their primary or secondary diagnosis. If several different specific diagnoses of diarrhea, together with the nonspecific diagnosis of diarrhea (A099), were reported during 1 episode of hospitalization, all the different specific diagnoses were counted as unique diagnoses, whereas the nonspecific diagnosis (A099) was ignored. The nonspecific diagnosis of diarrhea counted as a diagnosis only when the hospitalized patient was registered as having nonspecific diarrhea. If a person had been admitted for diarrhea several times, only episodes >7 days apart were included in the study. For children <2 and <5 years of age, we estimated hospitalization incidence rates per 1,000 person-years at risk by using age- and period-specific person-years at risk in the Danish population.

Other studies, mainly from the United States, have demonstrated how many rotavirus-associated hospitalizations are registered as other types of diarrhea (*9,13*). To achieve a more realistic estimate of the number of rotavirus admissions, we applied 2 different approaches based on the number of all-cause diarrhea admissions.

The first approach was an indirect method known as the Brandt estimation method (*14*), which uses external information on the proportion of rotavirus admissions among all-cause diarrhea admissions. In this approach, the monthly number of rotavirus-associated hospitalizations was estimated by multiplying the monthly number of all-cause diarrhea hospitalizations with the month-specific proportion of rotavirus infections identified at a Copenhagen County university hospital during 1977–1978 (*15*).

In the second approach, the number of monthly rotavirus-associated hospitalizations during the rotavirus season was estimated as the registered number of all-cause diarrhea admissions minus the expected level on the basis of the much lower average level of all-cause diarrhea admissions outside the season. The expected number was estimated by using a log-linear Poisson regression model; the dependent variable was the monthly number of all-cause diarrhea admissions outside the season. A Poisson regression model was used because the monthly number of hospitalizations traditionally can be assumed to be Poisson distributed. The log-linear regression form means that the logarithm of the mean parameter for the dependent variable is modeled by a linear combination of the independent variables. Two factors were included in the model as independent variables.

Table. Diarrhea-associated hospitalizations, by cause, for children <5 years of age, Denmark, 1994–2004*

Diagnostic category	ICD-10 code	Hospitalizations				
		Total no. (%)	Annual average	Incidence/1,000 child-years	% 0–23 mo. of age	% Boys
Etiology unspecified						
Presumed infectious	A09	22,475 (69.6)	2,043	6.7	67.8	55.9
Presumed noninfectious	A085	15 (0.1)	–	–	66.7	46.7
Etiology specified						
Viral, nonspecified	A084, A083	6,916 (21.4)	628	1.9	67.8	54.5
Norwalk virus and adenovirus	A081, A082	62 (0.2)	6	–	77.4	58.1
Rotavirus	A080	1,309 (4.1)	119	0.36	79.1	56.4
Bacterial	A00-A05	1,415 (4.4)	129	0.39	63.5	55.9
Parasitic	A06, A07	88 (0.3)	8	–	44.3	56.8
Total		32,280 (100)	2,935	8.9	67.8	55.6

*ICD-10, International Classification of Diseases, version 10; –, too few records to analyze.

The first factor took into account the varying monthly number of children at risk; this was done by including the logarithm of the risk time as a known factor (an offset). The second factor was a secular trend to allow for changes in the incidence during the study period; this was done by including time (months) as a continuous variable. In other words, we applied a log-linear Poisson regression model with number of all-cause diarrhea admissions as the dependent variable and logarithm of risk and time as independent variables. This regression model, based on the level outside the rotavirus season, was then used to estimate the monthly expected number of all-cause diarrhea admissions during the rotavirus season; i.e., the model for outside the season was extrapolated to the rotavirus season. The monthly observed number minus the expected number of all-cause diarrhea admissions during the rotavirus season was taken as an estimate of the monthly rotavirus-associated hospitalizations Estimation was performed within the age groups 0, 1, 2, 3, and 4 years and subsequently summed to achieve the total for children <5 years. The approach was based on 2 assumptions: first, that all rotavirus-associated hospitalizations were registered correctly in the months of July through December, when rotavirus is nonseasonal, and second, that the excess admissions during the annual peak season of diarrhea, January to June, were attributed to a pathogen believed to drive the intraseasonal all-cause diarrhea hospitalizations. The pathogen in this instance is rotavirus because no other gastrointestinal pathogen has yet been identified with the same seasonality.

According to the Danish National Board of Health, the price per hospitalization for diarrhea <4 days is US $1,420 (8,248 Danish krones [DKK] at a November 2006 exchange rate of 583 DKK to US $100). If the hospitalization is extended >3 days, cost is US $277 (1,608 DKK) per 24 hours of added stay (*16*). The costs include all expenditures related to the hospitalization (e.g., hospital bed, healthcare personnel, diagnostic testing, antimicrobial drugs, rehydration treatment, and intensive care). The costs are total costs and cannot be segregated further into the various above-listed expenditure categories (*16*). In Denmark, public hospital healthcare is free of charge. No private alternative is available for hospitalization of children with diarrhea.

Results

Epidemiology of All-Cause Diarrhea and Rotavirus-coded Hospitalizations

We found a total of 32,280 unique diarrhea-associated hospitalizations in Denmark among children <5 years of age from 1994 through 2004. Slightly more boys (55.4%) than girls were hospitalized with diarrhea; median age was 16 months. The number of hospitalizations, regardless of diarrhea agent, remained relatively constant over time (Figure 1). A total of 1,309 admission records (annual average ≈120) contained the rotavirus-specific ICD-10 code; proportions by sex were 56% boys and 44% girls (Table). In children <5 years of age, 79% of admissions for rotavirus had occurred before the age of 2, compared with only 68% of admissions for all-cause diarrhea. Incidence rates of rotavirus-coded admissions peaked twice during early childhood, at 7 and 12 months of age (Figure 2).

Viral infections constituted 85% of diarrhea-associated hospitalizations for which etiology was specified (Table). When studying the frequency of the main ICD-10 gastrointestinal disease categories (bacteria, virus, parasites, and presumed infectious) according to month, the seasonality of admissions coded as presumed infectious disease and virus without etiology each showed a seasonal pattern very similar to that of rotavirus, whereas admissions due to bacteria and parasites showed a more linear pattern throughout the year and bacterial infections showed a tendency to peak in the late summer and fall months of August through October (Figure 3). When comparing the trends for viral admissions without specified pathogen and trends for rotavirus admissions, we observed parallel seasonal trends throughout the entire study period (Figure 1). In all, 85% (1,115/1,309) of all rotavirus admissions and 63% (4,342/6,916) of nonspecified viral infections occurred from January through June, and the monthly numbers of admissions for these disease categories during the study period were significantly correlated (Spearman correlation coefficient 0.39, p = 0.007).

Estimates of Rotavirus-associated Hospitalizations and Direct Medical Costs

Using the Brandt indirect method, we estimated that ≈840 hospitalizations (28.7% of all diarrhea) annually were due to rotavirus. This finding corresponds to ≈2.5 rotavirus-associated admissions per 1,000 children <5 years of age and ≈5.3 per 1,000 children <2 years of age annually. By using Poisson regression, we estimated that ≈780 annual hospitalizations (≈26.5% of all admissions for diarrhea in children) were associated with rotavirus, resulting in ≈2.4 and ≈4.9 annual rotavirus-associated admissions per 1,000

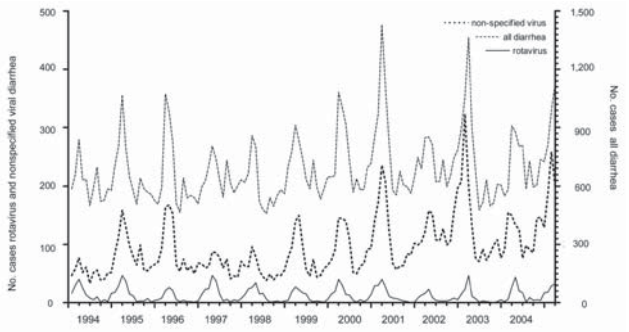

Figure 1. Monthly frequency of diarrhea-associated hospitalizations of children <5 years of age, Denmark, 1994–2005.

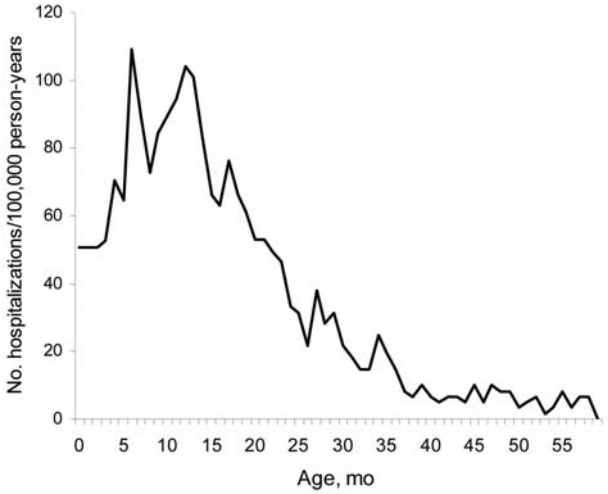

Figure 2. Incidence of rotavirus-coded hospitalizations in children <5 years of age, Denmark, 1994–2005.

children <5 years of age and <2 years of age, respectively. The estimate was only slightly affected by defining the rotavirus season as January through June. Adding December to the season increased the estimate by 24 annual hospitalizations, and removing June from the season decreased the estimate by 8 annual hospitalizations.

During 1994–2004, 82.4% of all-cause diarrhea admissions lasted ≤3 days (n = 26,586), and 17.6% (n = 5,694) lasted >3 days (range 4–30 days). Thus, the median cost for an all-cause diarrhea admission was US $1,375 (8,013 DKK), and total cost for 2,935 annual admissions was therefore ≈US $4.6 million (≈27.1 million DKK) per year.

In terms of duration, 63% of rotavirus-associated hospitalizations lasted ≤3 days; 21%, 4–6 days; 9%, 7–13 days; and 7%, >13 days. By using the Poisson regression model estimate of 780 annual rotavirus-associated hospitalizations, we found the total direct medical cost of rotavirus hospitalizations to be ≈US $1.7 million (≈9.9 million DKK) per year. By using the indirect method estimate of 840 annual rotavirus-associated hospitalizations, we found the direct medical costs to be US $1.8 million (≈10.4 million DKK).

Discussion

A decision to introduce new rotavirus vaccines into Denmark and most other European countries is likely to be based on a vaccine's ability to protect against severe disease and prevent hospitalizations. Due to a combination of underreporting, low prevalence of testing for rotavirus, and misclassification, hospital episode statistics are rarely sufficient for assessment of the extent of national rotavirus-associated disease. Studies from the United Kingdom (*17*) and United States that used hospital discharge data have shown how the proportion of children coded with the specific rotavirus disease code often represents a gross underestimate (*18*). These observations can be explained in part by the combination of poorly defined criteria at most hospitals for requesting rotavirus testing and the fact that physicians' diagnostic objective is often differentiation between the nosocomial highly active transmitting rotavirus and other less infectious agents for the purpose of isolation rather than therapeutic choices.

Our age-specific analyses demonstrated the magnitude of severe rotavirus infections among infants and young toddlers and showed disease peaks at 7 and 12 months of age. We believe the first peak is related to waning maternal antibody levels and the second peak to daycare attendance by Danish children (an effect of crowding and highly infectious transmissible environments).

Studies of seasonal trends of diarrhea-associated hospitalizations have shown that hospitalizations coded as diarrhea of nonspecified viral origin as well as diarrhea of presumed infectious origin have a marked winter peak, which suggests incomplete registration of rotavirus admissions. Our 2 different models estimated the annual rotavirus-associated hospitalizations to be between 780 and 840. We find it likely that the true contribution by rotavirus to all diarrhea-admissions is somewhere in between these numbers—or even higher, as the study from the late 1970s showed that rotavirus was identified among 37% of children admitted at a major Danish county hospital (*15*). A more recent estimate suggests that rotavirus infection constitutes an even higher proportion of diarrhea cases, ≈60% during the rotavirus seasonal months of December through April, but these data are based on a limited sample size of 69 (*11*). An updated prospective study of rotavirus and other diarrhea pathogens among children in Denmark is needed to further specify this estimate.

Despite underreporting and misclassification of rotavirus cases that results in gross underestimation of rotavirus disease, the coding of rotavirus diagnosis is relatively

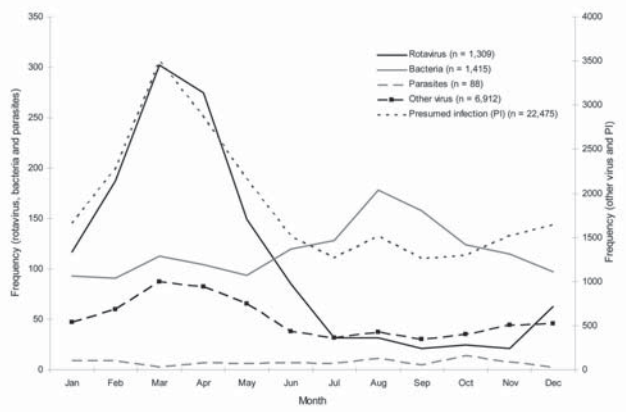

Figure 3. Seasonality of diarrhea-associated hospitalizations of children <5 years of age, Denmark, 1994–2005.

stable over time. The NPR system could be used as a timely and relatively sensitive tool by which to monitor the effectiveness of rotavirus vaccines. However, doing so would require improvements such as complete registrations of rotavirus infections, validation of diagnoses, and implementation of national guidelines for rotavirus sample collecting and testing.

A routine, universal rotavirus immunization program with a vaccine that is 75% effective against infection would prevent ≈45,000 cases of diarrhea annually among Danish children. If effectiveness were 95% against hospitalization, ≈700–800 hospitalizations could potentially be avoided per year, resulting in direct medical cost savings of ≈US $1.6 million. This estimate includes neither the number of nosocomial transmissions or outpatient visits prevented nor the indirect costs incurred when parents are forced to stay home from work to take care of sick children, factors that in western societies are likely more influential than the problem of hospitalization alone (*19*). Weighing the health benefits of vaccination against its costs requires a measure like quality-adjusted life years, which takes both reduced illness and death into account, and subsequent cost-effectiveness analyses in which the healthcare-associated as well as the societal costs are considered.

Our study provides updated information on the extent of disease and the cost of diarrhea- and rotavirus-specific hospitalizations in a European country. These data are often requested by health officials (*20*) to help increase the awareness of rotavirus disease in Europe and help health officials assess the potential benefits of disease prevention through vaccination.

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Strategies to Reduce Person-to-Person Transmission during Widespread *Escherichia coli* O157:H7 Outbreak

Edmund Y.W. Seto,* Jeffrey A. Soller,† and John M. Colford Jr*

During the *Escherichia coli* O157:H7 outbreak in 2006 in the United States, the primary strategy to prevent illness was to advise consumers not to eat spinach. No widespread warnings were issued about preventing person-to-person (secondary) transmission. A disease transmission model, fitted to the current data, was used to investigate likely reductions in illnesses that could result from interventions to prevent secondary transmission. The model indicates that exposure to contaminated spinach occurred early in the outbreak and that secondary transmission was similar to that in previous *E. coli* outbreaks (≈12%). The model also suggests that even a modestly effective strategy to interrupt secondary transmission (prevention of only 2%–3% of secondary illnesses) could result in a reduction of ≈5%–11% of symptomatic cases. This analysis supports the use of widespread public health messages during outbreaks of *E. coli* O157:H7 with specific advice on how to interrupt secondary transmission.

Widespread distribution of contaminated spinach was implicated in an *Escherichia coli* O157:H7 (*E. coli* O157) outbreak in the United States in 2006. As of September 24, 2006, a total of 173 cases had been reported in 25 states; 88% of cases were reported over an 18-day period from August 19 through September 5, 2006 (*1*). The outbreak strain was particularly virulent, resulting in 1 death, 53% of patients being hospitalized, and a 16% rate of hemolytic uremic syndrome. At the time of our analysis, the potential extent of the outbreak was unknown because new cases were still being reported. The Centers for Disease Control and Prevention (CDC) and the US Food and Drug Administration advised consumers not to eat spinach as the primary strategy for protecting against foodborne transmission of *E. coli* O157 (*2*). No warnings, however, were issued regarding the prevention of person-to-person (secondary) transmission.

According to recent studies on the extent of secondary transmission for *E. coli* O157 and other pathogens, the initially reported foodborne illnesses in the outbreak may have represented only a small fraction of a larger outbreak that included asymptomatic infections and secondary infections among household members of infected persons and other close contacts. Specifically, the *E. coli* O157 literature indicates that a large proportion (72%) of infections are asymptomatic (*3*), exposure to low doses can result in infection (*4*), and reported secondary transmission rates are on the order of 4%–16% (*5*). Further, outbreaks of shigellosis (*6*), cryptosporidiosis (*7*), and giardiasis (*8*,*9*) indicate that other highly infectious enteric pathogens can spread from person to person after being introduced into a community through water, food, or other sources (*9*). Recent adenovirus outbreak data indicate that persons with asymptomatic infection who are shedding virus can be a primary cause of continual transmission of infection (*10*). And in a prolonged giardiasis outbreak that occurred in late 2003 in a Boston, Massachusetts, suburb, 30 primary cases of giardiasis attributed to a children's swimming pool resulted in 105 secondary cases among persons from the same or socially related households. New cases of giardiasis continued to occur for up to 4 months after the pool was closed for the season (*9*).

Using an epidemiologically based disease transmission model, we investigated the potential for reducing the

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number of symptomatic infections (cases) of *E. coli* O157 by using interventions designed to reduce secondary transmission during the course of the 2006 *E. coli* O157 outbreak in the United States. We assumed that a combination of possible intervention strategies to interrupt secondary transmission would have a range of possible levels of effectiveness. These strategies would include strongly recommending handwashing, avoiding contact with persons with diarrhea (of any cause), meticulously preparing food, and avoiding work or school when ill with any gastrointestinal sign or symptom. Initiation of these strategies was assumed to occur at the same time as CDC's first press release on the outbreak on September 14, 2006, 1 week later, and 2 weeks later. We assumed that these strategies would reduce the transmission of infection to healthy persons from persons with both symptomatic and asymptomatic infections.

Methods

An existing epidemiologic model for disease transmission (*11*,*12*) was adapted to simulate the 2006 US *E. coli* O157 outbreak. The most recent data from CDC (*13*) were used, along with data from the published literature, to replicate the *E. coli* O157:H7 prevalence estimated by CDC in the United States and the reported outbreak conditions as of September 19, 2006 (*13*), and then to evaluate the potential effect of the timing and effectiveness of interventions on secondary infections.

Our modeling approach is consistent with a large base of literature that describes the use of dynamic population models in the study of epidemics (*14*–*16*) and environmental disease processes (*17*–*19*). Our model consists of 5 population-level epidemiologic states that account for persons who are susceptible (*S*), exposed (*E*), infected but asymptomatic carriers (*C*), diseased (*D*), and postinfection (*P*) (Figure 1).

There are 3 possible routes of exposure that move persons from the susceptible (*S*) to the exposed (*E*) state. These include environment-to-person infection (β_{ep}), person-to-person infection (β_{pp}), and infection through consumption of contaminated spinach ($\beta_{spinach}$). We assume that all infections take some time to manifest signs or symptoms (the incubation period). During this incubation period, persons are in the exposed state. Once sufficient time has passed for infections to become clinically apparent, persons are either carriers (in state *C*) or diseased (*D*). A proportion of the exposed persons move to the carrier state, *C,* at a rate inversely related to the duration of incubation (*20*,*21*). Symptoms develop in the remaining proportion of the exposed population that becomes infected, and these persons move to the diseased state, *D* (*3*). From both the asymptomatic and diseased states, *C* and *D*, persons recover and move to state *P* at a rate inversely proportional to the duration of infection (*22*–*24*). Finally, persons in state *P* become susceptible again, moving to a susceptible state, *S,* at a rate inversely proportional to the duration of immunity.

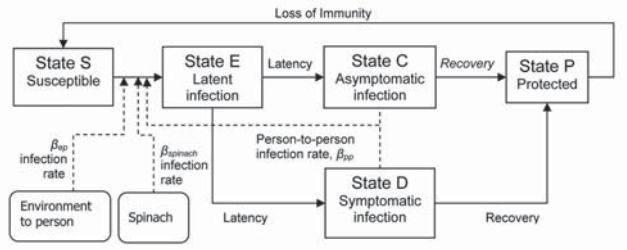

Figure 1. Conceptual model for disease transmission. Solid lines represent movement of persons between epidemiologic states; dotted lines represent routes of exposure.

The model was first calibrated to CDC's estimate of 73,480 annual US cases (*25*) and to reported rates of secondary transmission (*5*,*9*). With the model calibrated to transmission levels for endemic *E. coli* O157, we modeled the additional contribution of cases attributable to the outbreak. We assumed that exposure to contaminated spinach began on August 19, approximately when cases were first identified, and allowed the rate of transmission, ($\beta_{spinach}$), and the number of days of exposure to vary to fit the reported outbreak incidence of 131 cases from August 1 through September 19 with 122 of these cases (93%) occurring from August 19 through September 5 (*13*). The mathematical details of the model and its calibration are described in the online Appendix (available from www.cdc.gov/EID/content/13/6/860-app.htm).

The effect of the timing of interventions on person-to-person transmission with various levels of effectiveness was then evaluated. Three timings for the intervention were considered. The first was assumed to be initiated when CDC issued its first press release on the outbreak on September 14, 2006; the second and third timings were 1 and 2 weeks later, respectively. The interventions were assumed to reduce secondary transmission by 1%–25%. The number of averted cases was computed by comparing the number of cases with and without the intervention.

We also considered a range of possible levels of secondary transmission on the basis of prior reports that person-to-person transmission is responsible for 12% (*5*) and 75% (*9*) of the cases in a community during an outbreak from a highly infectious and transmissible pathogen. Intermediate values of secondary transmission, 25% and 50%, were also considered. Confidence intervals (CIs) for the number of averted cases due to the interventions were computed from 500 Monte Carlo simulations for the various secondary transmission rates. For each Monte Carlo simulation, we randomly sampled parameter values from the uncertainty ranges specified in the Table. Model simulations were implemented in Mathcad 13.0 (*26*).

Table. Disease transmission model parameter values

Description	Model parameter	Values used for base analysis (range*)
Mean duration of incubation†	ζ	5 d (2–8 d)
Probability of symptomatic response	P_{sym}	0.28
Mean duration of infection*	σ, δ	16 d (4–29 d)
Mean duration of protection from infection*	γ	56 d (35–77 d)
Hypergeometric dose response relation	α, β	0.08, 1.44
Nonoutbreak annual disease incidence in United States		73,480 cases
Population	N	275 million persons
Proportion of cases due to person-to-person transmission	k	0.12–0.75
Timing of interventions		0, 1, and 2 weeks after press release
Effectiveness of Interventions	$R(t)$	1%–25%

*Range used for uncertainty analysis.
†Inverse is used as a rate in the model (i.e., units of days^{-1}).

Results

To replicate the relatively large percentage (122 [93%]) of the 131 cases reported from August 19 through September 5, 2006, we assumed that exposure to contaminated spinach likely occurred comparatively early in the outbreak. Fitting the model to the case data was only possible when foodborne exposure occurred before August 22. This date is considerably earlier than CDC's initial press announcement (*27*), which was released 23 days later. If substantial foodborne transmission occurred after August 22, secondary infections would have greatly extended the outbreak period. Moreover, 4 days of exposure was sufficient to result in the outbreak, which suggests that exposures were largely limited to the earliest days of the outbreak. Generally, there was also a narrow window in which the outbreak could have started. For instance, we could also fit the calibration data if we assumed the outbreak started a day earlier, on August 18, with 1–4 days of ensuing exposure.

We were able to replicate the reported case data with our model under the assumption of 12% secondary infections. However, we could not replicate the case data under the assumption of 50% and 75% secondary infections. For both the 50% and 75% secondary infection assumptions, with a very short period of exposure, it was possible to fit 122 new cases of illness between August 19 and September 5; however, simulations of the model resulted in more secondary infections than suggested by the case data. Under the assumption of 25% secondary infections, the model fit the case data well, with only 5 more new infections after September 5 than reported. This result suggests that either secondary transmission was not as high as 50%–75% for this outbreak or the percentage of cases after September 5 was underreported.

Simulated interventions for person-to-person transmission decreased the number of ill persons, with greater reductions for higher levels of secondary transmission (Figure 2). With higher levels of secondary transmission (Figure 2B), the timing of interventions has a greater effect on the number of averted cases. However, with greater secondary transmission, the outbreak period is extended, which allows more time to organize a campaign against person-to-person transmission and a greater opportunity to reduce secondary hospitalizations and deaths.

Considering the number of cases that could be averted by an intervention such as a campaign to encourage handwashing and isolation of persons with diarrhea under a varying range of effectiveness (Figure 3), we found that such programs can be inefficient and still substantially reduce secondary transmission. Even if a campaign were initiated relatively late in the outbreak (day 51), the number of cases would be reduced. The reduction increases exponentially with increasing levels of secondary transmission (Figure 3). Specifically, with 12% secondary transmission, ≈6 (5%) of cases are averted. Averted cases increase to 16 (11%) and 61 (29%) with 25% and 50% secondary transmission, respectively. With 75% secondary transmission, a much larger number of illnesses can be averted (≈225 [57%]). The 95% CIs for the 12% secondary transmission rate scenario were 0.2–19 cases averted (0%–14% of total illnesses); for the 25% secondary transmission rate scenario, CI were 0.5–59 cases averted (0%–38% of total cases).

Discussion

The first mathematical models to analyze the spread and control of infectious diseases were developed in the early 20th century in attempts to understand measles (*28*) and malaria (*29*). This field grew exponentially in the middle of the 20th century. A tremendous variety of models have now been formulated, mathematically analyzed, and applied to infectious diseases (*16*). Mathematical models of disease transmission have become important tools that have led to understanding the transmission characteristics of infectious diseases in communities and better approaches to decreasing the transmission of these diseases (*16*,*30*). We applied such a model to the 2006 spinach-associated *E. coli* O175 outbreak to analyze data as they were still being collected to evaluate the effectiveness of strategies that might reduce person-to-person transmission of infection. The model as constructed allows for investigation across

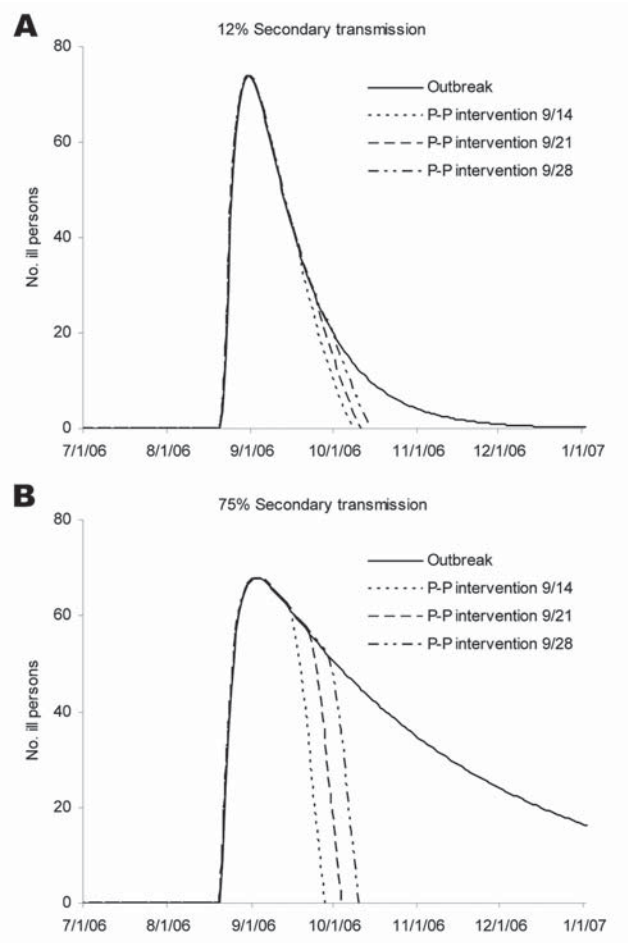

Figure 2. Number of ill persons over time, calibrated to the outbreak case data (131 cases from August 2 to September 19, 2006, with 93% of illnesses from August 19 to September 5, 2006) and to the timing of a person-to-person intervention program under the assumptions of 12% (A) and 75% (B) secondary transmission.

the full range of possible values for all relevant variables, including the rate of secondary transmission and the effectiveness of intervention strategies.

Public health messages about the importance of disease prevention methods (such as frequent handwashing, covering one's mouth when coughing or sneezing, and staying at home when ill) to prevent secondary transmission of infection routinely are conveyed during influenza outbreaks (*31*). Although the prevention of secondary transmission is a specific goal of hospital guidelines for the care of patients with diarrhea, such advice was not a focus of the public health messages disseminated for the 2006 *E. coli* O157 outbreak. We hypothesized that the interruption of secondary transmission might have had a useful role as an additional tool in managing this outbreak. A mathematical model that replicated the published data available as of September 19, 2006, was created. This model was used to examine the effect that various levels of effective interruption of secondary transmission would have on the course of the outbreak.

The model results suggested 2 findings. First, exposure to contaminated spinach apparently occurred early in the outbreak and was likely at low levels after that. Second, this *E. coli* outbreak appears to be more similar to previous *E. coli* outbreaks than to a large-scale 2003 giardiasis outbreak (*8,9*), in terms of person-to-person contribution to the overall outbreak-attributable incidence of infection. Despite wide confidence bounds on our estimates, our findings suggest that even a modestly effective strategy to interrupt secondary transmission (resulting in prevention of 2%–3% of secondary cases) could have resulted in a median reduction of ≈5%–11% of infected and symptomatic persons. Not all secondary infections are averted by the interventions because they are assumed to be initiated relatively late and because they are not completely effective. The number of averted cases, however, increases exponentially with increasing rates of secondary transmission, and the results suggest that these programs would have substantial benefits even if they are implemented relatively late in an outbreak.

Limitations

Several simplifying assumptions were needed to conduct the analysis. These assumptions relate to the form of the disease transmission model, interpretation of the available outbreak data, and treatment of the variability and uncertainty in the data used to inform the model.

With respect to the selected disease transmission model, a variety of model forms can be used to characterize infectious disease transmission and to evaluate the potential for effective interventions. Particular characteristics of each model form capture different aspects of the disease transmission system (*32*). In this analysis, the salient assumption was that the epidemiologic status of the population could be approximated reasonably well with the relatively simple structure of the disease transmission model. Other model structures also might yield additional or alternative insights. For example, if "super-spread" events are important determinants in characterizing the magnitude of disease transmission (*30*) during outbreaks such as this one, stochastic dynamic modeling may be necessary.

Another limitation of our study is the lack of data on efficacy of person-to-person reduction strategies specific for *E. coli* transmission. The person-to-person transmission rate and reduction due to handwashing were assumed to be the same for both symptomatic and asymptomatic persons, and the effect on the course of the epidemic (specifically, the number of cases averted) of these strategies was examined for a wide range of possible levels of effectiveness. Symptomatic and asymptomatic persons were assumed to

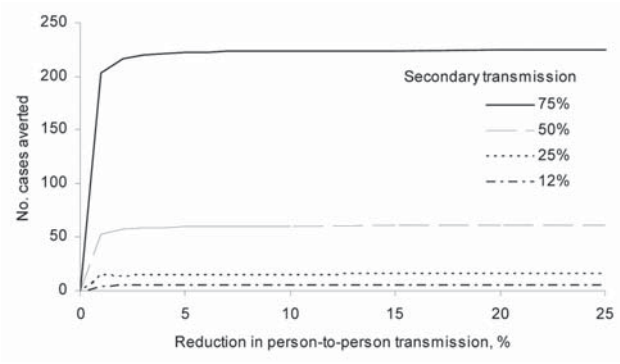

Figure 3. Number of illnesses averted because of a person-to-person transmission intervention for varying rates of secondary transmission and levels of intervention effectiveness. The intervention campaign is assumed to start 1 week after the press release, September 21, 2006 (day 51 of the outbreak).

transmit *E. coli* O:157 to susceptible persons in the same manner, since we have no data to suggest otherwise. It is possible that symptomatic-to-susceptible transmission may be greater than asymptomatic-to-susceptible transmission and that interventions may be more effective among persons with symptoms. Increasing the rate of symptomatic-to-susceptible transmission by 8% over the asymptomatic-to-susceptible rate indeed results in a small increase in averted cases. If new data on the differences between person-to-person transmission rates and/or person-to-person reduction efficacies become available, the parameterization of the model could be improved.

As noted previously, detailed information describing the timing of exposures to *E. coli* O157 through contaminated spinach and subsequent outbreak cases was not yet available at the time of our analysis. Thus, the model is limited by the precision and completeness of the case-reporting data. The model can be updated easily when additional data become publicly available. Despite these limitations, available data were sufficient to suggest that foodborne transmission was terminated early in the outbreak and that interventions to reduce secondary transmission could be very effective.

Finally, in the interest of producing timely results that might influence the control of the outbreak, median parameter values were used to calibrate exposure to the observed case data. Holding these exposures constant, Monte Carlo simulations were subsequently used to explore the variance and uncertainty in the estimates of averted cases. This resulted in large confidence bounds. When more complete case data become available, the data may reduce the uncertainty not only in the timing of the exposure but also in the values of the remaining parameters and the estimate of averted cases. In the past, we have explored such calibrations with Monte Carlo methods (*11*,*33*–*35*).

Public Health Implications

Public health strategies for preventing secondary transmission could include public media campaigns reminding the population of the importance of handwashing, avoiding contact with feces, minimizing nonessential contact with persons with diarrhea, meticulous care when preparing and consuming food, and staying at home from work or school when having any diarrhea during the outbreak period. Any of these strategies could be targeted to communities in which any cases of *E. coli* O:157 had been reported and scaled to regional or national audiences when appropriate. Messages for all of these strategies can be delivered inexpensively to large or targeted populations through a variety of media (television, radio, print, Internet). That public health officials already have the ability to launch rapid and successful infection control messages to the public was demonstrated during the outbreak of severe acute respiratory syndrome (*36*). In the future, public health officials might even rapidly deliver urgent health messages to a large population in a city or region through voluntary preregistration of email addresses as part of an emergency alert network that includes the media and public health officials. At the first appearance of evidence of an *E. coli* O157 outbreak, a message with clear instructions could be distributed to thousands or tens of thousands people at risk locally, regionally, or nationally, and to specific subgroups at high risk, such as the young, the elderly, or the immunocompromised.

We did not formally estimate in our model the economic tradeoffs between a public health campaign to reduce secondary transmission compared with the costs of hospitalizations and medical care for persons with this disease. However, because the hospitalization costs of a single *E. coli* O157 case complicated by death from hemolytic uremic syndrome are estimated to be as high as US $6.2 million per case, we believe that such a campaign would be highly cost-effective (*37*). Given the potential public health benefits to be gained by these actions, and the low costs associated with their implementation, these strategies also may be relevant for outbreaks from other highly infectious pathogenic microorganisms.

The individual effects of these intervention strategies when used alone or in combination to interrupt secondary transmission were not modeled. Rather, we assumed that a combination of strategies would be used and would have some combined benefit. We intentionally examined the possible benefits across a wide range of possible levels of effectiveness. As expected, higher levels of effectiveness resulted in greater impact on the outbreak. However, even fairly low levels of intervention effectiveness (such as 2%–3% interruption in secondary transmission) led to reductions (5%–11%) in the number of cases attributable to the outbreak. Further study is needed to select the individual

secondary control strategies to use if limiting the number of specific prevention strategies is necessary.

Implications for Future Outbreaks

Public health officials have the necessary authority to issue and widely distribute guidelines for preventing secondary transmission. Some readers might question whether our results are a sufficient demonstration to justify a large-scale campaign by public health officials to prevent secondary transmission. If additional proof were demanded, various study designs could be used to evaluate the effectiveness of media campaigns to help to control outbreaks. As 1 example, a relatively simple ecologic study could correlate incidence rates with local press coverage in different communities. A more definitive design, however, would be a randomized, controlled trial. Such a trial might, for example, randomize 50% of the affected areas to a "media-blitz" that explained the importance of handwashing, stool precautions, and other measures including those discussed above; the other communities would receive the public health messages currently delivered during outbreaks. The rapidity with which the outbreak is terminated would be the principal outcome of interest. However, obtaining approval to run such an experiment may be difficult because such a trial would only be ethically justified if the investigators could convince a review panel that sufficient uncertainty about the effectiveness of the intervention exists.

Conclusion

Our analysis of the 2006 *E. coli* O157 outbreak due to contaminated spinach in the United States supports the assertion that health officials should consider rapidly delivering widespread public health messages with specific advice on how to interrupt secondary transmission of *E. coli* O157. The results suggest that such an intervention, even if only modestly successful, could meaningfully reduce the number of cases.

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Bovine Spongiform Encephalopathy and Spatial Analysis of the Feed Industry

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In France, despite the ban of meat-and-bone meal (MBM) in cattle feed, bovine spongiform encephalopathy (BSE) was detected in hundreds of cattle born after the ban. To study the role of MBM, animal fat, and dicalcium phosphate on the risk for BSE after the feed ban, we conducted a spatial analysis of the feed industry. We used data from 629 BSE cases as well as data on use of each byproduct and market area of the feed factories. We mapped risk for BSE in 951 areas supplied by the same factories and connection with use of byproducts. A disease map of BSE with covariates was built with the hierarchical Bayesian modeling methods, based on Poisson distribution with spatial smoothing. Only use of MBM was spatially linked to risk for BSE, which highlights cross-contamination as the most probable source of infection after the feed ban.

In France, meat-and-bone meal (MBM) has been banned from cattle feed since July 30, 1990. However, through January 1, 2007, 957 cases of bovine spongiform encephalopathy (BSE) have been detected in cattle born after the ban. These cases provide evidence that BSE control has not been entirely effective, which poses a concern because BSE is a zoonotic disease, a source of variant Creutzfeldt-Jakob disease (vCJD). Until now, 158 definite or probable cases of vCJD in humans have been detected in the United Kingdom (www.cjd.ed.ac.uk/figures.htm, consulted March 12, 2007) and 21 in France (www.invs.sante.fr/recherche, consulted March 12, 2007). The risk for humans is controlled by removing specified risk materials from human consumption (since 1996 in France, later in other European countries) and testing all cattle at the abattoir with a rapid test (since 2001 in continental European Union). However, these measures are expensive. Achieving 100% control of the spread of BSE is a major challenge, important for human health but limited by economic constraints.

The main hypothesis concerning the source of infection in cattle born after the MBM ban still involves MBM; the BSE agent may have entered cattle feed by cross-contamination with feed for monogastric species (pigs and poultry) in which MBM was still authorized until November 2000. Cross-contamination could have occurred within factories, during feed delivery to the farm, or on mixed farms that have cattle and pigs or poultry. This hypothesis is supported by the finding of MBM traces in cattle feed (*1*) as well as by epidemiologic studies that showed a spatial link between density of monogastric species and risk for BSE (*2*–*7*).

Another hypothesis, however, suggests the role of other animal byproducts such as fat and dicalcium phosphate (DCP) derived from bones, which were not prohibited in cattle feed before 2000. Such components might have been contaminated by the BSE agent during cattle slaughter (*1*). Clauss et al. (*5*) found a statistically higher use of milk replacers (which contain animal fat) for calves on BSE-affected farms in Germany, and the same type of association was observed in France for scrapie in sheep (*8*).

More knowledge about these factors is critical for the management of the BSE risk, as the BSE epidemic decreases and pressure increases to release progressively more stringent control measures. Risk for BSE was spatially heterogeneous in France for the infected cattle born after the ban (*9*), meaning that the source of infection might be spatially heterogeneous. If animal byproducts were a source of BSE for cattle born after the ban, we would expect a higher risk for BSE in areas with higher use of those animal byproducts in feed. We therefore investigated geographic

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variations in the use of animal byproducts in feed factories (MBM for monogastric species, animal fat and animal DCP for cattle) and explored their spatial link with risk for BSE in the market areas of the factories.

Methods

Data Collection

BSE Cases

BSE cases are recorded in the BSE database of the Agence Française de Sécurité Sanitaire des Aliments (AFSSA) in Lyon, France. The period we considered for case detection was July 1, 2001, through December 31, 2005. This choice enabled us to obtain precise and comparable data because during this period detection of BSE was based on the mandatory reporting system and on the comprehensive active surveillance program (*10,11*); these 2 systems are complementary and ensure screening of every bovid ≥24 months of age, dead or slaughtered.

Because feed produced before the ban was not recalled and to account for possible stocks of feed, our study was restricted to BSE-infected cattle born after January 1, 1991. As shown in previous studies (*12,13*), the source of infection for cattle born after the ban and for those born after a complementary reinforced ban implemented in 1996 (www.agriculture.gouv.fr/esbinfo/esbinfo.htm, consulted September 7, 2006) appear to be the same. Therefore, to increase the statistical power of the study, we combined infected cattle born after the ban with those born after the complementary reinforced ban.

BSE cases considered in the analysis were in clinically suspected animals confirmed at AFSSA with Western blot or immunochemical tests and in animals with positive test results (same techniques) among the entire cattle population tested within the active surveillance program (*11*). The location of the BSE-infected cattle was defined by the center of the commune (French community, 36,582 units, average 15 km²) of the farm in which the animal had been raised during the 6–12 months after birth; according to modeling results, this period corresponds to the highest risk for infection (*14,15*).

Cattle Characteristics and Demographics

The background population was based on demography of female adult bovids available at the canton level (3,705 units, average 150 km²). Data were obtained from the Agricultural Census 2000 (CD-ROM, edited by Agreste, 251 rue de Vaugirard, 75732 Paris, France). Because BSE incidence varies according to production type, the cattle population was divided into dairy and beef (*3,16,17*); incidence was defined by breed of the animal or production type of the farm (when breed was not recorded or when animal was a mixed breed).

Factory Data

From March 2004 through June 2005, the Ministry of Agriculture (Direction Générale de l'Alimentation) used a questionnaire designed by the authors to investigate factories that produced compound feed for cattle. The period of interest for the questionnaire was January 1, 1991, through November 2000 (date of the total ban of MBM and byproducts in feed for all species). The 4 characteristics used in the analysis were market area (geographic area in which the factory was delivering feed), use of MBM in feed for monogastric species, use of animal fat in cattle feed, and use of animal DCP in cattle feed. Factories were located by the commune center. Canton perimeters and commune centers were provided by GEOFLA France Métropolitaine (Institut Géographique National, Paris, France; version 6; 2002).

Mapping Use of Byproducts in Feed Factories

Each factory had a unique market area; market areas varied in size and partly overlapped. To handle this complex situation, we listed all factories that delivered feed in each canton; then we used ArcView GIS (ESRI Inc. Redlands, CA, USA) to perform a spatial aggregation of all cantons that used the same set of factories and called this a delivery area. From 327 market areas, this process divided the territory into 943 delivery areas. Consequently, each delivery area could be considered homogeneous for the risk related to factories. For each byproduct (MBM, animal fat, animal DCP), exposure of cattle in a given delivery area was estimated by the proportion of factories that used this byproduct in the area (number of factories that used the byproduct divided by total number of factories in the area). The exposure of each area to each byproduct was then classified in quintiles and mapped. Use of quintiles enabled us to have a central class. To improve the legibility of the map, we smoothed it by using a spatial interpolation (kriging) of the values of the exposure in the delivery areas.

BSE Risk and Spatial Link with Use of Byproducts

To assess the relative risk for BSE—comparing risk in a given delivery area with average risk in the nation—we modeled the distribution of the number of BSE cases, taking into account the bovine demography in each area and the geographic adjacency of areas (*18–21*). The method is explained in detail in the online Technical Appendix, available from www.cdc.gov/EID/13/6/867-Techapp.htm. A basic model (model 0) without covariate (factor explaining the risk) was assessed to represent the risk for BSE in each area, following a method used previously (*9*); the relative risk for BSE in the area was then classified in quintiles, as was risk for exposure.

The crude link between exposure to each byproduct and risk for BSE was assessed by the crossing of the 5 lev-

els of the relative risk for BSE and the 5 levels of the exposure to MBM, animal fat, and animal DCP, respectively, which was reported in a 5×5 array table. The 25 cells of the table were classified in 5 groups on which the mapping of the crude link was based. Groups 1 and 2 represented a concordant relationship between the relative risk for BSE and exposure, i.e., high exposure with relative risk for BSE >1 on the one hand, and low exposure with relative risk for BSE <1 on the other. Groups 3 and 4 represented a discordant relationship, i.e., high exposure with relative risk <1 and the converse, and group 5 was intermediate. Furthermore, the crude link between the use of each byproduct in the area and the relative risk for BSE was tested with a Spearman rank correlation unilateral test.

Finally, the adjusted link between exposure to each byproduct and risk for BSE was assessed by including covariates in the hierarchical Bayesian model. We successively incorporated all covariates with a significant crude link with BSE, following a method used in a previous work (*22*) and shown in detail in the online Technical Appendix.

Sensitivity Analysis Regarding Missing Data

To test the effect of possible bias due to missing data, we conducted a sensitivity analysis. We assumed that missing data were randomly distributed for animal fat and animal DCP, which were still authorized in cattle feed until November 2000. For MBM, however, we hypothesized that lack of answer could be a way to escape the question; because MBM was prohibited for cattle, cross-contamination of cattle feed with MBM in feed for monogastric species should in fact be the responsibility of manufacturers that were not successful in controlling such a risk within their factories. So we tested a fictitious situation under a worst-case scenario: all missing data about MBM would be related to use of MBM in feed for monogastric species. The crude link between the use of MBM and the relative risk for BSE was mapped under this scenario and tested with a Spearman rank correlation unilateral test.

Results

Use of Byproducts in Feed Factories

We identified 327 factories that were producing compound feed for cattle during the study period (Figure 1). From the market areas, we divided the territory into 943 delivery areas (Figure 1), 7–9,734 km² (average 578 km²); 4–33 (average 16) factories delivered in a given area. Factories that did not answer questions on a given risk were excluded from analysis, except for the sensitivity analysis. The final sample with complete data was composed of 255 factories for the risk concerning MBM, 248 for animal fat, and 220 for animal DCP. In the delivery areas, the proportions of factories that used animal byproducts (Figure 2) were 16.7%–71.4% for MBM, 5.3%–68.8% for animal fat, and 13.3%–88.9% for animal DCP.

