Infectious Etiologies of Acute Febrile Illness among Patients Seeking Health Care in South-Central Cambodia

Matthew R. Kasper,* Patrick J. Blair, Sok Touch, Buth Sokhal, Chadwick Y. Yasuda, Maya Williams, Allen L. Richards, Timothy H. Burgess, Thomas F. Wierzba, and Shannon D. Putnam

United States Naval Medical Research Unit 2, Jakarta, Indonesia; Control of Communicable Disease Department, Ministry of Health, Phnom Penh, Cambodia; National Institute of Public Health, Ministry of Health, Phnom Penh, Cambodia; United States Naval Medical Research Unit No. 2, Phnom Penh, Cambodia; Naval Medical Research Center, Silver Spring, Maryland

Abstract. The agents of human febrile illness can vary by region and country suggesting that diagnosis, treatment, and control programs need to be based on a methodical evaluation of area-specific etiologies. From December 2006 to December 2009, 9,997 individuals presenting with acute febrile illness at nine health care clinics in south-central Cambodia were enrolled in a study to elucidate the etiologies. Upon enrollment, respiratory specimens, whole blood, and serum were collected. Testing was performed for viral, bacterial, and parasitic pathogens. Etiologies were identified in 38.0% of patients. Influenza was the most frequent pathogen, followed by dengue, malaria, and bacterial pathogens isolated from blood culture. In addition, 3.5% of enrolled patients were infected with more than one pathogen. Our data provide the first systematic assessment of the etiologies of acute febrile illness in south-central Cambodia. Data from syndromic-based surveillance studies can help guide public health responses in developing nations.

INTRODUCTION

Limited resources and the great diversity of acute febrile illness (AFI) etiologies in tropical regions challenge diagnosis, treatment, and public health responses to endemic and epidemic diseases. Further confounding this is the fact that a majority of the patients present with non-descript symptoms (e.g., low-grade fever, general malaise, headache, and muscle ache) and usually no focal point of infection. Health care providers lacking proper diagnostic tools are usually unable to determine specific etiologies, often diagnosing patients presumptively based on clinical features and assumptions regarding circulating pathogens.

Syndromic-based disease surveillance provides a useful methodology to systematically identify and document causes of acute fever. This approach has been used by TropNetEuropa1,2 to diagnose fevers of unknown origin in Turkey,3 China,4 and India and a project designed by the U.S. Centers for Disease Control and Prevention (CDC, Atlanta, GA) for the same purpose along the United States-Mexico border.

In Southeast (SE) Asia, a prospective study of Thai patients presenting with acute undifferentiated febrile illness determined the etiologies of over 40% of cases, with the most prevalent being influenza, dengue, and rickettsial infections.5 In Latin America, surveillance identified the etiology of acute undifferentiated febrile illness in Ecuador in 40% of enrolled patients, identifying a number of pathogens that have not previously been isolated in the country.6

Emerging and re-emerging diseases are a concern in SE Asia during a dynamic time of population growth, urbanization, and global migration. Infections from dengue virus, malaria parasites, influenza viruses, hepatitis viruses, rickettsiae, and Leptospira have previously been shown to impact populations across SE Asia. However, among the SE Asian nations, limited data exists on the etiologies of AFI in the Kingdom of Cambodia. Earlier studies reported illnesses among Cambodian refugee populations that had either immigrated to the United States or occupied refugee camps in Thailand.7,8

These works documented infections by agents such as malaria, hepatitis, cryptosporidium, and tuberculosis that presented in an often stressed and undernourished patient cohort. More recent studies conducted in Cambodia used both prospective and/or syndromic approaches. Infectious etiologies of encephalitic syndromes among patients admitted to a provincial hospital near Phnom Penh9 were identified in 35 of 88 (40%) human immunodeficiency virus (HIV)-seronegative patients; 11 bacterial, 1 fungal, and 23 viral infections (Japanese encephalitis virus [JEV] the principal causative agent of disease). A hospital-based study among children in Phnom Penh was conducted to diagnose hemorrhagic fever, encephalitis, or hepatitis in children,10 reporting that hepatitis A and JEV contributed greatly to the burden of disease in this population. In 2006, we initiated clinic-based surveillance to ascertain the etiologies of AFI among patients seeking treatment at peri-urban and rural health care clinics and hospital outpatient departments in Cambodia. Herein, we describe the demographics, symptoms, and identified etiologies of the patients seeking health care for AFI from December 2006 to December 2009.