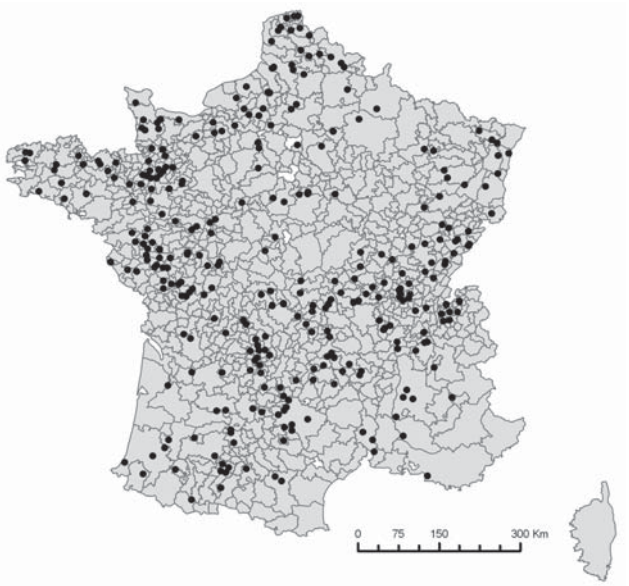

Figure 1. Location of the 327 feed factories that produce compound feed for cattle and location and size of the 943 delivery areas. Unshaded delivery areas were excluded from analysis because they did not contain cattle.

Relative Risk for BSE and Crude Link with Factory Variables

From July 1, 2001, through December 31, 2005, 525 BSE-infected cattle born after the ban and 104 born after the complementary reinforced ban were detected in France (Figure 3A). Among these 629, 505 were detected in the dairy cattle population (4.2 million) and 124 in the beef cattle population (4.3 million). The relative risk for BSE, based on the model without covariate (Table, model 0), varied between 0.49 and 2.29, depending on the area (Figure 3B). The maps of the crude link between relative risk for BSE and factory variables (Figure 4) show the concordant and discordant areas between the relative risk for BSE and use of each byproduct.

According to the Spearman rank correlation unilateral test, only use of MBM ($\rho = 0.43$, $p<0.001$) and use of animal fat ($\rho = 0.39$, $p<0.001$) appeared significantly linked to the relative risk for BSE. For MBM, the link remained the same ($\rho = 0.43$, $p<0.001$) when we performed the sensitivity analysis and replaced missing data with "yes"; we therefore eliminated missing data from the analysis. Because the link between animal DCP and the relative risk for BSE was not significant, this covariate was not incorporated into the disease mapping models.

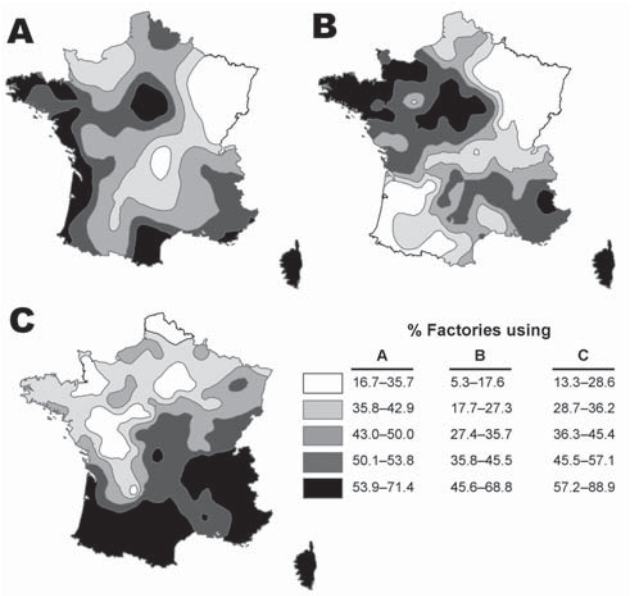

Figure 2. Mapping of the proportion of factories using meat-and-bone meal for monogastric species (A), animal fat for cattle (B), and animal dicalcium phosphate for cattle (C) in the delivery areas. The legibility of the maps was improved by smoothing with a spatial interpolation of the exposure level in the delivery areas.

Disease Mapping Models

The Table presents the set of models assessed with the covariates MBM and animal fat. We deduced from the lowest deviance information criterion (DIC) value of models 1 (ΔDIC = –5.0) and 3 (ΔDIC = –3.0) that only use of MBM significantly influenced the relative risk for BSE. The results of conformity tests $H_0 : \beta_1 = 0$ were significant ($p<0.03$). The model quantified the effect of the risk factor: the risk for BSE was 3.8× higher in a delivery area in which 100% of the factories used MBM compared with another area in which none of the factories used MBM.

Discussion

We used spatial analysis to explore the link between use of 3 byproducts (MBM, animal fat, animal DCP) in factories that produced cattle feed and the relative risk for BSE for animals born after the ban of MBM in cattle feed in France. Among 327 factories, questionnaires were incomplete for 72 (22%) for the use of MBM, 79 (24%) for animal fat, and 107 (33%) for animal DCP. Our hypothesis for missing data about animal DCP was that manufacturers did not know the answer because they often bought premix with preincorporated minerals. Therefore, lack of responses should not be biased and should not affect the analysis. We applied the same hypothesis to absence of information bias for the use of animal fat because this byproduct was allowed in cattle feed and manufacturers would have no reason to hide data. For MBM, the hypothesis of a possible information bias (because MBM was banned from cattle feed) was tested in a sensitivity analysis using a worst-case scenario; this scenario did not change the result, so a possible information bias, if any, should not have modified the results. The huge regional differences in the proportion of factories using MBM, animal fat, and animal DCP might have different explanations, including the local supply, which is linked to local production or import availability, and the differential interest in using each of these compounds for feed for different species whose densities vary in this French territory.

The main result of the spatial analysis provides evidence of a significant adjusted spatial link between factory use of MBM for monogastric species and the relative risk for BSE. This result favors the effect of cross-contamination of cattle feed with MBM-containing feed for monogastric species as a source of BSE for cattle born after the ban of MBM. A recent epidemiologic study in France (*23*) clearly showed that cattle that consumed feed from factories were at risk for BSE after the feed ban; it also showed that mixed farms were at a higher risk for BSE, which indicates that cross-contamination has possibly occurred on farms (by feeding monogastric-species feed to bovines). These findings are in agreement with our results; both studies complement each other and raise the question of effectiveness of the ban that was initially restricted to bovines and belatedly extended to other species to reduce cross-contamination.

Our study did not implicate animal fat as a source of infection. However, we cannot exclude a minor effect, which would be impossible to prove given the power of the study. Animal fat is considered potentially infectious because of the solubility of prions (*24,25*) and the possible contamination with protein impurities by contact with other infectious materials at the slaughterhouse. Animal fat is incorporated in cattle feed in milk replacer and in proprietary concentrates. Clauss et al. (*5*) identified milk replacer as a potential risk factor for BSE in Germany, of importance

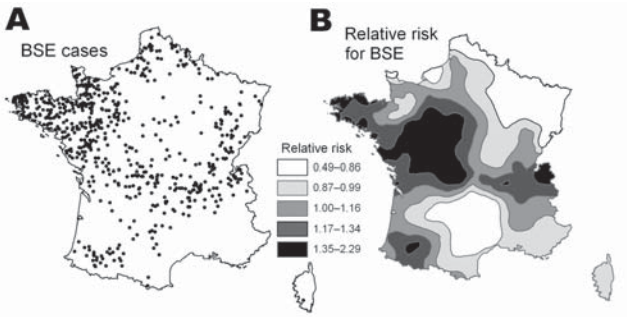

Figure 3. Location of the 629 bovine spongiform encephalopathy (BSE) cases under study (A) and disease mapping of the relative risk for BSE compared with the average national risk (B). For improved legibility, map B was smoothed using a spatial interpolation of the relative risk for BSE in the delivery areas.

Table. Estimations of regression parameters of the disease mapping model with covariates meat-and-bone meal and animal fat*

Model	$\log \lambda_i = e_i +$	Estimations of β	Prediction interval	Odds ratio†
0	u_i			
	DIC = 1596			
1	$u_i + \beta_0 + \beta_1 MBM_i$	$\hat{\beta}_0 = -0.679 \pm 0.03$	[–1.243, –0.116]	
	ΔDIC = -5.0	$\hat{\beta}_1 = 1.337 \pm 0.007$	[0.091, 2.562], p = 0.017	3.8
2	$u_i + \beta_0 + \beta_1 FAT_i$	$\hat{\beta}_0 = -0.329 \pm 0.002$	[–0.8 , 0.131]	
	ΔDIC = –1.0	$\hat{\beta}_1 = 0.786 \pm 0.007$	[–0.674, 2.230], p = 0.145	n.s.
3	$u_i + \beta_0 + \beta_1 MBM_i + \beta_2 FAT_i$	$\hat{\beta}_0 = -0.701 \pm 0.03$	[–1.315, -0.094]	
	ΔDIC = –3.0	$\hat{\beta}_1 = 1.296 \pm 0.008$	[0.01, 2.686], p = 0.035	3.7
		$\hat{\beta}_2 = 0.124 \pm 0.009$	[–1.456, 1.683], p = 0.438	n.s.

*λ_i,parameter of the Poisson distribution for area *i*; e_i expected number of bovine spongiform encephalopathy cases for area *i*; β, set of regression parameters to link the relative risk to the covariates; u_i, spatial component of smoothing for area *i*; *MBM*, meat-and-bone meal; *FAT*, animal fat; n.s., not significant. Variations of the deviance information criterion (ΔDIC) were calculated in comparison with the DIC value of model 0. The 95% prediction intervals were based on the quantiles of the Markov Chain Monte Carlo sample.
†Odds ratio was computed as exp(β); it compares the bovine spongiform encephalopathy risk for a 100% difference in the use of MBM in the area.

comparable to proprietary concentrates; the case-control study carried out in France (*23*) also found an effect of consumption of milk replacer, but to a lesser extent. Regardless, distinguishing the specific effect of milk replacer and proprietary concentrate in these studies was difficult.

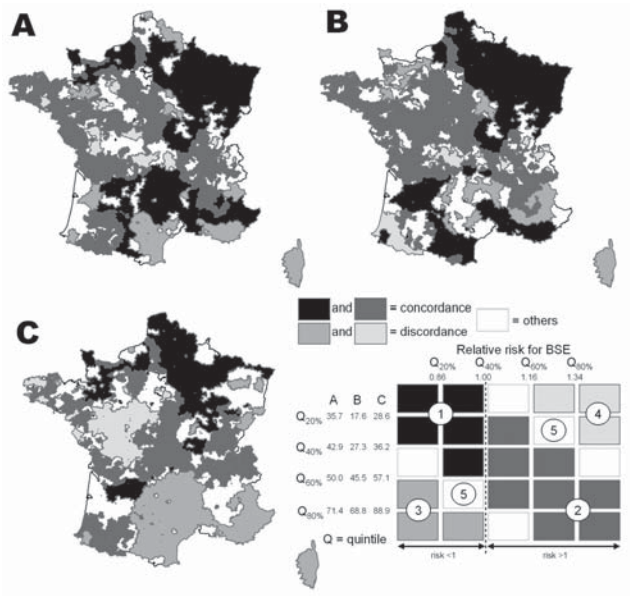

Figure 4. Mapping of the crude link between the relative risk for bovine spongiform encephalopathy (BSE) and the exposure to meat-and-bone meal (A), animal fat (B), and animal dicalcium phosphate (C). In the left part of the key are the limits of the quintiles for each type of exposure (expressed in percent of factories using the byproduct). In the rest of the key, for each type of exposure, groups 1 and 2 represent a concordant relationship between the relative risk for BSE and each type of exposure (high exposure with relative risk for BSE >1, and low exposure with relative risk for BSE <1); groups 3 and 4 represent a discordant relationship (high exposure with relative risk <1 and the contrary); group 5 is intermediate.

Concerning animal DCP, our study showed no effect of its use in compound feed for cattle; however, we did not take into account mineral and vitamin compounds fed to cattle, which can incorporate animal DCP as well. Our results agree with those of the case-control study (*23*), which did not provide evidence that use of mineral and vitamin compounds affect risk for BSE; the authors considered that the implication of animal DCP as a source of BSE, if it existed, should have been marginal. In contrast, a risk analysis by the European Food Safety Agency (www.efsa.europa.eu/en/science/biohaz/biohaz_opinions/1440.html, consulted 7 September 2006) highlighted the potential role of animal DCP in cattle infection, which might be the same order of magnitude as the residual risk from cross-contamination with MBM. Our results do not support this assessment; further studies would be useful.

Our spatial study highlighted the role of MBM as a source of BSE after the ban of MBM for cattle, through cross-contamination in feed factories. If we exclude deliberate use of MBM in feed for cattle (banned since 1990), our findings indicate that feed manufacturers did not implement sufficient measures to avoid cross-contamination during feed processing. Different key points were identified by the French Ministry of Agriculture (B. Thiebot and X. Delomez, pers. comm.) as minimizing risk for cross-contamination. Some can be implemented quickly, such as the sequence of processing, namely, banning the processing of feed for monogastric species just before feed for cattle. However, others are more difficult to implement, such as automatic computation of formula, automatic computation of the sequence of production, and automatic incorporation of unsold products in feed. The ultimate way to eliminate cross-contamination is to have a complete partition between the feed-processing chains dedicated to monogastric species and to ruminants, a huge investment for the feed

industry with low profit margins. Given the situation in the field, the results of our study indicate that the total ban of MBM for farm animals in November 2000 was essential for controlling the spread of BSE.

In the current context of the decreasing epidemic, economic pressure is increasing to release the ban of MBM in feed for monogastric species. The prerequisite, from an animal and human health perspective, is 100% efficient control of the risk for cross-contamination at the factory level and elsewhere. Releasing any control measure would need comprehensive cooperation with the feed industry to adapt their production units, which cannot be achieved in the short term.

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Dr Paul is a researcher at the French National Institute for Agricultural Research. Her primary research interest is spatial epidemiology and health geography applied to emerging animal diseases.

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Minority-Variant pfcrt K76T Mutations and Chloroquine Resistance, Malawi

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Genotyping of the chloroquine-resistance biomarker pfcrt (*Plasmodium falciparum* chloroquine resistance transporter gene) suggests that, in the absence of chloroquine pressure, *Plasmodium falciparum* parasites in Malawi have reverted to chloroquine sensitivity. However, malaria infections in Africa are commonly polyclonal, and standard PCRs cannot detect minority genotypes if present in <20% of the parasites in an individual host. We have developed a multiple site–specific heteroduplex tracking assay (MSS-HTA) that can detect pfcrt 76T mutant parasites consisting of as little as 1% of the parasite population. In clinical samples, no pfcrt 76T was detected in 87 pregnant Malawian women by standard PCR. However, 22 (25%) contained minority-variant resistant genotypes detected by the MSS-HTA. These results were confirmed by subcloning and sequencing. This finding suggests that the chloroquine-resistant genotype remains common in Malawians and that PCR-undetectable drug-resistant genotypes may be present in disease-endemic populations. Surveillance for minority-variant drug-resistant mutations may be useful in making antimalarial drug policy.

Drug-resistant *Plasmodium falciparum* malaria continues to be a growing health problem throughout most of the world (*1*). To combat this threat, governments and aid agencies need accurate drug resistance surveillance data. The World Health Organization has stressed the need for methods of detecting molecular markers of drug resistance that will be useful in predicting responses to both clinical and public health interventions (*2*). This has been difficult in highly malaria-endemic areas, where infections are almost always polyclonal (*3*,*4*). In patients with polyclonal infections, small drug-resistant parasite populations (minority variants) may be masked by larger drug-sensitive populations because standard PCRs are relatively insensitive to minority variants (*2*). Therefore, new methods capable of detecting these subpopulations may lead to better drug resistance surveillance and provide a better tool to predict outcome.

We describe a new multiple site-specific heteroduplex tracking assay (MSS-HTA) for detecting the pfcrt (*Plasmodium falciparum* chloroquine resistance transporter gene) K76T mutation. This mutation in a putative transporter gene is well-associated with chloroquine resistance in *P. falciparum* (*5*). The MSS-HTA was compared with a standard allele-restricted PCR (ARPCR) in clinical samples from Malawi, a country where standard PCR analyses and a recent clinical trial have suggested that chloroquine-resistant malaria has disappeared (*6*–*9*).

Materials and Methods

Study Samples

Informed consent, as approved by the ethics committees of the University of North Carolina and the Malawi College of Medicine/Ministry of Health, was obtained from all participants in this research study. The Malaria and HIV in Pregnancy Study (MHP) patient samples originated from a study of pregnant women attending Queen Elizabeth Central Hospital, an urban hospital in Blantyre. The complete characteristics of the cohort and study design have been described elsewhere (*10*). The Mpemba and Madziabango (MM) patient samples were also collected from pregnant women as part of a pilot randomized, open-label, efficacy study of intermittent preventive treatment in pregnancy at rural health clinics. The diversity of these infections, as well as the cohort and study design, has been described elsewhere (*3*). The characteristics of the patient samples used in this study are outlined in Table 1. All samples

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Table 1. Characteristics of patient samples*

Characteristic	Value (range)
Age (y)	
Total	21.9 (15–35)
MHP patients	23.7 (17–31)
MM patients	21.2 (15–35)
Parasite density (no. parasites/200 leukocytes)	
Total	243.6 (4–2,202)
MHP patients	599.7 (4–2,202)
MM patients	83.3 (5–750)
Gravidity	
Total	2.26 (1–9)
MHP patients	2.37 (1–3)
MM patients	2.21 (1–9)
Anemia (hemoglobin <11 g/dL)	
Total	64/87 (73.6%)
MHP patients	21/27 (77.8%)
MM patients	43/60 (71.7%)
HIV status	
Total†	38/58 (65.5%)
MHP patients	27/27 (100%)
MM patients	11/31 (35.5%)
Clinical symptoms‡	
Fever	14/27 (51.8%)
Headache	18/27 (66.7%)
General body pain	13/27 (48.1%)
Preventive measures‡	
Bed net use§	6/19 (31.6%)

*MHP, Malaria and HIV in Pregnancy; MM, Mpemba and Madziabango.
†29 patients declined testing.
‡Data only available for MHP patients.
§No data available for 8 patients.

analyzed in this study were from filter paper blood spots of peripheral blood.

Malaria DNA Stocks

All malaria DNA used in the experiments, other than clinical samples, was from MR-4 (www.mr4.org). Wild type (pfcrt K76) was from *P. falciparum* strain 3d7 (MR-4, MRA-102G). Two strains of mutant DNA (pfcrt 76T) were used in these experiments: *P. falciparum* strain K1 (MR4, MRA-159G) was used for the CVIET-resistant haplotype, based on the amino acid sequence from codon 72 to codon 76, and *P. falciparum* strain 7g8 (MR4, MRA-152G) was used for the SVMNT-resistant haplotype.

Generation of Heteroduplex Tracking Assay (HTA) Probe

Wild-type *P. falciparum* DNA was amplified with a Peltier thermal cycler (MJ Research, Waltham, MA, USA) in a volume of 50 μL. The reaction conditions consisted of 5 μL DNA, 2.5 U HotStar *Taq* DNA polymerase (QIAGEN, Valencia, CA, USA), 5 μL 10× PCR buffer, 1 μL deoxynucleoside triphosphate mix (catalog no. U1511, Promega, Madison, WI, USA), and 2,000 nmol/L forward and reverse primers CRT HTA F: 5′-GGAAATGGCTCACGTTTAGG-3′, and CRT HTA R: 5′-TGTGAGTTTCGGATGTTACAAAA-3′. This reaction was amplified by preheating to 95°C for 15 min, followed by 35 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min. The reaction was completed with a 10-min hold at 72°C. The 250-bp PCR product was cloned into a PCR2.1 TOPO plasmid as described in the protocol of the Topo TA cloning kit (Invitrogen, Carlsbad, CA, USA), and the sequence was confirmed at the University of North Carolina-Chapel Hill Automated DNA Sequencing Facility. Mutations were randomly introduced into the construct at the –3, –1, +1, and +3 nt relative to the single nucleotide polymorphism involved with the pfcrt K76T mutation using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) (*11*). Potential probes were then amplified by colony PCR under the conditions noted above.

Potential probes were screened against wild-type and resistant PCR amplicons by using a heteroduplex mobility assay on an 8% polyacrylamide gel in 1% Tris-borate-EDTA buffer. After an annealing reaction, probes were evaluated for differential migration between the lanes containing the different amplicons. The annealing reaction containing 4 μL PCR product from a colony of a potential probe mixed with 4 μL PCR product of control DNA, 1 μL 100-pmol CRT HTA F primer, 1 μL 100-pmol CRT HTA R primer, and 2 μL 6× loading dye (Promega) was heated to 95°C for 2 min and then allowed to cool at 25°C for 5 min. The annealing reaction was then loaded into the wells of a nondenaturing acrylamide gel and run at a constant current of 17 mA for 5 h per gel with an SE 600 Gel Electrophoresis Unit (Amersham Biosciences, Piscataway, NJ, USA). DNA was visualized by UV after staining in an ethidium bromide solution for 20 min and destaining in double-distilled H_2O for 15 min. In all, 77 potential probes were screened. The successful probe was sequenced and showed a –1 A to C mutation (GenBank accession no. submission in process).

The plasmid containing the probe was harvested by using Promega Wizard Minipreps (Promega) and then amplified according to the conditions noted above. The PCR product was the blunt-end cloned into the pT7Blue vector with the Perfectly Blunt Cloning kit (Novagen, Inc., Madison, WI, USA). The probe was radiolabeled according to the methods of Ngrenngarmlert et al. (*12*).

HTA

The MSS-HTA was performed under the conditions noted by Ngrenngarmlert et al. with some modifications (*12*). An annealing reaction consisting of 4 μL PCR product (either a control or sample DNA) was mixed with 1 μL 10× annealing buffer (1 mol/L NaCl, 100 mmol/L Tris-HCl, pH 7.5, 20 mmol/L EDTA), 2 μL 6× loading dye, 500 nmol/L CRT HTA F primer, 500 nmol/L CRT HTA R primer, and

1 μL radiolabeled probe in a total volume of 12 μL. The annealing reaction and electrophoresis were carried out under the same conditions as the heteroduplex mobility assay noted above. All MSS-HTA gels included the following controls: water, a nontemplate control PCR, and PCRs from the 3 genomic DNA stocks. The gels were dried onto filter paper (Whatman, Florham Park, NJ, USA) and exposed to BioMax MR X-ray film (Eastman Kodak, Rochester, NY, USA) for ≈48 h at 25°C. In addition, the gels were exposed to a phosphorimager screen for 48 h, and band intensities were quantified by using a GE Storm 860 Phosphorimager (Amersham Biosciences) and ImageQuant version 5.2 software (Molecular Dynamics, Sunnyvale, CA, USA).

ARPCR

ARPCR detection of pfcrt 76T was performed according to the methods described by Djimde et al. (*13*). The primers for the assay were modified according to Wilson et al. (*8*).

Minority Variant Detection

MSS-HTA and ARPCR were both run against mixtures of control DNA in quadruplicate. Differing proportions of wild-type genomic DNA and CVIET-resistant haplotype genomic DNA were mixed to a final sample concentration of 0.1 ng/μL. If a band was not visible to the eye, or only visible in 1 replicate, it was not counted.

Detection of Minor Variants in Clinical Samples

MSS-HTA was used to screen clinical samples in duplicate. All MHP samples were assayed by MSS-HTA and ARPCR. MM samples were all initially assayed by MSS-HTA. ARPCR was performed on all samples positive by HTA and on a random selection of 20 MSS-HTA–negative samples.

Ten samples that were positive by MSS-HTA were selected and Topo TA–cloned (Invitrogen). Twenty-five colonies from each of these 10 samples were screened by colony real-time PCR to determine if the plasmid construct contained a wild-type or resistant pfcrt insert (*8*,*12*). A selection of 2 mutant and 4 wild-type plasmids isolated from these colonies was then sequenced.

Results

DNA from standard culture strains was used. The MSS-HTA probe formed heteroduplexes with different mobilities when annealed to *P. falciparum* DNA amplicons from wild-type parasites (Figure, panel A, lane C) and from parasites containing each of the 2 major resistant haplotypes: SVMNT (Figure, panel A, lane D) and CVIET (Figure, panel B, lane E).

The sensitivities of MSS-HTA and ARPCR to detect subpopulations of resistant pfcrt were tested by using artificial mixtures of wild-type and mutant genomic *P. falciparum* DNA. MSS-HTA detected mutant CVIET variants comprising as little as 1% of the total population (Figure, panel A, lanes N and O). In contrast, the allele-restricted PCR could not detect mutants comprising <20% of the total population (Figure, panel B, lanes I and J). In addition, the MSS-HTA accurately and reproducibly quantified mutant populations comprising as little as 1% of the sample (Table 2).

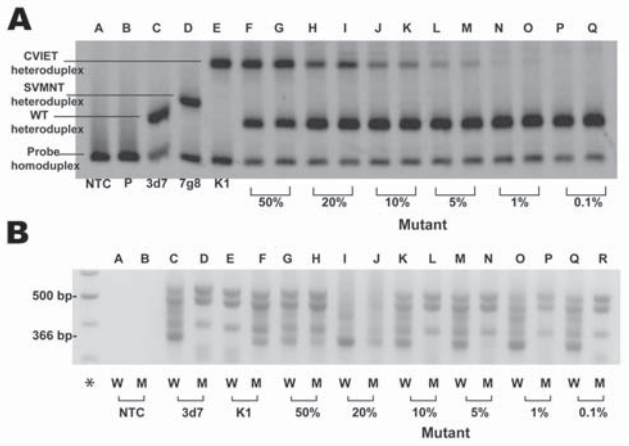

Figure. Evaluation of sensitivity of multiple site–specific heteroduplex tracking assay (MSS-HTA). A) MSS-HTA tested against known concentrations of *Plasmodium falciparum* DNA. Visible bands representing mutant DNA remain until the 1% population (lanes N and O). B) the same dilution series assayed with allele-restricted PCR, where visible mutant bands (366 bp) are not seen past the 20% mutant population (lanes I and J). The lanes marked with W and M represent wild-type and mutant restricted reactions, respectively. *, base pair ladder; P, probe alone.

The 2 assays were then applied to clinical samples from malaria-positive Malawian pregnant women (Table 3). In total, 87 clinical samples were screened. Twenty-seven samples (MHP) were collected as part of a study of pregnant women conducted in Blantyre. CVIET-resistant haplotype *P. falciparum* DNA was detected in 1 sample (3.7%) by MSS-HTA and in none by ARPCR. In addition, 60 samples (MM) from 2 rural health centers were initially screened with the MSS-HTA. This method detected CVIET-resistant haplotype DNA in 21 (35%) of the clinical samples. In these samples, the amount of mutant genotype was quantified with the phosphorimager and averaged 3.3% (SD 1.4, range 1.1%–8.3%) of the parasite population. ARPCR was conducted on all samples positive by MSS-HTA as well as 20 random samples that were negative by MSS-HTA and failed to detect any samples with mutant DNA.

To confirm the presence of mutant DNA in the samples, 10 samples positive by MSS-HTA were cloned and 25 colonies from each sample were screened by real-time PCR. Of the 250 screened colonies, 6 (2.4%) had the mutant genotype in the plasmid construct. Two mutant and 4

Table 2. Sensitivity testing of HTA on mixes of genomic DNA at known concentrations*

	Phosphorimager data			
% Mutant	Average wild-type (%)	SD weight	Average mutant (%)	SD mutant
50	48.1	2.39	51.9	2.39
20	77.0	2.56	23.0	2.55
10	88.9	2.34	11.1	2.34
5	94.8	0.95	5.2	0.95
1	98.7	0.22	1.3	0.22
0.1	100	0	ND	ND

*HTA, heteroduplex tracking assay; ND, none detected.

wild-type plasmid constructs were then sequenced to confirm the MSS-HTA results. All of the mutant plasmid constructs that were sequenced contained the CVIET-resistant haplotype, and none of the wild-type plasmid constructs contained the single nucleotide polymorphism associated with pfcrt 76T.

Discussion

Minority-variant drug-resistant parasite populations that were undetectable by PCR were found to be common in polyclonal Malawian *P. falciparum* infections. The presence of minority drug-resistant variants is consistent with results of other studies, which have shown patients with genotypicaly wild-type infections before therapy exhibiting genotypicaly mutant infections after unsuccessful chemotherapy (*14*,*15*). In Malawi, where chloroquine was replaced with sulfadoxine-pyrimethamine in 1993, the prevalence of the resistance marker pfcrt 76T, as determined by PCR, has been reported to have almost disappeared (*6*–*9*). However, our data suggest that the reversion to genotypically sensitive malaria is incomplete and that minority pfcrt 76T–bearing parasite strains are "lurking" within persons at levels undetectable by standard PCR. One caveat is that our study population comprised pregnant women with high HIV prevalence, so whether the results are applicable to the general population is unclear.

Minority-variant drug-resistant mutations are important in other diseases, such as HIV (*16*). The presence of minority-variant drug-resistant mutations in *P. falciparum* has been previously demonstrated by subcloning dihydrofolate reductase genes into yeast vectors and growing them under drug pressure (*17*). However, this technique cannot determine the frequency of minority variants either in a single host or in a population. To our knowledge, our results show, for the first time, that minority-variant drug-resistant mutations, representing several percentages of the parasites in a single host, are common in populations.

In response to the apparent reemergence of genotypically sensitive malaria, Laufer et al. recently completed a chloroquine efficacy trial in pediatric patients from urban Blantyre (*9*). The cumulative efficacy of chloroquine was 99% (95% confidence intervals 93%–100%) with only 1 treatment failure occurring in the chloroquine arm. The high efficacy rate of chloroquine therapy is not inconsistent with the results of our study. In urban Blantyre, we only found 1 patient with minority-variant pfcrt 76T. In addition, successful response to therapy requires not only susceptibility of the parasite to the drug but also factors such as acquired immunity, drug absorption, and nutrition. At this point, it is still unclear how minority-variant drug-resistant parasites will interact with these other factors. Further research in this area is needed.

Significantly more patients carried minority-variant pfcrt 76T (35%) at the rural sites ($p = 0.001$, Fisher exact test) than at the urban site (3.7%). Why such a marked difference was found in the prevalence of pfcrt 76T between the 2 sites is not clear. One possibility is that the transition from chloroquine to sulfadoxine-pyrimethamine may have occurred later in rural areas than in urban areas. Also, limited drug pressure may continue to be exerted on the parasites within Malawi because, as of early 2006, chloroquine was still available in local pharmacies (*11*). Another possible factor that may influence the prevalence of minority-variant drug-resistant parasites is external pressure from areas of high-level resistance such as Zambia and Mozambique. Mpemba and Madziabango lie on a major highway between Blantyre and the border with Zambia. Importation of cases of malaria by travel along this highway may lead to a gradient of resistance extending from the border to Blantyre. More studies on the epidemiology of minority-variant pfcrt K76T are needed to better understand the causes of this difference in the prevalence of pfcrt 76T.

In conclusion, MSS-HTAs can gather information on lurking drug resistance overlooked by standard PCRs.

Table 3. Detection of pfcrt 76T in clinical samples*

Clinical site	No. samples	No. positive samples by HTA (%)	No. positive samples by ARPCR (%)	Avg mutant population by HTA (%)	SD
MHP	27	1 (3.7)†	0 (0)	3.2	0.07
MM	60	21 (35)†	0 (0)	3.3	1.4

*HTA, heteroduplex tracking assay; ARPCR, standard allele-restricted PCR; MHP, Malaria and HIV in Pregnancy; MM, Mpemba and Madziabango.
†$p = 0.001$, Fisher exact test.

The method is currently performed by using radiolabeled probes, which may not be feasible in many underdeveloped countries. Implementing this method for public health purposes would require substitution of fluorescently labeled or biotinylated labeled probes for the radioisotope. MSS HTAs for other drug-resistance loci need to be developed so that the clinical and public health implications of minority variants can be fully assessed.

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Dr Juliano is an Infectious Disease Fellow in training at the University of North Carolina. His research interests focus primarily on drug resistance detection in malaria from Southeast Asia and Africa. He is also interested in clinical tropical medicine.

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Human Alveolar Echinococcosis after Fox Population Increase, Switzerland

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We analyzed databases spanning 50 years, which included retrospective alveolar echinococcosis (AE) case-finding studies and databases of the 3 major centers for treatment of AE in Switzerland. A total of 494 cases were recorded. Annual incidence of AE per 100,000 population increased from 0.12–0.15 during 1956–1992 and a mean of 0.10 during 1993–2000 to a mean of 0.26 during 2001–2005. Because the clinical stage of the disease did not change between observation periods, this increase cannot be explained by improved diagnosis. Swiss hunting statistics suggested that the fox population increased 4-fold from 1980 through 1995 and has persisted at these higher levels. Because the period between infection and development of clinical disease is long, the increase in the fox population and high *Echinococcus multilocularis* prevalence rates in foxes in rural and urban areas may have resulted in an emerging epidemic of AE 10–15 years later.

Human alveolar echinococcosis (AE), a hepatic disorder that resembles liver cancer, is a highly aggressive and lethal zoonotic infection caused by the larval stage of the fox tapeworm, *Echinococcus multilocularis* (*1*). The parasite is widely distributed in the Northern Hemisphere; the disease-endemic area stretches from North America through Europe to central and east Asia, including northern parts of Japan (*1–3*). Parasite eggs are shed into the environment in the feces of canid definitive hosts that harbor the adult parasite in their intestines. In addition, some of these eggs contaminate the fur of infected definitive hosts. Humans and intermediate host animals acquire the infection by ingesting *E. multilocularis* eggs in contaminated food or water or by having close physical contact with infected foxes, dogs, or host feces. In Europe, *E. multilocularis* exists predominantly in a cycle of wild animals that includes red foxes (*Vulpes vulpes)* as main definitive hosts and several vole species as intermediate hosts. In the United States and Canada, the coyote (*Canis latrans*) has also been shown to be a suitable host for this parasite; in Arctic regions, the artic fox (*Alopex lagopus*) is the principal definitive host (*1*). Domestic dogs are also highly susceptible definitive hosts (*4*); in some areas, such as Alaska (*5*), People's Republic of China (*6*), and Europe (*7*), they can play an additional or even the dominant role as an infection source for humans.

The documented area of *E. multilocularis* endemicity in Europe has recently increased (*3,7,8*). However, whether this increase results from a true extension of the geographic range or simply increased detection in populations of wildlife not previously investigated is still unclear (*1*). Similarly, new distribution ranges have been reported in North America. In addition to being found in the tundra zone of Alaska and Canada, the parasite has now been recorded in 3 Canadian provinces and an additional 11 contiguous US states (*9*).

Important changes have occurred in the population dynamics of foxes in central Europe. Between 1970 and the mid-1980s, fox populations decreased during an epidemic of rabies. After the successful establishment of anti-rabies vaccination programs, fox populations increased almost 4-fold (*10*). At the same time, fox habitat extended into urban areas and is still progressing. For example, large fox populations have now become established in all major cities and towns in Switzerland. In the largest city, Zurich, the number of foxes shot or found dead within the city

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boundaries increased 20-fold since 1985 (*10*). The reasons for the increase in the fox populations have been attributed to ecologic factors, the successful vaccination campaign against fox rabies, and the increase in anthropogenic food supplies (*7,10*).

In the core European area for AE, which includes Switzerland as well as neighboring France and Germany, high prevalence rates (35%–65%) of *E. multilocularis* in foxes have been consistently recorded (*3,7,8,11*). However, several studies have shown that the prevalence rates have been increasing in certain regions. For example, recent parasite density estimates in southwestern Germany were 10× higher than estimates before 1990 (*3,8*), and unexpectedly high prevalence rates in several urban fox populations have been reported (*7*). The combination of increased fox populations and increased parasite prevalence within these populations has led to a considerable increase in the overall parasite biomass per surface unit.

A question of major public health importance in central Europe is whether the growing fox populations that have high prevalence of infection and the colonization of densely populated urban areas by foxes could increase risk for transmission of AE to humans and lead to an increase of clinical cases. Until 2000, no statistically significant increase in AE had been recorded in Switzerland (*1*). We report countrywide annual incidence rates of human AE in Switzerland in recent years (1993–2005) and compare them with incidence data for previous years (1956–1992). The European Echinococcosis Registry (*11*) gives summary data for 559 cases of AE from central Europe between 1988 and 2000, which includes data from 112 of the Swiss cases in the present report. The incidence data were related to the fox population dynamics. Because Switzerland has been consistently collecting comprehensive data on AE in humans for 50 years, conditions for such long-term assessments are favorable.

Methods

We retrieved retrospective data of extensive AE case-finding studies covering all of Switzerland for 1956–1992 (Table). We included additional data from the Swiss National Center for Echinococcosis, collected when echinococcosis was a reportable disease (1987–1996), and data for 1996–2005, collected from databases of the University Hospitals of Zurich, Berne, and Lausanne, the 3 major centers for AE treatment in Switzerland.

Furthermore, we analyzed serodiagnostic data compiled from the 3 main diagnostic laboratories for parasitic diseases in Switzerland that offer reliable (and methodically comparable) immunodiagnosis of human AE (Institutes of Parasitology, Universities of Zurich and Bern, Swiss Tropical Institute in Basel). Cases that were diagnosed primarily on the basis of serologic testing results were further assessed by sending questionnaires to involved family doctors or by conducting retrospective analysis of the patients' history obtained from the treating hospitals.

Inclusion criteria were as applied previously (*11*). These were 1) diagnosis of AE by positive, species-specific serologic testing (*15*), 2) AE-characteristic imaging findings, and 3) if available, AE-characteristic histopathologic findings and species-specific molecular analysis by PCR. Seropositive persons who lacked characteristic imaging features or histologic or molecular diagnosis were excluded from the study. All data were entered by using only personal initials, birth date, and sex; patients remained anonymous. Data were collected in a single database (Microsoft Excel; Microsoft Corp., Redmond, WA, USA) to prevent multiple counting of patients.

For cases that occurred during 1993–2005, for which data on the clinical records were available, we used the PNM (primary, neighboring, metastasis) staging system, specifically, location and extension of the primary lesion, involvement of neighboring organs, and presence or absence of metastasis (*16*). We determined the stage of disease (stages I–IV) by using this PNM system. Stages I and II are more likely to represent subclinical disease and are often diagnosed by chance. Such cases, if detected early, are more amenable to curative resection. Patients with PNM stage III or IV are more likely to have advanced clinical disease. They have more limited treatment options, such as individualized interventional measures and lifelong chemotherapy (*17*). The stage of cases diagnosed during

Table. Data from reported case-finding studies on human alveolar echinococcosis, 1956–2005, Switzerland

Study (reference no.)	Years	No. cases	Mean annual incidence/100,000 population	Mean age ±SD, y	Sex, no. (%)	
					Male	Female
Drolshammer (*12*)	1956–1969	122	0.15	54.2 ± 18.2	65 (53)*	57 (47)
Gloor (*13*)	1970–1983	145	0.16	55.0 ± 16.0	79 (54)*	66 (46)
Eckert (*14*)	1984–1992	71†	0.12	52.0 ± 17.7	33 (46)	38 (54)
This study	1993–2000	60	0.10	52.5 ± 18.4	26 (43)	34 (57)
This study	2001–2005	96	0.26‡	54.5 ± 17.3	42 (44)	54 (56)
Total	1956–2005	494	0.15	54.0 ± 17.3	245 (49.7)	248 (50.3)

*Proportion of cases in male patients was significantly higher during 1956–1983 than 1984–2005 ($p<0.05$).
†Six more cases from 1984–1992 have been included in this study.
‡Significantly increased compared with1984–2000 (p for trend <0.01).

1993–2000 was compared with stage of cases diagnosed during 2001–2005 by converting the stage to a score. For example, patients with stage I disease were scored as 1 and those with stage IIIb disease were scored as 3.5. To determine whether earlier or later diagnosis during different periods might partly account for any trend in the change of incidence, the mean score of cases diagnosed during 1993–2000 was compared with mean score of those diagnosed during 2001–2005. PNM scores were available for 50% (51 cases) of the 2001–2005 cases and for 40 (68%) of the 1993–2000 cases. For 2001–2005, the average age of case-patients with available PNM scores was 54 years (SD 17 years). This average did not significantly differ from the average of 50 years (SD 18 years) of case-patients with PNM scores for 1993–2000 (p = 0.19, Student *t* test). Likewise, the proportion of male patients with scores for 2001–2005 (37%) did not significantly differ from the proportion of male patients with scores for 1993–2000 (44%) (p = 0.35, Fisher exact test). Therefore, we found no evidence of bias due to incomplete data.

We retrieved data regarding fox population sizes from the Swiss federal hunting statistics (www.wild.unizh.ch/jagdst). These data, based on annual hunting numbers, indicate trends over a long period of time (*18*). Human population data for Switzerland originated from the Swiss Federal Statistical Office (www.bfs.admin.ch).

To perform our statistical calculations, we used Microsoft Excel. Differences in human incidence rates and differences in numbers of reported foxes were analyzed by χ^2 test for trend. Proportions of male and female patients and the proportion of patients who had radical liver resection were analyzed by χ^2 test. Data for PNM scores were tested for normality and compared by using the Student *t* test. To smooth out annual fluctuations and better visualize longer term trends, we present data for number of foxes and incidence of human AE cases as 5-year moving averages.

Results

A total of 494 cases of human AE were diagnosed in Switzerland during 1956–2005. The mean age at time of diagnosis (54 years, range 12–89) did not change significantly over time (Table). The numbers of male and female patients were similar, although the trend was toward a decreasing proportion of male patients over the observation period. Male patients accounted for 53%–54% of patients during 1956–1983 compared with 43%–46% during 1984–2005 (p<0.05) (Table). The incidence of human AE during the observation period is shown in the Figure. The highest annual incidence per 100,000 was recorded in 2003 (0.38; 28 new cases); the lowest, in 1996 (0.04; 3 new cases). The mean annual incidence per 100,000 was 0.10 during 1993–2000 and increased to 0.26 during 2001–2005 (p<0.01)

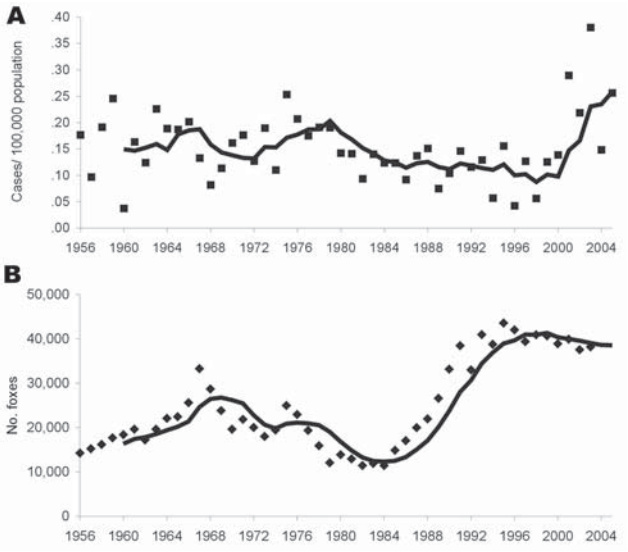

Figure. Actual data points with moving 5-year average for annual incidence of human alveolar echinococcosis in Switzerland (A) and annual number of foxes hunted per year in Switzerland (B), used as a fox population density marker.

(Figure, Table). The estimated fox population increased ≈4-fold during 1984–1993 (Figure) (p<0.001).

The mean PNM score for cases diagnosed during 1993–2000 was 2.41 (SD 1.3), which was not significantly different from the mean PNM score of 2.44 (SD1.2) for cases diagnosed during 2001–2005. The proportion of patients who had a radical liver resection was highest during 1996–2000 (75.3%) and lowest during 1991–1995 (37.5%) and 2001–2005 (54.9%) (p<0.001).

Discussion

The incidence of human AE in Switzerland has exhibited 2 trends during the past 50 years. After a slow but steady decline during 1956–1999, incidence has significantly increased since the year 2000 (Figure). A reasonable explanation for this finding may be the urbanization of the *E. multilocularis* cycle, which has resulted in an increase in the number and proportion of infected urban foxes in areas with high human populations, thereby increasing the infection risk for the human population (*7*). The increase of the fox population density started in ≈1985, some 10–15 years before the increased numbers of human AE cases (Figure). This temporal delay is consistent with the suggested latent or asymptomatic period of 5–15 years before development of clinically apparent AE in humans (*19*). Improved diagnostic accuracy, due to modern imaging technologies such as computerized tomography *(20),* is unlikely to account for this increase. Such improvements in diagnostic accuracy would be expected to lead to earlier

detection with a shift to PNM stages I and II at the time of diagnosis, but such a trend has not been recorded. In addition, earlier, more local investigations have already indicated an increase in AE seropositivity in defined human populations at risk in central Europe (*21,22*) without a concomitant increase in clinical AE cases. An increase in the rate of AE seropositivity that precedes that of clinical AE is therefore consistent with increased exposure and a temporally delayed rise in the number of clinical cases.

The use of foxes recorded in hunting statistics or hunting indices as a measure of the fox population is susceptible to bias and should be used only to describe trends from large areas (>1,000 km^2) over long periods (≥5 years) (*18*). For this reason, the temporal trends in the size of the fox population were estimated by using hunting returns compiled for 50 years for the entire territory of Switzerland (42,000 km^2). Therefore, we can conclude that the incidence of human AE appears to be increasing in Switzerland and that this increase was preceded 10 years earlier by a parallel increase and urbanization of the fox population.

The potential extent of this emerging epidemic of human AE cannot be predicted. Future trends will depend on the intensity of present and future contamination of the environment with *E. multilocularis* eggs as well as on the number of susceptible persons exposed to the parasite. In this respect, the human AE epidemic appears analogous to that of human variant Creutzfeldt-Jakob disease, for which predictions of future disease trends have been hampered by uncertain knowledge of incubation periods and unknown relationships between the risk for disease and host factors (*23,24*). Nevertheless, the temporal proliferation of *E. multilocularis* biomass in the main definitive host has increased the infection pressure for a large part of the human population in Switzerland. Likewise, other susceptible canid species involved in the life cycle of the parasite could present additional threats, in Europe and elsewhere. Within the past decade, for example, coyotes in the United States have become established in suburban areas with moderate to dense human populations (*25*). Because this species is a suitable definitive host of *E. multilocularis (9)*, risk for transmission to humans in the United States and Canada may increase markedly.

In conclusion, public health authorities in echinococcosis-endemic areas should establish coordinated systems of continuous surveillance and risk assessment, combined with measures to reduce illness and death from AE in human populations (*1*). Furthermore, new control strategies, including strategic deworming of foxes and other wild canids by using anthelminthic baiting options, should be further evaluated and developed. Such strategies should preferably target suburban areas that have high human and wild canid population densities (*7*).

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Risk Factors for Imported Fatal *Plasmodium falciparum* Malaria, France, 1996–2003

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Plasmodium falciparum malaria is a serious health hazard for travelers to malaria-endemic areas and is often diagnosed on return to the country of residence. We conducted a retrospective study of imported falciparum malaria among travelers returning to France from malaria-endemic areas from 1996 through 2003. Epidemiologic, clinical, and parasitologic data were collected by a network of 120 laboratories. Factors associated with fatal malaria were identified by logistic regression analysis. During the study period, 21,888 falciparum malaria cases were reported. There were 96 deaths, for a case-fatality rate of 4.4 per 1,000 cases of falciparum malaria. In multivariate analysis, risk factors independently associated with death from imported malaria were older age, European origin, travel to East Africa, and absence of chemoprophylaxis. Fatal imported malaria remains rare and preventable. Pretravel advice and malaria management should take into account these risk factors, particularly for senior travelers.

Imported malaria is increasingly reported in Europe and North America, with an estimated 30,000 cases yearly (*1,2*). In 2000, the countries with the highest rates of imported malaria were France (≈8,000 estimated cases), United Kingdom (2,069 cases), United States (1,402 cases), Italy (986 cases), and Germany (732 cases) (*3,4*). Imported *Plasmodium falciparum* malaria is a serious health hazard for travelers to malaria-endemic areas, owing to the potentially severe illness and high case-fatality rates (case-fatality rate per 1,000: France 4; Italy 6.5; UK 8.5; USA 13; Germany 30.4) (*4,5*). Risk factors associated with fatal imported malaria are poorly known. Limited series have suggested that the fatality rate is significantly lower for migrants from malaria-endemic areas than for patients from areas not endemic for the disease (*6–8*). Antimalarial chemoprophylaxis, even incomplete or inappropriate, may also confer a degree of protection (*9–11*). Better knowledge of the characteristics and risk factors for fatal imported malaria might help to improve prevention and patient management. We retrospectively analyzed the main features of fatal imported falciparum malaria observed in France during 1996–2003 and compared them with those for nonfatal cases.

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Methods

Description of Surveillance System (Data Sources)

Imported malaria is not a mandatory notifiable disease in metropolitan France. The data for this study were collected by a reporting network of 120 selected hospital laboratories and were analyzed by the French National Reference Center for Imported and Autochthonous Malaria Epidemiology (CNREPIA). Participants of the network were asked to report imported malaria cases whenever the laboratory observed asexual forms of *P. falciparum* in a patient's blood film. Data from the national medical informatics systems and from 2 exhaustive studies (National Quality Control Survey) suggested that these cases represented 50%–55%

of the total number of imported falciparum malaria cases in France during the study period (*12,13*). A standard 57-item questionnaire, completed by clinicians and biologists for each reported case, collected basic demographic, epidemiologic, clinical, and parasitologic information (including prophylaxis and treatment). In addition, a detailed clinical description was obtained for each fatal case.

Data Analysis

The study population consisted of all *P. falciparum*–infected patients reported to CNREPIA during 1996–2003. Deaths occurring during hospitalization for malaria were considered malaria related. The case-fatality rate per 1,000 patients was calculated for all relevant exposure variables. Various exposure categories were created for the analysis: patients were divided into European travelers (persons born and residing in areas not endemic for malaria), European expatriates (residing in malaria-endemic African countries), African travelers (persons born in Africa who reside mostly in France or another country not endemic for malaria), African residents (persons born and residing in Africa), and others. Use of malaria chemoprophylaxis, as reported by the patients, was categorized as follows: no use; use of ineffective drugs (e.g., chloroquine, proguanil, pyrimethamine, sulfadoxine-pyrimethamine); and use of effective drugs (mefloquine, atovaquone-proguanil, doxycycline, and chloroquine-proguanil). Logistic regression was used to identify factors associated with fatal malaria and to estimate odds ratios and 95% confidence intervals (CIs) for the association between exposure variables and death. Dummy variables were used for variables with >2 categories. Variables with $p<0.25$ were introduced in the multivariate logistic regression model. A manual backward stepwise approach was used to remove nonsignificant variables, and only variables with $p<0.05$ were retained in the final model. Interactions were sought by introducing interaction terms in the logistic regression model and testing for their significance ($p<0.05$). Because data were missing for the variables "region of malaria acquisition" (n = 9), "chemoprophylaxis" (n = 2,366), and "time between onset and diagnosis" (n = 3,845), multiple imputation for missing data was performed for the final model by using the multivariate imputation by chained equations (MICE) method described by Van Buuren et al. (*14*). The MICE method involves imputations of missing values by appropriate regression models and generation of multiple datasets (in our case, 5) to take into account the uncertainty involved in imputing the missing values. Standard complete-data methods are then used on each dataset, and results are combined to produce estimates with CIs and p values. Statistical analysis was performed by using EpiInfo, version 3.3 (Centers for Disease Control and Prevention, Atlanta, GA, USA) and Stata 8 (Stata Corporation, College Station, TX, USA).

Results

During the 1996–2003 period, 27,085 malaria cases were reported to CNREPIA; 21,888 of these patients had *P. falciparum* malaria, which constituted the study population. Included were 20,436 (93.4%) uncomplicated cases, 825 (3.8%) severe cases, 433 (2%) asymptomatic cases, 161 (0.7%) cases of hyper-reactive malarial splenomegaly, and 33 (0.1%) unspecified cases. Cases attributable to species other than *P. falciparum* and cases attributable to species combinations that included *P. falciparum* were not considered in the analysis (no fatal cases of imported malaria due to *Plasmodium* species other than *P. falciparum* were reported during the study period). The annual number of malaria cases reported by the network increased until 1999–2000, then stabilized at ≈3,000 cases per year (Table 1). Most patients were male (sex ratio M:F = 1.7), and the median (range) age was 29.0 (0–96) years. Most patients had acquired malaria in Africa: 59.2% in West Africa, 26.2% in central Africa, 11.2% in Madagascar and the Comoros Islands, and 0.9% in East Africa (Table 2 and online Appendix Table, available from www.cdc.gov/EID/content/13/6/883-appT.htm). Others (2.5%) had returned mainly from French Guiana, Haiti, India, Sri Lanka, Thailand, and Indonesia. African travelers were most numerous (44.6%), followed by European travelers (26.5%), African residents (12.9%), and European expatriates living in Africa (5.4%); "others" represented 10.6%. Few patients (30.4%) reported taking effective chemoprophylaxis, and more than half the patients had not taken any. The median duration of stay was 32 days (interquartile range 21–62). The median time from return to symptom onset was 6 days (interquartile range 1–12), and 10% of patients had their first symptoms before returning to France. The median time from symptom onset to diagnosis was 3 days (interquartile range 1–6). Compared with Europeans, Africans were more likely to seek care at a hospital (73.0% vs. 62.7%, $p<0.001$). Diagnoses were fairly evenly distributed between spring-summer (55.5%) and fall-winter (45.5%). At diagnosis, 7.9% of patients had high-level parasitemia (>5% of parasitized erythrocytes).

Description of Fatal Cases

Ninety-six patients died of malaria; 55 were European travelers; 12, European expatriates; 11, African travelers; 10, African residents; and 8, other. One case was diagnosed postmortem. Repatriations for medical reasons occurred in 13 of 96 fatal cases (1 African resident, 5 European travelers, and 7 European expatriates). Among the patients who died, the sex ratio (M:F) was 3.3, and the median (range) age was 47 (2–92) years. Three study participants who died were <15 years of age, and 5 were >70 years of age. Thirty (31.3%) of the patients who died had taken antimalarial chemoprophylaxis, and 2 were re-

Table 1. Distribution of falciparum malaria cases and deaths by calendar year, 1996–2003, France

Year	No. reported deaths	No. falciparum malaria cases	Case-fatality rate per 1,000
1996	8	1,804	4.4
1997	10	2,057	4.9
1998	9	2,459	3.7
1999	9	3,385	2.7
2000	12	3,355	3.6
2001	13	3,035	4.3
2002	15	2,919	5.1
2003	20	2,874	7.0
Total	96	21,888	4.4

ported to have correctly taken prophylaxis appropriate for the region visited.

Clinical data for patients who died are shown in Table 3. Fever was the most common initial symptom. All patients, except the one whose case was diagnosed postmortem, were hospitalized and received antimalarial therapy within 12 hours of diagnosis. Median parasitemia at admission was high (10%), although 10% of patients had parasitemia <1%. One case of black water fever and 1 case of splenic rupture were observed. Forty-three (44.8%) patients required mechanical ventilation, and 24 (25%) required hemofiltration. Three (3.1%) patients underwent exchange transfusion.

Risk Factors for Death

The case-fatality rate was 4.4 deaths per 1,000 cases during the study period and did not change over time (calendar years) (standard χ^2 for trends test; Table 1). Many factors were associated with an increased risk for death in univariate analysis, including older age, male sex, European origin, travel to East Africa, short stays (≤15 days), time to diagnosis, and initial visit to a general practitioner (online Appendix Table). However, male sex, short stays, visit to a general practitioner, and diagnosis during the fall-winter season were no longer predictive of death after age and ethnic origin were controlled for in multivariate analysis. Table 4 shows the results of multivariate analysis. Low hemoglobin levels (≤8 g/dL), low platelet counts ($\leq 50\times10^9$/L), high leukocyte counts ($>10\times10^9$/L), and high-level parasitemia (>5%) were all associated with increased risk for death among patients with measurements for these variables (online Appendix Table). The risk factors for death identified in this study were not different between Africans and Europeans (test of interaction not significant).

Discussion

To our knowledge, this is the largest retrospective study aimed at identifying risk factors for fatal imported malaria. France has large numbers of migrants of African origin. Those populations are particularly at risk of acquiring malaria when visiting friends and relatives (*15–17*).

Table 2. Distribution of falciparum malaria cases and deaths by country of acquisition, 1996–2003

Country	No. deaths	No. falciparum malaria cases	Case-fatality rate per 1,000
Cape Verde	1	4	250.0
Senegal	12	2,234	5.4
Mauritania	1	96	10.4
Guinea-Bissau	1	50	20.0
Guinea	3	823	3.6
Mali	8	2,124	3.8
Côte d'Ivoire	15	4,623	3.2
Burkina Faso	5	740	6.8
Ghana	1	194	5.2
Togo	3	604	5.0
Benin	3	1,012	3.0
Niger	3	152	19.7
Nigeria	2	123	16.3
Cameroon	12	2,707	4.4
Equatorial Guinea	1	31	32.3
Gabon	4	671	6.0
Congo	4	885	4.5
Central African Republic	1	728	1.4
Tanzania	1	38	26.3
Kenya	4	101	39.6
Djibouti	1	12	83.3
Mozambique	1	29	34.5
Comoros Islands	1	2,017	0.5
Madagascar	4	432	9.3
Others, several countries or unknown	4	1,458	2.7
Total	96	21,888	4.4

Table 3. Clinical data for 96 patients with fatal malaria, 1996–2003, France

Clinical data	No. travelers (%)
Initial symptoms	
Fever	80 (83.3)
Mental status changes	45 (46.9)
Jaundice	24 (25)
Diarrhea	18 (18.7)
Respiratory symptoms	13 (13.5)
Coma	11 (11.5)
Vomiting	9 (9.4)
Convulsions	6 (6.2)
Lethargy	5 (5.2)
Cough	4 (4.2)
Shock syndrome	4 (4.2)
Severity criteria* during clinical course	
Renal failure	70 (72.9)
Shock syndrome	60 (62.5)
Cerebral malaria	55 (57.3)
Acute respiratory distress syndrome	44 (45.8)
Acidosis	36 (37.5)
Disseminated intravascular coagulation	30 (31.2)
Pulmonary edema	12 (12.5)
Scleral icterus	11 (11.5)
Convulsions	9 (9.4)
Other conditions	
Nosocomial infection	24 (25)
Cardiac failure	49 (51)
Cerebral edema	10 (10.4)

*Severity criteria according to World Health Organization, 2000.

Migrants of African origin travel for longer periods in highly malaria-endemic areas (globally, 75% of malaria infections and 89% of *P. falciparum* infections in travelers are acquired in sub-Saharan Africa [*18*]), are less likely to have pretravel encounters with a healthcare provider, and are therefore unlikely to take antimalarial prophylaxis (*18*). These factors explain why France, in comparison with many other European countries or the United States, has so many malaria cases and why the country appears to receive disproportionally high numbers of malaria-infected returning travelers from Africa (*19*), rather than from Asia or South America. As a result, *P. falciparum* is overrepresented in imported malaria in France in comparison with other industrialized countries. The case-fatality rate (4.4 per 1,000 cases) is among the lowest in the World Health Organization (WHO) 2004 Computerized Information System for Infectious Diseases database, probably because of the large proportion of African migrants in our study population.

The following characteristics were independently associated with death from falciparum malaria, according to multivariate analysis: older age, origin in an area not endemic for malaria, infection in East Africa, and no effective chemoprophylaxis. Increasing age has also been a risk factor for fatal falciparum malaria in smaller studies (*10,11,13,20,21*). As has been reported elsewhere (*21*), we found a gradual increase in risk over the entire age spectrum, resulting in particularly high risk among elderly patients. This factor should be taken into account when offering pretravel health advice, particularly as the age of international travelers increases.

Severe malaria and death were particularly frequent among nonimmigrants, as previously reported in smaller series (*6–8,13,22*). These results are consistent with the hypothesis of persistent acquired immunity, even after several years of nonexposure, which may partially protect African immigrants from fatal malaria, as previously shown for severe forms of malaria (*23*). Genetic factors, selected at the population level over centuries of exposure to the parasite, may also partly explain the relative protection of African immigrants compared with Europeans (*24,25*).

Except for the Comoros Islands (0.5 cases per 1,000 cases of falciparum malaria), countries accounting for large numbers of cases in this study (Senegal, Cameroon, Mali, and Côte d'Ivoire) had similar case-fatality rates (3.2 to 5.4 cases per 1,000 cases of falciparum malaria). Most travelers returning from the Comoros Island were migrants; few were European tourists. East African countries such as Djibouti, Kenya, Mozambique, and Tanzania accounted for fewer cases but a disproportionate number of fatalities (34.1 cases per 1,000). Ben-Ami et al. recently reported a high rate of severe malaria (6 of 29 cases, including 1 death) among patients who visited Mombassa, Kenya (*26*). More generally, Krause et al. reported that falciparum malaria acquired in Africa had a higher case-fatality rate than falciparum malaria acquired elsewhere (*11*). During the period of our study, an increase in malaria deaths, probably related to higher levels of drug resistance, was seen in residents of East Africa but not in those of West Africa (*27*). Those observations are not necessarily linked, but particular attention should be paid to travelers returning from these areas. Further studies are needed to confirm and explain these findings.

The risk for death was higher when prophylaxis was absent or ineffective than when appropriate prophylaxis was taken. On the basis of interview data, only 2 authentic failures of prophylaxis were suspected among our patients, but drug and metabolite concentrations were not assayed. These results are consistent with those of Krause et al., who reported that study participants who had taken chemoprophylaxis with chloroquine-proguanil were less likely to die than those who had not taken chemoprophylaxis (*11*). These results once again underline the importance of recommending antimalarial prophylaxis for travelers to malaria-endemic areas (*28*).

Bruneel et al. reported that platelet counts were significantly lower in patients who eventually died of *P. falciparum*–infected patients than in survivors (*8*) and that leukocyte counts also tended to be higher. In our popula-

Table 4. Factors independently associated with deaths among patients treated for falciparum malaria in French hospitals, 1996–2003 (n = 21,888)*

Variable	Odds ratio	95% Confidence interval	p value
Age			
Per increase of 10 y	1.78	(1.56–2.02)	<0.001
Origin and residence			
African travelers	1		
African residents	3.15	(1.32–7.51)	
European travelers	6.79	(3.49–13.2)	<0.001
European expatriates	4.44	(1.91–10.3)	
Others	3.02	(1.21–7.57)	
Region of malaria acquisition			
West Africa	1		
Central Africa	0.86	(0.52–1.41)	
East Africa	3.39	(1.49–7.72)	0.02
Madagascar and Comoros Islands	0.61	(0.24–1.53)	
Others	0.47	(0.11–1.95)	
Chemoprophylaxis			
Effective drugs†	1		
No chemoprophylaxis	2.07	(1.19–3.61)	0.04
Ineffective drugs†	1.90	(0.91–3.95)	

*Multiple imputations were used for missing data for the variables "region of malaria acquisition" (n = 9) and "chemoprophylaxis" (n = 2,366) (see Methods).
†Effective drugs were mefloquine, atovaquone-proguanil, doxycycline, and chloroquine-proguanil; ineffective drugs were chloroquine, proguanil, pyrimethamine, and sulfadoxine-pyrimethamine.

tion, a platelet count $<50\times10^9$/L was associated with increased risk of dying; this effect was particularly marked at counts $<10\times10^9$/L. Disseminated intravascular coagulation, which is associated with marked thrombocytopenia, was frequent in patients who later died (Table 3). Leukocyte counts $>10\times10^9$ cells/L were also associated with increased mortality, and the effect was particularly marked at counts $>15\times10^9$ cells/L. Hyperleukocytosis in this setting may be related to cytokine or cortisol release or to bacterial (especially nosocomial) infections, which were frequent in our patients who eventually died (Table 3). Bruneel et al. found that hyperparasitemia (>5%), a WHO severity criterion, was weakly linked to death. Although the number of circulating parasitized erythrocytes may not accurately reflect the number of adherent red cells in deep organ capillaries, which are the source of most clinical complications (*29*), hyperparasitemia appeared to be an important predictor of death in our series. However, parasitemia and some biologic data (hemoglobin, platelet counts, and leukocyte counts) could not be included in our multivariate analysis because an excessive amount of data were missing. In particular, hemoglobin, platelet count, and leukocyte count data were missing for more than half the patients.

A limitation of our study is that our network for collecting data accounted for only 50%–55% of total malaria cases imported to France. Two annual exhaustive studies (National Quality Control Survey [*30*]; F. Legros, unpub. data) suggest that representativeness of our sites was correct. Thus, risk factors associated with death in imported malaria would not likely differ for cases not seen in our network.

Severe and fatal malaria, even though it is eminently preventable, continues to be seen in areas that are not endemic for malaria (*31,32*). Fatal cases are rare in patients who take appropriate prophylaxis. With the current increases in intercontinental travel, numbers of elderly travelers, risk for transmission in malaria-endemic areas, and drug-resistant strains of *P. falciparum* (*16,33*), the numbers of fatal cases of imported malaria should be carefully monitored in the coming years in France and other industrialized countries. Preventive measures remain necessary for all travelers, including those from Africa, for whom adherence is often poor (*34*). Posttravel care should also be reinforced to reduce the interval between symptom onset and diagnosis (*35,36*).

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Isolation and Characterization of Novel Human Parechovirus from Clinical Samples

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Using Vero cells, we isolated a virus (NII561-2000) from a cerebrospinal fluid specimen of a 1-year-old girl with Reye syndrome. The determined amino acid sequence of the virus indicated that the isolate was a human parechovirus (HPeV), a member of *Picornaviridae*. Neutralization test showed that the NII561-2000 virus had distinct antigenicity to HPeV-1, HPeV-2, and HPeV-3, and that the sequence was distinct from these types as well as from HPeV-4 and HPeV-5. Thus, we propose the virus (NII561-2000) as the prototype of HPeV-6. We isolated 10 NII561-2000–related viruses, 14 HPeV-1, 16 HPeV-3, and 1 HPeV-4 of 41 HPeVs from various clinical samples collected in Niigata, Japan. Clinical symptoms of the persons infected with the NII561-2000–related viruses were infectious gastroenteritis, rash, upper respiratory tract infection, and paralysis, in addition to Reye syndrome in the 1-year-old girl.

Human parechovirus (HPeV) is a small, nonenveloped RNA virus with a single-stranded genome of positive polarity, ≈7.3 kb in length; it is a member of the *Picornaviridae* family (*1–3*). On the basis of serologic and genetic studies, HPeV has been found to have 5 types, HPeV-1, HPeV-2, HPeV-3, HPeV-4, and HPeV-5, with 76.0%–80.9% similarity at the nucleotide level and 84.7%–90.0% similarity at the amino acid level (*1*,*4–7*). HPeV infections are commonly observed in general populations. For example, ≈20% of healthy children in Finland have antibodies against HPeV-1, and the percentage is as high as 97% in adults (*8*). In addition, these viruses are frequently isolated from patients with various human diseases, such as gastroenteritis, encephalitis, flaccid paralysis, and respiratory infections, and they are thought to be associated with these diseases (*2*,*3*,*5*,*8*,*9*).

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We report a 1-year-old girl who died with Reye syndrome, which is characterized as an acute, noninflammatory encephalopathy with hepatic dysfunction and fatty infiltration of the viscera; the syndrome is frequently associated with an antecedent viral infection, such as influenza or varicella (*10–12*). Inoculation of a cerebrospinal fluid (CSF) specimen from the patient into Vero cells identified a virus (NII561-2000) with similar properties to HPeVs. The nucleotide sequence of this virus showed it was closely related to HPeVs, especially HPeV-1, with 79.5% nucleotide and 90.7% amino acid (aa) similarities. Moreover, mutual neutralization assay showed that NII561-2000 has distinct antigenicity to HPeV-1, HPeV-2, and HPeV-3. In addition, the NII561-2000 virus was genetically distinct from HPeV-4 and HPeV-5. Thus, we propose that NII561-2000 is the prototype of HPeV-6.

Materials and Methods

Cell Lines and Culture Conditions

Eight adherent cell lines, MDCK, Caco-2, RD-18S, Vero, HeLa, HEp-2, LLC-MK2, and BSC-1, were used in our study. In brief, MDCK originated from a kidney of a normal adult cocker spaniel, Caco-2 was from a primary colorectal adenocarcinoma, RD-18S was from a rhabdomyosarcoma, Vero was from the kidney of a normal adult African green monkey, HeLa was from a cervical adenocarcinoma, HEp-2 was from an epidermoid carcinoma of the larynx, LLC-MK2 was from a kidney of a normal adult rhesus monkey, and BSC-1 was from a kidney of a normal adult African green monkey. These cell lines were cultured in Eagle minimum essential medium with 6 mmol/L L-glutamine, 1.1 g/L sodium bicarbonate, antimicrobial agents (0.2 g/L gentamicin, 0.25 g/L amphotericin B), and 8% fetal bovine serum at 37°C under 5% carbon dioxide.

Virus Isolation and Purification

MDCK, Caco-2, RD-18S, Vero, HeLa, HEp-2, LLC-MK2, and BSC-1 cells were cultured on 24-well plates for 3 days. Then, these cells were inoculated with a CSF specimen (100 μL per well) from a patient with a diagnosis of Reye syndrome and cultured at 33°C for 2 weeks. To check for cytopathic effect (CPE), we examined the cells under a light microscope. To purify the virus particles, we mixed 900 mL culture fluid of Vero cells inoculated with the NII561-2000 virus with 20 g NaCl and 76 g polyethylene glycol #6,000 at 4°C overnight. Next, the sample was centrifuged at 5,000 rpm for 30 min, and the pellets were suspended in 4.5 mL of phosphate buffer (pH 7.2). After treatment with 4.5 mL of chloroform at 4°C for 5 min, the sample was centrifuged at 2,500 rpm for 20 min. The supernatant was centrifuged at 120,000 × *g* for 24 h in cesium chloride (CsCl) solution at an initial density of 1.34 g/mL, and the virion-containing fraction was collected and used for viral RNA isolation.

Molecular Cloning

Double-stranded cDNA was synthesized from 5 μg of the viral RNA from the viruses purified by the CsCl density gradient ultracentrifugation method described above. The nucleotide sequences of the cDNAs from randomly picked-up bacterial colonies transfected with the cDNA-containing plasmids were determined by using the Big Dye sequencing kit (Applied Biosystems, Foster City, CA, USA). Two cDNAs isolated contained part of the NII561-2000 gene. To isolate the 3′ cDNA fragment of the isolated NII561-2000 cDNAs, the 3′ rapid amplification of cDNA ends (RACE) was performed by using the RNA PCR Kit (AMV) according to the instructions provided by the supplier (TaKaRa, Kyoto, Japan). The primers used for 3′ RACE were 35F-out (forward primer for the first PCR; 5′-GAT GCG GAA AAC TGC TGG ACA C-3′), 35F-in (forward primer for the second PCR; 5′-TGC CAA ATT TTT CTG CCC TAC TG-3′), and M13M4 (reverse primer for the first and second PCR; 5′-GTT TTC CCA GTC ACG AC-3′). Herculase Hotstart DNA Polymerase (Stratagene, La Jolla, CA, USA) was used. To isolate the 5′ cDNA fragment of the isolated NII561-2000, the cDNA fragment containing part of the 5′ untranslated region (UTR) and capsid precursor protein VP0 was amplified by PCR from the cDNA prepared from the NII561-2000 virus with degenerate primers corresponding to this region. The degenerate primers used were E23P1 (forward primer; 5′-CCG YAG GTA ACA AGW GAC AT-3′) (*5*) and 35R (reverse primer; 5′-TCT CAG CAC TAA TGA CCC TC-3′). To further extend the sequence information of 5′ UTR of the NII561-2000 virus, the 5′ RACE was performed with 5′ RACE System for Rapid Amplification of cDNA Ends (Version 2.0), by using the instructions provided by the supplier (Invitrogen, San Diego, CA, USA). The primers used for 5′ RACE were AAP (forward primer for the first PCR; 5′-GGC CAC GCG TCG ACT AGT ACG GGI IGG GII GGG IIG-3′) HPeV-GSP2 (reverse primer for the first PCR; 5′-AGA TGC ATC ATC TGC GAC TC-3′), UAP (forward primer for the second PCR; 5′-CUA CUA CUA CUA GGC CAC GCG TCG ACT AGT AC-3′), and HPeV-GSP (reverse primer for the second PCR; 5′GCC ATG TCT GCA ATG CTC TT-3′). *Taq* polymerase (Biotech International, Bentley, Western Australia, Australia) was used as a polymerase. Because 5′ RACE did not reach to the 5′ end of the NII561-2000 virus cDNA, the 5′ end cDNA fragment of the NII561-2000 virus was amplified from the NII561-2000 viral RNA by reverse transcription–PCR (RT-PCR). The primers used for RT-PCR were HPeV-head (forward primer; 5′-TTT GAA AGG GGT CTC CT-3′) and HPeV-mid (reverse primer; 5′-CAT AAG TTC CAC AAG CGT GG-3′) HPeV-head primer was designed as a conserved 5′ end sequence of the HPeVs 5′ UTR.

Neutralization Test

Twenty-five microliters (100 median tissue culture doses; 50% tissue infective dose [$TCID_{50}$]) of the indicated viruses and 25 μL of the serially diluted antisera (an initial 10-fold dilution and then 2-fold serial dilutions) were mixed in a 96-well plate and incubated at 37°C for 2 h. Then suspended Vero cells (100 μL/well) were added into these wells. Three to 10 days later the CPE of Vero cells was checked by light microscopy. To prepare the antiserum against the NII561-2000 virus, the virus particles grown in Vero cells were purified by CsCl centrifugation as described above. By using this purified virus, we prepared antiserum by Nippon Biotest Laboratory (Tokyo, Japan). In brief, the purified viruses were subcutaneously injected into rabbits 3× every 2 weeks. After we checked the antiviral titer by the Ouchterlony method, blood was collected from the vaccinated rabbits.

Comparisons of VP0 Amino Acid Sequences of Clinical Isolates

To determine the VP0 amino acid sequences of viruses isolated from clinical samples with cultured cell lines, we extracted the viral RNA from culture supernatant of cells injected with the clinical samples by using the High Pure Viral RNA Kit (Roche, Mannheim, Germany). cDNA was synthesized from 8 μL of the viral RNA by using 1 U Moloney murine leukemia virus reverse transcriptase (Invitrogen) and 20 U of recombinant RNAs in ribonuclease inhibitor (Promega, Madison, WI, USA). The 810-bp fragment containing part of 5′ UTR and VP0 was amplified by PCR, using 5 μL of cDNA in a 50-μL reaction mixture containing 50 mmol/L KCl, 10 mmol/L Tris/HCl (pH 8.5), 2.5 mmol/L $MgCl_2$, 0.2 mmol/L of each deoxynucleoside

triphosphates, 50 pmol of each primer, and 1 U of *Taq* polymerase (Biotech International). The amplification reaction consisted of 30 cycles at 95°C for 30 s, at 59°C for 30 s, and at 72°C for 1 min. The primer set used was E23P1 as a forward primer and HPV-N1 (5′-TAG GGG ATA CAT ARG TCR GCY T-3′) as a reverse primer (*5*).

Phylogenic Tree Analysis

The bootstrap values were calculated from P1 amino acid sequences of parechoviruses with the CLUSTAL X software program (*13*), and the phylogenetic tree of these P1 amino acid sequences was constructed by using the neighbor-joining method. The nucleotide sequences of the following parechoviruses were obtained from GenBank: and their accession numbers were L02971 for HPeV-1 (Harris strain), AJ005695 for HPeV-2 (Williamson strain), AB084913 for HPeV-3(A308/99), AJ889918 for HPeV-3 (Can82853-01), DQ315670 for HPeV-4 (K251176-02), AM235750 for HPeV-4 (T75-4077), AF055846 for HPeV-5 (CT86-6760), AM235749 for HPeV-5 (T92-15), and AF327920 for Ljungan virus (LV). The GenBank/EMBL/DDBJ accession numbers of NII428-2000, NII2392-2001, NII2667-2001, NII2694-2001, NII2729-2001, and NII561-2000 are AB252577–AB252582.

Results

Virus Isolation

In January 2000, a 1-year-old girl with croup and high fever (39.6°C) was hospitalized in a regional general hospital in Niigata Prefecture, Japan. The infant died, and her condition was diagnosed as Reye syndrome after postmortem pathologic examination. To identify the pathogenic agent, we added a CSF specimen collected before death to 8 cell lines: MDCK, Caco-2, RD-18S, Vero, HeLa, HEp-2, LLC-MK2, and BSC-1 cells. Only Vero cells inoculated with the specimen exhibited a CPE. The CPE titer of the culture fluid was 10^5–10^6 $TCID_{50}$ per 25 μL against Vero cells. Electron microscopic examination detected typical enteroviruslike virions in the culture fluid of the specimen (round, no envelop, ≈30 nm in diameter). Taken together, these results suggested that the CSF from the patient contained a virus, which we refer to here as NII561-2000.

Physical and Antigenic Properties

Neutralization tests were performed to examine whether the NII561-2000 agent is related to known viruses. The NII561-2000 virus infection of Vero cells was not neutralized by a pool of enterovirus typing antisera or 3 HPeV typing antisera [(HPeV-1, HPeV-2, and HPeV-3) (A308/99)] (Table 1). Conversely, the rabbit antiserum to the NII561-2000 virus did not neutralize the infection of prototype strains of echovirus (serotypes 1–6, 9, 11–15, 17–21, 24–27, 29, 30, and 33), enteroviruses (serotypes 68 and 69), and 3 HPeVs, while it efficiently inhibited the infection of the NII561-2000 virus. These results suggest that the NII561-2000 agent is distinct from the examined known enteroviruses and HPeVs.

Table 1. Neutralization activities of anti-HPeV antibodies using Vero cells*

	Virus			
Antiserum	NII561-2000	HPeV-1	HPeV-2	HPeV-3
NII561-2000	160	10	<10	<10
HPeV-1†	<10	≥1,280	<10	<10
HPeV-2	<10	<10	160	<10
HPeV-3	<10	<10	<10	≥1,280

*HPeV, human parechovirus.
†The HPeV strains used were Harris strain for HPeV-1, Williamson strain for HPeV-2, and A308/99 for HPeV-3.

We next examined the sensitivity of the virus to 5-iodo-2′-deoxyuridine (IUDR) (*14*). The NII561-2000 virus, treated with 10–4.5 μmol/L IUDR, was injected into Vero cells. The IUDR treatment did not affect the CPE in Vero cells, indicating that the virus has the RNA genome (data not shown). We next examined the acid-stability and thermostability of the NII561-2000 virus. The virus was treated at pH 3.0 for 3 h at room temperature, but the infectivity of the treated virus to Vero cells was little affected. Incubation of the virus at 50°C for 30 min reduced the infectivity, while incubation at 50°C for 1 h in the presence of 1 mol/L $MgCl_2$ did not reduce the infectivity. These results indicate that the NII561-2000 virus has similar properties to human enteroviruses and HPeVs.

Genetic Analysis of NII561-2000

To determine the nucleotide sequence of the NII561-2000 virus, the viral RNA was extracted from the purified virus. The partial nucleotide sequences of cDNA clones derived from this viral RNA were determined. BLAST (www.ncbi.nlm.nih.gov/blast) search identified that the nucleotide sequences of 2 isolated cDNAs showed high similarity with that of HPeV-2. By using these cDNAs as a starting material, the cDNAs containing the 5′ portion and 3′ portion of the NII561-2000 gene were isolated by RT-PCR with degenerated primers, 5′ RACE and 3′ RACE. The determined nucleotide sequence of NII561-2000 was 7,347 nt in length, excluding a poly (A) tract. Following a 709-nt 5′ UTR, a long open reading frame encoded a putative polyprotein precursor of 2,182 aa, which was followed by an 89-nt 3′ UTR. To verify the nucleotide sequence of the determined NII561-2000 genome, RT-PCR with a set of primers and RNA sample extracted from the virus-infected Vero cells was carried out. The nucleotide sequences of the NII561-2000 genome obtained by using this RT-PCR method were perfectly matched with those of the originally determined sequences (data not shown).

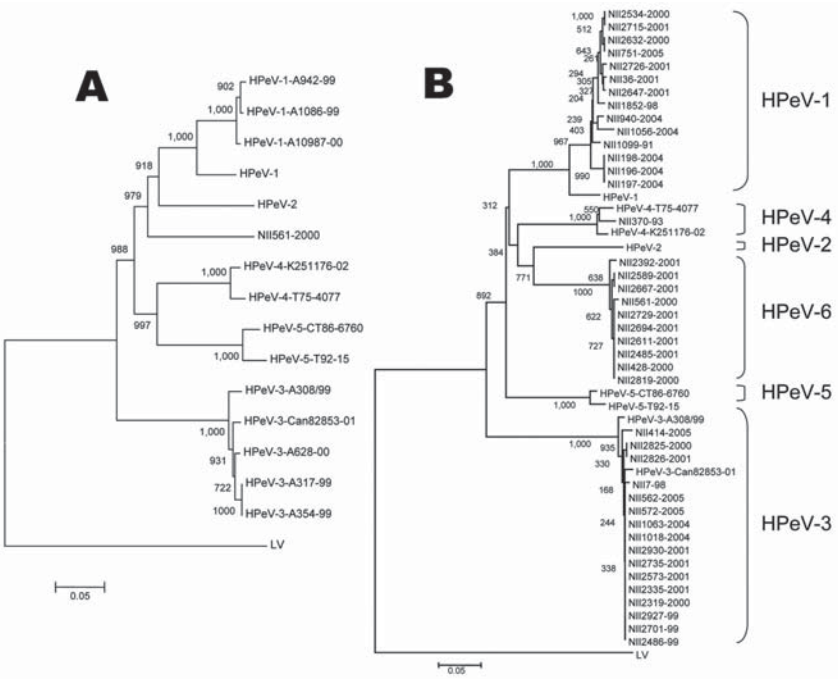

Figure. Phylogenetic tree analysis of the NII561-2000 virus and the NII561-2000–related viruses. A) The phylogenetic tree of P1 amino acid sequences was constructed as described in Materials and Methods. Bar shows a genetic distance of 0.05. The following amino acid sequences were obtained from GenBank: L02971 for HPeV-1 (Harris strain), AJ005695 for HPeV-2 (Williamson strain), AB084913 for HPeV-3 (A308/99), AJ889918 for HPeV-3 (Can82853-01), DQ315670 for HPeV-4 (K251176-02), AM235750 for HPeV-4 (T75-4077), AF055846 for HPeV-5 (CT86-6760), AM235749 for HPeV-5 (T92-15), and AF020541 for Ljungan virus (LV). B) Phylogenetic relationships among the NII561-2000 virus, the NII561-2000–related viruses, and other prototype HPeVs based on comparisons of amino acid similarities of capsid protein (the first 200 aa of VP0 sequences). The tree was constructed by the neighbor-joining method, using ClustalX. Bootstrap values are expressed in percentages after sampling 1,000×. Bar shows a genetic distance of 0.05.

Phylogenetic tree analysis with complete P1 (VP0, VP3, and VP1) amino acid sequences showed that the NII561-2000 virus was most similar to HPeV-1/HPeV-2 (Figure, panel A). The nucleotide sequence and amino acid similarities of NII561-2000 virus to HPeV-1 were 79.5% and 90.7%, those with HPeV-2 were 77.1% and 87.3%, those with HPeV-3 (A308/99) were 76.7% and 85.9%, those with HPeV-4 were 77.1% and 88.2%, and those with HPeV-5 (CT86-6760) were 77.0% and 86.9%, respectively (Table 2). Thus, the NII561-2000 virus is the most similar to HPeV-1 at the amino acid level. The VP1 capsid gene of NII561-2000 was the most divergent (33.8% at nucleotide level) from that of HPeV-5 (CT86-6760).

HPeVs have 9 polyprotein cleavage sites: VP0/VP3, VP3/VP1, VP1/2A, 2A/2B, 2B/2C, 2C/3A, 3A/3B, 3B/3C, and 3C/3D (Table 3). Comparison of these polyprotein cleavage sites among 6 HPeVs showed that those in VP3/VP1, 2A/2B, 2B/2C, 2C/3A, 3A/3B, 3B/3C, and 3C/3D were conserved among all 6 HPeVs. The cleavage site in VP1/2A was identical among HPeV-1, HPeV-2, HPeV-4, HPeV-5, and NII561-2000 but not HPeV-3. The cleavage site in VP0/VP3 of NII561-2000 was identical to that of HPeV-3 but not to those of others.

The NII561-2000 virus, HPeV-1, HPeV-2, HPeV-4, and HPeV-5, but not HPeV-3, had an RGD (arginine-glycine-aspartic acid) motif at the C terminus of VP1 (*15*).

Table 2. Comparisons of nucleotide and amino acid sequences among NII561-2000, HPeV-1, HPeV-2, HPeV-3, HPeV-4, and HPeV-5*

Sequence	% Nucleotide similarity (% amino acid similarity) with NII561-2000				
	HPeV-1†	HPeV-2	HPeV-3	HPeV-4	HPeV-5
5′UTR	85.3	87.3	86.9	88.5	86.9
VP0	73.6 (78.2)	76.0 (80.9)	70.6 (72.3)	72.6 (81.0)	73.1 (77.2)
VP3	74.4 (84.7)	73.0 (80.2)	67.0 (74.1)	70.9 (78.0)	68.7 (73.1)
VP1	71.7n(80.3)	67.3 (72.2)	71.1 (75.3)	70.0 (71.9)	66.2 (70.2)
2A	80.8 (90.7)	77.1 (88.0)	81.0 (85.3)	77.3 (91.2)	76.2 (88.7)
2B	78.6 (97.5)	77.8 (97.5)	82.0 (97.5)	79.8 (97.5)	78.7 (99.2)
2C	84.7 (97.3)	76.4 (86.9)	79.2 (92.7)	78.7 (92.4)	79.2 (93.6)
3A	85.7 (96.6)	77.4 (88.0)	78.0 (87.2)	75.5 (89.7)	78.6 (83.8)
3B	81.4 (80.0)	72.9 (80.0)	73.3 (75.0)	66.7 (85.0)	70.0 (85.0)
3C	84.2 (99.0)	82.7 (99.0)	82.3 (98.0)	82.5 (98.0)	80.5 (99.0)
3D	83.2 (95.9)	83.7 (95.5)	83.9 (95.7)	84.2 (96.8)	87.2 (97.0)
3′UTR	81.6	88.5	83.9	84.9	93.2
ORF	79.5 (90.7)	77.1 (87.3)	76.7 (85.9)	77.1 (88.2)	77.0 (86.9)

*HPeV, human parechovirus; UTR, untranslated region; ORF, open reading frame.
†The HPeV strains used were Harris strain for HPeV-1, Williamson strain for HPeV-2, A308/99 for HPeV-3, K2511876–02 for HPeV-4, and CT86–6760 for HPeV-5.

Table 3. Amino acid sequences of protein cleavage sites of HPeVs

Virus	VP0/VP3	VP3/VP1	VP1/2A	2A/2B	2B/2C	2C/3A	3A/3B	3B/3C	3C/3D
NII561-2000	N/G	Q/N	Q/S	Q/G	Q/G	Q/T	E/R	Q/R	Q/G
HPeV-1*	N/A	Q/N	Q/S	Q/G	Q/G	Q/T	E/R	Q/R	Q/G
HPeV-2	T/A	Q/N	Q/S	Q/G	Q/G	Q/T	E/R	Q/R	Q/G
HPeV-3	N/G	Q/N	E/S	Q/G	Q/G	Q/T	E/R	Q/R	Q/G
HPeV-4	N/N	Q/N	Q/S	Q/G	Q/G	Q/T	E/R	Q/R	Q/G
HPeV-5	N/S	Q/N	Q/S	Q/G	Q/G	Q/T	E/R	Q/R	Q/G

*The human parechovirus (HPeV) strains used were Harris strain for HPeV-1, Williamson strain for HPeV-2, A308/99 for HPeV-3, K2511876–02 for HPeV-4, and CT86–6760 for HPeV-5.

The RGD motif may be used for an entry receptor of these HPeVs to attach, penetrate, or both, into host cells. Mutant HPeV-1 viruses with 2 aa deletions in the RGD motif showed little infectivity, while an RGD-to-RGE (arginine-glycine-glutamic acid) change showed reduced infectivity, and the resultant viruses possessed a rescued RGD. In addition, mutations at the +1 and +2 positions downstream from the RGD motif produced small virus–inducing plaques, and an M-to-P change at +1 position was lethal (*15*). The amino acids (+1 and +2 positions) downstream from the RGD motif of NII561-2000 were identical to those of HPeV-1.

The N terminal ends of VP4 of many picornaviruses are myristoylated, and they have a consensus myristoylation motif (*16*). HPeVs, including NII561-2000, lacked a myristoylation motif in the corresponding VP0 sequence. Myristoylation of capsid proteins is suggested to play a role in virion assembly. Thus, the virion assembly mechanism of HPeVs, including NII561-2000, might be distinct from other picornaviruses, including poliovirus.

Isolation of HPeVs from Clinical Samples

By using cultured cells (Caco-2, RD-18S, Vero, HeLa, HEp-2, LLC-MK2, and BSC-1), we have isolated 8,195 CPE-inducing agents from 13,656 clinical samples (stool, throat swab, and CSF) collected between 1991 and 2005, at Niigata, Japan (Table 4, Table 5). The CPE morphologic features and CPE-inducing cell types suggested that 1,521 isolates are likely to be enteroviruses, HPeVs, rhinoviruses, or other viruses, and the others are likely to be influenza viruses or adenoviruses. Neutralization tests that used antisera against enteroviruses and HPeV-1 showed that 1,365 were enteroviruses and 12 were HPeV-1, and the remaining 144 viruses were not neutralized by these agents. RT-PCR that used degenerate primers against HPeVs, enteroviruses, and rhinoviruses identified 29 HPeVs, 72 enteroviruses, and 26 rhinoviruses, respectively. The remaining 17 viruses were not identified by these methods. Thus, a total of 41 HPeVs were isolated from the samples collected in Niigata.

Phylogenetic tree analysis showed 14 HPeV-1, 16 HPeV-3, and 1 HPeV-4, but no HPeV-2 and HPeV-5 among the examined 41 HPeVs. In addition, 10 viruses, including NII561-2000, formed a distinctive tree from those of the other HPeVs (Figure, panel B). Of note, in our search from 1991 through 2005, the NII561-2000–related viruses were isolated only in 2000 (3 cases) and 2001 (7 cases); HPeV-1 were isolated in 1991, 1998, 2001, 2004, and 2005; HPeV-3 were isolated in 1998, 1999, 2000, 2001, 2004, and 2005; and HPeV-4 was isolated only in 1993 (Table 4). The clinical symptoms of the patients infected

Table 4. Numbers of isolated HPeVs in 1991–2005 at Niigata, Japan*

Year	HPeV subtype				No. isolated HPeVs	No. examined samples	No. isolated viruses
	Type 1	Type 3	Type 4	Type 6			
1991	1	0	0	0	1	303	255
1992	0	0	0	0	0	142	53
1993	0	0	1	0	1	166	50
1994	0	0	0	0	0	151	55
1995	0	0	0	0	0	167	116
1996	0	0	0	0	0	133	54
1997	0	0	0	0	0	277	84
1998	1	1	0	0	2	1,607	1,117
1999	0	3	0	0	3	2,118	1,203
2000	2	2	0	3	7	1,974	1,127
2001	4	5	0	7	16	1,699	989
2002	0	0	0	0	0	2,783	1,787
2003	0	0	0	0	0	986	597
2004	5	2	0	0	7	584	329
2005	1	3	0	0	4	566	379
Total	14	16	1	10	41	13,656	8,195

*HPeV, human parechovirus.

with NII561-2000–related viruses were gastroenteritis, respiratory symptoms, rash, and flaccid paralysis in addition to Reye syndrome (Table 5), and these disease categories were similar to those of other HPeVs (*2*,*3*).

Discussion

In this study, we isolated a novel HPeV (NII561-2000) from a 1-year-old girl with Reye syndrome and determined the nucleotide sequence. Nucleotide sequence analysis and mutual neutralization test indicated that the NII561-2000 virus was distinct from 5 known HPeVs (Figure, panel A) (*17*). Thus, we propose that the NII561-2000 virus is the prototype of HPeV-6.