MATERIALS AND METHODS

Ethical considerations. Eligible subjects voluntarily enrolled in accordance with an Institutional Review Board protocol approved by U.S. Naval Medical Research Unit Two (NAMRU2) in compliance with all applicable Federal regulations governing the protection of human subjects and the National Ethics Committee of the Royal Kingdom of Cambodia, Ministry of Health. Written consent was obtained from patients 18 years of age and older. For patients younger than 18 years of age, written consent was obtained from a parent or legal guardian. Additionally, written assent was obtained from patients between 8 and 17 years of age. For all individuals, the informed consent was verbally explained and questions solicited.

Site selection and study enrollment. Clinic-based acute fever surveillance was initiated in December 2006. Patients were initially recruited from outpatient clinics of two referral hospitals, but during the course of the study, additional clinics...
Operational district is a designation used by the Cambodian government to denote a continuous geographic region under the purview of a single government office.

**Specimen collection.** For each enrollee, a blood specimen was obtained by venipuncture and one throat and one nasal swab were collected. For nasal swabs, a dry polyester swab was inserted into the nostril, parallel to the palate, slowly withdrawn, and placed in a vial containing 2–3 mL of virus transport medium. For throat swabs, both tonsils and the posterior pharynx were swabbed vigorously, and the swab placed in 2–3 mL of virus transport medium. All inoculated vials were kept at 4°C until transported to NAMRU2/NIPH’s laboratory. All specimens were accessioned at the laboratory between 24 and 72 hours post collection.

**Malaria smears.** At the time of enrollment a blood smear is prepared and transported (daily) to the laboratory for staining by Giemsa stain and interpretation by microscopy.

**Blood cultures.** A single blood culture was obtained from all enrolled patients in an aerobic BacT/Alert 3D (bioMérieux, Hazelwood, MO) bottle. Daily, inoculated bottles were transported to the NAMRU2 laboratory in Phnom Penh and then incubated for up to 7 days in the BacT/Alert blood culture system. A sample of blood from positive blood cultures was inoculated onto chocolate, MacConkey’s, and blood agar plates and incubated at 37°C, 5% CO2, for up to 48 hours. Bacterial identification was done using the API 20E biochemical identification system (bioMérieux).

**Serological assays.** All commercial enzyme-linked immunosorbent assay (ELISA) test assays were performed and interpreted according to the manufacturer’s instruction as Positive, Equivocal, and Negative. Dengue IgM serological assays were performed on all acute serum samples from all study participants using the Dengue IgM Capture ELISA Dx Select (Focus Diagnostics, Cypress, CA). Hepatitis A IgM was performed with the Hepalisa Anti-HAV IgM (PT INDEC DIAGNOSTICS, Jakarta, Indonesia). Hepatitis B surface antigen was detected using the Hepalisa HBsAg (PT INDEC DIAGNOSTICS). An IgM serological response to hepatitis E was detected using the HEV IgM ELISA 3.0 (MP Biomedicals, Singapore). An acute immunological response was detected to *Leptospira* using the *Leptospira* IgM ELISA (PANBIO, Brisbane, Australia).

The IgG serology was performed for dengue, Hantavirus, chikungunya, and *Rickettsia* on all convalescent serum samples. All commercial ELISA test assays were performed and interpreted according to the manufacturer’s instruction as Positive, Equivocal, and Negative. Convalescent specimens that were found to be IgG positive were paired with the acute serum and retested. A 4-fold increase in titer from acute to convalescent sample, or a result change going from negative to positive, was considered indicative of seroconversions.