The NII561-2000 virus was originally isolated from a patient with Reye syndrome, an acute noninflammatory encephalopathy characterized by an antecedent viral infection, such as influenza or varicella (*10*–*12*). The significance of the NII561-2000 virus in this syndrome is not clear, because our samples did not include any other samples from this patient. We also isolated 9 NII561-2000–related viruses from clinical samples collected from other patients. The clinical symptoms of the persons infected with NII561-2000–

Table 5. Diseases associated with HPeVs isolated 1991–2005 in Niigata, Japan*

Strain	HPeV subtype	Specimen	Clinical symptom	Sex	Age, y	Cell line(s)
NII1099-91	1	Stool	Gastroenteritis	F	1	BSC-1
NII1852-98	1	Throat swab	Hand-foot-mouth disease	M	<1	CaCo2, RD-18S
NII2534-2000	1	Stool	Fever of unknown origin	M	<1	CaCo2
NII2632-2000	1	Stool	Gastroenteritis	M	1	RD-18S, Vero
NII36-2001	1	Stool	Gastroenteritis	M	1	BSC-1
NII2647-2001	1	Throat swab	Upper respiratory tract infection	M	1	RD-18S
NII2715-2001	1	Stool	Gastroenteritis	M	<1	CaCo2, RD-18S
NII2726-2001	1	Stool	Gastroenteritis	F	1	CaCo2
NII196-2004	1	Stool	Gastroenteritis	M	9	LLC-MK2
NII197-2004	1	Stool	Gastroenteritis	M	<1	LLC-MK2
NII198-2004	1	Stool	Gastroenteritis	M	<1	LLC-MK2
NII940-2004	1	Throat swab	Bronchitis	M	<1	CaCo2, RD-18S, Vero
NII1056-2004	1	Stool	Gastroenteritis	F	<1	LLC-MK2
NII751-2005	1	Stool	Gastroenteritis	M	<1	CaCo2
NII7-98	3	Stool	Gastroenteritis	F	1	BSC-1
NII2486-99	3	Stool	Rash	F	<1	Vero
NII2701-99	3	Throat swab	Rash	F	1	Vero
NII2927-99	3	Stool	Aseptic meningitis	M	<1	Vero
NII2319-2000	3	Stool	Upper respiratory tract infection	M	<1	BSC-1
NII2825-2000	3	Throat swab	Upper respiratory tract infection	M	1	Vero, LLC-MK2
NII2335-2001	3	Throat swab	Rash	F	<1	Vero, LLC-MK2
NII2573-2001	3	Throat swab	Rash	F	<1	Vero, LLC-MK2
NII2735-2001	3	Stool	Aseptic meningitis	F	<1	Vero, LLC-MK2
NII2826-2001	3	Throat swab	Upper respiratory tract infection	M	<1	Vero, LLC-MK2
NII2930-2001	3	Throat swab	Myositis	M	8	CaCo2, Vero
NII1018-2004	3	CSF	Fever of unknown origin	F	<1	Vero, LLC-MK2
NII1063-2004	3	Stool	Gastroenteritis	M	<1	CaCo2, LLC-MK2
NII414-2005	3	Throat swab	Influenzalike illness	M	4	Vero, LLC-MK2
NII562-2005	3	Throat swab	Influenzalike illness	F	35	LLC-MK2
NII572-2005	3	Throat swab	Influenzalike illness	M	5	Vero, LLC-MK2
NII370-93	4	Stool	Lymphadenitis	M	5	RD-18S, Vero, LLC-MK2
NII428-2000	6	Stool	Gastroenteritis	F	1	Vero
NII561-2000	6	CSF	Reye syndrome	F	1	Vero
NII2819-2000	6	Throat swab	Rash	M	2	BSC-1
NII2392-2001	6	Stool	Flaccid paralysis	M	1	CaCo2
NII2485-2001	6	Throat swab	Upper respiratory tract infection	F	<1	RD-18S
NII2589-2001	6	Throat swab	Gastroenteritis	M	8	RD-18S
NII2611-2001	6	Stool	Gastroenteritis	F	3	CaCo2, Vero
NII2667-2001	6	Stool	Gastroenteritis	M	6	HeLa
NII2694-2001	6	Throat swab	Rash	M	2	CaCo2, RD-18S
NII2729-2001	6	Stool	Gastroenteritis	F	2	BSC-1

*HPeV, human parechovirus; CSF, cerebrospinal fluid.

related viruses were gastroenteritis, upper respiratory tract infection, rash, and flaccid paralysis. These disease categories of the NII561-2000–related virus are similar to those of other HPeVs, but the pathologic roles of the NII561-2000 viruses in these diseases, including Reye syndrome, need further etiologic and biologic studies.

In our search at Niigata from 1991 through 2005, the NII561-2000 and related viruses were isolated only in 2 consecutive years (2000 and 2001), HPeV-1 were isolated in 5 years, and HPeV-3 were isolated in 6 years. The genetic variations of these NII561-2000–related viruses were small (Figure, panel B). These results suggest that a 2-year outbreak of NII561-2000–related virus may have occurred in Niigata, Japan.

Thirteen of 16 HPeV-3 isolated in Niigata were from the patients <3 years of age, consistent with the previous report (*18*). Sepsislike illness and central nervous system involvement were more frequently reported in children infected with HPeV-3 than HPeV-1 (*18*). Consistent with those findings, the HPeV-3 infections in Niigata included 2 aseptic meningitis patients, whereas no such illness was associated with HPeV-1.

HPeV-4 was isolated from a 5-year-old patient with lymphadenitis in Niigata (Table 5). Thus, this virus is prevalent and is likely to be pathogenic in at least 3 countries. The diseases associated with HPeV-4 in the Netherlands and the United States were fever and TORCH (toxoplasmosis; other infections; namely, hepatitis B, syphilis, herpes zoster, rubella, cytomegalovirus, and herpes simplex virus infections) (*6*,*19*). Further analysis is required to establish an association of HPeV-4 with these diseases.

The degenerate primer set we used here was originally developed by Ito et al. (*5*) to amplify 3 known HPeV cDNAs. Here, we successfully amplified the NII561-2000 viral cDNA and HPeV-4 from culture supernatants of the infected cells, indicating that this primer set can amplify the cDNA fragments of at least 5 HPeVs from culture supernatants of infected cells. Thus, this primer set is a useful tool to determine the subtypes of HPeVs.

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Melioidosis Outbreak after Typhoon, Southern Taiwan

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From July through September 2005, shortly after a typhoon, 40 cases of *Burkholderia pseudomallei* infection (melioidosis) were identified in southern Taiwan. Two genotypes that had been present in 2000 were identified by pulsed-field gel electrophoresis. Such a case cluster confirms that melioidosis is endemic to Taiwan.

Melioidosis is an infectious disease caused by *Burkholderia pseudomallei* and is found in northern Australia and tropical countries in Southeast Asia (*1*). The incidence of melioidosis cases increases during the rainy season (*2*). Melioidosis often affects adults who have chronic underlying diseases, especially diabetes mellitus, and is often associated with illness and death. The mortality rate varies geographically, ranging from 19% in northern Australia to 68% in northeastern Thailand (*1*).

The first case of melioidosis in Taiwan, acquired in the Philippines, was reported in 1985 (*3*); since then several sporadic cases have been reported. In a recent comprehensive review, Taiwan was categorized as an area in which melioidosis may be endemic (*1*). However, this conclusion was deduced from a limited number of clinical cases reported in the literature. A total of 13 cases have been reported in Taiwan in patients who never traveled to melioidosis-endemic areas (*4*–*12*). These patients likely have indigenous cases of melioidosis, and no common source of *B. pseudomallei* isolates has been identified. We report a cluster of 40 cases of melioidosis after a typhoon hit Taiwan, which confirms that melioidosis is endemic in this country.

The Study

Heavy rains from Typhoon Haitang on July 16, 2005, caused mudslides and flooding in central and southern Taiwan. The first clinical isolate of *B. pseudomallei* was found on July 29, 2005. Demographic and clinical data for melioidosis case-patients at 3 hospitals were collected.

Clinical information was collected on a case record form. For each patient, demographic data, including location, clinical signs, underlying illness, laboratory data, radiologic images, antimicrobial drug therapy, and clinical outcome, were obtained from medical records. Information about patients' functional levels, recent exposure to mud or water before admission, and prior travel to Southeast Asia or Australia was obtained by telephone from the patients or their families if such information was incomplete or not available on medical charts. The study was reviewed and approved by the Institutional Review Board of Chi-Mei Foundation Medical Center.

Forty oxidase-positive, nonfermentative, gram-negative bacilli grew in Ashdown selective medium and showed characteristic dry, rough, blue-purple colonies. These isolates were then tested for flagellin genes by PCR. The paired primers used for PCR amplification were PMA-1 (5′-CTG TCG TCG ACG GCC GT-3′) and PMA-2 (5′-GGT TCG AGA CCG TTT GCG-3′) (*13*). The amplicons, ≈190 bp, were sequenced by using an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). These isolates showed 99% identity with the homologous region of *B. pseudomallei* ATCC23343 in the GenBank database.

In addition to the 40 isolates obtained in 2005, a total of 14 *B. pseudomallei* isolates from 14 sporadic cases were available for molecular typing, including 1 isolate found in 2000, 4 in 2001, 4 in 2002, 1 in 2003, and 4 in 2004. Only the first isolate from each case was studied. Two genetically distinct *B. pseudomallei* strains isolated in northern Taiwan (*9*) were used as reference strains. For pulsed-field gel electrophoresis (PFGE), bacterial plugs were digested with restriction endonucleases *Xba*I and *Spe*I. Digests were subjected to gel electrophoresis using the Chef Mapper System (Bio-Rad Laboratories, Hercules, CA, USA) with a bacteriophage λ DNA ladder. Gels were stained with ethidium bromide, viewed under UV light, and analyzed by using the Molecular Analyst System (Bio-Rad Laboratories).

Of 40 patients, 30 (75%) were male. Their mean age was 64.6 years (range 38–87 years). A total of 37 (92.5%) patients never traveled abroad, and 28 (70%) denied recent contact with mud or dirty water before their illness. Because 3 patients could not walk, infection by cutaneous contact with contaminated dirt or water was less likely. Twenty-seven (67.5%) patients had an underlying debilitating illness, predominantly diabetes mellitus (20 patients).

The most common initial symptoms were fever (29 patients, 72.5%) and cough (13 patients, 32.5%). Relevant prodromes lasting ≤72 hours before admission were noted in 25 (62.5%) patients, and 13 (32.5%) patients visited hospitals within 24 hours after onset of the illness, which suggests acute illness. The earliest onset of symptoms related

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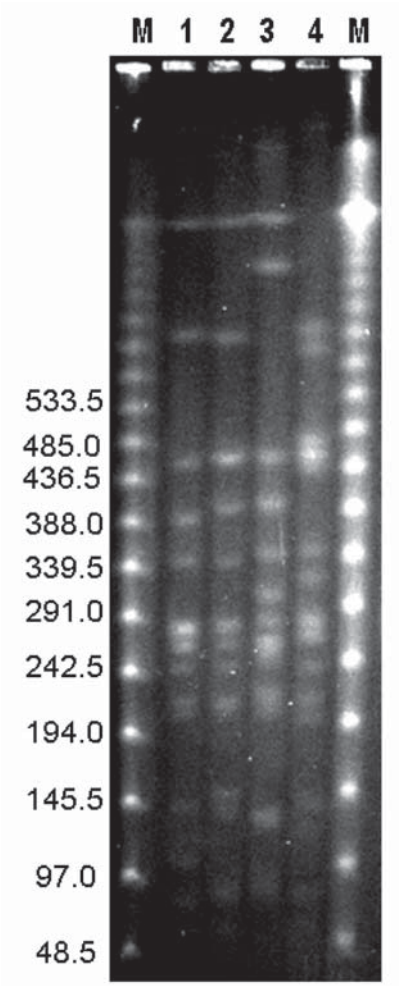

Figure 1. Pulsed-field gel electrophoresis of DNA from *Burkholderia pseudomallei* isolates digested with *Spe*I from patients with melioidosis in Taiwan. Lane M, bacteriophage λ DNA ladder (48.5 kb–970 kb). Lane 1, isolate from Kaohsiung County, 2005 (type A); lane 2, isolate from Tainan County, 2005 (type B); lane 3, isolate from northern Taiwan (type C); lane 4, isolate from northern Taiwan (type D). Values on the left are in kilobases.

to melioidosis was July 20, 2005. This onset was 4 days after the arrival of Typhoon Haitang, which suggests an incubation period of 4 days. Most patients (30, 75%) had *B. pseudomallei* bacteremia, and 20 of these patients had concomitant pleuropulmonary infections. Of 10 (25%) patients without bacteremia, pulmonary infections remained the predominant foci in 6. Eight (20%) patients died during hospitalization.

*Xba*I restriction profiles in PFGE provide a level of resolution similar to that obtained with multilocus sequence typing for *B. pseudomallei* isolates (*14*), but the discriminative sensitivity of *Spe*I was greater than that of *Xba*I in differentiation of our *B. pseudomallei* isolates. Thus, genotyping information obtained from PFGE *Spe*I profiles is useful in epidemiologic studies. Among the 54 isolates, 2 PFGE genotypes (types A and B) were identified in *Spe*I macrorestriction profiles (Figure 1). These genotypes were genetically distinct from the 2 reference isolates (types C and D). Genotypes A and B were found in isolates obtained as early as 2000 (Figure 2).

Conclusions

Melioidosis became a reportable disease in Taiwan in 2000; through 2004, the Center for Disease Control in Taiwan received reports of 43 cases of *B. pseudomallei* infections (*15*). A total of 33 (77%) cases were reported in southern Taiwan. Increased rainfall in conjunction with Typhoon Haitang was observed from July through September 2005. At the end of September 2005, a total of 40 cases of melioidosis were identified. From October 2005 through March 2006, only 3 cases were identified, but these were not included in our study.

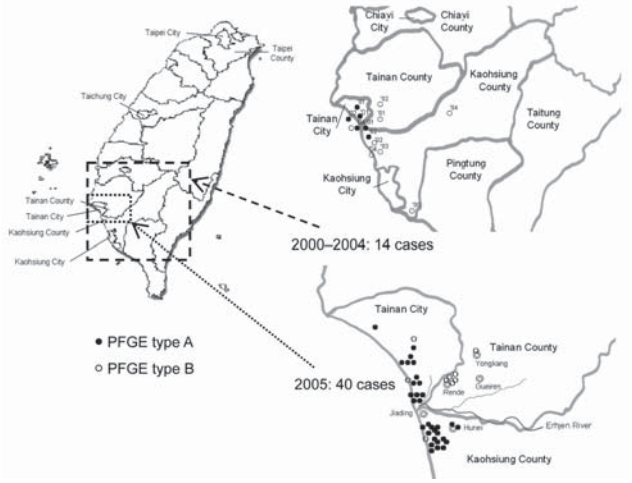

Figure 2. Geographic distribution of 14 sporadic cases of melioidosis, 2000–2004, and 40 clustered cases, 2005, Taiwan. Two pulsed-field gel electrophoresis (PFGE) genotypes (types A and B) of *Burkholderia pseudomallei* were present in southern Taiwan. The numbers in the upper right panel indicate year of isolation.

In conclusion, after widespread flooding caused by a typhoon, an outbreak of melioidosis occurred in southern Taiwan from July 2005 through September 2005. Two genotypic strains were found in clustered cases in 2005 and in sporadic cases found in Tainan and Kaohsiung counties in 2000. These findings confirm that environmental sources of *B. pseudomallei* are likely present in southern Taiwan and that melioidosis is endemic in this country.

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Loop-Mediated Isothermal Amplification for Influenza A (H5N1) Virus

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We describe a 1-step reverse-transcription loop-mediated isothermal amplification assay for detection of highly pathogenic avian influenza A (H5N1) viruses. The assay was tested by using a panel of highly pathogenic H5N1 subtypes isolated over the past 10 years and clinical specimens. The assay produced negative results for all non-H5N1 subtypes.

Highly pathogenic avian influenza A (H5N1) virus has had a significant global effect on the poultry industry, human healthcare, and many other sectors (*1*). Several molecular tests have been developed for the rapid detection of influenza (H5) virus subtypes (*2*), but they often require sophisticated equipment (e.g., PCR machine) and are difficult for researchers and clinicians to perform in resource-limited settings. Loop-mediated isothermal amplification (LAMP) provides a molecular testing option for this scenario (*3*). The LAMP mechanism has been described (*3*,*4*). Using this approach, nucleic acids are amplified under isothermal conditions (e.g., in a water bath) with high specificity, efficiency, and speed (*3*). The assay is highly specific due to recognition of target DNA by 6 independent sequences. An attractive feature of LAMP is its ability to generate large amounts of white magnesium pyrophosphate precipitate in positive reactions (*4*). Examples of positive and negative reactions are shown in online Appendix Figure 1 (available from www.cdc.gov/EID/content/13/6/899-appG1.htm). LAMP enables easy visual identification of positive reactions (*4*,*5*) and avoids additional cost and labor for postamplification analysis. This closed-tube method can also minimize the problem of carryover contamination in less controlled environments.

The Study

We aimed to develop a reverse transcription (RT) LAMP assay that could detect a variety of highly pathogenic influenza (H5N1) viruses. For primer design, we used highly pathogenic influenza (H5N1) sequences publicly available from the Influenza Sequence Database (www.flu.lanl.gov/index.html) in August 2006. We used hemagglutinin (HA) from an influenza (H5N1) prototype vaccine strain (A/Vietnam/1203/2004, GenBank accession no. AY651334) as the reference for this study. The sequences included highly pathogenic influenza (H5N1) viruses identified in the past 2 years. In addition, sequences from all the other HA subtypes (e.g., 1,127 for H1 and 1,472 for H3) in the above database were used for the analysis. Because highly pathogenic influenza (H5N1) gene sequences are genetically diverse, all studied H5 sequences (n = 711) were aligned, and a highly conserved region at the 5′ end of HA2 encoding sequence (corresponding to nt 1,065–1,298 of the reference sequence) was selected as the target (online Appendix Figure 2A, available from www.cdc.gov/EID/content/13/6/899-appG2.htm, and online Appendix Figure 3, available from www.cdc.gov/EID/content/13/6/899-appG3.htm). Of all the downloaded highly pathogenic influenza (H5N1) sequences, 115 did not contain the target site and were excluded from analysis. The GenBank accession numbers for the highly pathogenic influenza (H5N1) sequences used for the primer design are listed in the online Appendix Table (available from www.cdc.gov/EID/content/13/6/899-appT.htm).

Previous demonstration that degenerate primers could be used in LAMP assays (*6*) led us to use the same approach for designing a set of degenerate primers for the targeted region (online Appendix Figure 2B). Our HA sequence analysis also showed that HA sequences from influenza (H5N2) viruses of North American lineage and other viruses with other HA subtypes contain extensive mismatches to the primers used in the LAMP assay (online Appendix Figure 2A, B). In the initial phase of the study, we used the reference strain (A/Vietnam/1203/2004) to determine the sensitivity of the assay. Purified RNA from a viral supernatant with a known titer was serially diluted and subjected to the LAMP assay. As shown in the Figure, panel A, the detection limit of the assay was found to be 2×10^{-3} plaque-forming units (pfu) per reaction. The sensitivity of this assay was also compared with that of an optimized RT-PCR assay recommended by the World Health Organization (*7*). As shown in the Figure, panel A, the detection limit of the RT-PCR assay was identical to that from the LAMP assay. We also spiked various amounts of influenza (H5) viruses into non-H5 nasopharyngeal aspirate samples and tested the purified RNA from these clinical specimens by the LAMP and RT-PCR assays. The results from these assays were identical (data not shown). These findings are in accordance with previous findings that the sensitivity of LAMP assays is comparable to that of conventional PCR methods (*5*,*6*,*8*). In addition, all positive reactions were vi-

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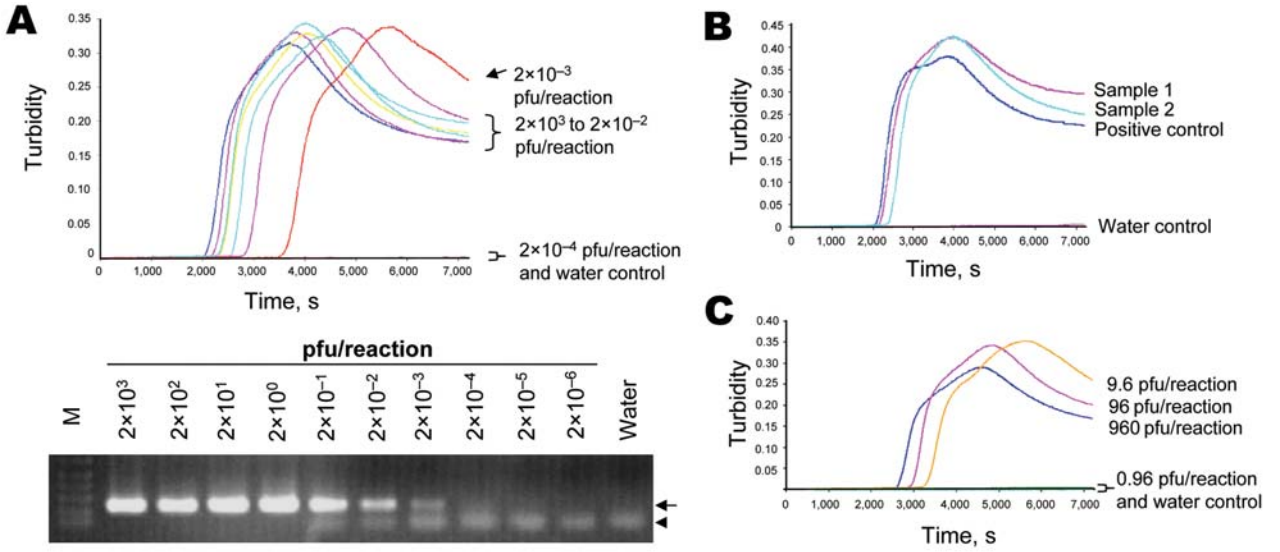

Figure. Detection of influenza (H5) virus by loop-mediated isothermal amplification (LAMP). A) Serially diluted RNA from A/Vietnam/1203/2004 was tested by the reverse transcription (RT)–LAMP (upper panel) and RT-PCR (lower panel) assays. The viral titers used in these reactions are indicated. Viral RNA was extracted by using the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. For a typical 25-μL reaction, 2 μL of sample was mixed with 2× in-house reaction buffer (40 mmol/L Tris-HCl, pH 8.8; 20 mmol/L KCl; 16 mmol/L $MgSO_4$; 20 mmol/L $[NH_4]_2SO_4$; 0.2% Tween 20 [v/v]; 1.6 mol/L betaine; 2.8 mmol/L each dNTP), 50 U *Bst* DNA polymerase (New England Biolabs, Ipswitch, MA, USA), 8 U avian myeloblastosis virus reverse transcriptase (Invitrogen, Gaithersburg, MD, USA), 40 pmol/L primers FIP and BIP, 20 pmol/L primers LPF and LPR, and 5 pmol/L primers F3 and B3. Reaction mixtures were incubated at 60ºC for 120 min, and the turbidity of these reactions was examined by use of a turbidity meter (LA-200, Treamecs; Kyoto, Japan) in real time. The turbidities of these reactions 5–20 min after incubation were taken as the baseline. The threshold value for a positive reaction was set to be 10× above the standard deviation of the baseline. For the H5-specific RT-PCR assay, primers H5-1 (5′-GCCATTCCACAACATACACCC-3′) and H5-3 (5′-CTCCCCTGCTCATTGCTATG-3′) were used according to the protocol optimized by the World Health Organization H5 Reference Laboratory Network (*7*). Positive (219 bp) and nonspecific products from the PCR reaction are highlighted by the arrow and arrowhead, respectively. B) Detection of H5 virus in postmortem lung tissues from a patient with influenza (H5). Signals from the tested samples, positive control, and water control are indicated. C) Direct detection of influenza (H5) viruses from culture supernatants. Heat-treated supernatant from cells infected with A/Vietnam/1203/2004 were serially diluted and directly used as input in the LAMP assay. The plaque-forming units (pfu) of influenza (H5) virus in these reactions are shown.

sually examined for the presence of white precipitate at the end of the reaction incubation. As expected, all results correlated exactly with those deduced by the real-time turbidity meter (data not shown).

We also tested the feasibility of using this assay to detect influenza (H5) viruses in clinical specimens from a patient with influenza (H5). RNA from 2 different postmortem lung tissue samples from a patient infected with influenza A/HK/212/03 was subjected to the LAMP assay. As shown in the Figure, panel B, both RNA samples had positive results for the influenza (H5) viruses.

To evaluate the specificity of the LAMP assay for influenza (H5) viruses, a comparative study including all 16 HA subtypes and a variety of H5N1 strains was performed (Table). All 14 H5N1 strains isolated from different geographic regions during the past 10 years were positive, whereas none of the non-H5 samples were positive (Table). All the reactions were visually examined, and the results corresponded with those generated from the real-time turbidity meter. Influenza A/chicken/Wajo/BBVM/2005, A/duck/Vietnam/568/2005, and A/bar-headed goose/Qinghai/5/2005 are clade 2 influenza (H5N1) viruses that have recently emerged from different geographic regions (*9*), and these 3 viruses are phylogenetically distinct (*10*,*11*). Our results demonstrate that this assay is applicable to a wide variety of highly pathogenic influenza (H5N1) viruses. In addition, other virus reassortants with the HA of this lineage are expected to be positive in the assay.

We previously demonstrated that heat-treated blood samples could be directly tested by a LAMP assay specific for DNA of a bloodborne pathogen (*5*). These modifications could avoid the need for nucleic acid purification, thereby reducing the cost and turnaround time for molecular diagnosis. We tested the feasibility of detecting the H5 sequence from viral cultures without RNA extraction. In a Biosafety Level 3 facility, 50 μL of viral culture of the prototype virus was heat inactivated (99°C for 10 min), the heat-treated sample was serially diluted with standard viral culture medium, and 2 μL of serially diluted samples was added to the LAMP assay. As shown in the Figure, panel C,

Table. RT-LAMP assay results for highly pathogenic influenza A (H5N1)*

Virus subtype	Strain	Result
H1	A/HK/54/98	–
H2	A/Singapore/57	–
H3	A/HK/1174/99	–
H4	A/duck/HK/MPA892/06	–
H5N1	A/HK/483/97	+
H5N1	A/HK/486/97	+
H5N1	A/chicken/HK/61.9/2002	+
H5N1	A/goose/HK/739.2/2002	+
H5N1	A/HK/213/03	+
H5N1	A/HK/212/03	+
H5N1	A/Thailand/MK2/04	+
H5N1	A/Vietnam/1203/04	+
H5N1	A/chicken/Indoneasia/4/2004	+
H5N1	A/chicken/Thailand/1/2004	+
H5N1	A/chicken/Vietnam/33/2004	+
H5N1	A/chicken/Wajo/BBVM/2005	+
H5N1	A/duck/Vietnam/568/2005	+
H5N1	A/bar-headed goose/Qinghai/5/2005	+
H6	A/teal/HK/W312/97	–
H7	A/env/HK/MPB127/05	–
H8	A/duck/HK/MP4275/2005	–
H9	A/duck/HK/G1/97	–
H10	A/env/HK/MPB839/05	–
H11	A/env/HK/MPB1679/06	–
H12	A/red necked stint/WA/5745/1984	–
H13	A/gull/Maryland/704/1977	–
H14	A/mallard/Gurjev/244/1982	–
H15	A/shelduck/WA/1762/1979	–
H16	A/gull/Denmark/68110/2002	–

*RT-LAMP, reverse transcription loop-mediated isothermal amplification; –, negative; +, positive.

and in online Appendix Figure 1, H5N1 subtype could still be detected by the assay. However, the detection limit (9.6 pfu/reaction) was ≈1,000× less sensitive than the limit with purified RNA as an input.

Conclusions

The RT-LAMP assay is highly specific, and its sensitivity is comparable to that of an optimized RT-PCR assay for influenza (H5N1) viruses. The detection limit is equivalent to that of a similar LAMP assay (*12*), but our assay was extensively tested by using a wide variety of influenza (H5) viruses, including recent clade 2 influenza (H5) viruses (*9*). LAMP does not require thermocyclers and gel electrophoresis. Reactions can simply be incubated in a water bath or heating block, and the results can be confirmed by direct visual inspection. Because early identification of influenza (H5) is crucial for the containment of the disease, this novel assay can provide an efficient option for the preliminary molecular detection of highly pathogenic influenza (H5) viruses in basic laboratory or clinical settings. Combined with the use of a turbidity meter, which costs much less than a real-time RT-PCR system, RT-LAMP can provide quantitative data for viral load studies. In conclusion, the LAMP assay is a promising tool for the detection of influenza (H5) viruses.

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Nosocomial Buffalopoxvirus Infection, Karachi, Pakistan

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During 5 months in 2004–2005, buffalopoxvirus infection, confirmed by virus isolation and limited nucleic acid sequencing, spread between 5 burns units in Karachi, Pakistan. The outbreak was related to movement of patients between units. Control measures reduced transmission, but sporadic cases continued due to the admission of new patients with community-acquired infections.

Buffalopoxvirus, a strain of *Vaccinia virus* in the genus *Orthopoxvirus* of the family *Poxviridae,* has been associated with sporadic cases and outbreaks of infection in Asian buffalo (*Bubalus bubalis*) in Pakistan, India, Bangladesh, Russia, Indonesia, Egypt, and Italy (*1–13*). The virus causes pock lesions on the udder, which adversely affect milk production and can be a source for human infection characterized by transient fever, regional lymphadenitis, and pock lesions, usually on the hands, from contact with infected buffalo.

The Study

Karachi is the largest city in Pakistan, with a population of 12 million. Healthcare is provided by public and private hospitals, and there are 5 major burns units. In January 2005, pustular lesions were observed on the foot of a patient in 1 of the burns units, and similar lesions subsequently appeared on other patients. Local health authorities were informed of the outbreak, and an investigatory team confirmed reports of similar infections in the city's other burns units, with retrospective identification of at least 19 probable cases occurring over a 5-month period.

Most patients had a fever (39.0°C–40.5°C) for 2–3 days, followed by the appearance of an eruption(s). Lesions were typically small, rounded, umbilicated, and nodular in appearance with an erythematous base (Figure 1A). They contained cheesy pustular material and increased in size (from 1–2 mm to >1 cm in diameter) and severity over 7–8 days. The lesions involved burn wounds and intact skin surrounding them, with the unhealed margins of the wounds being covered by a layer of thick yellow secretion. Some patients had a sparse rash; others had closely spaced lesions that produced a cobblestone appearance. The lesions developed crusts, which shrank and sloughed without residual scars. A single lesion developed on a paramedical staff member's finger, and lesions developed around 1 patient's insertion site for an intravenous line (Figure 1B). In all instances, the disease was self limiting, and patients recovered in 3–4 weeks.

Results of bacteriologic and mycologic examination of biopsy samples, impression smears, and swab samples from lesions were negative. Histopathologic examination showed extensive ulceration and granulation, with epidermal necrosis and subepidermal edema plus acute and chronic inflammatory cell infiltration. No molluscum bodies were observed, but eosinophilic cytoplasmic inclusions were present in keratinocytes. Impression smears and biopsy tissues were sent to the Special Pathogens Unit, National Institute for Communicable Diseases (NICD), Sandringham, South Africa, and to the Health Protection Agency (HPA), Centre for Emergency Preparedness and Response, Porton Down, Salisbury, United Kingdom. Electron microscopy of negative-stained grids prepared at HPA and NCID laboratories from pustular material showed orthopoxvirus particles, and examination of ultrathin sections prepared from infected Vero cell cultures at HPA found classic orthopox intracytoplasmic virus factories and particle maturation sites (Figure 1C, D).

PCR was performed on nucleic acid extracted from the samples, using primers specific for regions of the orthopoxvirus hemagglutinin gene (at NICD) and B5R membrane protein gene (at HPA). After nucleotide sequences were determined for the PCR products, phylogenetic analyses were conducted in relation to corresponding orthopoxvirus sequences obtained from GenBank, using methods described elsewhere (*14*,*15*). The causative agent was found to cluster with buffalopoxvirus isolates within the vaccinia subgroup of orthopoxviruses (Figure 2), and 3 patients from 2 separate burns units were shown to be infected with the identical virus, which was distinct from other known buffalopoxvirus isolates. To investigate the possibility of a shared source of infection, 17 samples of saline, antimicrobial drug ointments, petroleum jelly, cotton dressings, and swabs in common use were obtained from the 5 burns units and tested by PCR at NICD; no results were positive. Inquiries led to the suggestion that the outbreak was probably propagated by transfer of infected patients between burns units. This hypothesis was confirmed when a policy to isolate all new admissions, including referrals from other

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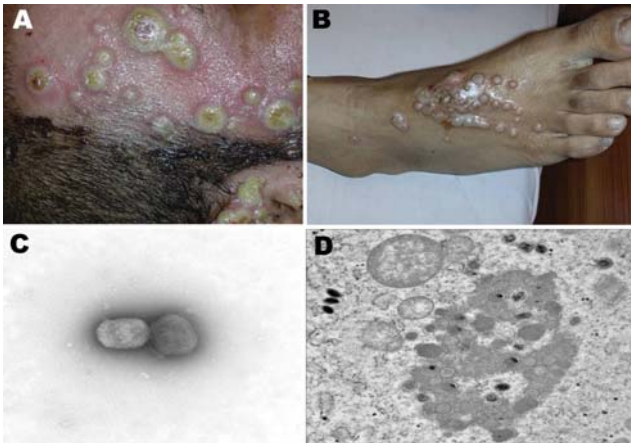

Figure 1. Nosocomial buffalopoxvirus infection of patients in burns units. A) Lesions involving intact skin around a burn wound and the wound itself. B) Lesions around an insertion site for an intravenous line. C) Orthopoxvirus particles detected by electron microscopy (EM) examination of negatively stained grids prepared from pustular material (magnification ×73,000). D) Transmission EM examination of ultrathin sections of infected Vero cell cultures showing classic intracytoplasmic orthopoxvirus factories and maturing virus particles (magnification ×21,000).

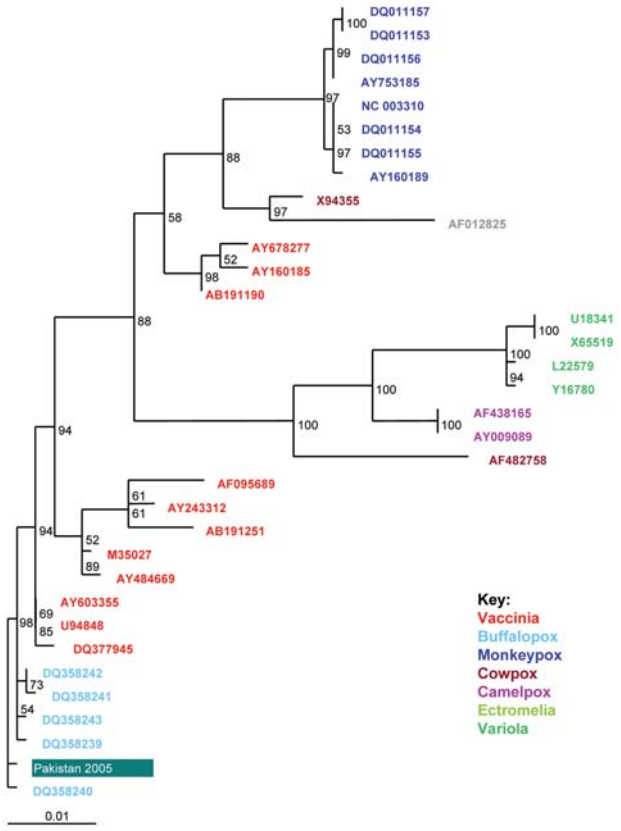

Figure 2. Maximum likelihood phylogenetic tree based on a 955-nt alignment of the Karachi isolate and 33 orthopoxvirus sequences of the B5R gene from GenBank constructed with ClustalW (www.ebi.ac.uk/clustalw/index.html) and TREE-PUZZLE (http://bioweb.pasteur.fr/seqanal/interfaces/puzzle.html); figures at nodes represent PUZZLE support values. The orthopoxvirus types are indicated to the right. The Karachi isolate sequence (Pakistan 2005) groups within the buffalopox B5R genes.

burn centers, for their first 2 weeks in a unit successfully controlled transmission of new endogenous cases.

Control measures included education of staff, single-room or cohort isolation of patients infected or suspected of being infected with buffalopoxvirus, and reinforcement of infection-control practices, such as hand disinfection after contact with any patient. To reduce virus load in the environment, the facilities were cleaned more frequently and hypochlorite disinfectant was used for cleaning. The measures proved effective in reducing transmission within burns units, but they did not prevent the sporadic arrival of newly infected patients.

Conclusions

Buffalopoxvirus outbreaks reported to date have been geographically restricted, and human cases have been limited to persons with direct exposure to infected animals, usually in rural communities (*1–11*). This reported outbreak uniquely involved nosocomial infections in 5 widely separated burns units in Karachi, Pakistan. However, buffaloes are the most common dairy animal in Pakistan, even within the city limits of Karachi, and buffalo fat, particularly in the form of butter or ghee, sometimes is used at home as a dressing for burns. Thus, burn patients newly infected with buffalopoxvirus may periodically arrive at burns units. Due to disparity in the sophistication and cost of the care provided at the burns units in Karachi, patients are often transferred or move themselves between units, thus facilitating the possible spread of infection. In this outbreak, 6 of the 19 patients with putative cases of buffalopoxvirus infection are known to have transferred between burns units during treatment. Fortunately, the infection was of low virulence for humans.

Delay in recognizing and investigating the outbreak is cause for concern and can be ascribed to poor awareness and lack of resources. Clearly, improvements are needed in disease surveillance, diagnostics, and infection control.

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Tickborne Encephalitis in Naturally Exposed Monkey (*Macaca sylvanus*)

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We describe tickborne encephalitis (TBE) in a monkey (*Macaca sylvanus*) after natural exposure in an area at risk for TBE. TBE virus was present in the brain and could be identified as closely related to the European subtype, strain Neudoerfl.

Tickborne encephalitis (TBE) is a zoonotic disease caused by TBE virus (TBEV), a flavivirus. There are 3 subtypes of TBEV, the European subtype, transmitted by the hard tick *Ixodes ricinus*, and the Far Eastern and Siberian subtypes. Except in Austria, where mass vaccination campaigns were organized, the incidence of TBE in humans has increased in the past 30 years in all European countries with regions with risk for the disease. The average increase of TBE in 10 European countries was 311% from 1974–1983 to 1994–2003 (*1*,*2*). In Germany TBE incidence has increased enormously during recent years.

Most TBE group viruses use rodents as maintenance and amplifying hosts. TBE is predominately reported in humans, seldom in dogs and horses, and is as yet unknown in monkeys.The clinical progress of TBE is typically biphasic. In humans, a nonspecific influenzalike illness develops as the first phase of illness 7-14 days after they are bitten by a TBEV-positive tick. A second phase, with central nervous system involvement, develops in ≈30% of patients. Initial signs and symptoms include meningitis, encephalitis, and radiculitis. The case-fatality rate is 1%–2% in central Europe and 20%–40% in Siberia and the Far East.

The Study

On July 14, 2006, staggering paresis of the hind legs, incoordination, and intermittent opisthotonos developed in a female barbary macaque (*Macaca sylvanus*). No nystagmus was present. The monkey, born April 27, 2005, was from a group of ≈200 animals living in 3 social groups. The animals were kept in a large, outdoor enclosure of a monkey park situated in a TBE-risk area in southern Germany. Four days after the onset of clinical signs, the animal became comatose and was euthanized. Blood was collected before euthanasia, and serum was prepared.

At necropsy, no macroscopic lesions were observed. The brain was removed and immediately frozen on dry ice and sectioned in 2-cm slices. Alternating slices were placed in 3.5% neutral buffered formalin at 4°C. After 48 h, representative areas, including cerebral cortex, hippocampus, basal ganglia, and cerebellum, were embedded in paraffin. Sections (3–5 μm) were cut from each block and stained with hematoxylin and eosin for standard histopathologic evaluation. For immunohistochemical detection of TBEV antigens, a noncommercial rabbit polyclonal hyperimmune serum (dilution 1:1,000) was used as described previously (*3*).

Histologic examination of the brain tissue could be satisfactorily performed despite moderate artifacts caused by freezing (Figure, Panel A). Moderate perivascular inflammatory cuffs and slight diffuse infiltration of brain parenchyma by mononuclear cells were observed in almost all brain areas, including basal ganglia and cerebellum (Figure, Panel B). In addition, slight mononuclear inflammatory infiltrates were present in the meninges. Microglial nodules were not detected. Immunohistochemical testing demonstrated several anti-TBEV immunoreactive neurons and processes, mainly in Purkinje cells of the cerebellar cortex (Figure, Panel B), and to a lesser extent in pyramidal neurons of the temporal cortex. Single neuronophagias were also observed.

From the frozen material, 10 samples of brain tissue were selected for PCR analysis, including cerebrum, cerebellum, and brain stem (Table). Viral RNA was extracted from brain tissue with RNeasy Kit and from cerebrospinal fluid with QIAamp Viral Kit (both from QIAGEN, Hilden, Germany). The brain tissue was homogenized by beadmilling (Retsch, Haan, Germany) with 3-mm stainless steel beads in 0.5 mL of lysis buffer, and the QIAshredder system (QIAGEN) was used to improve homogenization. A modified nested reverse transcription–PCR (nRT-PCR) was conducted with primer pairs Pp1, Pm1 (reverse transcription and first PCR), and Pp2, Pm2 (nested PCR) (*4*). Amplification was done in a 50-μL reaction volume containing 10 μL 5× buffer, 2 μL deoxynucleotide triphosphate mix (10 mmol/L), 2 μL enzyme mix (all from QIAGEN), 2 μL RNase inhibitor (Promega, Mannheim, Germany), 1.25 μL primers Pp1 and Pm1 (20 pmol/μL; Jena-Bioscience, Jena, Germany), 27.5 μL diethyl pyrocarbonate (DEPC)–treated water, and 4 μL RNA extract. The reaction was performed in an Eppendorf (Hamburg, Germany) thermal cycler for 30 min at 60°C for RT and 15 min at 95°C for denaturation as the initial step, followed by 40 cycles of PCR with 30 s

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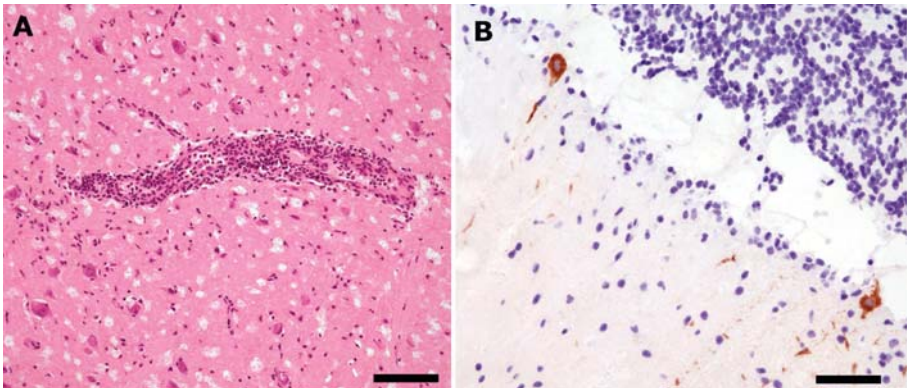

Figure. A) Moderate perivascular inflammatory infiltrates and slight diffuse infiltration of brain parenchyma by mononuclear cells in basal ganglia (hematoxylin and eosin stain, bar = 110 μm). B) Immunohistochemical findings for tickborne encephalitis virus (TBEV): strong immunolabeling of cerebellar Purkinje cell perikaryon and apical dendrites (anti-TBEV, bar = 60 μm).

at 94°C, 30 s at 66°C, and 1 min at 72°C. Final extension was 10 min at 72°C.

The second amplification reaction was carried out with 4 μL of amplification product in a 50-μL reaction (25 μL *Taq* PCR Master Mix [QIAGEN], 1.25 μL of each primer Pp2 and Pm2 [20 pmol/μL], and 18.5 μL DEPC-treated water). After a denaturation step of 2 min at 95°C, 30 cycles of 30 s at 94°C and 30 s at 65°C were performed, followed by 10 min at 72°C.

PCR products (178 bp) were visualized under UV light after electrophoresis on 1.5% agarose gel and ethidium bromide staining. Bands were cut out, and DNA was extracted by using the QIAquick Gel Extraction Kit (QIAGEN). DNA sequencing was conducted by cycle sequencing, using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's instructions. Amplification primers Pp2 and Pm2 were also used as sequencing primers. Nucleotide sequences were determined on an ABI Prism 310 Genetic Analyzer (Applied Biosystems).

Six of the10 brain samples were positive and CSF was negative for TBEV by nRT-PCR (Table). The sequences of these 6 PCR products (178 bp) differed by only 1 nt from that of strain Neudoerfl, the prototype strain of the European virus subtype.

The serum was tested for whole specific TBE antibodies (immunoglobulin [Ig] G and IgM) by ELISA (*5*). Solid-phase bound antigen and antigen conjugate were from a commercially available test kit (Immunozym FSME, Progen, Heidelberg, Germany). As standards, negative and positive test serum samples were used, and the following limit values were defined: <5 U/L, negative; 5–7 U/L, border line; 9–14 U/L, weakly positive; >14 U/L, positive; and >50 U/L, strongly positive. The serum of the macaque described in this paper was positive for specific TBE antibodies (24 U/L)

Conclusions

Experimental infections of macaques with TBEV and related flaviviruses (Kyasanur Forest disease virus, Powassan virus) have been reported (*6–8*), but natural infections with TBE virus have not been reported previously. In our case, clinical signs, neuropathologic findings, and immunohistochemical detection of TBEV antigen in neurons and of TBEV by nRT-PCR indicate that the macaque succumbed to natural TBEV infection. Although the classical multi-

Table. Results of nested reverse transcription–PCR (nRT-PCR) analysis of the tickborne encephalitis virus (TBEV)–infected macaque brain

Sample no.	Specimen	TBEV results by nRT-PCR*	TBEV strain (sequencing)
06F0927			
T653	Cerebellum	+	Neudoerfl†
T654	Cerebellum	–	–
T655	Neocortex	+	Neudoerfl†
T656	Neocortex	–	–
T659	Neocortex	+	Neudoerfl†
T660	Neocortex	–	–
T657	Brain stem	+	Neudoerfl†
T658	Brain stem	–	–
T661	Brain stem	+	Neudoerfl
T662	Brain stem	+	Neudoerfl†
06F0926			
T663	Cerebrospinal fluid	–	–
Total		6/11	

*+, virus detected; –, no virus detected.
†Differs by 1 nt from strain Neudoerfl, the prototype strain of the European virus subtype.

nodular pattern of lesions in the brain was not observed, the distribution of viral antigens was comparable to that observed in fatal human TBEV infection with a short clinical course. The TBEV was characterized as closely related to the European prototype strain Neudoerfl, which suggests that the infection was acquired locally by infected ticks. This fact is surprising because macaques generally quickly remove ticks during social grooming.

The monkey park where the animal became infected is situated in southern Germany, close to the Bodensee (Bodenseekreis). This area, flanked on the west by an area at high risk for TBE (Kreis Konstanz), is also at risk for TBE. From 1999 to 2006, a total of 29 autochthonous clinical cases of TBE in humans were reported in the Bodenseekreis and 35 in the Kreis Konstanz (Hellenbrand W., pers. comm.); 19% of unvaccinated forestry workers in the Bodenseekreis and 15% in the Kreis Konstanz were seropositive for TBEV (*9*). The prevalence of TBEV in ticks was 1.2%–2.3% in the Bodenseekreis (*10*).

Retrospective analyses of anamnestic data from the affected monkey park show that TBE may have appeared sporadically in macaques in the past. A monkey died in September 1995 and another in May 2006, and TBEV antigen was subsequently detected in brain tissue from the first animal and antibodies to TBEV were detected in both animals (12 U/L and 46 U/L, respectively, by ELISA). These 2 cases have not been systematically evaluated. Clinical signs of encephalitis were observed in another animal in 1999, but it seroconverted (42 U/L, ELISA test) and recovered after 2 months. Thus, TBE should be considered as a differential diagnosis in cases of encephalitis in monkeys kept outdoors in areas at risk for TBE. Further seroepidemiologic studies are planned to determine the status of TBEV infection among animals in this German monkey park. Vaccination against TBEV should be an option to protect other macaques in the group.

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Norovirus Infection in Children with Acute Gastroenteritis, Madagascar, 2004–2005

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Of 237 children with acute gastroenteritis in Antananarivo, Madagascar, during May 2004–May 2005, 14 (≈6%) were infected with norovirus. Seasonality (November–December peak) was detected. Reverse transcription–PCR identified GII as the most common genogroup. GIs belonged to GI.1, GI.3, and GI.4. Noroviruses in Madagascar show extensive genetic diversity.

Gastroenteritis is a major public health issue worldwide. Noroviruses are now considered emerging pathogens (*1*) and are recognized as the leading cause of nonbacterial, acute gastroenteritis in humans (*2*). The 2 genera, *Norovirus* and *Sapovirus,* are members of the family *Caliciviridae* and have a positive-sense, single-stranded RNA genome ≈7.5 kb long. Because no readily available cell culture system exists, characterization and classification of noroviruses are based on reverse transcription (RT)–PCR, genomic sequencing, and phylogenetic analysis (*2*–*5*). According to the latest scheme for norovirus nomenclature, the 29 genetic norovirus clusters or genotypes are classified into 5 genogroups (GI–V) (*6*). GI and II infect humans, and GII.4 has been the most highly prevalent genotype worldwide during the past decade (*2*,*5*,*7*).

The saying "Madagascar is not an island, but an archipelago" captures an important aspect of the country, whose geography and history have combined to produce a society of considerable diversity and uniqueness (*8*). In recent years, many studies have investigated the role of human noroviruses in childhood diarrhea and found worldwide distribution (*4*,*7*,*9*,*10*). However, no studies have reported the prevalence and molecular epidemiology of noroviruses in Madagascar, which we report here.

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The Study

From May 2004 to May 2005, a study of acute gastroenteritis in children ≤16 years of age was undertaken by the Institut Pasteur, Antananarivo, Madagascar. Children with a diagnosis of acute dehydrating watery diarrhea who were seen at the rehydration clinics and hospitals of Antananarivo were eligible for the study. Antananarivo, the capital city of Madagascar, has a population of ≈4 million. The study was approved by the Ethical Review Board of the Institut Pasteur, Antananarivo, Madagascar.

Fecal samples were collected from the children and stored at –80°C until analysis was undertaken at the University of Liverpool, UK. There, viral RNA was extracted from 150 μL of 10%–20% fecal suspensions in phosphate-buffered saline by using a guanidine and silica method (*11*). Sapoviruses were not sought in the initial screening. RT-PCR was performed in a 50-μL reaction mixture with primers that targeted the capsid N terminus/shell gene. For GI, forward primer G1SKF (5′-CTGCCCGAATTYGTAAATGA-3′) and reverse primer G1SKR (5′-CCAACCCARCCATTRTACA-3′) were used, yielding a PCR product of 330 bp. For GII, the primers used were forward primer G2SKF (5′-CNTGGGAGGGCGATCGCAA-3′) and reverse primers G2SKR (5′-CCRCCNGCATRHCCRTTRTACAT-3′) and G2ALSKR (5′-CCACCAGCATATGAATTGTACAT-3′), yielding a 344-bp product (*12*). The PCR was performed with an initial denaturation at 94°C, followed by 40 cycles of 60-sec denaturation at 94°C, 60-s primer annealing at 50°C, an extension for 2 min at 72°C, followed by a final extension stage of 15 min at 72°C. Amplification products were examined under ultraviolet light after electrophoresis through a 2% agarose gel with ethidium bromide staining. Products were extracted by using the QIAquick PCR purification kit (QIAGEN, Basingstoke, UK) and were sequenced by Macrogen Inc. (Seoul, South Korea).

Phylogenetic relationships were examined by aligning sequences with the ClustalW multiple alignment program (European Molecular Biology Laboratory, Heidelberg, Germany). A phylogenetic tree was constructed according to the neighbor-joining method by using ClustalX (version 1.83) and the alignment file obtained by analysis with ClustalW. Bootstrap values on a scale from 1 to 1,000 were also calculated. An unrooted phylogram of norovirus isolates from the present study and prototype strains was plotted in the PHYLIP format (http://evolution.genetics.washington.edu/phylip.html) output by using TreeView software, version 3.1 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). Assignment of norovirus to genotype was made according to the scheme proposed by Zheng et al. (*6*). The nucleotide sequences of the Malagasy strains have been deposited at GenBank (accession nos. EF213624–EF213635, EF213638, and EF213640.) Norovirus-positive samples

were screened for other viruses by negative-stain electron microsopy and by RT-PCR for rotavirus and astrovirus (*13*,*14*).

During this 12-month study, 258 children with acute gastroenteritis in Madagascar were screened for norovirus infection. Because 21 samples contained insufficient stool, 237 samples were analyzed (142 from boys and 95 from girls). Overall, 85% of children were <3 years of age, 77% were <2 years, 43% were <1 year, and 3% were newborns. The median age of the study population was 20 months (range 1 day to 16 years).

Fourteen noroviruses (5.9%) were detected in 237 children (Table 1). Noroviruses were found in all age groups. Infection rates did not differ between boys and girls (6.3% and 5.2%, respectively, p>0.1). No coinfections with other viruses (rotavirus, astrovirus, and adenovirus) were detected. Ten (71%) of the noroviruses detected were identified as belonging to genogroup GII; the remaining 4 (29%), to GI. Most GII noroviruses belonged to a potentially novel cluster (Figure 1). GI noroviruses were further classified into 3 genotypes: GI.1 (1 isolate), GI.4 (1 isolate), and GI.3 (2 isolates).

The median age of children with norovirus infection was 18 months (range 3–51 months). Most infections (≈86%) occurred in children <36 months of age. No infections were recorded in newborns or children >5 years of age. All infections with genogroup GI noroviruses were found in children <24 months of age (Table 2). Noroviruses were detected throughout the year; however, infections peaked during the wet season in Madagascar. November and December were the months of major norovirus prevalence (35.7% each month). GI noroviruses preceded GII infections by a few days (Figure 2).

Conclusions

To our knowledge, ours is the first study that has used molecular detection methods to investigate the role of noroviruses in pediatric gastroenteritis in Madagascar. We showed that infections with GI and GII noroviruses are relatively common. In a 1-year collection of stool samples, we detected noroviruses by RT-PCR in ≈6% of children with acute gastroenteritis in Antananarivo. This rate establishes norovirus as the second most commonly detected enteric virus in this population, behind rotavirus (38%) and followed by astrovirus (2.5%) (data not shown). These findings are consistent with those of studies elsewhere (*7*,*10*).

The median age of children with norovirus infection (18 months) was higher than previously reported (*7*) and higher than that of the rotavirus-infected group (median 10 months, range 1 day to 48 months) and that of the astrovirus-infected group (median 10 months, range 5–20 months) (data not shown). Noroviruses were detected throughout the year, but the number peaked in November and

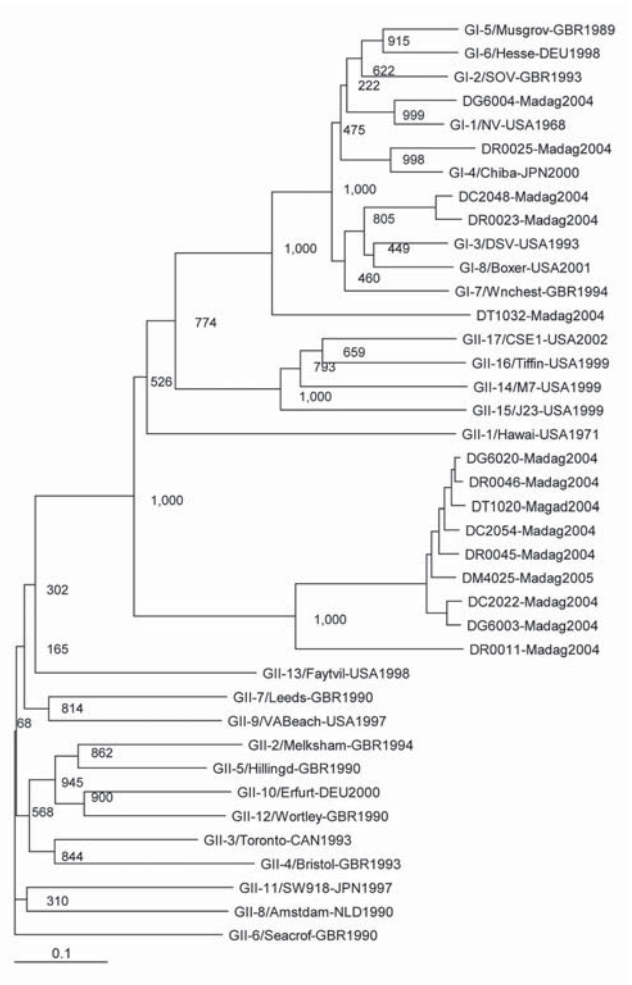

Figure 1. Phylogenetic tree of noroviruses based on the 330-bp region (for GI) and 344-bp region (for GII) of the capsid N terminus/shell gene. Fourteen novel sequences were included, designated according to isolate code, place, and year (e.g., DR001-Madag04); 25 sequences of reference norovirus strains (*6*) were included, designated according to genogroup-genotype, place, country, and year (e.g.,GII-2/Melksham-GRB1994). Comparative strains are GI-1/NV-USA1968 (Norwalk, M87661), GI-2/SOV-GBR1993 (Southampton, L07418), GI-3/DSV-USA1993 (Desert Shield, U04469), GI-4/Chiba-JPN2000 (AB042808), GI-5/Musgrov-GBR1989 (Musgrove, AJ277614), GI-6/Hesse-DEU1998 (AF093797), GI-7/Wnchest-GBR1994 (Winchester, AJ277609), GI-8/Boxer-USA2001 (AF538679), GII-1/Hawai-USA1971 (U07611), GII-2/Melksham-GBR1994 (X81879), GII-3/Toronto-CAN1993 (U02030), GII-4/Bristol-GBR1993 (X76716), GII-5/Hillingd-GBR1990 (Hillingdon, AJ277607), GII-6/Seacrof-GBR1990 (Seacroft, AJ277620), GII-7/Leeds-GBR1990 (AJ277608), GII-8/Amstdam-NLD1990 (Amsterdam, AF195848), GII-9/VABeach-USA1997 (AY038599), GII-10/Erfurt-DEU2000 (AF427118), GII-11/SW918-JPN1997 (AB074893), GII-12/Wortley-GBR1990 (AJ277618), GII-13/Faytvil-USA1998 (Fayetteville, AY113106), GII-14/M7-USA1999 (AY130761), GII-15/J23-USA1999 (AY130762), GII-16/Tiffin-USA1999 (AY502010), and GII-17/CSE1-USA2002 (AY502009). Bootstrap values based on 1,000 generated trees are displayed at the nodes. The scale bar represents nucleotide substitutions per site.

Table 1. Characteristics of viruses from children with acute gastroenteritis in Madagascar*

Identification no.	GenBank accession no.	Sample† date	Age, mo	Sex	Norovirus PCR	Norovirus sequencing	EM for NoV, RV, AV‡
DG6004-Madag04	EF213640	May 2004	14	M	GI pos	GI-1	Neg
DC2022-Madag04	EF213626	July 2004	14	F	GII pos	GII novel	Neg
DG6003-Madag04	EF213634	Oct 2004	44	M	GII pos	GII novel	Neg
DR0011-Madag04	EF213628	Nov 2004	12	M	GII pos	GII novel	Neg
DR0025-Madag04	EF213625	Nov 2004	17	M	GI pos	GI-4	Neg
DC2048-Madag04	EF213624	Nov 2004	3	M	GI pos	GI-3	Neg
DR0023-Madag04	EF213638	Nov 2004	9	F	GI pos	GI-3	Neg
DR0045-Madag04	EF213627	Nov 2004	19	F	GII pos	GII novel	Neg
DC2054-Madag04	EF213629	Dec 2004	16	M	GII pos	GII novel	Pos (putative NoV)
DG6020-Madag04	EF213630	Dec 2004	30	M	GII pos	GII novel	Neg
DR0046-Madag04	EF213631	Dec 2004	14	F	GII pos	GII novel	Neg
DT1020-Madag04	EF213632	Dec 2004	12	F	GII pos	GII novel	Neg
DT1032-Madag04	EF213633	Dec 2004	8	M	GII pos	GII novel	Neg
DM4025-Madag05	EF213635	Mar 2005	51	M	GII pos	GII novel	Neg

*EM, electron microscopy, NoV, norovirus; RV, rotavirus; AV, astrovirus; pos, positive; neg, negative.
†Fecal samples.
‡All norovirus-positive samples were negative for AV and RV when tested by reverse transcription–PCR.

December. Such seasonality in a tropical country is not really expected, as year-round circulation has been previously documented (*15*).

Our findings confirm the finding of previous studies that GII is the predominant norovirus genogroup circulating in communities worldwide. Considerable genetic diversity was observed among the norovirus GII isolates, and some were identified as belonging to a potentially novel cluster. The closest reference strain to the potentially novel cluster was the recombinant Hu/NoV/GII.1/Hawaii/1971/US. In contrast, norovirus GI isolates were clustered with prototype strains; Hu/NoV/GI.3/DSV395/1990/SA (Desert Shield) was predominant (2 strains), followed by Hu/NoV/GI.1/Norwalk/1968/US (Norwalk) and Hu/NoV/GI.4/Chiba407/1987/JP (1 each).

This study has several limitations. First, it is a preliminary study. The sample size was small, and we examined samples collected over a 13-month period. Longer, longitudinal studies are required to address issues such as norovirus seasonality and temporal genetic variability. In addition, we restricted our analysis to specimens collected from patients at rehydration clinics and hospitals, so prevalence of norovirus infections in the general population may have been underestimated. Furthermore, the use of short conserved sequences, although successful for diagnosis of norovirus infection, should be used with caution for classification and phylogenetic analyses. Further analysis by full capsid sequencing might be required. Nevertheless, continued norovirus surveillance is needed to monitor the spread and persistence of the various genotypes infecting children in Madagascar.

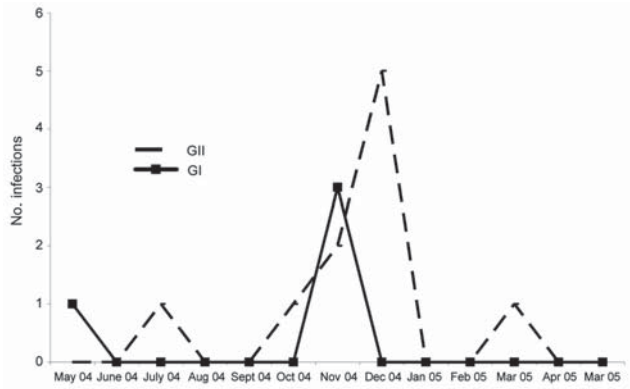

Figure 2. Seasonality of GI and GII norovirus infections, Antananarivo, Madagascar, May 2004–May 2005.

Table 2. Distribution of viruses and norovirus genogroups in children with acute gastroenteritis, by age, Madagascar, May 2004–May 2005

Virus	Age group, mo			
	0–12	13–24	25–36	>36
Other than norovirus	96	75	18	34
Norovirus GI	2	2	0	0
Norovirus GII	3	4	1	2

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Reemergence of Oropouche Fever, Northern Brazil

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Oropouche fever has reemerged in Parauapebas and Porto de Moz municipalities, Pará State, Brazil. Serologic analysis (immunoglobulin M–ELISA) and virus isolation confirmed *Oropouche virus* (OROV) in both municipalities. Nucleotide sequencing of 2 OROV isolates from each location indicated genotypes I (Parauapebas) and II (Porto de Moz) in Brazil.

Oropouche virus (OROV), the cause of Oropouche fever, belongs to the family *Bunyaviridae*, genus *Orthobunyavirus,* Simbu serogroup (*1*), and is transmitted between humans in urban areas by the biting midge *Culicoides paraensis* (*2*,*3*). This virus was first isolated from febrile forest workers in Trinidad in 1955. The first isolation in Brazil was in 1960 from the blood of a sloth (*Bradypus tridactylus*) (*4*). The epidemic potential of OROV was recognized during an outbreak in Belém, Pará State, Brazil, in 1961, where ≈11,000 persons were infected (*4*). Over the past 45 years, many outbreaks of Oropouche fever, ≈500,000 cases, have been described in the Americas. OROV has been isolated in Trinidad, Panama, Peru, and Brazil, and in the past 40 years Oropouche fever has emerged as a public health problem in tropical areas of Central and South America (*3*).

Members of the genus *Orthobunyavirus* have a tripartite, single-stranded, negative-sense RNA genome of small (S), medium (M), and large (L) RNAs that encode nucleocapsid, glycoproteins, and RNA polymerase, respectively. Phylogenetic analysis of nucleocapsid genes of different OROV strains identified 3 distinct genotypes (I, II, and III) currently circulating in Central and South America; genotypes I and II have been detected in the Brazilian Amazon (*5*). Recently, an OROV isolate from a marmoset (*Callithrix* sp.) was characterized as a member of genotype III (*6*).

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The Study

Two outbreaks of Oropuche fever occurred during 2003 and 2004. The first occurred in April–May 2003 in 2 communities (Vila Sansão, 140 inhabitants, and Vila Paulo Fontelles, 835 inhabitants).in the municipality of Parauapebas (6°4′S, 49°54′W). The second outbreak occurred in July–August 2004 in 1 community (Vila Tapara, 2,000 inhabitants) in the municipality of Porto de Moz (1°45′S, 52°14′W) (Figure 1).

A total of 125 and 109 serum samples were collected from residents of Parauapebas and Porto de Moz, which represented 12.8% and 5.45% of all inhabitants, respectively. Criteria for sampling were a history of acute fever several weeks before or during the survey or clinical symptoms similar to those of Oropouche fever. All serum samples were analyzed by hemagglutination inhibition (HI) test (*7*) and immunoglobulin M–ELISA (*8*) for specific HI and IgM antibodies to OROV. HI titers ≥20 and ELISA results greater than the cut-off value (optical density ≥0.200) were considered positive (*8*).Virus isolation was conducted by intracranial injection of newborn mice with a 1:10 (v/v) suspension of serum samples in phosphate-buffered saline, pH 7.4, as described elsewhere (*9*). Fifty-four and 11 serum samples from Parauapebas and Porto de Moz, respectively, were used for virus isolation. Identification of isolates was performed by complement fixation test as reported (*9*). Two OROV strains were isolated from patients in Parauapebas, and 2 strains were isolated from patients in Porto de Moz.

To genetically characterize the viruses, 2 isolates were selected from Parauapebas (Brazil 2003a and Brazil 2003b) and 2 from Porto de Moz (Brazil 2004a and Brazil 2004b). Viral RNA was extracted from Vero cells infected with human samples, and S RNA was amplified by using a 1-step reverse transcription–PCR assay as described (*5*,*6*).

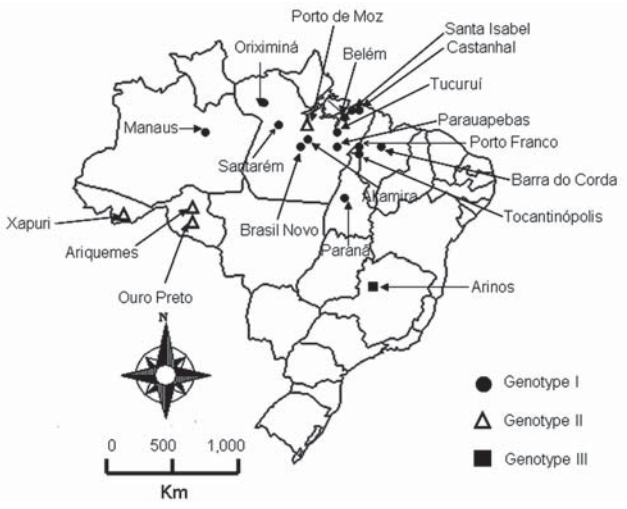

Figure 1. Map of Brazil showing locations where Oropouche fever outbreaks were identified up to 2003–2004.

Phylogenetic trees were constructed for nucleocapsid gene nucleotide sequences by comparison with other OROV nucleocapsid gene sequences in GenBank (Table 1); neighbor-joining analysis (*10*) implemented in Mega version 2.1 (*11*) was used. Bootstrap analyses were performed on 1,000 replicates to generate confidence for groupings (*12*).

Of 125 serum samples from patients in Parauapebas, HI results were positive for 16 (12.7%) from Vila Sansão, 6 (4.8%) from Paulo Fontelles, and 4 (3.2%) from other localities. IgM was detected in 16 (12.7%), 8 (4.8%), and 6 (4.8%) serum samples from these 3 areas, respectively. Of 117 serum samples from patients in Porto de Moz, 56 (46.7%) had HI antibodies and 61 (52.1%) had IgM to OROV.

A total of 71.9% of female patients in Parauapebas and 59% in Porto de Moz had symptoms suggestive of Oropouche fever. Although all age groups were affected, persons 5–14 years of age had the highest frequency of symptoms (30.4%) and those <1–4 years of age had the lowest frequency (4.8%) (Table 2). Symptoms most frequently reported were fever (100%), headache (79.3%), joint pain (68.7%), and muscle pain (30%). Seventy percent of patients reported ≥1 episode of recurrence of fever, characterized by fever, headache, and other symptoms ≈2–3 weeks after onset of initial symptoms (*2*,*3*).

Full-length S RNA of the 4 OROV strains contained 754 nt and encoded 2 overlapping open reading frames, the

Table 1. Characteristics of *Oropouche virus* strains used for small RNA phylogenetic analyses

Strain	Source	Sample	Year	Location	GenBank strain identification	Accession no.
TRVL 9760	Human	Blood	1955	Trinidad	Trinidad 55	AF164531
BeAn 19991	*Bradypus trydactylus*	Blood	1960	São Miguel, Brazil	Brazil 60	AF164532
BeH 271815	Human	Blood	1975	Santarém, Brazil	Brazil 75	AF164533
BeAn 206119	*Bradypus trydactylus*	Blood	1971	Maracanã, Brazil	Brazil 71a	AY993909
BeAn 208402	*Bradypus trydactylus*	Blood	1971	Maracanã, Brazil	Brazil 71b	AY993910
BeAn 208819	*Bradypus trydactylus*	Blood	1971	Maracanã, Brazil	Brazil 71c	AY993911
BeAn 208823	*Bradypus trydactylus*	Blood	1971	Maracanã, Brazil	Brazil 71d	AY993912
BeH 390233	Human	Blood	1980	Manaus, Brazil	Brazil 80c	AF164536
BeH 381114	Human	Blood	1980	Belém, Brazil	Brazil 80b	AF164535
BeH 379693	Human	Blood	1980	Castanhal, Brazil	Brazil 80a	AF164534
BeH 472200	Human	Blood	1988	Porto Franco, Brazil	Brazil 88a	AF164537
BeH 472204	Human	Blood	1988	Tocantinópolis, Brazil	Brazil 88b	AF164538
BeAr 473358	*Culicoides paraensis*	Pool	1988	Porto Franco, Brazil	Brazil 88c	AF164539
BeH 475248	Human	Blood	1988	Tucuruí, Brazil	Brazil 88d	AF164540
GLM 444477	Human	Blood	1989	Panama	Panama 89a	AF164555
GLM 444911	Human	Blood	1989	Panama	Panama 89b	AF164556
GLM 445252	Human	Blood	1989	Panama	Panama 89c	AF164557
GLM 450093	Human	Blood	1989	Panama	Panama 89d	AF164558
BeH 505514	Human	Blood	1991	Santa Isabel, Brazil	Brazil 91a	AF164541
BeH 505442	Human	Blood	1991	Ouro Preto d'Oeste, Brazil	Brazil 91b	AF164542
BeH 505663	Human	Blood	1991	Ariquemes, Brazil	Brazil 91c	AF164543
IQT 1690	Human	Blood	1992	Peru	Peru 92	AF164549
MD 023	Human	Blood	1993	Peru	Peru 93a	AF164550
DEI 209	Human	Blood	1993	Peru	Peru 93b	AF164551
BeH 521086	Human	Serum	1993	Barra do Corda, Brazil	Brazil 93	AY704559
BeH 541863	Human	Blood	1996	Altamira, Brazil	Brazil 96a	AF164544
BeH 543033	Human	Blood	1996	Oriximiná, Brazil	Brazil 96b	AF164545
BeH 544552	Human	Blood	1996	Brasil Novo, Brazil	Brazil 96c	AF164546
BeH 543087	Human	Blood	1996	Xapuri, Brazil	Brazil 96d	AF164547
BeH 543618	Human	Blood	1996	Oriximiná, Brazil	Brazil 96e	AF164548
BeH 543733	Human	Serum	1996	Oriximiná, Brazil	Brazil 96f	AY704560
IQT 4083	Human	Blood	1997	Peru	Peru 97	AF164552
01–812–98	Human	Blood	1998	Peru	Peru 98a	AF164553
IQT 7085	Human	Blood	1998	Peru	Peru 98b	AF164554
BeAn 626990	*Callithrix sp*.	Viscera	2000	Arinos, Brazil	Brazil 00	AY117135
BeH 622544	Human	Blood	2002	Paranã, Brazil	Brazil 02	EF467368
BeH 669314	Human	Blood	2003	Parauapebas, Brazil	Brazil 03a	EF467370
Be H 669315	Human	Blood	2003	Parauapebas, Brazil	Brazil 03b	EF467369
BeH 682426	Human	Blood	2004	Porto de Moz, Brazil	Brazil 04a	EF467371
BeH 682431	Human	Blood	2004	Porto de Moz, Brazil	Brazil 04b	EF467372

Table 2. Distribution of serum samples positive for immunoglobulin M to *Oropouche virus* in 2 municipalities, Pará State, Brazil, 2003–2004

Patient age, y	Porto de Moz, no. positive/no tested		Parauapebas, no. positive/no tested	
	Male	Female	Male	Female
<1–4	1/6	3/4	0/2	1/9
5–14	11/21	7/19	3/21	7/24
15–24	2/7	7/14	0/4	4/13
25–34	4/7	6/10	0/3	5/11
35–44	4/5	3/5	4/4	1/7
45–54	2/3	4/8	1/7	3/8
≥55	2/3	5/5	1/6	2/5
Total	26/52	35/65	9/47	23/77

nucleocapsid (693 nt and 231 aa) and nonstructural protein (273 nt and 91 aa). Two small noncoding regions were also found at the 3′ and 5′ ends of these reading frames, spanning nt positions 1–44 and 741–754, respectively. Phylogenetic analysis of Brazil 2003 and 2004 isolates grouped strains from Parauapebas (Brazil 2003a and Brazil 2003b) into OROV genotype I and strains from Porto de Moz (Brazil 2004a and Brazil 2004b) into OROV genotype II (Figure 2).

Conclusions

Oropouche fever is the second most common arboviral disease (after dengue fever) in the Brazilian Amazon region. From 1960 to 1980, Oropouche fever outbreaks were detected only in Pará State, mainly in Belém and neighboring areas, where thousands of people were infected (*2*,*3*). OROV was then detected in other Amazonian states including Amazonas, Amapá, Acre, Rondônia, and Tocantins; and non-Amazonian states, including Maranhão in northeastern Brazil and Tocantins in central Brazil (*3*,*8*). Recently, OROV isolated from *Callithrix* sp.in Arinos, Minas Gerais State, southeastern Brazil was characterized as genotype III, which indicated the presence of this genotype in Brazil (*6*). OROV from this species has been identified only in Panama (*5*). From 1980 to 2005, sporadic cases or self-limited outbreaks of Oropouche fever were reported in areas of the Brazilian Amazon, which suggested silent endemic circulation of the virus (*13*). In 2003 and 2004, several cases of Oropouche fever were detected in Parauaebas and Porto de Moz in Pará State. Parauaebas is located in the Carajás mineral province and Porto de Moz is located in the Altamira region.

Genetic characterization of strains indicated the presence of genotype II in the eastern Amazon region. This genotype had been associated with cases of Oropouche fever in restricted western Amazonian areas (Rondônia State), as well as in Peru (*5*). This finding suggests movement of OROV genotype II across the Amazon region from western to eastern areas or emergence of this genotype after silent circulation for several years. Genotype I (Brazil 2003a and Brazil 2003b) found in Parauapebas was closely related to Trinidadian and Brazilian isolates obtained from 1955 through 1960 (Trinidad 55 and Brazil 60) (*5*). Genotype II strains isolated in Porto de Moz were genetically related to strains isolated in Peru during the 1990s (Peru 92, 93, 97, 98a, 98b) and Rondônia State in 1991 (Brazil 91a, 91b), as reported by Saeed et al. (*5*). These data indicate that Parauapebas and Porto de Moz OROV isolates are genetically distinct and have different ancestor viruses (Figure 2). Recognition of different OROV genotypes in the Brazilian Amazon, as well as new genetic information, is useful for understanding the epidemiology and genetic diversity of this emergent human pathogen.

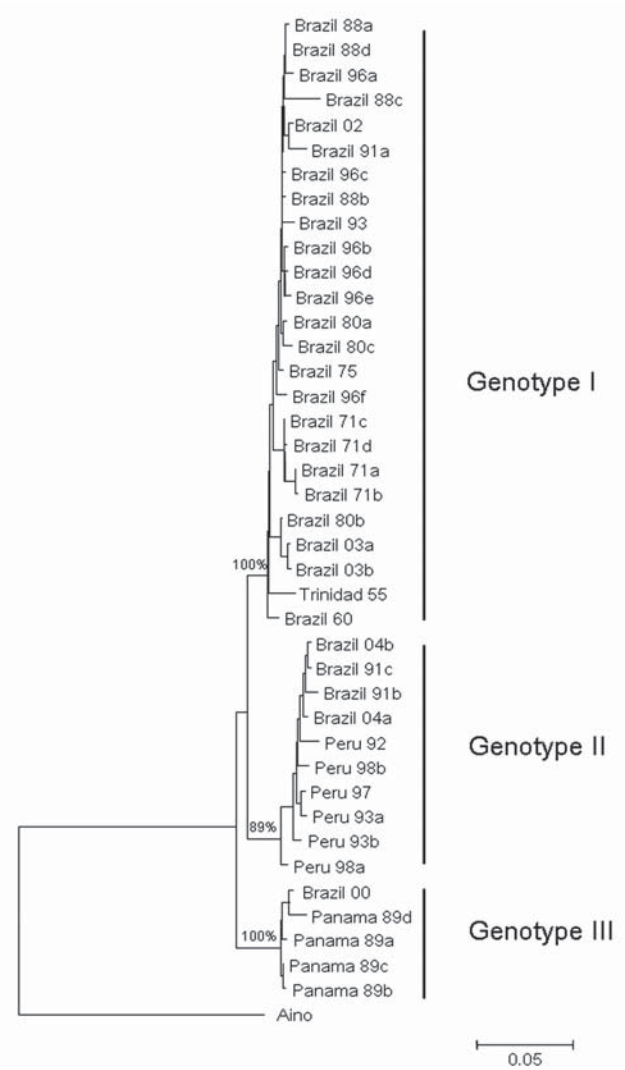

Figure 2. Comparative small (S) RNA phylogenetic tree constructed by using the neighbor-joining method for *Oropouche virus* strains isolated in Parauapebas and Porto de Moz, Pará State, Brazil. Bootstrap values were placed over the 3 nodes for each main group (I, II, and III). Aino virus S RNA sequence was used as an outgroup. Scale bar indicates a divergence of 5% in the nucleotide sequence.

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Emergence of Serotype G12 Rotaviruses, Hungary

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We describe the emergence of serotype G12 rotaviruses (67 [6.9%] of 971 specimens tested) among children hospitalized with rotavirus gastroenteritis in Hungary during 2005. These findings are consistent with recent reports of the possible global spread and increasing epidemiologic importance of these strains, which may have implications for current rotavirus vaccination strategies.

Group A rotaviruses are the leading cause of acute severe gastroenteritis in infants and young children worldwide. Approximately 130 million children are infected with rotavirus, and nearly 450,000–750,000 die of disease caused by this agent each year (*1*). Group A rotaviruses are classified into G and P serotypes and genotypes on the basis of antigenic and genetic diversity of the outer capsid proteins, VP7 and VP4, respectively. At present, 11 of 15 G types and 12 of 26 P types are known to infect humans. On a global basis, most severe infections are caused by 5 G types (G1–G4 and G9) and 3 P types (P1A[8], P1B[4], and P2A[6]), although considerable differences exist in some areas, especially in tropical countries (*2*,*3*).

One year after its introduction in 1998, RotaShield, the first licensed rotavirus vaccine, was withdrawn from use due to an association with intussusception. Recently, 2 new candidate vaccines, RotaTeq and Rotarix, were licensed for use in >40 countries and introduced into the vaccine market of several nations. RotaTeq was developed with the aim of providing serotype-specific immunity against the 4 common G types (G1–G4) and 1 common P type (P1A[8]). In contrast, Rotarix vaccine, a monovalent vaccine containing a single P1A[8],G1 strain, is expected to induce heterotypic immunity to a variety of strains that have epidemiologic and clinical importance. However, the increasing number of reports of the emergence of novel G and P types in various countries raises concerns about the adequacy of current vaccination strategies (*2*,*3*). Strain surveillance studies during and after introduction of these vaccines are needed to gauge their impact on circulating strains and monitor for possible emergence of rotavirus types that escape the immunity provided by the vaccines.

As part of the Hungarian rotavirus strain surveillance program, we obtained fecal samples from community-acquired rotavirus gastroenteritis patients <15 years of age who were admitted with acute dehydrating diarrhea in 2005 to the "St. Laszlo" Hospital, Budapest. The rotavirus-positive samples (confirmed by use of an immunochromatographic assay with Rota Uni-Strip [Coris BioConcept, Gemblaux, Belgium]) were delivered to our laboratory on a monthly basis. Routine strain characterization included polyacrylamide gel electrophoresis of the viral genome (electropherotyping) and genotyping of the outer capsid genes, VP7 and VP4, by reverse transcription–PCR (RT-PCR), as described previously (*4*). The G-typing algorithm included the use of 3 primer sets (specific for G1–G4, G6, and G9). In spite of the use of a variety of primer sets, 85 (8.7%) of the 971 analyzed specimens were nontypeable G serotypes. A relatively high number of these untypeable strains (n = 67) had identical RNA patterns (long electropherotype; data not shown), which suggested that they might belong to the same serotype. To investigate this possibility, we sequenced a 501-bp stretch of the VP7 gene (nt 79–579, amino acids [aa] 11–177) from 4 of these strains, using procedures we described elsewhere (*4*). These data showed that the 4 strains share 100% nt sequence identity to each other, >90% nt identity and 91% aa similarity to serotype G12 strains, and ≤78% similarity to other serotypes, a finding that strongly suggested that they belonged to serotype G12 (data not shown).

To test the remaining G-nontypeable strains, we set up a genotype-specific RT-PCR assay, using a G12-specific primer designed by Samajdar et al. (*5*), homologous to the 4 sequenced strains, in combination with a consensus VP7 gene oligonucleotide, 9Con1, to yield an amplicon of 464 bp. We first tested the specificity of this primer pair by using the Hungarian G12 strains confirmed by nucleotide sequencing and a variety of other strains (including 8 G1, 2 G2, 2 G3, 4 G4, 15 G9, and 4 untypeable strains) unrelated to the Hungarian G12 strains that were isolated during the same period. None of the non-G12 strains produced an amplicon after RT-PCR with this primer pair. In contrast, all 63 strains with the same electropherotype as the 4 sequenced G12 strains gave a 464-bp amplicon when tested with this primer pair, suggesting that they also belonged to G12. Including the 4 sequenced strains, the 67 G12 strains identified represented 6.9% of all typeable by electropheresis strains collected in the study period. Although most of the rotavirus gastroenteritis cases were identified during the peak activity of group A rotaviruses (March–April),

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the G12 strains also showed relative abundance in summer and autumn, representing 15%–28.6% of the total rotavirus strains detected during this period (Figure 1). The emergence of these strains in Hungary during 2005 raises the question of whether they were able to overcome the immunity of older children or neonates to common rotavirus strains, as demonstrated earlier when serotype G9 rotaviruses emerged in Europe. The mean and median ages of children infected with G12 rotaviruses (2.7 and 2.2 years, respectively) were, however, not significantly different from those of children infected with type G1 (3.0 and 2.5 years, respectively), G2 (2.4 and 2.0 years, respectively), G4 (2.9 and 2.4 years, respectively), and G9 (2.9 and 2.3 years, respectively) rotaviruses. Only children infected with G3 and G6 rotaviruses showed significant differences in mean and median ages compared with those for children with G12 rotavirus (G3: 4.8 and 3.8 years, respectively, $p = 0.009$; G6: 5.3 and 4.3 years, respectively, $p = 0.047$). A subset of G12 samples subjected to P genotyping ($n = 14$) tested positive for P[8]; this result was confirmed by sequencing results for 3 strains. No other RNA patterns were associated with G12 specificity.

The first description of G12 rotaviruses dates back to the 1990s, when 2 unusual human strains (L26 and L27) from the Philippines were characterized serologically and by nucleotide sequencing as P[4],G12 with subgroup I specificity and a long electropherotype (*6*,*7*). The origin of these strains was obscure because they were apparently very rare in humans over the next 10 years and had not been previously detected in animals. Beginning in 2002, reports of the detection and increased prevalence of G12 strains have appeared from Asia (Thailand, India, Korea, Japan, Bangladesh, Nepal, and Saudi Arabia) and the Americas (the United States, Argentina, and Brazil) (*5*,*8*–*14*), including strains with as yet unpublished G12 VP7 sequences in the GenBank database. Although these strains are closely related to each other (>96.9% nt identity), they have diverged substantially from early human G12 isolates (≤91% nt identity).

While the origin of G12 strains is unknown, a G12 rotavirus was recently identified in a pig, representing a possible animal source of this serotype (*15*). However, this single porcine isolate was not highly related to any known human G12 isolate (<92% nt identity), a finding that leaves gaps in our knowledge about the source of the modern lineages of serotype G12 rotaviruses from humans.

The global emergence of rotavirus G12 shares several features with the worldwide spread of serotype G9 in the mid to late 1990s (*2*,*3*). First, both serotypes were identified in humans ≈2 decades ago and subsequently were rarely detected for many years despite intensified surveillance activity and the introduction of sensitive and specific molecular typing methods. Second, sequence analysis and serologic

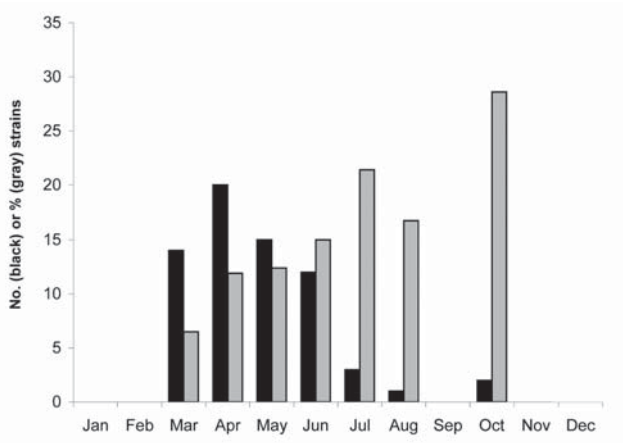

Figure 1. Temporal distribution of serotype G12 human rotaviruses in Budapest, Hungary, 2005. Black columns indicate the number (N) of strains identified; gray columns represent the percentage of total strains for each month that were type G1.

studies demonstrated substantial genetic and antigenic differences between early and modern variants within both serotypes, which suggested a new or separate introduction into humans. Third, of these coexisting genetic lineages, only 1, the modern lineage of each serotype, has been recognized as being capable of spreading globally. Four, various associated P types have been detected and display geographic differences in their distribution. For example, G12 strains in South America and far eastern Asia contain the globally rare P[9]; the only identified G12 strain in the United States bears the regionally common P[6]; Hungarian strains are associated with the most common genotype, P[8]; and Indian strains have been found in association with globally or regionally distributed P[4], P[6], or P[8] specificities. It will be interesting to determine, whether, as with the spread of G9 strains (*3*), emergence of G12 rotavirus will occur predominantly through reassortment of a single gene, VP7, into a background of globally common human Wa genogroup strains, as suggested by our finding of G12 in Hungarian P[8] strains with a typical long electropherotype. Last, identification of clearly related VP7 genes in pigs suggests that we should continue to search for possible ancestors for both serotypes in the porcine species. This finding also reaffirms that pigs act as reservoirs of newly recognized human rotavirus antigenic types.

At the time of manuscript preparation, ≈40 G12 VP7 sequences were available in the DNA databases. Most sequence data have been published from India, where G12 strains showing minor genetic variation in their VP7 gene sequences (<3% nt difference) were isolated between 2001 and 2005 in various geographic regions. In contrast, all 4 Hungarian G12 strains share 100% sequence identity over a stretch of 501 nt. This finding suggests that G12 rotaviruses circulated for some years in India before their iden-

tification. By contrast, the G12 rotavirus detected in 2005 in Hungary is likely the result of a very recent introduction. One possibility is the importation of a single G12 strain into Hungary, which subsequently spread in the population. Although few data are available on the sequence of P[8] VP4 genes of G12 strains, we found that the P[8] VP4 of the Hungarian G12 strains was most closely related to the VP4 of a Saudi Arabian strain, MD844 (>99.4% nt similarity along with corresponding sequence length), confirming the epidemiologic linkage among these strains that was suggested by the nearly complete sequence identity for the VP7 gene (>99.4%, Figure 2).

Detailed molecular characterization of the entire genome is needed to help determine the extent of genetic variation and the relatedness of these Hungarian G12 strains to other, recently emerged G12 rotaviruses. More intensified investigation of animal rotavirus strains may identify the possible animal ancestor of the new genetic lineage. Continuous monitoring of human rotavirus strains circulating in local communities will be important to determine if we are again detecting the early stages of the global emergence of a novel genetic lineage of an "old" rotavirus serotype. This was seen several years ago for the global spread of a modern lineage of serotype G9 rotaviruses. This finding may have important implications for vaccine use in planned or already launched rotavirus immunization programs in numerous countries worldwide.

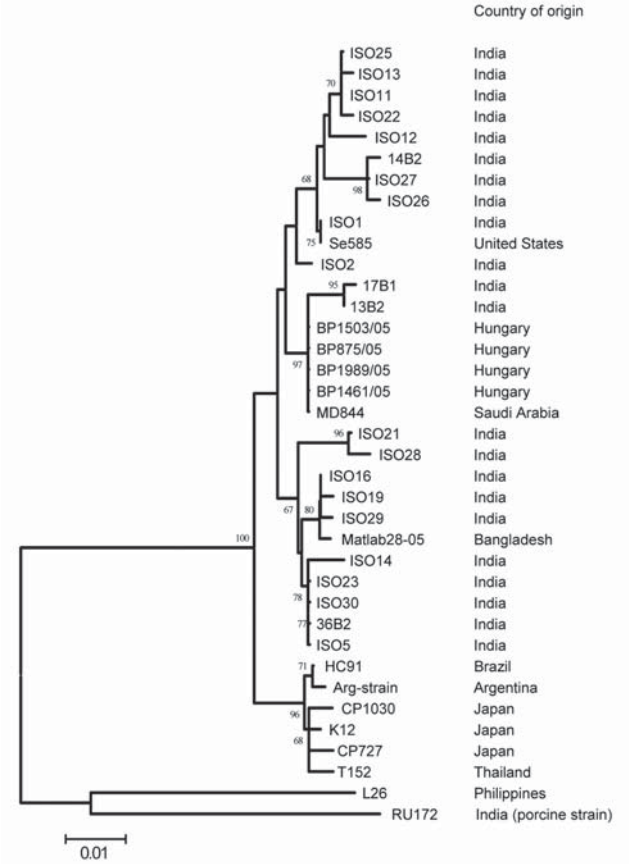

Figure 2. Phylogenetic relationship among Hungarian and other G12 rotaviruses. The tree was generated by the neighbor-joining algorithm by using a 501-nt fragment of VP7 (nt 79–579). Scale bar represents the nucleotide distance. Bootstrap values >60% are shown in the branch nodes. The country of origin is shown parallel to the strain names. ARG strain is an unnamed G12 isolate from Argentina.

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Nucleotide sequences deposited in GenBank are VP7, AM397926–AM397929; VP4, AM397930–AM397932.

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Meningitis Serogroup W135 Outbreak, Burkina Faso, 2002

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In 2002, the largest epidemic of *Neisseria meningitidis* serogroup W135 occurred in Burkina Faso. The highest attack rate was in children <5 years of age. We describe cases from 1 district and evaluate the performance of the Pastorex test, which had good sensitivity (84%) and specificity (89%) compared with culture or PCR.

Meningococcal epidemics in sub-Saharan Africa have been caused, until recently, mainly by *Neisseria meningitidis* serogroup A (*1*); strains of serogroup W135 have been isolated sporadically (*2*). In 2000 and 2001, serogroup W135 was associated with outbreaks in pilgrims to Mecca, Saudi Arabia, followed by several clusters of cases worldwide (*3*–*5*).

Laboratory confirmation of meningococcal meningitis is conducted by using antigen detection in cerebrospinal fluid (CSF), culture, or PCR techniques (*6*,*7*). The Pastorex latex agglutination test (Bio-Rad Laboratories, Marnes-La-Coquette, France) is the most common rapid test used in the field to detect *N. meningitidis* serogroup W135 antigen, although it cannot differentiate serogroups W135 and Y.

In January 2002, a preventive mass-vaccination campaign with a bivalent A-C polysaccharide vaccine started in Burkina Faso in districts with low vaccine coverage in 2001. In the week of January 28, 2002, Pama District crossed the epidemic threshold of 10 cases/100,000 per week (*8*). This district had achieved 100% vaccination coverage of the target population (2–29 years of age) in 2001 (Médecins sans Frontières internal report). Four weeks later, 4 other districts that had achieved ≥80% vaccination coverage in 2001 (Epicentre internal comm.) crossed the epidemic threshold (*8*). By mid-March 2002, the World Health Organization (WHO) Collaborating Centre for Reference and Research on Meningococci (CCRRM) in Oslo, Norway, confirmed most of these cases as caused by serogroup W135. Because the A-C vaccine could not provide protection against serogroup W135, the Ministry of Health ended the vaccination campaign.

The epidemic in Pissy District (population 520,314 in 2002) was investigated by the Burkina Faso Ministry of Health and WHO. We evaluated the Pastorex test for detecting *N. meningitidis* serogroup W135 in patients at Pissy Medical Health Centre (MHC).