**PCR amplification.** *Influenza.* The RNA was extracted from nasal and throat swabs using QIAamp viral RNA mini...
kits (QIAGEN, Hilden, Germany) following manufacturer’s instruction and stored at −80°C. Influenza virus genome was detected using a standardized real-time reverse transcription (rRT)-PCR assay developed by the U.S. CDC to detect influenza A and B viruses as well as influenza A viruses of H1, H3, and H5 subtypes. Real time assays were developed at the CDC and assays and primer sequences are available upon request under a Material Transfer Agreement. One-step rRT-PCR was performed in a final volume of 25 μL containing 5 μL of extracted RNA, 12.5 μL of buffer mix, and 0.5 μL Superscript III/Platinum Taq-Enzyme mix, 20 U of RNase-out (Invitrogen, Carlsbad, CA), 0.8 μM of probe. The Rotor-Gene 6000 real time thermocycler (Corbett Life Science, Sydney, Australia) was used for all pH1N1 PCR reactions. The thermocycling parameters for all targets were performed in 25 μL containing 2.5 μL of ABI PCR buffer, 1.5 μL of 25 mM MgCl2, 2.0 μL of 10 mM dNTP (final concentration 200 μM), 1.0 μL of 10 μM forward primer, and 1.0 μL of reverse primer, 0.25 μL of 5 units of AmpliTaq DNA Polymerase, and 5 μL of diluted (1:50) RT-PCR product. The cycle conditions of the nPCR reaction were as follows; 94°C for 2 minutes, and 40 cycles with 94°C for 1 minute, 60°C for 1 minute, 68°C for 1 minute, and then 68°C for 7 minutes. All thermal cycling was performed with Applied Biosystems type 9700 machine. Inner primer pairs are then used in separate nPCR reactions using GeneAmp PCR Core reagent (Applied Biosystems). The nPCR reactions were performed in 25 μL containing 2.5 μL of ABI PCR buffer, 1.5 μL of 25 mM MgCl2, 2.0 μL of 10 mM dNTP (final concentration 200 μM), 1.0 μL of 10 μM forward primer, and 1.0 μL of reverse primer, 0.25 μL of 5 units of AmpliTaq DNA Polymerase, and 5 μL of diluted (1:50) RT-PCR product. The cycle conditions of the nPCR reaction were as follows; 94°C for 2 minutes, and 40 cycles with 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, and then 72°C for 10 minutes. Outer and inner primer pairs are designed to amplify three structural gene regions, Capsid (C), Envelope-2 (E2), and an area overlapping the 6K and E1 genes (6K/E1).14 The PCR products were visualized on 2% agarose gels stained with ethidium bromide under UV light. A positive result was indicated upon observation of a 200 base pair PCR product, representing the capsid, and at least one of the envelope targets is present.

Quantitative real-time PCR (qPCR) for rickettsiae and Orientia tsutsugamushi (scrub typhus). Deoxyribonucleic acid (DNA) was extracted from serum using QIAamp DNA mini kits (QIAGEN) following manufacturer’s instruction and stored at −80°C. The qPCR assays developed to detect Rickettsia spp. (Rick17), tick-borne rickettsiae (Trick), Rickettsia typhi (Rtyph), Rickettsia felis (Rfelis), and O. tsutsugamushi (Otsu47) were performed as previously described.19,20 Briefly, primer pairs were used in separate qPCR assays in a 25 μL final volume: Rick17, Trick, and Rtyph assays contained 2 μL of DNA, 12.5 μL of 2× TaqMan Universal PCR Master Mix (Cat. no. ABI 4304437), 0.4 μM for each primer, and 0.3 μM of probe. Orientia tsutsugamushi qPCR assay contained 5 μL polymerase (Promega), and 5 μL of RNA template. The PCR thermal cycling was performed as follows: 48°C for 45 minutes, 94°C for 2 minutes, and 40 cycles with 94°C for 1 minute, 60°C for 1 minute, 68°C for 1 minute, and then 68°C for 7 minutes. All thermal cycling was performed with Applied Biosystems type 9700 machine. Inner primer pairs are then used in separate nPCR reactions using GeneAmp PCR Core reagent (Applied Biosystems). The nPCR reactions were performed in 25 μL containing 2.5 μL of ABI PCR buffer, 1.5 μL of 25 mM MgCl2, 2.0 μL of 10 mM dNTP (final concentration 200 μM), 1.0 μL of 10 μM forward primer, and 1.0 μL of reverse primer, 0.25 μL of 5 units of AmpliTaq DNA Polymerase, and 5 μL of diluted (1:50) RT-PCR product. The cycle conditions of the nPCR reaction were as follows; 94°C for 2 minutes, and 40 cycles with 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, and then 72°C for 10 minutes. Outer and inner primer pairs are designed to amplify three structural gene regions, Capsid (C), Envelope-2 (E2), and an area overlapping the 6K and E1 genes (6K/E1).14 The PCR products were visualized on 2% agarose gels stained with ethidium bromide under UV light. A positive result was indicated upon observation of a 200 base pair PCR product, representing the capsid, and at least one of the envelope targets is present.