The Study

A suspected case was defined as a febrile syndrome of sudden onset, associated with headache, stiff neck, or vomiting. A probable case was any suspected case with either a positive or doubtful result on direct microscopic examination of CSF. A confirmed case was a probable case with serogroup identification in CSF by culture, Pastorex test, or PCR. Patients with suspected cases were hospitalized and treated with a suspension of chloramphenicol in oil or another antimicrobial drug, as appropriate (*9*). Patients with severe cases were routinely transferred to Yalgado Ouédraogo National Hospital in Ouagadougou. Attack rates by age group were calculated for cases reported during weeks 6–18 (February 4–May 5) by using population data for Pissy District and standard age-group distributions for developing countries (*10*).

CSF samples from patients with suspected cases during weeks 17–20 (April 21–May 15) were examined at Pissy MHC by direct macroscopic and microscopic techniques, including Gram stain and leukocyte counts (as long as the CSF was not bloody). Pastorex rapid agglutination test was also used following the manufacturer's instructions.

A positive result for direct microscopic examination was indicated by numerous organisms or ≥10 leukocytes/mm^3 CSF. A doubtful result was indicated by a rare organism and <10 leukocytes/mm^3 CSF (or count not made). Any other result was considered negative. If results of direct microscopy were positive or doubtful, the remaining CSF sample was placed in 2 bottles of trans-isolate medium (provided by WHO CCRRM in Oslo). One bottle was sent for culture to the Charles de Gaulle Paediatric Hospital Laboratory in Ouagadougou. For quality control, the other was sent to WHO CCRRM for culture or PCR.

In Oslo, 100 μL of each CSF sample in trans-isolate medium was plated onto chocolate agar and chocolate agar containing 7.5 mg/L colimycin, 0.5 mg/L linocmycin, 1.0 mg/L amphotericin B, and 5.0 mg/L trimethoprim. Plates were incubated at 35°C in an atmosphere of 10% CO_2 for ≤3 days, and meningococci were identified by standard

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methods (*11*). PCR was performed as previously described (*6*,*7*) on samples that were either contaminated or culture negative for meningococci.

Performance of the Pastorex test was measured by calculating sensitivity and specificity, by using culture or PCR results from WHO CCCRM as the comparison standard. Samples with contaminated cultures and inhibited PCR (clinical specimens may contain inhibitory substances [*12*]) were considered negative, as were undetermined results. Positive and negative predictive values (PPV and NPV, respectively) were also calculated.

Of 2,130 patients with suspected cases reported in Pissy District during weeks 6–18, the conditions of 1,325 (65%) were diagnosed and treated at Pissy MHC (Figure 1); 44 died (case-fatality rate [CFR] 3%). Age was available for 1,307 (99%) of 1,325 patients. The highest attack rate was in patients <1 year of age (1,092/100,000), followed by patients 1–4 years of age (660/100,000). The attack rate continued to decrease with age (Table 1). Vaccination history was provided by 1,137 patients with suspected cases (86%), of whom 791 (70%) had been vaccinated against meningitis; information on year of vaccination was unknown.

Confirmed case-patients showed typical clinical features (*13*) (Table 2) and a CFR of 10%. Their ages ranged from 5 months to 19 years (median 4 years); the male:female ratio was 1.6:1. The 3 classic clinical signs of meningitis (headache, fever, and stiff neck) were present in 10 case-patients (33%).

During weeks 17–20, successful lumbar punctures (LPs) were performed in 260 patients with suspected cases at Pissy MHC. Thirty-one were positive for meningitis serogroup W135 by culture, PCR, or Pastorex test. CSF was clear in 6 (19%) samples, cloudy in 22 (71%), and bloody in 3 (10%). Among 6 clear CSF samples, 3 had doubtful results by direct microscopy and were confirmed only by Pastorex test.

Eighty-two CSF samples from all probable case-patients were sent to WHO CCCRM. These samples were tested by direct microscopy, and most were tested by Pastorex test in Burkina Faso. Sixty samples had doubtful results, and 22 had positive results by direct microscopy (Figure 2).

The Pastorex test on 64 samples tested by culture or PCR showed a sensitivity of 84% (95% confidence interval [CI] 60%–97%) and a specificity of 89% (95% CI 76%–96%) for detection of serogroup W135. PPV and NPV for this test were 76% (95% CI 53%–92%) and 93% (95% CI 81%–99%), respectively.

Conclusions

The meningitis epidemic in Burkina Faso in 2002 was the largest reported outbreak caused by *N. meningitidis* serogroup W135 to date (*3*,*13*), with nearly 13,000 suspected cases (*9*). We report a portion of this epidemic at 1 health center, which represented ≈10% of suspected cases nationwide. Attack rate was highest in patients <5 years of age and decreased with age. Symptoms and CFR of confirmed case-patients were typical for meningitis. The Pastorex test had adequate sensitivity (84%) and specificity (89%) for detecting the W135 serogroup, similar to those found under ideal laboratory conditions (85% and 97%, respectively [*14*]).

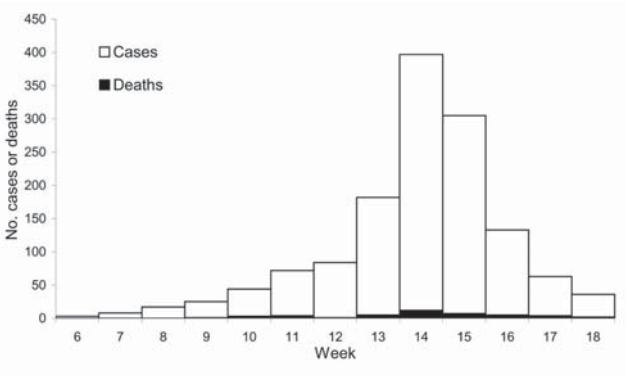

Figure 1. Number of meningitis cases (N = 1,325) and deaths reported during weeks 6–18 (February 4–May 5) at Pissy Medical Health Centre, Burkina Faso, 2002.

An effective public awareness campaign and fear in the population (because of lack of suitable vaccine) resulted in large numbers of patients with suspected cases arriving at health centers throughout the country, and more LPs were conducted than expected. This situation—and the case definition, which was sensitive but not specific—explained why of 260 LPs performed in a 4-week period at the end of the epidemic, only 31 were positive. Routine transfer of severe case-patients from Pissy MHC to the national hospital explained the lower CFR reported from Pissy MHC (3%) than for the whole epidemic (12%; [*9*]).

During this study, 25% of CSF samples analyzed with Pastorex test were unreadable, which may have been caused by differences in the serogroup W135/Y reaction in this test. In addition, difficulties in reading this test (possibly because of a lack of expertise in reading agglutination test results) have been reported in the field during epidemics.

The Pastorex test provides faster results than either

Table 1. Attack rates per 100,000 population by age group for suspected meningitis cases, Pissy Medical Health Centre, Burkina Faso*

Age group, y	Population	No. cases	Attack rate/100,000
<1	19,772	216	1,092
1–4	68,681	453	660
5–14	145,688	289	198
15–29	145,688	220	151
≥30	140,485	129	92
All	520,314	1,307	251

*Weeks 6–18 (Feb 4–May 5), 2002 (n = 1,307). Population data are for the entire district of Pissy.

Table 2. Characteristics of patients with confirmed cases of infection with *Neisseria meningitidis* W135, Pissy District, Burkina Faso, Apr–May 2002 (n = 31)

Characteristic	No. (%)
Age group, y	
<1	1 (3)
1–4	16 (52)
5–14	13 (42)
15–30	1 (3)
Male sex	19 (61)
Symptom onset, d*	
<1	2 (7)
1–2	18 (60)
3–4	10 (33)
Received antimicrobial drug†	25 (89)
Temperature, °C*	
≤38	8 (27)
38.1–39.9	12 (40)
≥40	10 (33)
Other clinical signs	
Headache	21 (70)
Vomiting	22 (73)
Anorexia	22 (73)
Stiff neck	16 (53)
Fever, stiff neck, and headache	10 (33)

*Data missing for 1 case-patient.
†Data missing for 3 case-patients.

culture or PCR (minutes vs. days) and requires less training and no specialized equipment other than a refrigerator, centrifuge, and water bath. It is thus more appropriate for developing countries with limited resources (*15*), despite relatively high costs (in 2005 kits cost ≈€11 per CSF sample analyzed). The high NPV of this test and its rapidity make it an important case management tool because cases of nonmeningococcal meningitis during an outbreak require different treatment. Other studies have shown this test to have high sensitivity and specificity under ideal conditions for both serogroups A (*14*,*15*) and W135 (*14*). Further study is needed to confirm the validity of this test under epidemic conditions in the field, particularly readibility of results for serogroup W135.

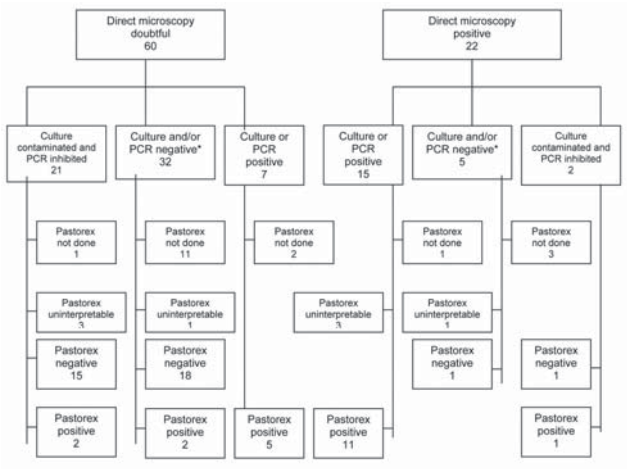

Figure 2. Schematic representation of results of culture or PCR performed on 82 cerebrospinal fluid samples with doubtful or positive direct microscopic results. See text for definitions of doubtful and positive direct microscopic results. *With the other result contaminated (culture) or inhibited (PCR).

Acknowledgments

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Dr Nathan was a medical epidemiologist at the Epicentre in Paris until his unexpected death in 2004. His research interests included meningococcal meningitis, trachoma, adult malnutrition, and yellow fever.

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Age and Clinical Dengue Illness

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The relationship between age and risk for classic dengue fever has never been quantified. We use data from clinical patients to show that the relative risk of having classical disease after primary dengue virus infection increases with age. This relationship has implications for strategies aimed at controlling dengue fever.

Dengue fever has emerged as a serious international public health threat with almost half of the world's population at risk for infection (*1*). Although >50 million cases of dengue fever are estimated to occur each year (*2*), a large proportion of infections are asymptomatic (*3*).Why infection progresses to clinical disease in some persons, but not in others, is not clear. Some evidence suggests that risk for disease, with both classic dengue fever and the more severe dengue hemorrhagic fever, varies by age (*4*); however, the relationships have never been rigorously quantified. We used data from patients with laboratory-confirmed clinical dengue to describe the relationship between age and the relative risk of becoming ill with classical disease after primary infection with dengue virus. A clearer understanding of this relationship has implications for strategies aimed at controlling dengue fever.

The Study

Clinical dengue incidence data that have been serologically confirmed were abstracted from a survey conducted in 1997 in the communities of Belém and Ananindeua in Pará State, Brazil (*5*). In 1996, these communities reported their first cases of dengue fever (dengue serotype l) in >50 years, after the successful control of *Aedes aegypti* mosquitoes during the 1940s (*5*). We assumed, therefore, that persons ≤50 years of age were susceptible to all 4 dengue serotypes at the time of the survey and, as a result, that most reported cases were due to primary dengue infection. Age-stratified population data from the 2000 Brazilian census were used to estimate the total population of Belém and Ananindeua for the following age classes: 0–4, 5–9, 10–14, 15–24, 25–34, 35–44, and 45–54 years. The midpoints of these age classes were used in subsequent statistical analyses. Survey data describing the number of serologically confirmed clinical dengue cases were then used to estimate the minimum proportion of all persons in each age class who had clinical dengue (Figure, Panel A).

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We further investigated the relationship between age and probability of having clinical disease by calculating the risk for each age class relative to the age class that had the highest proportion of clinical cases. Unlike the absolute proportion, the relative risk is independent of transmission intensity. In calculating relative risk, we assumed that the risk for dengue infection was independent of age, which was corroborated by a seroepidemiologic study performed in Fortaleza, Brazil, in 1994 (*6*). A logistic regression model was developed to describe the relationship between age and the relative probability of disease after primary dengue infection. Model fitting was performed with Stata 8.0 (Stata Corporation, College Station, TX, USA), and robust standard errors were calculated for each regression coefficient.

The logistic model provided a significant fit to the data (McFadden $R^2 = 0.512$, $\chi^2 = 4.86$, df = 1, $p<0.028$) and described a clear positive relationship between age and relative risk for clinical disease ($\beta = 0.164$; bootstrap

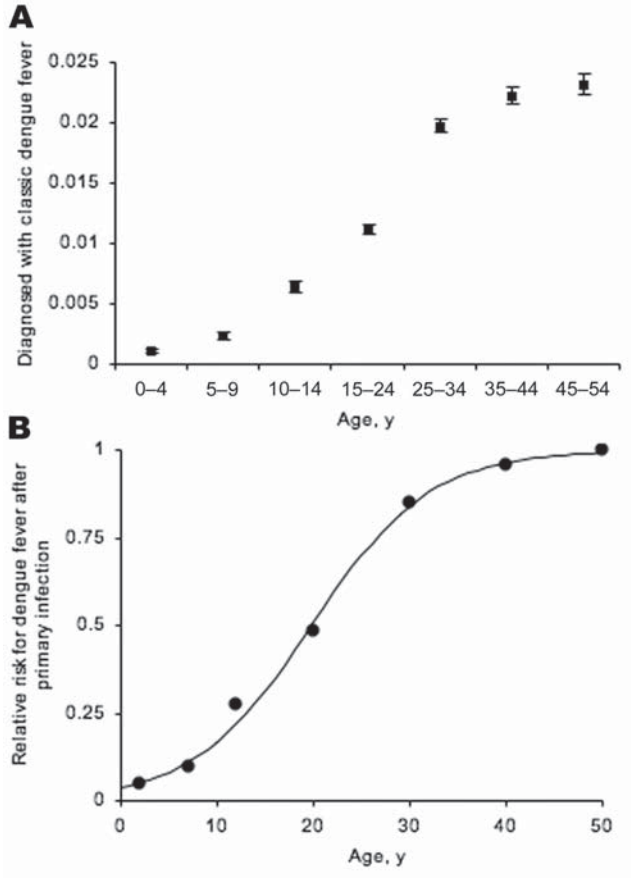

Figure. Estimated minimum proportion of the population, by age, with laboratory-confirmed classical dengue, showing exact 95% binomial confidence intervals. A) Fitting a logistic regression model (not shown) to the absolute proportion produced a significant age estimate: McFadden $R^2 = 0.762$, $\chi^2 = 5{,}196.13$, df = 1, $p<0.001$. B) Relative risk, by age, of having classical dengue after primary infection. Black circles, observed; line, model fit. See text for details of statistical analysis.

95% confidence interval 0.1470–0.1769), as shown in the Figure, Panel B. The results suggest that the risk for clinical disease after primary dengue infection is relatively low throughout childhood and then increases rapidly through adolescence and early adulthood.

Conclusions

To our knowledge, this is the first time data have been used to empirically derive the quantitative relationship between age at time of primary dengue infection and risk of having clinical dengue fever. These findings are consistent with results of earlier studies that suggest that adults are more likely than young children to have clinical dengue (*7*–*9*).

Several factors should be considered when interpreting these results. First, because dengue virus serotypes l and 2 were circulating in the population during the study period, some persons may have been infected with both serotypes during the 1-year period and, therefore, clinical signs may have resulted from a secondary infection. This proportion is probably small. Second, several factors other than age are thought to influence severity of classic dengue illness, including viral serotype and strain (*4*,*10*). Data from a dengue epidemic (dengue virus type 3) in Puerto Rico showed the attack rate to be independent of age (*11*). Although the proportion of these cases that were due to primary infection was uncertain, the different infecting serotype may be partly responsible for the conflicting findings between that study and ours. Further research should be conducted to determine whether the relationship between age and classic dengue fever is similar in epidemics involving all 4 dengue virus serotypes. Finally, whether all age groups in the study population had equal access to participating health facilities is not known. However, if a reporting bias were introduced, it would likely be in adults (because of child-rearing duties and difficulty taking time off work). Therefore, because adults represent a higher proportion of total patients with clinical cases in this study, underreporting in this age group would suggest that our relative-risk estimates in the adult age classes are conservative.

Despite the complexities of dengue epidemiology, these findings provide strong empirical evidence that age is an important factor in determining risk for disease severity after primary dengue virus infection. As such, these findings have important implications for initiatives aimed at controlling dengue. Interventions focused on reducing the number of *Aedes* mosquitoes are the mainstay of dengue control worldwide. Such approaches, however, have proved incapable of interrupting dengue transmission (*12*). At best, vector control may result in a partial reduction in the rate at which dengue virus is transmitted, which consequently increases the average age of the population susceptible to dengue infection. If age is a risk factor for clinical dengue fever, as our results suggest, then while partial control will decrease the rate of dengue infection, it may have the adverse effect of increasing clinical incidence.

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Murine Typhus in Children, South Texas[1]

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Children from South Texas were evaluated for immunoglobulin G to *Rickettsia typhi,* the causative agent of murine typhus. Of 513 children, 8.6% of those 1–5 years of age, 13.3% of those 6–11 years of age, and 13.8% of those 12–17 years of age had positive results.

Rickettsia typhi causes murine typhus in humans, a febrile illness with headache and rash. Despite the disappearance of *R. typhi* from most of the United States, 9–72 cases per year of murine typhus were reported to the Texas Department of State Health Services from 1994 to 2003 (*1*). An earlier study of 200 cases reported in Texas from 1980 through 1984 found that 29% of the patients resided in Nueces County (*2*). Children with murine typhus often have nonspecific signs and symptoms that mimic those of common viral infections, and the illness usually resolves without antimicrobial drug therapy (*3*,*4*). We believe that many cases go unrecognized and unreported. The objective of this study was to determine the seroprevalence of *R. typhi* in children residing in Nueces County and to assess whether the seroprevalence increases with age due to a greater chance of exposure over time.

The Study

Driscoll Children's Hospital is a tertiary care pediatric teaching hospital located in Corpus Christi, Texas, the seat of Nueces County. A convenience sample of serum residuals was obtained from blood samples of children seen in the hospital, its clinic, and its emergency department. Serum was evaluated by use of an indirect immunofluorescence antibody (IFA) test kit for immunoglobulin G (IgG) to *R. typhi* and *R. rickettsii* (Focus Diagnostics, Cypress, CA, USA). Specimen preparation, testing, quality control, and interpretation were done as described in the package insert (www.focusdx.com). Reciprocal titers ≥64 were considered positive. Endpoint titers were not determined. Testing was performed by the laboratory technician who does all rickettsia assays for the hospital.

No patient information was collected other than age, sex, and county of residence. Only serum residuals from children residing in Nueces County who were 1 to 17 years of age were included. A minimum of 150 serum residuals were obtained from children in each of 3 age groups (1–5, 6–11, and 12–17 years), with ≈50% from each age group being boys. χ^2 analysis and the Fisher exact test were used to compare frequencies between groups. The Institutional Review Board at Driscoll Children's Hospital approved this research project; informed consent was not required.

Samples (n = 513) were obtained between May 1, 2005, and August 31, 2006; 47.2% were from boys. There were 152 samples from children 1–5 years of age (mean age 2.7 years; 75 boys), 180 from children 6–11 years of age (mean age 8.2 years; 91 boys), and 181 from children 12–17 years of age (mean age 14.4 years; 76 boys).

Of the 152 samples from children 1–5 years of age, 13 (8.6%) were positive for *R. typhi* IgG and 6 (3.9%) were positive for *R. rickettsii* IgG. Four (67%) of the 6 patient samples positive for *R. rickettsii* IgG were also positive for *R. typhi* IgG. Of the 180 samples from children 6–11 years of age, 24 (13.3%; p = 0.18 compared with children 1–5 years of age; power = 0.21) were positive for *R. typhi* IgG and 13 (7.2%) were positive for *R. rickettsii* IgG. Eleven (85%) of 13 patient samples positive for *R. rickettsii* IgG were also positive for *R. typhi* IgG. Of the 181 samples from children 12–17 years of age, 25 (13.8%; p = 0.18 compared with children 1–5 years of age; power = 0.25) were positive for *R. typhi* IgG and 1 (0.6%) was positive for *R. rickettsii* IgG. The 1 patient sample that was positive for *R. rickettsii* IgG was also positive for *R. typhi* IgG. Thus, 62 (12%) of 513 samples tested had IgG reactive to *R. typhi,* and 20 (3.9%) had IgG reactive to *R. rickettsii.* χ^2 analysis for trend showed no difference in *R. typhi* seroprevalence between the 3 age groups (p = 0.28; power = 0.27).

Conclusions

On the basis of this study, ≈9%–14% of children in Nueces County have antibodies reactive to *R. typhi*. Seroprevalence appeared to increase with age. This trend did not reach statistical significance, but the power was insufficient to resolve a difference between the 3 age groups. Our results are similar to those of seroepidemiologic studies of *R. typhi* conducted in Texas and other areas of the world. Wiggers and Stewart (*5*) found that 15.7% of serum samples from an adult population in East Texas were positive for *R. typhi*.

Of the 20 samples positive for *R. rickettsii* IgG, 16 (80%) were also positive for *R. typhi* IgG and probably represent cross-reactivity, which can occur within and between the typhus fever and spotted fever groups (*6*). Because *R. rickettsii* is not endemic in South Texas, the 4 samples

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positive for *R. rickettsii* IgG and negative for *R. typhi* IgG may represent cases of *R. felis,* for which no test kit was available. *R. felis* can cause murine typhus–like illness, as reported, for example, in a patient from South Texas (*7*). In addition, opossums and cat fleas in South Texas demonstrate a higher infection rate with *R. felis* than *R. typhi* (*8*). It is also possible that the children in our study with test results positive for *R. rickettsii* IgG but negative for *R. typhi* IgG may have traveled outside South Texas to an area where *R. rickettsii* is endemic. They may also represent cases of infections caused by other *Rickettsia* spp., such as *R. prowazekii*, *R. parkerii*, and *R. amblyommii.*

Our study had several limitations and potential sources of bias due to the testing and sampling methods used, and these may have led to an overestimation of *R. typhi* seroprevalence. First, the reading of slides is subjective for indirect IFA assays; thus, it is possible that some negative results were deemed positive. We did not have >1 observer validate the results, but we did use a laboratory technician who was experienced at performing the tests. Second, a reciprocal titer ≥64 was considered positive per the test kit instructions. Use of a higher reciprocal titer for the cutoff may increase the specificity of the test and reduce the number of false-positive results. However, IgG titers decline over time (*9*), and we wanted to make sure we detected low-level titers that may have resulted from infections that occurred years ago. Third, we did not obtain medical or travel histories for the children. It is possible that some with positive test results may have had contact with other rickettsia and that their test result was positive due to cross-reactivity. Last, the convenience sample of specimens may not be representative of the Nueces County population as a whole because the specimens were not obtained through a randomized process.

Endemic murine typhus continues to occur frequently in South Texas children, as shown by the high rate of *R. typhi* seroprevalence that we found. Most cases probably go undiagnosed and spontaneously resolve. During the 1930s and 1940s, when murine typhus was more common, investigators evaluating the seroprevalence of *R. typhi* estimated that ≈700 people per year in San Antonio, Texas, were infected; whereas, the peak number of cases reported by the San Antonio Health Department in 1944 was only 91 (*10*). Physicians practicing in or near *R. typhi*–endemic areas need to consider murine typhus in the differential diagnosis of children with a febrile illness without a clear source of infection. *R. typhi* can be a cause of fever of unknown origin in hospitalized children who live in or travel to areas where this rickettsia is endemic, and it is important to know that effective antibiotic treatment is available (*3*,*4*).

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Full Recovery from *Baylisascaris procyonis* Eosinophilic Meningitis

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Infection by *Baylisascaris procyonis* is an uncommon but devastating cause of eosinophilic meningitis. We report the first case-patient, to our knowledge, who recovered from *B. procyonis* eosinophilic meningitis without any recognizable neurologic deficits. The spectrum of illness for this organism may be wider than previously recognized.

Eosinophilic meningitis is defined by >10 eosinophils/mm^3 in the cerebrospinal fluid (CSF) or >10% eosinophils of the total CSF leukocyte count. In the United States, the most common cause of eosinophilic meningitis is the presence of a ventriculoperitoneal shunt, but worldwide it is infection by *Angiostrongylus cantonensis* (*1*). Other infectious causes of eosinophilic meningitis include *Toxocara* spp., *Gnathostoma spinigerum*, neurocysticercosis, and *Baylisascaris procyonis*. The latter is an intestinal roundworm endemic to the US raccoon population (*2*). *B. procyonis* has not been described in New Orleans but is known to occur in raccoons in northern Louisiana (D. Bowman, pers. comm.) and in the nearby states of Texas, Georgia, Oklahoma (*2*,*3*), and Mississippi (C. Panuska, unpub. data).

Previously, all reported human cases of *B. procyonis* eosinophilic meningitis have resulted in death or severe neurologic sequelae. We describe a patient with clinically apparent *B. procyonis* eosinophilic meningitis, who appears to have completely recovered. This patient's recovery supports the notion that a wide spectrum of clinical disease exists for this parasite and that it may be a more common human infection than previously recognized.

The Case

A 4-year-old boy from New Orleans, Louisiana, who had sickle cell disease and a history of a splenectomy, was admitted to our hospital with 1 day of headache, right arm pain, and emesis. He was alert and oriented; his oral temperature was 38°C; and physical examination found only mild upper extremity tremors, dysmetria, and bilateral extensor plantar response. His blood leukocyte count was 16,000/mm^3, with 12% eosinophils; blood cultures yielded no growth. During the next 3 days, his headache and vomiting worsened and ataxia developed. Magnetic resonance imaging of the brain, performed on day 5 of illness, demonstrated cerebellar edema (Figure). CSF analysis showed the following: 5 erythrocytes, 1,734 leukocytes/mm^3 (55% of which were eosinophils), protein 290 mg/dL, and glucose 53 mg/dL. The boy was given 1 dose of mannitol (0.25 g/kg) and a loading dose of dexamethasone (1 mg/kg), followed by 0.25 mg/kg dexamethasone every 6 hours for 2 weeks. On day 9, a parasitic infection was suspected, and albendazole (10 mg/kg every 12 hours for 5 days) was begun. The boy's history was negative for travel outside the United States, raw food consumption, household pets, developmental disability, or pica. His mother reported household rodent infestation and fecal droppings on the patient's bed; raccoons had been seen in the neighborhood, but the boy had not been directly exposed to them. Three days after corticosteroid therapy began, headache and vomiting stopped

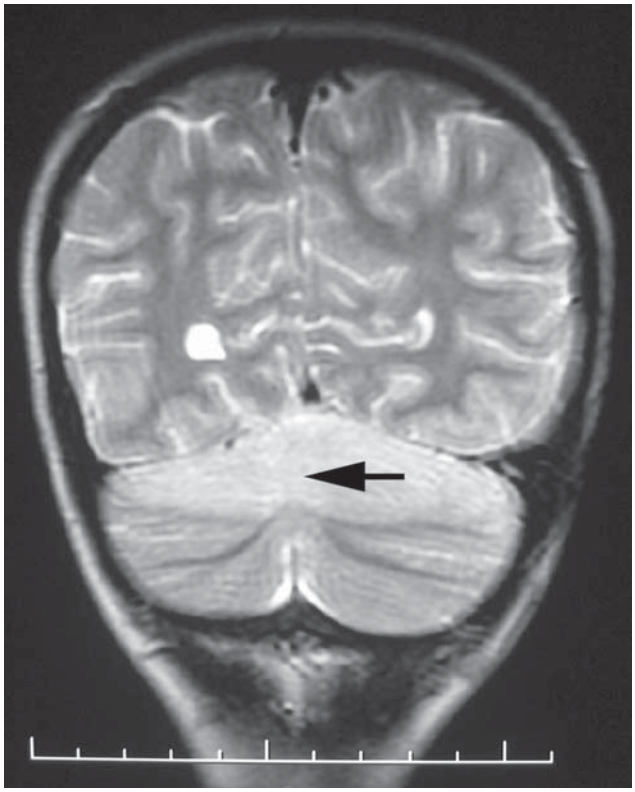

Figure. Coronal T2-weighted magnetic resonance imaging of the brain in a 4-year-old child with *Baylisascaris procyonis* eosinophilic meningitis. Arrow shows diffuse edema of the superior cerebellar hemispheres. Scale bar increments = cm.

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and only mild ataxia remained. Repeat magnetic resonance imaging and lumbar puncture on day 12 showed improvement in cerebellar edema and CSF eosinophilia (107 leukocytes/mm^3 with 6% eosinophils). Upon discharge (day 16), the patient's only abnormal finding was mild dysmetria of the right hand. One month later he was asymptomatic, and results of a neurologic examination were within normal limits; 24 months later he remained well.

Serologic test results for *Toxocara canis* (Centers for Disease Control and Prevention, Atlanta, GA, USA), *Treponema pallidum*, *Mycoplasma*, Epstein-Barr virus, and lymphocytic choriomeningitis virus were all negative. Acute-phase and convalescent-phase serum specimens (obtained 10 days and 7 weeks after illness onset, respectively) were negative for *A. cantonensis* (Western blot performed at Mahidol University, Bangkok, Thailand) (*4*); both were positive for *B. procyonis* (ELISA performed at Purdue University, West Lafayette, IN, USA) with optical density (OD) values of 0.547 and 0.976, respectively (positive cut-off OD ≥0.250; K. Kazacos, pers. comm.). CSF (acute specimen only) was negative for *A. cantonensis* and *B. procyonis* (OD = 0.006). A stool sample was negative for ova and parasites.

Conclusions

Comprehensive reviews of the epidemiology and clinical features of *B. procyonis* infection have been recently published (*2*,*3*,*5*,*6*). The definitive host for this roundworm is the raccoon (*Procyon lotor*), which each day sheds millions of eggs in feces, which heavily contaminate parks and neighborhoods (*7*). Humans are infected by ingesting the eggs; the median age of infected humans is 13 months, reflecting the propensity of young children to explore the environment orally (*2*,*3*). Subclinical infection has been suggested by a study in Chicago, which found 30 (8%) of 389 children 1–4 years of age were seropositive for *B. procyonis,* although none had experienced symptoms (*8*). After *B. procyonis* larvae hatch, they penetrate the intestinal wall and disseminate; the ensuing manifestations depend on infecting dose, location of migrating larvae, and degree of inflammation produced (*2*,*3*). The larvae are not neurotropic, but some may reach the central nervous system and, because of their large size (1.5–2.0 mm), cause major tissue damage (*2*,*3*). All previously described 13 patients who had clinically apparent *B. procyonis* eosinophilic meningitis died or were left with severe neurologic sequelae (Table); to our knowledge, our patient represents the first to fully recover. *B. procyonis* could cause low-level infections with mild and nonspecific clinical manifestations, similar to covert toxocariasis, (*9*,*10*) that can go unrecognized. Our patient underwent an extensive evaluation because of his underlying sickle cell disease and the suspicion for a cerebrovascular event; otherwise, his eosinophilic meningitis may have been missed.

Table. Reported human cases of eosinophilic meningoencephalitis caused by *Baylisascaris procyonis**

Year of onset	Location	Age	Sex	Blood eos/ mm^3, n (%)†	CSF eos/ mm^3, n (%)†	Diagnostic method	Outcome
1975	Missouri	18 mo	Female	5,139 (30)	209 (80)	Serologic (cross-reacting)	Persistent weakness and spastic right arm and leg
1980	Pennsylvania	10 mo	Male	4,698 (27)	63 (68)	Autopsy, serologic	Death
1984	Illinois	18 mo	Male	3,700 (37)	220 (80)	Autopsy, serologic	Death
1990	New York	13 mo	Male	6,513 (39)	75 (60)	Serologic	Neurologic deficits, cortical blindness, brain atrophy
1993	Michigan	9 mo	Male	Unknown	Unknown	Serologic	Neurologic deficits, cortical blindness
1993	California	13 mo	Male	12,780 (45)	Not done	Brain biopsy, serologic	Neurologic deficits, blindness, seizures, brain atrophy
1996	Illinois	6 y	Male	605 (5)	2 (<1)	Serologic	Neurologic deficits, seizures
1996	Minnesota	13 mo	Male	7,035 (35)	3 (54)	Serologic	Death
1997	Minnesota	19 mo	Male	2,232 (18)	5 (4)	Serologic	Death
1998	California	11 mo	Male	3,111 (17)	1 (7)	Serologic	Neurologic deficits, seizures, profound visual impairment
2000	Illinois	2.5 y	Male	5,880 (28)	26 (32)	Serologic	Neurologic deficits, blindness, generalized spasticity
2000	California	17 y	Male	2,385 (15)	7 (37)	Brain biopsy, serologic	Death
2002	California	11 mo	Male	Unknown	Unknown	Serologic	Neurologic deficits, cortical blindness, seizures
2004‡	Louisiana	4 y	Male	1,920 (12)	954 (55)	Serologic	Full recovery

*Modified from references (*5*) and (*6*), with permission.
†Highest within first 5 days of admission; eos, eosinophils; CSF, cerebrospinal fluid.
‡Case reported in this article.

Confirmation of *B. procyonis* infection requires identification of the larvae in tissues. A biopsy specimen was not obtained from our patient because of his benign clinical course; his case thus remains probable, rather than confirmed. Probable cases can be ascertained by a rise in serum or CSF antibody, as detected by an ELISA (*11*) performed at the Department of Comparative Pathobiology, Purdue University. Our patient's acute-phase serum sample was moderately positive and the convalescent-phase titer increased, which supports *B. procyonis* infection. Stool testing, however, is not useful because in humans the parasite does not complete its life cycle and thus does not produce eggs (*3*).

Treatment with albendazole after egg ingestion but before the onset of symptoms can prevent development of clinical baylisascariasis (*2*,*10*). However, after central nervous system symptoms appear, whether albendazole confers clinical benefit or instead worsens outcome due to parasite death and the resultant inflammatory response is unclear. Because of the latter possibility, corticosteroids should be used (*3*). Whether our patient's favorable outcome was due to a low burden of infection (as suggested by mild symptoms and modest serum antibody levels), host factors, or prompt initiation of therapy (mannitol, corticosteroids, and albendazole) is not known.

In conclusion, eosinophilic meningitis is a syndrome with many causes; in the United States, an important cause to consider is *B. procyonis*. All previously reported cases of eosinophilic meningitis resulted in death or severe neurologic disability. We report the likely first case-patient who fully recovered from *B. procyonis* eosinophilic meningitis. Although the potential for long-term sequelae in this patient is unknown, his short-term recovery has been excellent, and clinical and radiologic improvements suggest resolution of the disease process. This case demonstrates that milder presentations of *B. procyonis* infection are possible and suggests that infection by this parasite may be more frequent than previously recognized.

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Plasmodium malariae in Haitian Refugees, Jamaica

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Since 1963, reported malaria transmission in Haiti has been restricted to *Plasmodium falciparum*. However, screening of Haitian refugees in Jamaica in 2004, by microscopic examination, identified *P. falciparum, P. vivax,* and *P. malariae.* PCR confirmed the *P. malariae* and *P. falciparum* but not *P. vivax* infections. DNA sequencing and rRNA gene sequences showed transmission of *P. malariae*. This report confirms that *P. malariae* is still being transmitted in Haiti.

Malaria remains an important disease in Latin America and the Caribbean, where 30.4% (264 million) of the 869 million persons live in areas where ecologic conditions have been propitious for the transmission of malaria (*1*). Endemic transmission of malaria in Jamaica was interrupted from 1960 through 1965, when the last cases (16 *Plasmodium malariae* and 1 *P. vivax*) were reported (*2*). The island remains at risk for reintroduction of malaria because the population is immunologically naive and the vector, *Anopheles albimanus,* is endemic there (*3*). One possible route of reintroduction is through infected persons from a malaria-endemic area such as Haiti. Transmission of endemic malaria in Haiti has been reported to be restricted to *P. falciparum;* the last cases of *P. vivax* were reported in 1983 and the last case of *P. malariae* in 1963 (*1*,*2*,*4*,*5*). *P. falciparum,* which was associated with Haitian immigrants, was the sole etiologic agent of reintroduced malaria, which caused recent an epidemic in Great Exuma Island, Bahamas (*6*).

The Study

From February through April 2004, 429 Haitian refugees arrived by boat in Jamaica. Included in their health status screening was a microscopy examination for *P. falciparum* because malaria is well established in Haiti but is not endemic in Jamaica.

The refugees landed on Jamaica's northeast shore, where they received chloroquine and primaquine and had blood was drawn for thick and thin blood film preparation. The smears were then transported over land to the National Public Health Laboratory in Kingston for microscopic examination.

Of the samples from the 429 refugees, a subset of 274 (which included posttreatment duplicates) was chosen by the Ministry of Health for inclusion in this aspect of the study. These samples included 30 *P. falciparum* isolates, 13 *P. vivax* isolates, and 1 isolate of *P. malariae* based on microscopy examination; 31 samples were identified only as *Plasmodium* sp. The lack of a definitive diagnosis may have been influenced by the quality of the smears made at first contact with the patients and the fact that some smears were taken after initial treatment and contained only dying or dead parasites.

Samples from 94 patients (including some negative by microscopy examination as well as posttreatment duplicates) were sent to the Heath Protection Agency, Malaria Reference Laboratory, London School of Hygiene and Tropical Medicine, for species confirmation by PCR. DNA was extracted from 105 filter-paper blood spots and tested for DNA from *P. falciparum*. *P. malaria,* or *P. vivax* by using either the single-round PCR protocol of Padley et al. (*7*) or the more sensitive nested protocol of Snounou et al. (*8*). Of 15 isolates that were positive for *P. malariae* DNA by either or both PCR procedures, 7 also harbored *P. falciparum* DNA. Only 1 of the PCR-confirmed *P. malariae* cases had been identified as such by microscopy. Of the remainder, 2 samples contained *P. vivax;* 1 contained *P. falciparum,* and 4 were free of malaria parasites. Of the 105 isolates tested, 57 were positive for *P. falciparum* DNA.

No cases of *P. vivax* were found with PCR. The absence of *P. vivax* was verified by species-specific real-time PCR, as described by de Monbrison et al. (*9*)

Four *P. malariae* isolates were selected for DNA sequencing, and the rRNA gene sequences were amplified by using a hybrid nested PCR approach. The first-round PCR products were produced by using the first stage of the nested PCR protocol; in the second round, the *P. malariae*–specific primers of Padley et al. (*7*), which lie wholly within the first-round PCR product, were used to amplify a final product of 425 bp. This was done because the much smaller nested PCR product produced by the protocol of Snounou et al. (*8*) (125 bp) was not suitable for direct sequencing. Products were purified by using the QIA Quick DNA extraction kit (QIAGEN, Crawley, UK), and directly sequenced on both strands by using the ABI BigDye sequencing kit (Applied Biosystems, Warrington, UK) according to the manufacturer's instructions, except that the reaction mix was diluted 8-fold before use. The forward and reverse PCR primers were used to prime the sequencing reaction. Reaction products were fractionated on an ABI capillary sequencer (Applied Biosystems), and sequencing data were proofread with Chromas software (available from www.technylesium.com).

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The full sequence obtained from both strands of 1 representative isolate was compared with 3 *P. malariae* rRNA gene sequences in GenBank, and to that of *P. brasilianum* that had been isolated from a monkey in French Guiana ([10]; GenBank accession no. AF130735) by using the program T-Coffee (http://ca.expasy.org/tools/#translate). The alignment produced is presented in the Figure. The Haitian sequence obtained matched very closely with all 4 *P. malariae* rRNA gene sequences in GenBank and with that of the indistinguishable simian parasite, *P. brasilianum* (*10*). Two single-nucleotide substitutions were observed in the sequence at positions 228 and 337, and each of these also occurred in at least 1 of the other 4 Haitian sequences we obtained (data not shown). Available sequence data for *P. malariae* are very scanty, and currently no well-established loci exist for examining genetic diversity in this species, apart from the small subunit rRNA genes (*11*).

Conclusions

Our results provide conclusive evidence that *P. malariae* is still being transmitted on the island of Hispaniola, which contains the countries of Haiti and the Dominican Republic. *P. malariae* and *P. brasilianum* have been reported in both human and simian hosts in the continental South American states of Suriname, French Guiana, and Brazil (*8*,*12*,*13*), which are located on the northeastern coast of the continent, facing Hispaniola. Although a recent introduction of *P. malariae*, due to movement of persons, is possible, *P. malariae* has likely been present but unreported for a long period because it has been incorrectly diagnosed as *P. vivax*, as has occurred in Suriname (*12*). Indeed, Garnham (*14*) described the ring forms of *P. malariae* in blood films as "rather like those of *P. vivax*," although less amoeboid and with a more dense rim of cytoplasm.

The movement of *P. malariae* to Jamaica has implications for surveillance throughout the Caribbean and the southeastern United States. *P. malariae* infections can persist without symptoms for long periods, and thus the absence of recent travel history to a malaria-endemic area is not a reliable criterion for ruling out a diagnosis of malaria caused by this species. Our study shows that molecular diagnostic methods can provide the sensitivity and discriminating power required to identify *P. malariae* in such infections.

Furthermore, Jamaica is especially at risk for reintroduction of malaria (as illustrated by the Great Exuma outbreak) because the population is immunologically naive and the competent vector, *Anopheles albimanus,* is endemic there. A reassessment of the risk for *P. malariae* infection in Haiti should be undertaken before control and elimination programs are implemented.

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Figure. Comparison of *Plasmodium malariae* (Pm) rRNA gene sequences (GenBank accession nos. AF487999, AF145336, AF488000) and *P. brasilianum* (Pb) sequences from a monkey from French Guiana (Fr Gu) (AF130735) with isolates from *P. malariae* of humans from Haiti (Haiti Pm).

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Endemic Human Monkeypox, Democratic Republic of Congo, 2001–2004

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By analyzing vesicle fluids and crusted scabs from 136 persons with suspected monkeypox, we identified 51 cases of monkeypox by PCR, sequenced the hemagglutinin gene, and confirmed 94% of cases by virus culture. PCR demonstrated chickenpox in 61 patients. Coinfection with both viruses was found in 1 additional patient.

Monkeypox (MPX) virus is an orthopoxvirus that causes human MPX, a smallpoxlike disease reported in the African rainforests. Humans acquire the virus through direct contact with infected animals or patients (*1*). To determine whether MPX virus could potentially occupy the niche vacated by smallpox virus, the World Health Organization (WHO) conducted an active surveillance program during 1981–1986 in the Democratic Republic of Congo (DRC) and identified 338 MPX cases (67% confirmed by virus culture). Epidemiologic data led to the conclusion that MPX was a sporadic disease with a low potential for person-to-person transmission and that infection could not sustain itself in the human population (*1*). In the following years (1986–1995), only 13 MPX cases were reported (*2*). However, during 1995–1996, >500 cases of suspected MPX were reported, although only a small number were laboratory confirmed (*3*). In contrast to the earlier findings by WHO, the percentage of secondary cases was much higher (78%) and the mortality rate was much lower (1.5%). The question was raised as to whether a number of these cases were actually chickenpox, caused by the varicella-zoster virus (VZV), which is characterized by a high rate of secondary transmission and a low mortality rate (*4*). Although MPX is the only known severe orthopoxvirus infection today, the current dynamics of MPX infection are poorly understood and little information is available to improve WHO recommendations for the prevention of human MPX. We report laboratory data obtained from patients with suspected MPX infection.

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The Study

As part of the DRC Ministry of Health national disease surveillance program, 2,734 cases of suspected human MPX were reported from all 11 DRC provinces during January 2001–December 2004: 380 cases in 2001, 545 in 2002, 783 in 2003, and 1,026 in 2004. However, because civil war severely hampered surveillance activities, only 171 clinical specimens were obtained from 136 patients, who represent 4.9% of all reported cases. Ethical approval for this study was obtained from the Kinshasa School of Public Health, DRC, and the University of North Carolina, Chapel Hill, NC, USA.

All 171 specimens (crusted scabs and vesicle fluids) were inoculated onto MA104-cells by using standard procedures in a Biosafety Level 3 laboratory. Results yielded 56 MPX virus isolates from 48 patients (Table 1); scab and fluid specimens from the same patient were virus-positive for 8 patients. Identity of all isolates as MPX virus was confirmed by sequencing the entire open reading frame of the hemagglutinin (HA) gene.

DNA of all specimens was analyzed by using the RealArt Orthopox LightCycler PCR kit (QIAGEN, Hilden, Germany), which amplifies sequences of the fusion protein gene present in all orthopoxviruses, including MPX virus (*5*). Subsequent melting-curve analysis enables further differentiation of either variola or nonvariola orthopoxviruses; however, specific identification of MPX virus is not possible. We amplified nonvariola orthopoxvirus sequences in 65 specimens from 52 patients (Table 1); all specimens from which MPX virus had been isolated were also positive by PCR. For all 13 patients from whom crusts and vesicle fluids were available, both specimens were PCR-positive.

Of 171 specimens examined by using RealArt VZV LC PCR (QIAGEN), 78 showed specific amplification that indicated that 62 patients had VZV infection (Table 1). Of 136 patients investigated, 1 was coinfected with both, MPX (PCR and virus isolation) and VZV (PCR). Coinfections were reported during the 1996–1997 outbreak (*3*) and in a patient who died in 2001 (*6*). Whether these coinfections resulted from simultaneous circulation of both viruses or

Table 1. Orthopoxviruses and varicella-zoster virus, Democratic Republic of Congo, 2001–2004*

Specimen (no. patients)	MPX virus isolated	PCR result	
		Orthopoxvirus	VZV
Crusts (50)	16	18	21
Vesicle fluid (51)	19	21	24
Crusts and vesicle fluid (35)	8 and 5†	13‡	17‡
Total (136)	48	52	62

*MPX, monkeypox; VZV, varicella-zoster virus.
†Virus was isolated from both specimens from 8 patients and from either specimen from 5 patients.
‡Both specimens were positive by PCR.

to a mutual influence in the pathogenesis of either virus is not known.

Data on sex and age were available for 134 patients. The male-to-female ratio was approximately equal: 66 males and 68 females (Table 2). Ages ranged from 2 months to 54 years; average was 15.4 years (median 11.0). The average age of patients with confirmed MPX was 10.0 years (median 7.0 years), whereas the mean age of patients with VZV infection was 20.6 (median 17.0, $p \leq 0.001$, $t = 4.625$, $df = 109$). That most MPX cases (94%) occurred in patients <25 years of age suggests that cross-protective immunity may still exist; a potential for continued immunity has been demonstrated (*7*). However, this difference could also reflect different exposures in adults >25 years of age.

The DRC is divided into 512 administrative health zones that are within 11 provinces (Figure 1). Consistent with earlier reports (*1*), all MPX patients identified in this study lived mainly in small villages located in or near the tropical rainforest, where populations have ample opportunities for multiple close contacts with animals. No MPX cases have been identified in the area of Kinshasa. In some health zones, MPX virus and VZV circulate simultaneously (data not shown).

The entire sequence (942 nt) of the open reading frame of the HA gene was determined (*8*) for 48 virus isolates derived from 48 patients after PCR amplification. In addition, the HA gene sequence was successfully determined directly from lesion material of 2 MPX patients from whom no virus isolate was available; for a third patient, only a part of the HA sequence could be sequenced. All HA nucleotide sequences were deposited in GenBank (accession nos. DQ443476 through DQ443525). Sequencing identified 2 distinct groups consisting of 29 and 21 identical sequences, which differed by a single nucleotide. Geographic data analysis demonstrated that this difference was distributed consistently between patients living in east and west DRC (Figure 1). There were no sequence differences in the MPX virus hemagglutinin gene (942 nt) of isolates from the same patient. The 2 sequences determined here were identical to sequences reported previously (*9*) from MPX patients in the DRC (AF375099 and AF375096, respectively). The sequences represent 2 of 5 branches of the Central African MPX virus clade (Figure 2), which is distinguishable from a second clade comprising isolates from 1) West Africa, 2) outbreaks in primate-holding facilities in Europe and the United States, and 3) the recent 2003 US outbreak.

For our study, we used 2 commercially available kits to amplify orthopoxvirus and VZV sequences. The assays contain an internal control to monitor inhibition and use 4 positive controls to enable quantification of the amount of input genomic viral DNA. On the basis of our results, one can calculate that MPX lesion material usually contains several million viral genomic copies (data not shown). Whereas WHO surveillance confirmed 67% by virus culture (*1*), we isolated MPX virus in 94% of the MPX patients who had positive PCR results. The rather high rate of virus culture–confirmed cases points to the robust and highly concentrated amounts of virus in lesions; virus remained viable in lesions over an extended period despite suboptimal collection and transportation conditions.

Conclusions

Among 136 patients, 51 (37.5%) had laboratory-confirmed MPX infection, 61 (44.8%) had laboratory-confirmed VZV infection, and 1 (0.7%) had coinfection. MPX virus is considered to be the most important orthopoxvirus

Table 2. Age and sex distribution of patients with monkeypox or chickenpox, Democratic Republic of Congo, 2001–2004*

Age, y	No. cases investigated male/female, n = 134	MPX-positive male/female, n = 51	VZV-positive male/female, n = 61
≤4	12/21	8/7	3/6
5–14	22/19	12/9	7/8
15–24	17/13	5/7†	10/5†
25–34	9/8	2/0	5/6
≥35	6/7	1/0	5/6
Total	66/68	28/23	30/31

*MPX, monkeypox; VZV, varicella-zoster virus.
†Coinfection with MPX and VZV in a 17-year-old female patient.

Figure 1. Distribution of 52 confirmed cases of human monkeypox (MPX) by health zone in the Democratic Republic of Congo (DRC), 2001–2004. The cumulative number of cases per province is in parentheses. Confirmed cases of MPX originated from a total of 26 different health zones located in 5 provinces of DRC; most (70.5%) were reported from Kasai Oriental and Equateur Provinces. Two groups can be differentiated on the basis of sequence of the hemagglutinin gene: light gray, group 1; dark gray, group 2. Note: The boundaries of the DRC health zones have since been redrawn. Although the health zones Tshopo and Mweka are not shown (located in provinces Orientale and Kasai Occidental, respectively), the general area is highlighted to represent MPX cases in these regions.

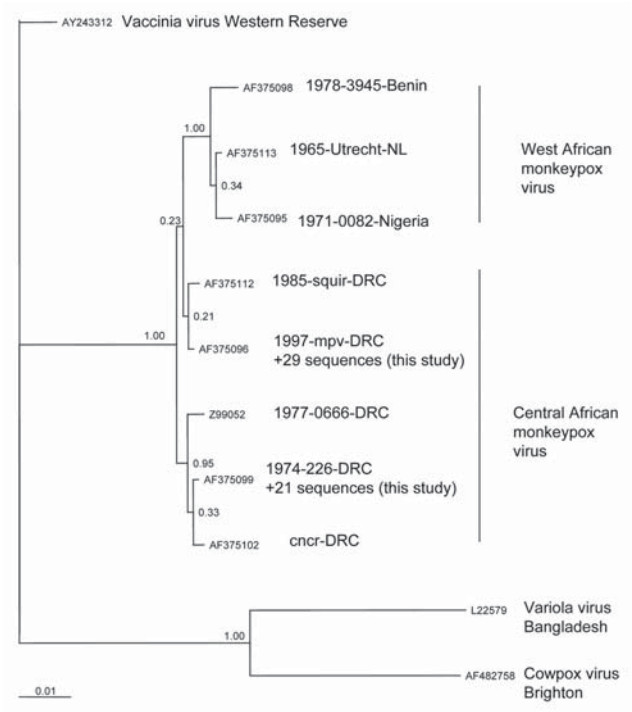

Figure 2. Phylogenetic inference relationships of the open reading frames encoding the hemagglutinin protein of selected strains of vaccinia, variola, cowpox, and monkeypox viruses and monkeypox virus isolates described in this study. Sequences used were cowpox virus Brighton (AF375089), variola virus Bangladesh (AF375129), vaccinia virus Lister (AY678276), monkeypox virus mpv 1997 (AF375096), mvp-squir (AF375112), mpv Zaire 77-0666 (Z99052), mpv-cncr (AF375102), mpv 74-226 (AF375099), mpv-082 (AF375095), mpv-utc (AF375113), and mpv-3945 (AF375098). ClustalW, version 1.83 (*10*), was used to generate amino acid multiple sequence alignments (pairwise gap opening = 35 and gap extension = 0.75; multiple alignment gap opening = 15 and gap extension = 0.30; Gonnet series). Each alignment was processed using RevTrans (*11*). Bayesian posterior probability inference of phylogeny used MrBayes, version 3.084. MrBayes settings for the best-fit model (GTR+I+G) were selected by hierarchies for the likelihood ratio test in MrModeltest 2.0 (*12*). Bayesian analysis was performed with MrBayes; the maximum likelihood model used 6 substitution types (nst = 6). Rate variation across sites was modeled by using a gamma distribution, with a proportion of sites being invariant (rates = invgamma). The Markov chain Monte Carlo search was run for 1 million generations; trees were sampled every 100 generations (the first 4,000 trees were discarded as burn-in). NL, Netherlands; DRC, Democratic Republic of Congo.

infection in humans because it causes disease clinically indistinguishable from smallpox. Recent outbreaks of MPX in the United States (*13*), the Republic of Congo (*14*) and Sudan (*15*) highlight the capacity of this virus to appear where it has never before been reported. Because clinically, MPX is often confused with chickenpox, laboratory diagnosis is critical to determine whether a suspected case is indeed MPX. In our study, we identified the causative agent for a rash-causing illness in 83% of all patients. We have recently strengthened the MPX surveillance program to improve understanding of the epidemiology of human MPX.

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Bartonella Species in Blood of Immunocompetent Persons with Animal and Arthropod Contact

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Using PCR in conjunction with pre-enrichment culture, we detected *Bartonella henselae* and *B. vinsonii* subspecies *berkhoffii* in the blood of 14 immunocompetent persons who had frequent animal contact and arthropod exposure.

Attempts to isolate *Bartonella* sp. from immunocompetent persons with serologic, pathologic, or molecular evidence of infection are often unsuccessful; several investigators have indicated that *Bartonella* isolation methods need to be improved (*1*–*4*). By combining PCR and pre-enrichment culture, we detected *B. henselae* and *B. vinsonii* subspecies *berkhoffii* infection in the blood of immunocompetent persons who had arthropod and occupational animal exposure.

The Study

From November 2004 through June 2005, blood and serum samples from 42 persons were tested, and 14 completed a questionnaire, approved by the North Carolina State University Institutional Review Board. Age, sex, animal contact, history of bites, environment, outdoor activity, arthropod contact, travel, and medical history were surveyed. Bacterial isolation, PCR amplification, and cloning were performed by using previously described methods (*5*–*7*). Each blood sample was tested by PCR after direct DNA extraction, pre-enrichment culture for at least 7 days, and subculture onto a blood agar plate (Figure). An uninoculated, pre-enrichment culture was processed simultaneously as a control. Methods used for DNA extraction and conventional and real-time PCR targeting of the *Bartonella* 16S-23S intergenic spacer (ITS) region and heme-binding protein (Pap31) gene have been described (*7*,*8*). Conventional PCR amplicons were cloned with the pGEM-T Easy Vector System (Promega, Madison, WI, USA); sequencing was performed by Davis Sequencing, Inc. (Davis, CA, USA). Sequences were aligned and compared with GenBank sequences with AlignX software (Vector NTI Suite 6.0 (InforMax, Inc., Bethesda, MD, USA) (*7*,*8*). *B. vinsonii* subsp. *berkhoffii*, *B. henselae*, and *B. quintana* antibodies were determined by using a modification of a previously described immunofluorescence antibody assay (IFA) procedure (*9*).

Study participants included 12 women and 2 men, ranging in age from 30 to 53 years; all of them reported occupational animal contact for >10 years (Table). Most had daily contact with cats (13 persons) and dogs (12 persons). All participants reported animal bites or scratches (primarily from cats) and arthropod exposure, including fleas, ticks, biting flies, mosquitoes, lice, mites, or chiggers. All participants reported intermittent or chronic clinical symptoms, including fatigue, arthralgia, myalgia, headache, memory loss, ataxia, and paresthesia (Table). Illness was most frequently mild to moderate in severity, with a waxing and waning course, and all but 2 persons could perform occupational activities. Of the 14 participants, 9 had been evaluated by a cardiologist, 8 each by an infectious disease physician or a neurologist, and 5 each by an internist or a rheumatologist. Eleven participants had received antimicrobial drugs.

When reciprocal titers of ≥64 were used, 8 persons were seroreactive to *Bartonella* antigens (online Appendix Table, available from www.cdc.gov/eid/content/13/6/938-appT.htm). *B. henselae* or *B. vinsonii* subsp. *berkhoffii* was detected or isolated from all 14 participants. At the time of initial testing, *Bartonella* DNA was amplified directly from 3 blood samples, from 7 pre-enrichment liquid cultures, and from 4 subculture isolates (Online Table). For 5 persons, results of PCR and culture of initial samples were negative. Overall, *Bartonella* DNA was amplified from 11 (28%) of 40 extracted blood samples, 13 (33%) of 40 pre-enrichment cultures, and 5 isolates. For 7 persons, *B. henselae* DNA was amplified at multiple time points. *Bartonella* DNA was never amplified from any PCR control or uninoculated culture control.

By using the ITS target region, 2 distinct *B. henselae* ITS and Pap31 strains were sequenced, *B. henselae* Hous-

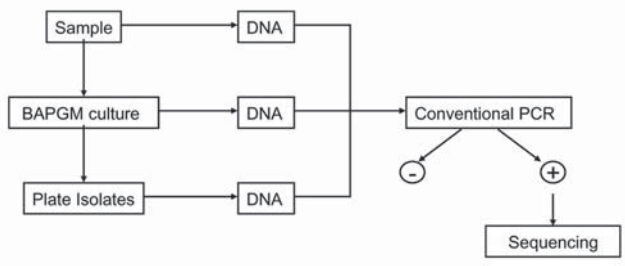

Figure. Diagram of sample processing and testing.

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Table. Selected demographic, epidemiologic, and medical information reported by 14 immunocompetent persons infected with *Bartonella henselae* or *B. vinsonii* subsp. *berkhoffii**

Characteristic/ symptom	Study participant no.														Total, N = 14
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Sex	F	F	F	F	F	F	M	F	F	F	F	F	M	F	
Age, y	51	30	48	44	53	50	32	33	48	53	52	39	52	44	
State of residence	NC	NC	NC	CO	VA	CA	NC	VA	CA	CA	CA	CA	VA	MN	
Occupational animal exposure	V	VtA	AHR	V	V	CR	VtA	VtA	VtA	VtA	VtA	V	WB	WB	
Daily contact with dogs/cats	Y/Y	Y/N	N/Y	Y/Y	Y/Y	Y/Y	Y/Y	Y/Y	Y/Y	Y/Y	Y/Y	Y/Y	N/Y	Y/Y	
Contact with fleas/ticks†	2/1	3/3	4/4	4/4	3/3	2/3	3/3	2/2	4/4	2/4	3/1	3/2	NA/3	4/3	
Self-health assessment‡	CI	CI	II	II	II	CI	CI	CI	CI	II	II	CI	II	CI	
Fatigue	+	+	–	+	+	+	+	+	+	+	+	+	+	+	13
Joint pain	+	+	–	+		+	+	+	–	+	+	+	+	U	10
Difficulty sleeping (insomnia)	+	+	–	–	+	–	+	+	+	+	+	+	–	–	9
Muscle pain	+	+	–	–		U	+	+	–	+	U	+	+	+	8
Difficulty remembering	+	+	–	–	+	–	+	+	+	+	+	–	–	U	8
Loss of sensation or numbness	+	+	+	–		+	–	+	–		+	+	–	U	7
Balance problems	+		–	–	+	+	+	+	–		+	+	–	–	7
Headache	+	+	–	–		+	+	+			–	+	+	U	7
Tremors	+		–	–		–	+	+	–	+	+	+	–	–	6
Irritability	+		–	–		–	+	+	+		+	–	–	+	6
Bowel or bladder dysfunction	+		–	+		+	–	+	–		+	–	+	–	6
Eye pain	+		–	+		+	+	+	–		–	–	–	–	5
Blurred vision	+		–	–		–	+	+	–	+	+	–	–	–	5
Sleepiness	+		–	–		–	+	–	–	+	+	–	–	+	5
Syncope or fainting episodes	+	+	+	–	+	+	–	–	–		–	–	–	–	5
Shortness of breath	+		–	+		–	+	+	–		+	–	–	U	5
Muscle weakness	+		–	–			+		–	+	+	+	–	U	5

*F, female; M, male; NC, North Carolina, CO, Colorado, VA, Virginia; CA, California, MN, Minnesota; V, veterinarian; VtA, veterinary assistant; AHR, animal health researcher; CR, cattle rancher; WB, wildlife biologist; Y, yes; N, no, with respect to the study participant's daily contact with dogs/cats; CI, chronically ill; II, infrequently ill; +, yes; –, no; blank, no answer reported; U, unknown.
†Reported as frequencies and defined as follows: 1, daily; 2, infrequently (weekly); 3, occasionally (monthly); 4, almost never (yearly).
‡Self-health assessment: As part of the questionnaire, study participants were asked to rate their own health status: healthy, infrequently ill, or chronically ill.

ton I (HI) (GenBank NC-005956) and *B. henselae* San Antonio 2 (SA2) (GenBank AF369529). Within the noncoding ITS region, *B. henselae* SA2 strains have a 30-bp insertion (ATT GCT TCT AAA AAG ATT GCT TCT AAA AAG) located 518 bases downstream from the 16S gene. Only *B. vinsonii* subsp. *berkhoffii* types I and II were detected (*8*).

Conclusions

Persistent human infection with *B. bacilliformis* and *B. quintana* has been previously documented, whereas infection with *B. henselae* (cat-scratch disease [CSD]) is generally considered self-limiting (*1*,*2*,*10*). Recently, *B. henselae* DNA was amplified from the blood of a child 4 months after CSD diagnosis (*11*). Our study indicates that *B. henselae* and *B. vinsonii* subsp. *berkhoffii* can induce occult infection in immunocompetent persons and that detection can be enhanced by combining PCR with pre-enrichment culture. Considering only the results from initial blood samples, PCR detected *Bartonella* DNA in 3 samples, all of which were subsequently PCR positive by subculture or enrich-

ment culture. In samples from 5 persons, pre-enrichment was necessary, and in 5 other persons, sequential sampling was necessary to detect *Bartonella* infection. Intermittent bacteremia, as occurs in *B. henselae*–infected cats (*12*), antimicrobial drug administration, low bacterial copy numbers, and low inoculum volume (1 mL) may have contributed to intermittent detection or inability to isolate *Bartonella* spp. from some participant samples. Although our approach is an improvement over historical isolation approaches, our results emphasize ongoing limitations associated with the detection of *Bartonella* infection. Obtaining stable *Bartonella* subcultures (n = 5 in this study) has proven problematic for other specialized laboratories that routinely culture for *Bartonella* spp. (*3*,*4*). To our knowledge, the *B. vinsonii* subsp. *berkhoffii* type II isolate described in our study is the only type II human isolate reported to date (*8*). Various combinations of *B. henselae* and *B. vinsonii* subsp. *berkhoffii* strain types were detected in the same blood sample or sequential blood samples. The coexistence of *B. henselae* genetic variants has been described among primary patient isolates, which suggests that multiple genotypes may emerge within the same person (*13*).

Overall, 57% of persons tested were seroreactive to 1 or all 3 *Bartonella* test antigens. Previous reports from the United States identified a *B. henselae* seroprevalence of 3% in healthy blood donors and a cumulative seroprevalence of 7.1% to both *B. henselae* and *B. quintana* antigens in veterinary professionals (*1*). In this and other studies, serologic test results did not correlate with PCR amplification or isolation results. Antigenic variability among *B. henselae* test strains can cause false-negative IFA results in persons with suspected CSD. Also *B. henselae*, *B. quintana*, or *B. elizabethae* antibodies were not detected in some persons with DNA evidence of active infection (*1*,*3*,*4*).

Animal contact, often to a wide spectrum of domestic and wild animal species, is an obvious consequence of the daily activities of the study population, which is biased by veterinary occupational exposure and by self-selection (volunteer bias). Cats are considered the primary reservoir host for *B. henselae*, whereas coyotes and foxes are considered reservoir hosts for *B. vinsonii* subsp. *berkhoffii* (*1*,*2*,*8*). Detection of *B. vinsonii* subsp. *berkhoffii* in 4 of 5 Californian participants could be related to the high prevalence of bacteremic coyotes in this region as well as to the potential transmission by a tick vector (*1*,*2*). All 14 participants reported frequent arthropod exposure. Although *Bartonella* spp.transmission by ticks has not been proven, several recent studies have identified *Bartonella* DNA in questing ticks, ticks attached to animals, and ticks attached to humans (*1*,*2*,*14*).

Despite reporting chronic or episodic illness, most participants continued to effectively maintain daily professional and personal activities. The symptoms described in the study patients are very similar to those described in a community and hospital-based surveillance study of CSD patients, in whom CSD-associated arthropathy was an uncommon chronic syndrome affecting mostly young and middle-age women (*15*). Our study was initiated to investigate the feasibility of combining PCR with pre-enrichment culture. Prospective studies, with appropriate controls, are needed to characterize the prevalence and clinical relevance of persistent *Bartonella* infection in immunocompetent persons.

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Wound Botulism in Injection Drug Users

To the Editor: Infections are the most frequent and serious wound complications in injection drug users (IDUs). Wound botulism is primarily caused by *Clostridium botulinum* (*1*) and was first observed in IDUs in New York in 1982 (*2*). It results from the introduction of *C. botulinum* spores into a wound and their multiplication, germination, in situ synthesis, and secretion of toxin under anaerobic conditions. Of 7 designated toxin types, neurotoxins A, B, E, and F result in human disease. During the 1990s, wound botulism cases among IDUs increased in the United States in conjunction with the use of black-tar heroin (*3*). Since 2000, wound botulism cases in IDUs have been reported in Europe (*4*). To our knowledge, molecular epidemiologic analyses have not been performed to confirm suspected outbreaks.

Within a 6-week period in October and November 2005, 12 clinical cases were recognized in the metropolitan area of Cologne, Germany (*5*). Six patients were successfully treated at teaching hospitals of the University of Cologne. On admission, all socially nonrelated patients had signs of bilateral symmetric cranial neuropathies such as ptosis, diplopia, blurred vision, dysphagia, dysarthria associated with symmetrical descending weakness of the upper extremities, and no sensory deficiencies. Treatment of patients included administration of trivalent A, B, and E antitoxin; antimicrobial drugs such as penicillin G or mezlocillin with metronidazole; and surgical drainage of any existing abscesses.

Patient 1, a 31-year-old female IDU, had multiple abscesses on both legs. Four days after her admission, wound botulism was suspected and antitoxin administered. Respiratory failure required mechanical ventilation for 11 weeks. Patient 2, a 51-year-old male IDU, had 1 large abscess on the left lower leg. Antitoxin was administered within 3 days of hospital admission. Mechanical ventilation was required for 5 weeks. Patient 3, a 25-year-old male IDU, had a large abscess on the left forearm. Patient 4, a 43-year-old man who used heroin intramuscularly, had an abscess of moderate size on the left forearm. Antitoxin was administered within 12 hours of admission to patients 3 and 4, and both patients required 2 weeks of respiratory support. Patient 5, a 32-year-old male IDU who was positive for hepatitis C virus, had purchased heroin from the same dealer as patient 2. Abscesses were absent. Antitoxin was administered within several hours of admission. Within 10 days, the patient recovered fully without need for mechanical ventilation. Patient 6, a 44-year-old male IDU, had several skin lesions at injection sites on his arms, but no abscesses. He received antitoxin treatment within several hours of admission and was discharged with minimal residual neck weakness after 7 days.

Serum specimens were obtained from patients 1, 2, 5, and 6. Botulinum toxin detected by the mouse bioassay in serum of patients 1 and 2, but not of patients 5 and 6, was neutralized by polyvalent antitoxin (Novartis Behring, Marburg, Germany). Abscess specimens were available from patients 2, 3, and 4. Anaerobic cultures grew *C. botulinum,* which was identified by Gram stain, culture morphologic features, Rapid ID 32A (bioMérieux, Marcy l'Etoile, France), and 16S rDNA sequencing. All strains were susceptible to penicillin G and metronidazole, as determined by the E-test (AB Biodisk, Solna, Sweden). PCR assays performed for *C. botulinum* type A, B, E, and F neurotoxin genes (*6*,*7*) identified the single toxin B. Toxin B production was confirmed by the mouse bioassay. Pulsed-field gel electrophoresis (PFGE) after *Sma*I, *Sac*II, and *Xho*I restriction (*8*) showed indistinguishable strains from patients 2, 3, and 4 (shown for *Sma*I in the Figure).

To our knowledge, this is the first outbreak of wound botulism in IDUs that was confirmed by molecular epidemiologic typing. PFGE suggests a

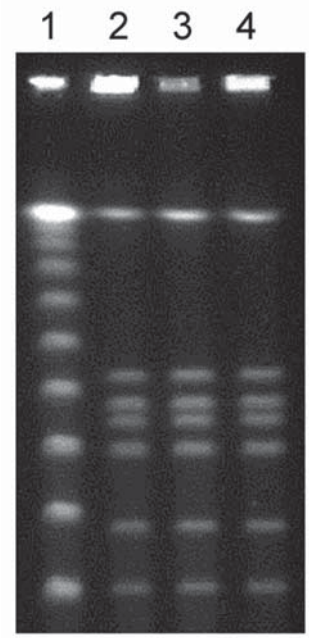

Figure. Fingerprint patterns obtained for *Clostridium botulinum* isolates following pulsed-field gel electrophoresis after *Sma*I restriction show identical strains. Lane 1, 100-bp ladder; lanes 2–4, abscess fluid isolates from patients 2, 3, and 4, respectively.

single-source exposure with *C. botulinum* type B in at least 3 IDUs; this implies that the heroin was obtained from a common source, where contamination with *C. botulinum* spores may have been introduced when mixed with adulterants or diluted with substances such as dextrose or dyed paper. Skin popping (subcutaneous and intramuscular injection), which may increase the odds of wound botulism by a factor >15 (*9*), was used by all patients for drug delivery. This study confirms previous observations that the duration of clinical symptoms before antitoxin administration affects the need for and duration of mechanical ventilation (*10*). Here, the time from hospital admission to antitoxin treatment ranged from several hours to 4 days and correlated with the mechanical ventilation interval ranging from 0 days to 11 weeks. In addition, the extent of abscesses, which ranged from no abscesses to multiple abscesses, seems to affect clinical outcome. As soon as an index case of wound botulism in IDUs is diagnosed, a coordinated public health case-management effort, including hospitals, outpatient clinics, and information centers for drug addicts, is mandatory to alert the medical community and the drug users to consider wound botulism if typical symptoms occur and to enable the prompt administration of antitoxin. Obtaining tissue samples or abscess fluid for culture and molecular epidemiologic studies of *C. botulinum* isolates is necessary to facilitate identification of the source of the contaminated heroin.

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Multidrug-Resistant *Acinetobacter baumannii*

To the Editor: In the January 2007 issue of Emerging Infectious Diseases, Sunenshine et al. (*1*) described their finding of an independent association between patients with multidrug-resistant (MDR) *Acinetobacter* infection and increased hospital and intensive care unit (ICU) length of stay compared with that for patients with antimicrobial drug–susceptible *Acinetobacter* infection. The authors did not, however, find a statistically significant difference in mortality rates between the 2 groups of patients.

Acinetobacter infections frequently occur in severely ill ICU patients with other chronic illnesses or prolonged hospitalizations. We analyzed data for 27 neutropenic cancer patients with *A. baumannii*–associated bacteremia (15 with MDR and 12 with drug-susceptible *A. baumannii* infections) but no other chronic illness. We considered *A. baumannii* strains to be MDR if they were resistant to amikacin, meropenem, and ciprofloxacin. Univariate analysis (Epi Info 2000; Centers for Disease Control and Prevention, Atlanta, GA, USA) showed that most of the bacteremic episodes were associated with certain risk factors, such as catheter insertion, neutropenia, acute leukemia, and previous prophylactic treatment with quinolones or therapeutic treatment with cephalosporins or carbapenems (meropenem or imipenem) (Table).

Table. Risk factors and outcome for 27 neutropenic cancer patients with bacteremia due to multidrug-resistant (MDR) or drug-susceptible *Acinetobacter baumannii* infection

Characteristic	All patients, no. (%) (N = 27)	Patients with drug-susceptible *A. baumannii,* no. (%)* (n = 12, 44%)	Patients with MDR *A. baumannii*, no. (%)* (n = 15, 56%)
Risk for bacteremia			
Central venous catheter	19 (70.4)	9 (75.0)	10 (66.7)
Acute leukemia	11 (40.7)	6 (50.0)	5 (33.3)
Previous prophylaxis with quinolones	14 (51.9)	8 (66.7)	6 (40.0)
Previous therapeutic treatment with cephalosporins	15 (55.6)	8 (66.7)	7 (46.7)
Previous therapeutic treatment with carbapenems	8 (29.6)	4 (33.3)	4 (26.7)
Outcome			
Septic shock	4 (14.8)	2 (16.7)	2 (13.3)
Death	2 (7.4)	1 (8.3)	1 (6.7)

*Insignificant difference between patients with drug-susceptible infection and those with MDR infection ($p \leq 0.05$ by univariate analysis).

Septic shock developed in 4 (14.8%) of the 27 neutropenic patients with *A. baumannii*–associated bacteremia, and 2 (7.4%) of the 27 died (Table). However, we did not find a statistically significant association between death among patients with bacteremia caused by MDR *A. baumanni* (1 death) compared with death among those with bacteremia caused by *A. baumannii* strains susceptible to the carbapenems, ciprofloxacin, and amikacin (1 death) (Table). This finding is similar to that described by Sunenshine et al. (*1*) in the general ICU population and in neutropenic cancer patients with bacteremia; however, multivariate analysis was not conducted to control for severity of illness and coexisting illness. In conclusion, neutropenic cancer patients with bacteremia due to MDR *A. baumannii* infection do not appear to be at increased risk for death compared with patients with bacteremia due to antimicrobial drug–susceptible *A. baumannii*.

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Letters

Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have one Figure or Table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Serogroup X in Meningococcal Disease, Western Kenya

To the Editor: Although >12 different serogroups of *Neisseria meningitidis* exist, most disease outbreaks across the African meningitis epidemic belt are caused by serogroup A and, less frequently, by serogroups C and W135 (*1*). *N. meningitidis* serogroup X was first described in the 1960s and has been found to cause a few cases of invasive disease across North America, Europe, and Africa (*2*). In Africa, small serogroup X outbreaks have been described in Ghana (9 cases over a 2-year period) and in Niger (134 cases between 1995 and 2000) (*3*,*4*). In 2006, however, 51% of 1,139 confirmed cases of meningococcal meningitis in Niger were found to be caused by serogroup X (*5*). Before the 2005-06 meningococcal epidemic season, no published reports had described serogroup X isolates in East Africa. We report the involvement of *N. meningitidis* serogroup X in an outbreak of meningococcal disease in Western Kenya.

In January 2006, the Ministry of Health of Kenya and Médecins sans Frontières were notified of a suspected meningococcal disease outbreak in West Pokot District, bordering Uganda, in Western Kenya. On the basis of the initial outbreak investigation, the outbreak was assessed to have begun in late December 2005. Subsequent active surveillance, using the same clinical case definition of sudden fever onset with stiff neck, altered mental status, or both, showed 74 suspected cases through mid-March 2006, with a case-fatality rate of 20%. No cases were reported after March 2006.

Over the course of the outbreak, cerebrospinal fluid samples were obtained from 18 patients. Due to low population density, poor access to seminomadic populations, and the

limited nature of the outbreak (relatively small numbers dispersed over a wide geographic region), obtaining specimens from untreated patients in West Pokot proved difficult. Three of the 5 first samples were found to show gram-negative diplococci on staining, the next 2 were negative on Pastorex rapid latex agglutination test (Bio-Rad Laboratories, Hercules, CA, USA) (during the outbreak investigation), and a subsequent 13 were sent to the African Medical and Research Foundation (AMREF) laboratory in Nairobi, Kenya, for culture and susceptibility testing. From these 13 specimens, 2 yielded a pure growth of *N. meningitidis* serogroup X, while no growth was observed for the remaining 11 specimens. These 2 cultures were subsequently confirmed as serogroup X by the World Health Organization Collaborating Centre for Meningococci in Oslo, Norway. Multilocus sequence typing and sequencing of the *porA* and *fetA* genes as described (http://pubmlst.org/neisseria/), showed that the infecting strain belonged to a new sequence type, ST-5403, and that it was subtype P1.19,26 and FetA type F3-27. This sequence type is unrelated to other serogroup X isolates from Africa, including those from the latest serogroup X outbreak in Niger, but it resembles a sequence type isolated in the United States in the 1970s. In addition to the testing at AMREF and in the Oslo laboratory, the 13 samples were also analyzed by PCR at the US Naval Medical Research Unit No. 3 in Cairo, Egypt. Overall, 5 of these 13 specimens were positive for serogroup X (including the 2 samples found to be serogroup X at AMREF and confirmed by PCR in Oslo) and 1 each was positive for serogroups C, W135, and Y.

At the same time as this outbreak in Western Kenya, a meningococcal meningitis outbreak was being monitored across the border in the Karamoja region of northeastern Uganda. Seminomadic populations move freely across the 2 countries, and we can assume that there was 1 meningitis outbreak that started in eastern Uganda and spread to Western Kenya. Although initial laboratory testing in Uganda suggested the presence of serogroup A, among 23 specimens subsequently sent to the Oslo laboratory, 11 were identified as serogroup X by PCR and 3 were serogroup W135 (*6*). Therefore, the outbreaks in both Kenya and Uganda involved multiple *N. meningitidis* serogroups. In West Pokot, Kenya, the Ministry of Health and Médecins sans Frontières conducted a vaccination campaign using the trivalent polysaccharide vaccine against serogroups A, C, and W135.

Before 2006, previous disease outbreaks caused by serogroup X had not reached the magnitude of those caused by serogroups A, C, or W135; they tended to evolve independently of the occurrence of both serogroups A and C and to be self-limited (*3*,*4*). Although most of Kenya is not included in the African meningitis belt, large epidemics of meningococcal disease have been reported previously (*7*). In conclusion, we would like to highlight the presence of *N. meningitidis* serogroup X in East Africa, its potential involvement in disease outbreaks, and the difficulties it may cause for laboratory confirmation and, consequently, for making an appropriate epidemic response.

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Imported Cutaneous Melioidosis in Traveler, Belgium

To the Editor: In some tropical areas, melioidosis, a disease caused by infection with *Burkholderia pseudomallei*, results in sepsis (*1*). This disease affects mostly adults with an underlying predisposing condition (*2*). With the increase in international travel, melioidosis has been identified in patients returning from disease-endemic areas (*3*). We report a case of a travel-associated cutaneous melioidosis without any systemic involvement.

A 90-year-old woman came to the Hôpital Erasme in Brussels with a nonhealing erythematous and ulcerated cutaneous lesion on the side of her left elbow. The lesion was a papule that gradually increased in size. The patient had diabetes mellitus but was otherwise healthy when she had traveled to Bangladesh 8 weeks earlier. She stayed 3 weeks in a village in the northwestern area of Rangpur District during the rainy season. She reported multiple insect and mosquito bites that evolved into intensely pruritic papules. This led to uncontrolled scratching and repeated washing of bite lesions with untreated well water.

Two weeks after her return, the lesion developed; it increased steadily in size, despite application of topical antimicrobial ointment. Three weeks later, after three visits to a physician, she was admitted to our institution. She did not report any fever, rigors, sweating, malaise, weight loss, or respiratory symptoms. Skin examination showed an irregular (3.0 cm × 4.0 cm), erythematous, fluctuant, tender, painful plaque (online/Appendix Figure, panel A, available from www.cdc.gov/EID/content/13/6/946-appG.htm). She did not have palpable regional lymph nodes. Results of a physical examination and laboratory investigations were normal. Five blood cultures at different times failed to isolate any microorganism.

A skin biopsy specimen from the plaque showed an inflammatory granulomatous reaction. Gram staining of biopsy specimens showed scanty lymphocytes, no polymorphonuclear leukocytes, and no microorganisms. Specimens were ground and placed on Columbia agar containing 5% horse blood and Schaedler enrichment broth and incubated aerobically for 3 days and on Schaedler agar containing 5% horse blood and incubated anaerobically for 10 days. After 72 hours, Schaedler broth showed a few colonies of an aerobic gram-negative bacillus that was identified as *B. pseudomallei* on the basis of typical biochemical characteristics. The strain was mobile at 37°C; grew at 42°C, oxidized but did not ferment glucose; produced cytochrome oxidase, arginine dihydrolase, and gelatinase; and was resistant to 300 IU polymyxin B 300 (DiaTabs; Rosco, Taastrup, Denmark). The isolate had a negative reaction for metabolism of arabinose.

Antimicrobial drug testing showed susceptibility to temocillin, amoxicillin-clavulanic acid (MIC 2 mg/L), piperacillin-tazobactam, ceftazidime, cefepime, meropenem (MIC 0.75 mg/L), doxycycline, and cotrimoxazole, and resistance to cefazolin, cefoxitin, ampicillin, gentamicin, ciprofloxacine, and amikacin. Results of tests for systemic involvement, as well as sputum and urine cultures, were negative. The patient was discharged and received oral doxycycline, 100 mg twice a day, and amoxicillin/clavulanic acid, 875 mg twice a day, for 32 weeks. The lesion dramatically improved 8 weeks after treatment was started (Appendix Figure, panel B) and had disappeared by 20 weeks after treatment was started (Appendix Figure, panel C). At 24 months after the diagnosis, no relapse had occurred.

Our patient with imported melioidosis had an unusual clinical course. She had never been febrile and had an uncomplicated localized skin infection skin despite her predisposing diabetes. A similar course has been reported in 2 tourists from Finland after the tsunami in Thailand in December 2004 (*4*), but most imported cases have pulmonary or systemic involvement associated with a severe prognosis (*5*,*6*).

The mode of acquisition in our patient herein was probably by an insect bite, contaminated water, or direct contact with wet soil during the rainy season. This mode of acquisition reinforces the hypothesis of a predominant role of percutaneous *B. pseudomallei* infection (*7*). Although the lesion healed, the patient was advised to have lifelong follow-up because relapses have been observed several years after infection.

Imported melioidosis is no longer a rare disease. With the increase in international travel and adventure tourism to disease-endemic regions, melioidosis is more likely to develop among travelers, even in those with short-term exposure. Recent reports suggest that melioidosis is probably widespread but poorly recognized throughout Bangladesh (*5*). Clinicians who treat patients returning from disease-endemic tropical areas, including the Indian subcontinent, should consider the disease in the differential diagnosis of febrile illnesses and isolated skin ulcers. Diagnosis is based on isolation of *B. pseudomallei* from blood, sputum, or biopsy specimens from lesions. Microbiologists should also be aware of the characteristics of the agent, and cultures should be handled under laboratory biosafety level 3 containment. Moreover, *B. pseudomallei* is a potential bioterrorism agent (*8*). Assessment of geographic and seasonal exposure is needed for identifiying this polymorphic exotic disease. Furthermore, travel advertisements to disease-endemic countries should include prophylactic measures to avoid contact with wet soils and contaminated water.

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Coronaviruses in Children, Greece

To the Editor: Two recently detected human coronaviruses (HCoVs), NL63 and HKU1, increased the number of coronaviruses known to infect humans to 5 (*1*–*3*). HCoV-229E and HCoV-NL63 belong to antigenic group 1, HCoV-OC43 and HCoV-HKU1 belong to antigenic group 2, and severe acute respiratory syndrome (SARS)–associated coronavirus (SARS-CoV) is most closely related to group 2 coronaviruses. In 2005, an optimized pancoronavirus reverse transcription–PCR assay was used to explore the incidence of HCoV-NL63 infection in children in Belgium who had a diagnosis of respiratory tract infection (*4*). We report the results of an epidemiologic study that used a universal coronavirus RT-PCR assay to detect coronaviruses among children in Greece with acute respiratory tract infections.

We tested throat swab specimens obtained from children hospitalized in Greece during June 2003 through May 2004 (200 children 2 months to 14 years of age, mean 4.09 years) and during December 2005 through March 2006 (44 children 1.6–8.5 years of age, mean 5.05). Specimens were obtained the first day of each child's hospitalization, and all specimens were included in the study, regardless whether other respiratory microorganisms were detected.

The 25-μL reaction contained 200 μM dNTPs, 0.2 μM primer PC2S2 (equimolar mixture of 5′-TTATGGGTTGGGATTATC-3′ and 5′-TGATGGGATGGGACTATC-3′), 0.8 μM primer PC2As1 (5′-TCATCAGAAAGAATCATCA-3′), 1 μL of enzyme mix from the QIAGEN One-Step RT-PCR Kit (QIAGEN GmbH, Hilden, Germany), and 5 μL of RNA. The initial 30-min reverse transcription step at 48°C was followed by 10 cycles of 20 sec at 94°C, 30 sec at 62°C with a decrease of 1°C per cycle, 40 sec at 72°C; 40 cycles of 20 sec at 94°C, 30 sec at 52°C, 40 sec at 72°C; and a final extension step at 72°C for 10 min. To determine the sensitivity after optimization, we tested quantified RNA in vitro transcripts that included the natural primer binding sites of the respective coronavirus genomes. Sensitivities for SARS-CoV, HCoV-OC43, HCoV-229E, and HCoV-NL63 were 61.0, 800.0, 8.2, and 82.3 nominal RNA copies per assay, respectively. A separate test was not done for HCoV-HKU1 because it had the same primer binding sites as HCoV-OC43. A phylogenetic tree based on a 400-bp genome fragment of the polymerase gene was constructed (online Appendix Figure, available from www.cdc.gov/EID/13/6/947-appG.htm).

Of 200 samples collected in 2003–2004, 5 (2.5%) were positive for coronaviruses (2 each for HCoV-NL63 and HCoV-229E and 1 for HCoV-OC43), and of 44 samples collected in 2005–2006, 2 (4.5%) were positive for coronaviruses (1 for HCoV-229E and 1 for HCoV-OC43) (GenBank accession nos. EF103180–EF103184, EF394298, and EF394299). CoV-HKU1 was not detected.

The amplified genome region is one of the most conserved regions of the coronavirus genome. However, sequences for HCoV-NL63 strains isolated in Greece are genetically closer to the sequence for a strain (AY567487) isolated in Amsterdam in 2003 (*1*) than to a strain (AY518894) from a specimen collected in Rotterdam in 1988 (*2*) (0.6% vs. 1.1% nucleotide divergence). Sequences for HCoV-229E and HCoV-OC43 strains isolated in Greece differ from sequences for strains isolated elsewhere by 0.5%–1.7%.

The HCoV-NL63–positive specimens in our study were obtained from a 9- and a 14-month-old child during winter 2003–2004; no cases were identified during 2005–2006. Specimens positive for HCoV-229E and HCoV-OC43 were detected during both study

Table. Epidemiologic and laboratory data for patients with coronavirus infection, Greece*

Specimen no., HCoV strain	Age, sex	Sample date	Symptoms	WBC (cells/ mm^3)	Granulocytes, %	ESR (mm/h)	Days in hospital	Coinfection
10/03, 229E	3 y, F	Jun 3, 2003	Fever (39°C), cough, pharyngitis	10,400	87	40	3	RSV
16/03, 229E	8 y, M	Jun 14, 2003	Fever (41°C), headache, rhinitis, sinusitis	18,900	86.4	30	4	ND
109/03, NL63	14 mo, F	Nov 30, 2003	Fever (39°C), cough, severe pneumonia	18,700	44.0	85	12	ND
173/04, NL63	10 mo, M	Feb 10, 2004	Fever (38.5°C), cough, rhinitis, tachypnea, bronchiolitis	7,100	57.9	55	3	ND
185/04, OC43	17 mo, F	Feb 25, 2004	Pharyngitis, rhinitis, respiratory distress, bronchiolitis	10,100	63.2	30	2	ND
12A/06, OC43	6 mo, F	Jan 11, 2006	Fever (38.8°C), cough, tachypnea, bronchiolitis	19,950	80.3	35	6	RSV
14A/06, 229E	7.5 y, M	Feb 13, 2006	Fever (40.5°C), cough, rhinitis	20,600	83.1	98	4	*Mycoplasma pneumoniae*

*HCoV, human coronavirus; WBC, white blood cell count; ESR, erythrocyte sedimentation rate; RSV, respiratory syncytial virus; ND, not detected.

periods (Table). HCoV-OC43 affected children with a mean age of 3.1 years (median, 1.4 years), and HCoV-229E affected children with a mean (and median) age of 5.5 years. However, no general conclusions can be drawn from these data because number of cases is too few.

None of the patients in Greece had an underlying disease, and all recovered completely. Patients infected with HCoV-229E had been hospitalized for upper respiratory tract infections, and those with HCoV-OC43 had lower respiratory tract infections; all cases were mild. Both children infected with HCoV-NL63 had symptoms of lower respiratory tract infections: 1 child had severe pneumonia and was hospitalized for 12 days, while the other had a mild course of bronchiolitis.

HCoV-NL63 was first identified in Amsterdam, the Netherlands, by van der Hoek et al. (*1*) from a nasopharyngeal specimen obtained in 2003 from a 7-month-old child with bronchiolitis, conjunctivitis, and fever. One month later, Fouchier et al. (*2*) reported the characterization of the same virus isolated from a specimen collected in 1988. The specimen had been obtained from an 8-month-old child with pneumonia in Rotterdam, the Netherlands. Later, HCoV-NL63 was detected in 2.5% of bronchiolitis patients <2 years of age in Japan (*5*) and in most children hospitalized with bronchiolitis in Australia and Canada (*6*,*7*).

Coinfection with HCoVs and other respiratory viruses is frequently observed and is associated with severe clinical syndromes, especially in infants and young children (*6*,*8*). Coinfection was observed in 3 of the 7 HCoV-positive patients in our study. The 3 patients were infected with HCoV-OC43 or HCoV-229E; coinfection with respiratory syncytial virus was found in 2 patients, and coinfection with *Mycoplasma pneumoniae* was found in 1 patient. It was not possible to determine the role of the HCoVs in these coinfections. In addition, because coronaviruses can be detected even 3 weeks after an acute episode, some cases of coinfection might represent former rather than current HCoV infection (*9*).

In conclusion, we detected 3 types of HCoVs in Greece: 229E, OC43, and NL63. This finding provides initial insight into the epidemiologic features of coronaviruses in Greece. Further studies are needed to find the exact clinical effect of these HCoVs in humans and to elucidate the epidemiology of coronaviruses worldwide.

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Bartonella DNA in Loggerhead Sea Turtles

To the Editor: *Bartonella* are fastidious, aerobic, gram-negative, facultative, intracellular bacteria that infect erythrocytes, erythroblasts, endothelial cells, monocytes, and dendritic cells, and are transmitted by arthropod vectors or by animal scratches or bites (*1*–*6*). Currently, 20 species or subspecies of *Bartonella* have been characterized, of which 8 are known zoonotic pathogens (*7*). *B. henselae* has been recently identified from canine blood (*8*) and from harbor porpoises (*9*). Pathogenic bacteria are an important threat in terrestrial and marine environments, and in the case of *B. henselae,* reservoir hosts may be more diverse than currently recognized.

The purpose of this study was to determine whether sea turtles are infected with *Bartonella* spp. Blood samples were obtained from 29 free-ranging and 8 captive, rehabilitating loggerhead sea turtles (*Caretta caretta*) from North Carolina coastal waters. Reptilian erythrocytes are nucleated, and commercial lysis methods clogged filtration columns because of the high DNA content of whole blood. Consequently, DNA was extracted from frozen whole blood by using a modified alkaline lysis method adapted from an avian cell culture DNA extraction method (*10*). PCR screening for *Bartonella* was performed by using primers for the 16S-23S internal transcribed spacer (ITS) region (Table). *Bartonella* ITS–positive samples were further screened by using primers for a consensus sequence of the phage-associated gene Pap31 (*9*). Primers for the 28S rRNA were used as a housekeeping gene. The PCR-positive control contained 0.002 pg/μL of *B. henselae* H1. Water was the negative PCR control. Amplicons of the expected sizes were consistently obtained from housekeeping gene and positive control reactions, while amplicons were never obtained from negative controls. ITS amplicons were obtained from 16 (43%) of 37 sea turtle blood samples tested, including samples from 13 free-ranging and 3 rehabilitated turtles. Pap31 PCR was performed for *Bartonella* ITS-PCR–positive samples. Pap31 amplicons were obtained from 5 samples of which 3 were successfully sequenced. Amplification and sequencing of the 16S-23S ITS region detected 2 *Bartonella* species: a *B. henselae*–like organisms and 1 more similar to *B. vinsonii* subsp. *berkhoffii*. The 3 Pap31 amplicons successfully sequenced confirmed *B. henselae* infection. Sequences obtained from 1 sample matched *B. henselae* strains H1-like, the *B. henselae* SA2-like strain, and *B. vinsonii* subsp. *berkhoffii* genotypes II and IV, which suggests that this turtle was co-infected with multiple *Bartonella* spp. and strains. Three other samples yielded amplicons 99%–100% identical with *B. henselae* strain SA2, and 3 yielded sequences most similar to *B. vinsonii* subspecies *berkhoffii* genotypes II and IV. Two samples contained an ITS region sequence most similar to *B. henselae* SA2, but with a 15-bp deletion beginning 617 bases downstream from the 16S rRNA gene. Whether these ITS sequence differences represent distinct strains or nonrandom translocation events is uncertain.

Four sea turtle blood samples contained partial ITS sequences most similar to *B. vinsonii* subsp. *berkhoffii*. However these amplicons were much shorter than expected for *B. vinsonii* subspecies *berkhoffii* genotype II and genotype IV sequences in GenBank. Because Pap31 gene amplification was unsuccessful for these samples, it is unclear whether small amplicons represent a species related to *B. vinsonii* subsp. *berkhoffii* or a new *Bartonella* sp.

Table. Primers used for PCR amplification

Primer	Sequence
28s s	5′-AAACTCTGGTGGAGGTCCGT-3′
28s as	5′-CTTACCAAAAGTGGCCCACTA-3′
ITS 325s	5′-CTTCAGATGATGATCCCAAGCCTTTTGGG-3′
ITS 1100as	5′-GAACCGACGACCCCCTGCTTGCAAAGCA-3′
Pap 31 1s	5′-ACTTCTGTTATCGCTTTGATTTCRRCT-3′
Pap 31 688(as)	5′-CACCACCAGCAAAATAAGGCAT-3′

To our knowledge, detection of *Bartonella* spp. DNA in sea turtle blood represents the first molecular evidence of *Bartonella* infection in nonmammalian vertebrates. *B. henselae* infection, now reported in porpoises and sea turtles, may represent an emerging infection of marine animals. According to previous studies, immune status appears to affect disease severity, variation in clinical manifestations, the pattern of histopathologic features, and the relative ease of diagnostic detection of the organism (*4*,*7*). Although healthy at the time of sample collection, the captive rehabilitated sea turtles were known to have been sick or injured before sampling, potentially reflecting immunocompromise. Whether detection of *Bartonella* spp. in blood of sea turtles is a function of prior immunosuppression induced by stressors is not known. Such stressors could include mechanical injury, malnutrition, environmental toxins, parasites, or concurrent bacterial or viral infections. Alternatively, sea turtles may be a natural marine reservoir for *B. henselae* or for a *Bartonella* sp. genetically related to *B. vinsonii* subsp. *berkhoffii.*

In summary, documentation of *B. henselae* and an organism genetically similar to *B. vinsonii* subsp. *berkhoffii* in the blood of loggerhead sea turtles provides evidence that this genus is not ecologically limited to terrestrial reservoirs. The geographic distribution, prevalence of infection, carrier potential, mode of transmission, and pathogenicity of bloodborne *Bartonella* spp. in sea turtles await additional studies.

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Human *Oestrus* sp. Infection, Canary Islands

To the Editor: Myiasis due to *Oestrus ovis* is a well known zoonosis that affects a variety of animals. Human myiasis has also been described and affects mainly persons in rural areas such as shepherds (*1*) and farmers (*2*). Although this disease has been reported in both humans and mammals in Spain (*3*,*4*), no human case has been described on the Canary Islands. We describe what we believe is the first confirmed case on the islands and discuss the potential utility of serologic diagnosis for this disease.

A 55-year-old farmer from the island of El Hierro, with a medical history of hypercholesterolemia, Q fever, and murine typhus, but currently not being treated, consulted a physician in August 2005 concerning a wormlike sensation in his nose and sinuses that had lasted 2 days. Three days before noticing this sensation, he had been working in his neighbor's barn, when he noticed that a passing fly "dropped" something in his nose. He also reported sneezing and watery rhinorrhea. These symptoms were self-treated with nasal anticongestants, which provided temporary relief. He finally sought medical attention when a severe cough de-

veloped and the wormlike sensation extended to his throat.

On physical examination, the patient's vital signs were normal, although a turbinate hypertrophy and mild redness of the throat were noted. No foreign objects or insects were seen on otorhinolaryngologic examination. The patient's blood count showed 8,480 leukocytes/μL with 6.1% (520/μL) eosinophils. Because of his stated symptoms, myiasis was suspected, and symptomatic treatment was started, consisting of antihistamines, nasal anticongestants, cough suppressants, and asphyxiant methods, i.e., swallowed olive oil. The patient was monitored closely and had complete remission of his symptoms after 6 days. No relapse has occurred.

In the meantime, we discovered that a serologic test for *O. ovis* was available (*5*). We requested and obtained a convalescent-phase serum sample from the patient on day 14 of his illness. Blood was also obtained from different "healthy" animals in the patient's neighborhood, including 2 dogs, 4 sheep, and 5 goats. This serologic assay had not previously been used in testing humans. Excretory and secretory antigens from *O. ovis* L2 (OL2ES) were obtained as previously described (*6*), and samples were analyzed by an immune enzymatic assay technique (*7*). Appropriate testing with different dilutions of the antigens, sera, and immunoconjugates was conducted. Immunoglobulin G (IgG) was detected in the patient, sheep, goats, and dogs following a similar protocol. OL2ES concentrations were 1, 1, 3, and 5 μg/mL, respectively. Serum samples were diluted 1:100 for the patient and the dogs and 1:50 for the goats; immunoconjugates were diluted 1:1,500 for all species. *O. ovis* IgG was found in the patient's sera, as well as in sera of the 2 dogs, 2 of 4 sheep, and all 5 goats (Table).

Human infection by *O. ovis* is generally considered to be an accidental occurrence (*8*). This case confirms, however, that myiasis caused by *O. ovis* must be considered in the differential diagnosis of a patient with typical symptoms and eosinophilia. Most farmers in this area have reported similar symptoms. Most, however, do not seek medical attention because they prefer to use homemade remedies, such as topical oil.

The diagnosis of oestrosis is usually made by direct visualization of the larvae, since the most frequent symptoms are pharyngeal myiasis and ophthalmomyiasis. Immunodiagnostic methods, however, could be a viable alternative to the clinical examination when no larvae are directly seen but a high degree of suspicion exists. The ELISA was noted to have a sensitivity of 96.1% and a specificity of 55.8% (positive predictive value of 86.7% and negative predictive value of 82.8%) in various investigations made with sheep and goats (*6*).

Although allergic symptoms are frequent in animals, the pathophysiologic process seems to be different in humans (*8*). Nevertheless, other authors have also described coughing and sneezing (*1*), probably attributable to irritation of the mucosa. In animals, a primary peak in eosinophil numbers has been noted 4 days after infection with a primary increase 48 hours after infection (*9*). In humans this pattern has not been described, but we did note a mild eosinophilia that disappeared after the patient recovered from his symptoms.

Outcome of the disease in humans is generally benign. Treatment includes removal of the larvae and, in some cases, prevention of local infections. Ivermectin has also been found useful in animal and human infections (*10*).

To our knowledge, this is the first case of human oestrosis on the Canary Islands, as well as the first human case described with eosinophilia. Physicians should be aware of the possibility of this disease in our region and of the fact that a serologic test is available for its diagnosis.

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Table. Results and interpretation, *Oestrus* sp. infection, Canary Islands*†

Human		Dogs		Sheep		Goats	
OD	Interpretation	OD	Interpretation	OD	Interpretation	OD	Interpretation
0.658	Positive	0.677	Positive	0.639	Positive	0.838	Positive
		0.824	Positive	0.685	Positive	0.535	Positive
				0.226	Negative	0.594	Positive
				0.187	Negative	0.673	Positive
						0.622	Positive

*****Results are expressed as optical density (OD), and interpretation (positive/negative) was made by using the following cut-offs: in sheep: 0.369 (0.1718 + 3× 0.066); goats: 0.406 (0.211 + 3 × 0.065); human 0.32 (0.17 + 3 × 0.049); dogs: 0.493 (0.37 + 3 × 0.041).
†One sample of positive and negative control samples was added to each plate. Sheep and goat sera from animals with a known history of *O. ovis* exposure were used. When positive sera were not available (human and dogs), we used only negative sera, and the cut-off was estimated as the mean OD of the negative sera plus 3 SDs (*7*).

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European Hedgehogs as Hosts for *Borrelia* spp., Germany

To the Editor: The European hedgehog, *Erinaceus europaeus*, is known to host a variety of tickborne pathogens, including the virus that causes tickborne encephalitis (*1*) and at least 3 species of the *Borrelia burgdorferi* sensu lato group: *B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii* (*2*). Members of the *B. burgdorferi* s. l. group are the most common vectorborne pathogens of humans in central Europe (*3*). The role of hedgehogs as hosts for these pathogens is, therefore, of considerable epidemiologic interest. Hedgehogs are a common synanthropic species that live in urban, suburban, and rural environments (*4*) and are known to carry not only the hedgehog tick, *Ixodes hexagonus*, but also the most common European tick, *I. ricinus* (*2*,*5*). Both of these ticks are known vectors of *B. burgdorferi* s. l. and tickborne encephalitis virus; *I. ricinus* is the most important vector of both throughout Europe (*1*,*5*). To date, however, only limited information has been available on the role of the hedgehog as a host or reservoir for *B. burgdorferi* s. l. in Germany.

We report the presence of 3 species of the *B. burgdorferi* s. l. group in European hedgehogs from Germany. To our knowledge, this is the first report of these species in hedgehogs in this country and the first report of *B. spielmanii* (A14S) (*6*) from this host.

The investigated hedgehogs came from 2 sources: 9 from the ≈40 in an experimental plot in the city of Karlsruhe, state of Baden-Wuerttemberg, and the remainder from wild hedgehogs that had been brought to hedgehog care centers from various areas of Germany. All hedgehogs had died naturally, and tissue samples were taken from 43 animals (kidneys from 43, heart from 22, bladder from 33). The bodies had been frozen at –17°C before the samples were taken.

DNA isolation was done by using the Maxwell 16 Instrument and System (Promega, Madison, WI, USA). Tissue samples were 3×3×3 mm. To detect *B. burgdorferi* s. l., we used 2 PCR protocols. The first was a nested PCR done according to the method of Rijpkema et al. (*7*). The target for the PCR was the spacer region between 5S and 23S rRNA genes of *B. burgdorferi* s. l. The nested primers generated a product of 226 bp. The amplified products were analyzed by agarose gel electrophoresis. The second protocol, a LightCycler-PCR hybridization assay (Roche Diagnostics, Mannheim, Germany) (*8*), simultaneously detects and genotypes the 3 genomic groups of *B. burgdorferi* s. l. This assay was specific for *B. burgdorferi* senso stricto, *B. garinii*, and *B. afzelii* (*8*) but also amplified *B. spielmanii* and *B. valaisiana*. The target for the PCR was the OspA gene.

The PCR products of both systems were sequenced. For DNA sequencing reaction, the fluorescence-labeled didesoxynucleotide technology (Applied Biosystems, Darmstadt, Germany) was used. The sequenced fragments were separated, and the data were collected with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The obtained sequences were then analyzed and compared by using BLAST (www.ncbi.nlm.nih.gov/BLAST).

For 6 hedgehogs, *Borrelia* spp. could be clearly defined by using both gene sequences. Two additional animals had positive results, but sequencing was not possible because of either too little DNA or a mixed infection. *B. spielmanii* DNA was detected in the kidneys of 2 hedgehogs: 1 from Karlsruhe and 1 from 30 km west of this city in the German federal state of Rhineland-Palatinate. When sequences were compared by using BLAST, 4 BLAST sequences (AM055823, AM055822, DQ133518, AY 995900)

showed 100% similarity with *B. spielmanii*. *B. garinii* was detected in the heart of 2 animals (from Berlin and Karlsruhe); *B. afzelii* in 3 animals (in the kidney of 2 from Hamburg and Karlsruhe and in the bladder of 1 from Rhineland-Palatinate). A single animal (from Karlsruhe) had *B. afzelii* in the kidney and bladder and *B. garinii* in the heart. Preliminary results have also shown that ticks collected from hedgehogs from the Karlsruhe site were infected with *B. afzelii* (an *I. hexagonus* nymph and an *I. ricinus* female) and with *B. spielmanii* (an *I. ricinus* female, a nymph, and a larva) (Skuballa et al., unpub. data).

These results show, that hedgehogs harbor at least 3 of the 5 recognized *Borrelia* genospecies found in Germany, all of which are known (*B. afzelii*, *B. garinii*) or are strongly suspected (*B. spielmanii*) of being pathogens for humans (*9*,*10*). To our knowledge, ours is the first report of *B. spielmanii* from hedgehogs, a *Borrelia* sp. that is usually associated with rodents, especially with garden and hazel dormice (*10*). That *Borrelia* spp. infections commonly occur in European hedgehogs is likely. However, questions remain about the role of these pathogens in regulating the populations of European hedgehogs and about the status of these common synanthropic mammals as a reservoir host of *B. burgdorferi* s. l. in periurban and rural environments.

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Invasive Cryptococcosis and Adalimumab Treatment

To the Editor: Tumor necrosis factor-α (TNF-α) antagonists are immunosuppressants that have shown efficacy in treating inflammatory disorders. However, a recent meta-analysis of controlled trials has shown evidence of increased risk for serious infections in patients with rheumatoid arthritis treated with TNF-α antagonists (*1*).

Adalimumab is a human monoclonal antibody to TNF-α approved by the US Food and Drug Administration (FDA) for treatment of rheumatoid arthritis. The Spanish registry of adverse events of biologic therapies in rheumatic diseases reported that 1,080 patients were treated with adalimumab from 2003 through 2006 and no cases of cryptococcosis were recorded (*2*). No cases of cryptococcosis have been detected in 10,050 treated patients in the US postmarketing database for adalimumab (*3*). We report invasive cryptococcosis in a patient receiving adalimumab. This case underscores the relationship between TNF antagonists and emergence of severe and difficult-to-treat opportunistic infections.

A 69-year-old woman with rheumatoid arthritis diagnosed in 2002 was referred to our hospital for severe acute inflammation of the second finger of the left hand. She had been treated with oral corticosteroids (prednisone, 7.5 mg/day) and several disease-modifying antirheumatic drugs, including chloroquine, methotrexate,

and sulfasalazin, without improvement. One year before the current episode, therapy with adalimumab, 40 mg subcutaneously every 2 weeks for 52 weeks, was started and she showed an acceptable clinical response. She had no recent trauma.

Examination showed severe tenosynovitis of the digital flexor tendon with intense edema and compartmental signs (online Appendix Figure, available from www.cdc.gov/EID/content/13/6/953-appG.htm). She had an axillary temperature of 36.7°C and an admission leukocyte count of 5,900 cells/μL. Results of a neurologic examination and a chest radiograph were normal. Early surgical decompression was performed. Intraoperative findings indicated extensive subcutaneous celullitis with infiltration of vasculonervous bundles and flexor tendon synovitis. Culture of extracted material from 4 samples, including a biopsy specimen of subcutaneous tissue, identified *Cryptococcus neoformans* susceptible to amphotericin B, azoles, and flucytosine. Results of cerebrospinal fluid analysis were normal. A cranial computed tomographic scan showed no focal lesions. Results of a serum cryptococcal latex test and HIV serologic analysis were negative. Magnetic resonance imaging of the finger showed inflammation of soft tissues, including the flexor tendon, but no signs of arthritis or osteomyelitis. Treatment with adalimumab was discontinued.

Intravenous liposomal amphotericin B, 300 mg once a day, and intravenous flucytosine, 2.5 g 3× a day, were administered for 7 days. Treatment with intravenous fluconazole, 400 mg twice a day for 21 days, was then started. Inflammatory signs decreased. Because residual soft tissue necrosis was extensive, reconstructive surgery was not performed, and her second finger was amputated during the third week after admission. A pathologic examination showed chronic necrotizing granulomatous inflammation with typical encapsulated fungal forms of *Cryptococcus* spp. inside multinucleated giant cells. These forms were observed by staining specimens with hematoxylin and eosin and Mayer mucicarmine (Figure). After an uneventful postoperative period, the patient was discharged and received oral fluconazole, 200 mg once a day for 6 months. Two years later, the patient remains asymptomatic and receives therapy with methotrexate, salazopyrin, and prednisone.

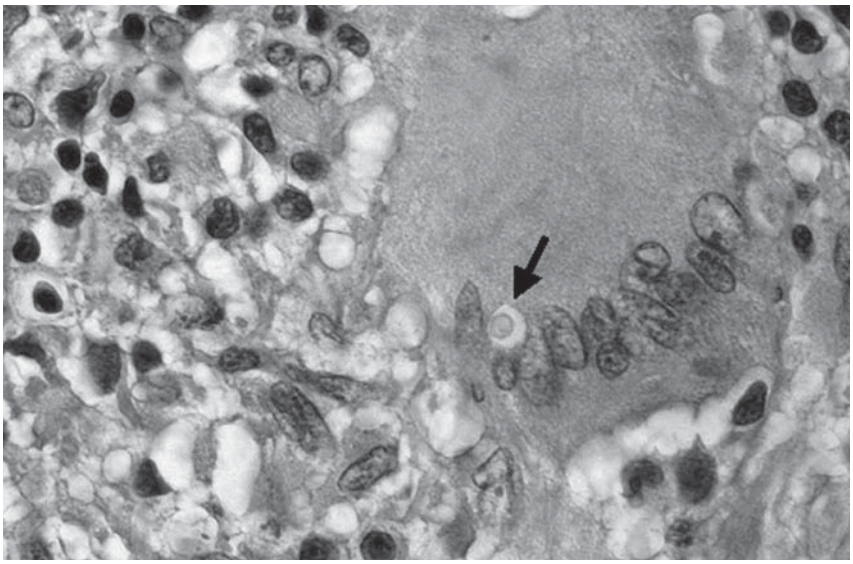

Figure. Histiocytic granuloma with lymphocytes and multinucleated giant cells and an encapsulated intracytoplasmic mucicarmine-positive structure identified as a *Cryptococcus* sp. (arrow) (hematoxylin and eosin– and Mayer mucicarmine–stained, magnification ×400).

The rate of serious infections in the US clinical trial safety database of adalimumab as of April 2005 was 5.1/100 patient-years. This rate is similar to that reported in the general population with rheumatoid arthritis. However, as in our case, some infections associated with adalimumab are severe and difficult to treat (*3*). Cryptococcosis has not been previously associated with use of adalimumab. Cryptococcal infections have been described in 19 patients receiving TNF-α antagonists other than adalimumab (infliximab or etanercept) in the FDA Adverse Event Reporting System from 1998 to 2002 (*4*). Three cases of cryptococcosis in patients receiving TNF-α antagonists have been reported (*5*–*7*).

The association between cryptococcosis and use of TFN-α antagonists can be explained by the immune response to *C. neoformans*, which relies on effective T-cell host defenses and in which TNF-α has an essential role. TFN-α is involved in maintaining a T-helper cell type 1 immune response because it induces production of interleukin-12 (IL-12) and IL-18, with subsequent production of fungicidal interferon-γ (*8*). In animal models, TNF-α blockers are associated with reduced recruitment of inflammatory cells to the area of infection and an increased risk for cryptococcal dissemination (*9*). Moreover, *C. neoformans* impairs production of TNF-α, IL-1β, and IL-6 and increases levels of IL-10, which induce a T-helper cell type 2 immune response (*10*). Cryptococcal virulence factors impart greater dependence upon TNF-α for a sufficient host response (*9*). Adalimumab may increase immunosuppression, which is required for a cryptococcal infection.

Our patient received a low dose of prednisone. Although corticosteroids are a risk factor for cutaneous cryptococcosis, cases with serious outcomes rarely occur. However, the

risk for fungal infection related to low doses of steroids is minimal. Active surveillance, as well as analysis of associated risk factors, is required to detect concurrence of severe opportunistic infections in patients treated with TNF antagonists and to identify patients who could benefit from these therapies with fewer risks.

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Determining Risk Factors for Infection with Influenza A (H5N1)

To the Editor: Novel antigenic subtypes of influenza viruses have been introduced periodically into the human population, resulting in large-scale global outbreaks (*1*). Highly pathogenic avian influenza (H5N1) viruses reemerged in 2003. Since then, they have reached endemic levels among poultry in several Southeast Asian countries, and across Asia, they have caused nearly 300 human infections, with a high rate of mortality (*1*,*2*). The results of many studies, including those for one recently conducted by Dinh et al. (*3*), have been published in an effort to identify the source(s) and modes of transmission of influenza A (H5N1) to humans and to guide the control and prevention of influenza infection.

Although new data regarding influenza A (H5N1) are urgently required, scientific rigor must be maintained during research and analysis to prevent misidentification of exposures as a risk factor for the disease and to prevent creation of iatrogenic panic among the exposed population and the scientific community (*4*). One point of scientific rigor that must be maintained is the use of adequate statistical analysis. The multivariate model in the study by Dinh et al. (*3*) was constructed by using a backward, stepwise variable selection strategy, in which variables with $p<0.20$ were included in the initial model. However, such a strategy has resulted in a first model and subsequent steps with far more than 10 variables per outcome (e.g., 28 persons with avian flu), resulting in model overfitting (i.e., a statistical model that is too complex for the amount of data), which could result in imprecise estimates or spurious associations (*5*).

We believe that scientific methods must be meticulously applied when planning, executing, analyzing, and interpreting the results of influenza (H5N1) studies to prevent identification of false risk factors for acquiring infection.

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In Response: Lukrafka et al. (*1*) warn against the dangers of overfitting a regression model when the number of outcomes is <10 per variable, "which could result in imprecise estimates or spurious associations." This warning is valid, but it is equally important to consider the relative merits of multiple analysis options given the data available, the difficulties in collecting the data, and the objective of the study. The objective of our study (*2*) was to explore possible risk factors for human infection with influenza A (H5N1) rather than to test an explicit a priori hypothesis or to obtain precise estimates of risk. We were limited to a finite number of cases, and had we slavishly followed criteria to avoid overfitting, we would not have run a regression model at all because we could have included only 2 variables, for which a stratified analysis would have been preferable. The regression model was run to confirm that the variables identified in the bivariate analysis retained their importance in the context of other variables; it was not intended to confirm or refute an a priori hypothesis, to be a predictive model, or to obtain precise and adjusted measures of risk. Despite the sample size limitations, we felt that looking at independence in a multivariable analysis was still valuable.

We explicitly acknowledge the limitations imposed by a small study size and were cautious in our interpretation, stating that the findings are the "basis for formulating new hypotheses." The wide confidence intervals clearly indicate the low level of precision. The 3 variables in the final regression model were all statistically significant in bivariate analysis, and we do not believe they are spurious associations arising solely from an overfitted regression model.

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Ilheus Virus Isolate from a Human, Ecuador

To the Editor: *Ilheus virus* (ILHV) (genus *Flavivirus* in the Ntaya antigenic complex) is most closely related to *Rocio virus*. However, antibodies produced during ILHV infection cross-react in serologic assays to other flavivirus antigens, and ILHV was originally classified in the Japanese encephalitis antigenic complex (*1–3*). ILHV is transmitted in an enzootic cycle between birds and mosquitoes. Since the first isolation of ILHV from a pool of *Aedes* spp. and *Psorophora* spp. mosquitoes collected in 1944 at Ilheus City, on the eastern coast of Brazil (*4*), isolates have been obtained in Central and South America and Trinidad, primarily from *Psorophora ferox* mosquitoes (*5*,*6*). ILHV is not associated with epidemic disease and has been only sporadically isolated from humans (*5*,*7–9*). The clinical spectrum of human infections documented by virus isolation ranges from asymptomatic to signs of central nervous system involvement suggestive of encephalitis. Most commonly, patients exhibit a mild febrile illness accompanied by headache, myalgia, arthralgia, and photophobia, symptoms that may result in clinical diagnosis of dengue, Saint Louis encephalitis, yellow fever, or influenza (*7*). Laboratory diagnosis of ILHV infection may be difficult, unless a virus isolate can be obtained, because of the cross-reactivity in serologic assays to other flaviviruses that circulate in the same area, such as Rocio, dengue, yellow fever, and Saint Louis encephalitis viruses.

On March 1, 2004, after 4 days of symptoms, a 20-year-old male soldier stationed in Lorocachi, Ecuador, was admitted to the Hospital de la IV División del Ejercito "Amazonas" in Puyo, Ecuador. Lorocachi is in the Amazonian province of Pastaza, of which Puyo is the capital. The patient

had fever, rash, epistaxis, headache, myalgia, retroocular pain, nausea, vomiting, jaundice, sore throat, and abdominal pain.

A blood specimen (containing isolate FSE800) was collected, and an acute-phase serum sample was processed for virus isolation in C6/36 (*Aedes albopictus*) and Vero cells. After 3 days, cytopathic effects were observed, and cells were screened by an immunofluorescence assay for reactivity against alphaviruses and flaviviruses by using polyclonal antibodies. The cells gave positive results with yellow fever, Saint Louis encephalitis, Rocio, and Ilheus antisera. Viral RNA was then extracted from the patient's acute-phase serum and cell supernatants and processed for virus sequencing. Amplification of an ≈250-bp product by SyBRgreen real-time reverse transcriptase PCR and subsequent sequencing of the amplicon were conducted with the flavivirus consensus primers FU1 and cFD2 (*2*). Viruses were identified by BLAST search (www.ncbi.nlm.nih.gov/blast) and alignment to GenBank sequences. FSE800 had 96% identity (182 of 188 bp) with the nonstructural (NS) 5 region of the original ILHV strain AY632539 (*1*). An ILHV original 1944 isolate used as positive control had 100% sequence identity with AY632539.

Limited sequence information is available in GenBank for ILHV outside of the conserved NS5 region. Therefore, further sequencing of the NS5 region using flavivirus consensus primers FU1 and cFD3 (*2*) and the complete envelope (E) gene region, based on the complete open reading frame ILHV nucleotide sequence AY632539 (*1*), was conducted on FSE800, as well as a low-passage original 1944 isolate (R. Tesh, University of Texas Medical Branch [UTMB]), 2 mosquito isolates from Iquitos, Peru (R. Tesh, UTMB), and 2 human isolates from Brazil (CDC) (*8*) (Figure). There was 100% sequence identity among the 2 Brazilian and 2 Peruvian isolates at both gene regions. In the NS5 region, there was 100% identity between Brazilian and original isolates; 95% identity between FSE800 and Brazilian-original isolates; 98% identity between FSE800 and Peruvian isolates; and 96% identity between Peruvian and original-Brazilian isolates. At the protein level, 3 amino acid (aa) differences were found between FSE800 and original-Brazilian isolates, 2 between FSE800-Peruvian and original-Brazilian isolates (aa 3086, Lys-Gln; aa 3138, Ala-Val) and 1 between FSE800 and original-Brazilian-Peruvian isolates (aa 3309, Asp-Asn).

In the E gene region, there was 99.9% identity between original and Brazilian isolates; 95.1% and 95% identity between FSE800 and original and Brazilian isolates, respectively; 98.5% identity between FSE800 and Peruvian isolates; and 95.3% identity between Peruvian and original isolates. At the protein level, 2 aa differences were found between original-Brazilian and Peruvian-FSE800 isolates (aa 432, Ile-Thr; aa 652, Lys-Asn), 1 aa change only in Brazilian isolates (aa 570, His-Tyr), and 1 only in Peruvian isolates (aa 675, Asn-Ser). With the exception of the Ala-Val change, all were nonconservative changes. The effect of the amino acid changes has yet to be determined. However, nucleotide sequence comparison between the 6 ILHV isolates has shown that these gene regions are highly conserved geographically and temporally.

Ilheus virus is not usually associated with human disease, and human ILHV infections may not be correctly identified without a virus isolate because of the similar clinical symptoms and cross-reactivity in serologic assays to other flaviviruses. As laboratory surveillance is enhanced to monitor the emergence of West Nile virus in Central and South America, detection of ILHV and other flaviviruses may increase.

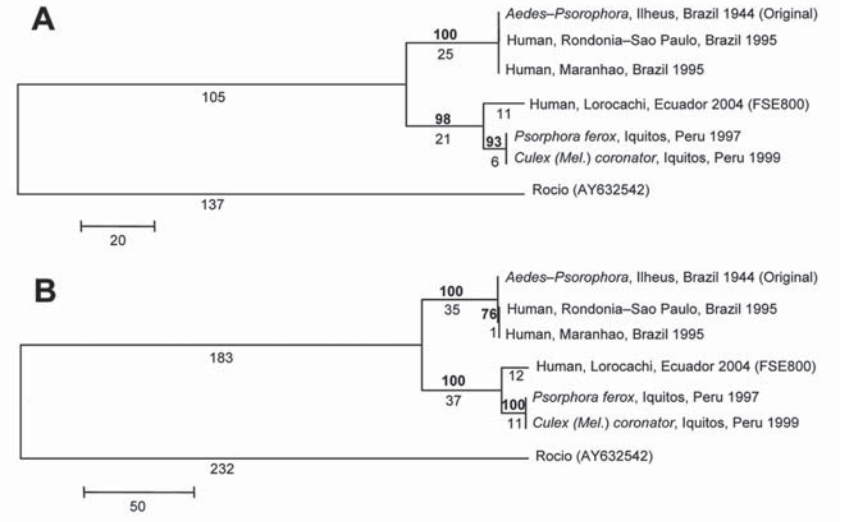

Figure. Phylogenetic analysis of the NS5 (A) and E gene (B) regions of 6 *Ilheus virus* (ILHV) isolates. The sequences were aligned in MegAlign (DNASTAR, Inc., Madison, WI, USA); the alignments were then analyzed by using the maximum parsimony method with 500 bootstrap replicates in the program MEGA 3.1 (*10*). *Rocio virus* (GenBank accession no. AY632542) was included as an outgroup in the analysis, based on the phylogram of Kuno and Chang (*1*). Bootstrap values, shown in **boldface** above the branch, are percentages derived from 500 samplings; branch lengths are shown below the branch. Scale bars show nucleotide changes.The sequences generated from our study were deposited in GenBank under accession nos. EF396941–EF396952.

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Prions: The New Biology of Proteins

Claudio Soto

CRC Press, Taylor & Francis Group, Boca Raton, Florida, USA, 2005
ISBN: 9780849314421
Pages: 184; Price: US $139.95

Prions are believed to be the causative agents of a group of rapidly progressive neurodegenerative diseases called transmissible spongiform encephalopathies, or prion diseases. They are infectious isoforms of a host-encoded cellular protein known as the prion protein. Prion diseases affect humans and animals and are uniformly fatal. The most common prion disease in humans is Creutzfeldt-Jakob disease (CJD), which occurs as a sporadic disease in most patients and as a familial or iatrogenic disease in some patients. Whether prions are infectious proteins that act alone to cause prion diseases remains a matter of scientific debate. However, mounting experimental evidence and lack of a plausible alternative explanation for the occurrence of prion diseases as both infectious and inherited has led to the widespread acceptance of the prion hypothesis.

Interest in prion disease research dramatically increased after the identification in the 1980s of a large international outbreak of bovine spongiform encephalopathy (BSE, also known as mad cow disease) in cattle and after accumulating scientific evidence indicated the zoonotic transmission of BSE to humans causing variant CJD. In recent years, secondary bloodborne transmission of variant CJD has been reported in the United Kingdom.

Prions: The New Biology of Proteins describes the current state of knowledge about the enigmatic world of prion diseases. The book is organized into 12 mostly brief chapters, which nicely summarize the various types of prion diseases and the challenges associated with their diagnosis and treatment. These sections review the biology of prions, the underlying hypotheses for prion replication, and the biochemical basis for strain diversity. Chapters 2 through 5 describe the various characteristic features of prions, including the historical evolution of the prion hypothesis, a detailed description of the possible mechanisms by which the normal prion protein is converted into the pathogenic form, and the cellular biology and putative functions of the normal prion protein. The author's lucid descriptions of the various topics are supported by diagrams and key references. Subsequent chapters describe prion disease laboratory diagnostic tools that are available or under development. Chapter 9 succinctly summarizes the most likely target sites, from the formation of the infectious agent to its effects on neurodegeneration, which can be exploited for likely therapeutic development. The same chapter describes the various antiprion compounds that have been or are being tested as therapeutic interventions for prion diseases.

The book is unusual because its entire content was exclusively authored by 1 person, resulting in a paucity of in-depth information in some areas, which may have been provided by multiple authors. However, all things considered, the book can be a valuable resource for scientists beginning to understand the world of prion diseases, the underlying biochemical mechanism of disease occurrence, and the challenges associated with the diagnosis and treatment of prion diseases.

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Battle of the Genomes: The Struggle for Survival in a Microbial World

H.M. Lachman

Science Publishers, Enfield, New Hampshire, USA, 2006
ISBN: 1578084326
Pages: 334; Price: US $29.95

Although this book's title promises the excitement of a 21st-century computer game, the cover photograph of Robert Koch in 1883 provides a better clue to the contents. The general plan is a survey of 20th-century genetics, illustrated by insights into human coevolution with microbial pathogens. Early chapters focus on familiar examples, including G6PD deficiency and sickle cell trait as adaptations to malaria, as evidence for pathogen-driven natural selection. Later chapters discuss more recent research findings, varying from female preference for the scent of males with dissimilar human leukocyte antigen types to the role of human CFTR membrane protein in infection with *Salmonella* Typhi. All of these are such good stories that science writer Matt Ridley included briefer versions in Chapter 9 of his popular book Genome: The Autobiography of a Species in 23 Chapters (*1*).

Battle of the Genomes: The Struggle for Survival in a Microbial World discusses in some detail how catastrophic epidemics of cholera, bubonic plague, and smallpox could explain the emergence of certain common human genetic mutations. Some of these mutations are deleterious; for example, CFTR ΔF508, which reduces the risk for typhoid, causes cystic fibrosis in persons who inherit 2 copies. Other mutations are beneficial, such as CCR5 Δ32, which may have protected carriers from smallpox and now reduces the risk for HIV infection. In general, the author's review

of the evidence for and against these hypotheses, which remain speculative, is evenhanded and up-to-date. His accounts of the human and social effects of epidemic diseases and the origins of public health are full of lively anecdotes and colorful detail. Interspersed throughout are personal asides, clinical pearls, and lengthy tutorials on basic science topics, such as DNA replication and gene splicing.

Although this book is far more information dense than are popular books for the lay public, its many shortcomings in terms of organization, depth, and documentation (including surprisingly few references) diminish its value to scholarly readers. More than anything else, it resembles an intellectually inspired but somewhat disorganized professor's medical school lecture, which would probably be more fun to hear in person than to read. Meanwhile, those who are interested in a 21st-century account of the battle of the genomes may want to wait. Rapid advances in genomic science and technology are opening the way to better understanding of biology, evolution, and medicine, but the full integration of these disciplines is still at a relatively early stage. The idea that genes of 1 species can influence whole ecosystems, described by Richard Dawkins in 1982 as the "extended phenotype" (*2*), is only now giving rise to new perspectives on community genetics (*3*).

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Atlas of Human Parasitology, 5th Edition

Lawrence R. Ash and Thomas C. Orihel

American Society for Clinical Pathology Press, Chicago, Illinois, USA, 2007
ISBN: 0891891676
Pages: 525; Price: US $200.00

The 5th edition of Ash and Orihel's Atlas of Human Parasitology is a superb, up-to-date compendium of protozoan and metazoan parasites. It also covers vectors and uncommon parasites found in humans. The authors present the material in a clear and concise manner that encourages one to delve more deeply into the structure and function of these unique and fascinating organisms. It is a must for persons interested in medical zoology and geographic medicine. Laboratory personnel, directors, and teachers who need a refresher course or additional training will find the book very valuable.

The Atlas of Human Parasitology is an essential treatise for helping to protect our citizens at home, deployed military personnel, and global travelers from parasitic infections. The quick keys to the identification of protozoans, helminths, and arthropods are helpful for distinguishing pseudoparasites from harmful ones. The labeling of various stages of the color images with letters, numbers, and arrows is extremely useful.

Attention has been given to opportunistic infections found in patients with AIDS. This book opens new vistas in helping to understand the global impact of AIDS and parasitic infections. The glossary and current references provide a ready resource for those interested in learning more about host–parasite relationships.

As an extra bonus, readers will find this edition a visual feast that integrates science and the arts. This book is highly recommended reading.

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Food Safety: Old Habits, New Perspectives

Phyllis Entis

ASM Press, Herndon, Virginia, USA, 2007
ISBN: 9781555814175
Pages: 414; Price: US $49.95

Anyone who works in food safety sooner or later discovers that one of the most valuable tools for prevention is simply reading about and understanding how past outbreaks have occurred. Using major and frequently famous or at least newsworthy outbreaks, Phyllis Entis in Food Safety: Old Habits, New Perspectives illustrates how critical factors come together to produce tragic and largely preventable results. This nicely written reference book reads more like an engaging novel in some ways, complete with bad guys (pathogens and sometimes careless corporations) and good guys (intrepid and resourceful outbreak investigators). The author's unique style, usually avoided

in science writing but appropriately used here, tells the tale of modern food safety issues so well that the book, literally, is difficult to put down.

Each of the 17 chapters covers a different food safety principle, illuminating how modern microbes often team up with old practices, shortsighted decisions, or current consumer trends to produce an outbreak. Chapters conclude with a concise "lessons learned" summary, such as this conclusion from Chapter 3: "Whether it's serotype Enteritidis in eggs or *C. botulinum* in eggplant, the challenge is the same. Recipes that do not include an adequate final cooking step have become increasingly popular with consumers and can be a significant source of food-borne illness."

One of the few downsides of this book is that it does leave the reader with the somewhat sensational impression that most food businesses are out to get the consumer. While the examples of greed and negligence are true, positive examples of good corporate behavior could have illustrated prevention and better balanced the portrayal of the food industry. Despite this small drawback, the tables are "one-stop shopping" for anyone looking for lists of outbreaks, and the fact boxes inserted here and there provide marvelous tidbits of information. A "who's who" of microbes at the end of the book is an added bonus.

Whether someone is preparing to teach a food safety course, looking for information about how and why outbreaks occur, or trying to get the facts on a critical food safety event, this author has already done all of the homework. For any food safety professional who has ever dreamed of the ultimate literature search, the references at the end of each chapter are breathtaking. This book is a must-have for any serious food safety professional.

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Jacob Lawrence (1917–2000). Marionettes (1952). Tempera on panel (46.4 cm x 62.2 cm). High Museum of Art, Atlanta, Georgia, USA. Purchased with funds from the National Endowment for the Arts and Edith G. and Philip A. Rhodes, 1980.224.

"What Did I Do to Be so Black and Blue?"[1]

—Louis Armstrong

Polyxeni Potter*

"Our homes were very decorative, full of…pattern…color….The people used this as a means of brightening their life," said Jacob Lawrence, attributing his love of vibrant color design to his youth in Harlem (*1*). When asked if anyone in his family was artistically inclined, he would say no, "It's only in retrospect that I realized I was surrounded by art. You'd walk Seventh Avenue and look in the windows and you'd see all these colors in the depths of the Depression, all these colors!" (*1*). "Most of my work depicts events from the many Harlems that exist throughout the United States. This is my genre. My surroundings. The people I know…the happiness, tragedies, and the sorrows of mankind…" (*2*).

Lawrence was born in Atlantic City, New Jersey, "But I know nothing about it," he always said, because his family soon moved, to Pennsylvania (*3*). He moved again in his early teens, with his mother, after his parents separated. "And we came to New York and of course this was a completely new visual experience" (*3*). Lawrence showed artistic talent at an early age. "I liked design….I used to do things like rugs by seeing the pattern…in very bright primary and secondary colors…and papier-mâché masks…not for play or anything…I just liked to make them….My first exposure to art which I didn't realize was even art at the time was at an after-school settlement house….The Utopia Children's Settlement House" (*3*).

"I never saw an art gallery until I was about eighteen years of age…. And going to the settlement house I was exposed to arts and crafts; soap carving, leather work, woodwork and painting…" (*3*). In the early 1930s, Depression relief programs sprang up all over the United States. Lawrence met Augusta Savage, already a well-known sculptor, at a center across the street from where he lived. He met writers Alain Locke, Richard Wright, and Ralph Ellison and worked with many prominent artists of the day, Norman Lewis, Charles Alston, Romare Bearden, Henry Bannarn.

Encouraged by Augusta Savage, he participated in the Works Progress Administration's Federal Arts Project, a program founded in 1935 to create jobs in the arts, "…[I]t was like a very informal schooling. You were able to ask

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[1]Lyrics, Andy Razaf. Music, "Fats" Waller and Harry Brooks.

questions of people who had more experience …about technical things in painting" (*3*). For inspiration, he visited the 135th Street Branch of the New York Public Library and walked 60 blocks to the Metropolitan Museum of Art.

Lack of academic training did not thwart Lawrence's artistic development. Rather, his individual style, borne of his personal view of the world and nourished by the community around him, flourished early and well. His flat patterns and colors and bold narrative scenes showed the influence of Mexican painters José Clemente Orozco, David Alfaro Siqueiros, and Diego Rivera, and of Polish artist Käthe Kollwitz, all of whom espoused a social realist philosophy.

At age 21, Lawrence attracted attention with his series of 41 paintings on Toussaint Louverture (c.1743–1803), a hero of the Haitian Revolution. He read voraciously and researched his topics thoroughly. He painted a story over a series of panels planned and executed as a cluster. Each scene was outlined in pencil, each color applied to all panels simultaneously to ensure consistent tonal quality across the series (*4*).

"The Human subject is the most important thing. My work is abstract in the sense of having been designed and composed, but it is not abstract in the sense of not having human content…I want to communicate. I want the idea to strike right away" (*2*). Lawrence freed his figures of detail, exacting the essence, which he punctuated with saturated color recalling Henri Matisse, and he repeated patterns and breaks, creating a rhythmic quality reminiscent of jazz syncopations. In gouache and tempera, he recreated the "hard, bright, and brittle" feel of Harlem during the Great Depression (*2*).

The work that brought Lawrence national recognition was The Migration of the Negro, a series of 60 panels recounting the mass movement of African Americans from the rural South to northern urban centers. The series was featured in Fortune magazine in 1941. He continued to paint for decades, at times doubting the strength of his style within modernism and questioning the influence of popularity on his work. He taught at the Pratt Institute, the New School for Social Research in New York, the Skowhegan School of Painting and Sculpture in Maine, and the University of Washington in Seattle. When he died at 82, Lawrence the chronicler of major cultural events of the 19th and 20th centuries had created an American aesthetic, and his expressive style, crafted in Harlem workshops, had made a lasting impression.

In Marionettes, on this month's cover, Lawrence revisited a topic addressed in The Dancing Doll (1947), an earlier work he had described as "mostly autobiographical" (*5*).

Marionettes, controlled with strings by a puppeteer from above, predate live theater. They were found in the tombs of ancient Egypt and the works of Archimedes and Plato.

Their inherent inability to stand alone makes marionettes an irresistible artistic and literary metaphor. In Invisible Man (1947), his epic of self-discovery, Ralph Ellison describes his encounter with a marionette, "I'd seen nothing like it before. A grinning doll of orange-and-black tissue paper with thin flat cardboard disks forming its head and feet and which some mysterious mechanism was causing to move up and down in a loose-jointed, shoulder-shaking, infuriatingly sensuous motion, a dance that was completely detached from the black, mask-like face" (*6*).

Viewed en masse at the bottom of the painting, Lawrence's marionettes seem dwarfed under the dark backdrop and drooping tents, the inevitable strings a reminder of their attachment to a set. In what seems a makeshift theater, they await the next move. To paint the lifeless dolls, the artist was prompted by social ills, which strip people of control over their lives, causing them to withdraw because, as Ellison put it, "…ain't nothing I can do but let whatever is gonna happen, happen" (*6*).

As with all his work, Lawrence touched a universal nerve: human vulnerability against forces beyond one's control. "I'm so forlorn, Life's just a thorn/My heart is torn/Why was I born?" lamented Louis Armstrong (1901–1971), speaking for Lawrence and for all of us. Faced with overwhelming social injustice or with recurring insults of a more biologic nature—microbial resistance to drugs, mutating viruses, emerging prions, migrating hazards—we may at times seem little more than hapless marionettes, caught in a degrading tangle at the foot of a large set.

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

Look in the July issue for the following topics:

Brazilian Vaccinia Viruses and their Origins

Water Management and Schistosomiasis, Dongting Lake Region, China

Thottapalayam Hantavirus

Antimicrobial Drugs and Community-Acquired MRSA

Klebsiella pneumoniae Bloodstream Infections and Virulence Characteristics

Antiretroviral Therapy during Tuberculosis Treatment of HIV-Infected Patients

Ongoing Genome Reduction in *Mycobacterium ulcerans*

Rift Valley Fever Outbreak in West Africa and East-Central African Virus Lineage

Linezolid-Resistant Enterococci

Person-to-Person Transmission of Nipah Virus

Effects of Internal Border Control on Spread of Pandemic Influenza

Emergency Authorization for Product Use in Civilian and Military Emergencies

Influenza Pandemics in Singapore

Live Poultry Exposures, Hong Kong and Hanoi

Cutaneous Leishmaniasis, Sri Lanka

Norovirus in a Captive Lion (*Panthera leo*)

Complete list of articles in the July issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

June 17–23, 2007
Options for the Control of Influenza VI
Toronto, Ontario, Canada
http://www.optionsviconference.com

June 24–28, 2007
Association for Professionals in Infection Control and Epidemiology
34th Annual Conference and International Meeting
San Jose, CA, USA
Contact: 202-454-2638
http://www.apic.org

June 25–27, 2007
National Foundation for Infectious Diseases (NFID) 2007 Annual Conference on Antimicrobial Resistance
Hyatt Regency Bethesda
Bethesda, MD, USA
http://www.nfid.org/conferences/resistance07

June 27, 2007
The Interagency Task Force on Antimicrobial Resistance presents: The Annual Report of Progress on a Public Health Action Plan to Combat Antimicrobial Resistance
Hyatt Regency Bethesda
Bethesda, MD, USA
http://www.nfid.org/conferences/resistance07

July 29–August 1, 2007
17th ISSTDR Meeting | 10th IUSTI World Congress
Seattle, WA, USA
http://www.isstdr.org

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EMERGING
INFECTIOUS DISEASES
A Peer-Reviewed Journal Tracking and Analyzing Disease Trends

Editorial Policy and Call for Articles

Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles see below and visit http://www.cdc.gov/eid/ncidod/ EID/instruct.htm.

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.

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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.

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Types of Articles

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

Synopses. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch of first author—both authors if only 2. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research Studies. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary, and a brief biographical sketch of first author—both authors if only 2. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and brief biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., “The Study” and “Conclusions.” Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and a brief biographical sketch of first author—both authors if only 2. Dispatches are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no figures or tables.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. All letters should contain material not previously published and include a word count.

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

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted on the journal Web page only, depending on the event date.)

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