Nested conventional PCR for dengue. Dengue virus genome and serotype detection was based on previously published procedures with modifications to the Dengue-1 primer.17,18

Nested reverse real-time PCR for chikungunya virus. The RNA was extracted from serum using QIAamp viral RNA mini kits (QIAGEN) following manufacturer’s instruction and stored at −80°C. Chikungunya virus genome was detected using a nested RT-PCR assay, which comprises two assay steps; RT-PCR and nested PCR (nPCR). The RT-PCR reactions were performed in 25 μL containing 5 μL of extracted RNA, 12.5 μL of buffer mix, and 0.5 μL Superscript III/Platinum Taq-Enzyme mix, 20 U of RNase-out (Invitrogen, Carlsbad, CA), 0.8 μM for each primer, and 0.2 μM of probe. The Rotor-Gene 6000 real time thermocycler (Applied Biosystems, Foster City, CA) was used for all PCR reactions. The thermocycling parameters for all targets consisted of 50°C for 30 minutes, 95°C for 2 minutes, and 45 cycles with 95°C for 15 seconds, 55°C for 30 seconds (U.S. CDC). In August 2009, a real-time PCR assay distributed by the U.S. CDC was introduced for the detection of pandemic H1N1 influenza virus. The Applied Biosystems 7500 real time thermocycler (Applied Biosystems, Foster City, CA) was used for all pH1N1 PCR reactions.

FIGURE 2. Catchment area (gray) of febrile surveillance study.
of DNA, 12.5 μL of 2× TaqMan Universal PCR Master mix (Cat. no. ABI 4304437), 0.4 μM for each primer and 0.3 μM of probe; and the R. felis PCR assay contained 2 μL of DNA, 12.5 μL of 2× TaqMan Universal PCR Master mix (Cat. no. ABI 4304437), 0.4 μM for each primer and 0.4 μM of probe. The Applied Biosystems 7500 real time thermocycler (Applied Biosystems) was used for all real-time PCR assays. The thermocycling program used consisted of the following parameters: 50°C for 2 minutes, 95°C for 10 minutes, and 60 cycles with 95°C for 15 seconds, 60°C for 30 seconds.

rRT-PCR for hantaviruses. Hantavirus RNA was extracted from serum using QIAamp viral RNA mini kits (QIAGEN) following manufacturer’s instruction and stored at −80°C. An rRT-PCR assay was used to detect Hantavirus serotypes Hantaan (HTN), Puumala (PUU), Seoul (SEO), and Dobrava-Belgrade (DOB). One-step rRT-PCR was performed in a final volume of 25 μL containing 5 μL of extracted RNA, 12.5 μL of buffer mix, 0.5 μL of MgSO₄, and 0.625 μL Superscript III/Platinum Taq-Enzyme mix, 0.5 μL of RNAase-out (Invitrogen), 0.4 μM for each primer, and 0.25 μM of probe. The Rotor-Gene 6000 real time thermocycler (Corbett Life Science, Sydney, Australia) was used for all PCR reactions. The thermocycling parameters for all targets consisted of 50°C for 15 minutes, 95°C for 2 minutes, and 45 cycles with 95°C for 15 seconds, 55°C for 2 minutes, and 72°C for 1 minute.

Nested RT-PCR for detection of hepatitis E viruses. The RNA was extracted from serum using QIAamp viral RNA mini kits (QIAGEN) following manufacturer’s instruction and stored at −80°C. The nonstructural (NS) gene of Hepatitis E virus was detected using a nested reverse real-time PCR assay. The RT reactions were performed in 25 μL containing 5 μL of Qiaqen buffer mix (Qiagen, Cat. no. 210212) and 1 μL of enzyme mix, 1 μL of 10 mM dNTP, 0.63 μM of each primer, 0.15 μL of 40 U RNase inhibitor (Promega, Cat. no. N2511), and 5 μL of RNA template. The PCR thermal cycling was performed as follows: 45°C for 30 minutes, 94°C for 15 minutes, and 40 cycles with 94°C for 1 minute, 45°C for 1 minute, 72°C for 1 minute, and then 72°C for 7 minutes. The PCR reactions were performed using the Applied Biosystems 9700 (Applied Biosystems). An nPCR reaction was carried out at a total volume of 25 μL. The reaction contained 2.5 μL of ABI PCR buffer (Applied Biosystems, Cat. no. N808-0009), 1.5 mM MgCl₂, 2 μL of 10 mM dNTP, 0.75 μM forward primer and reverse primer, 0.125 μL of 5 U of AmpliTaq DNA Polymerase, and 5 μL of diluted (1:50) RT-PCR product. The cycle conditions of nPCR reaction are as follows: 94°C for 2 minutes, and 40 cycles with 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute, and then 72°C for 7 minutes. Products were visualized on 2% agarose gels stained with ethidium bromide under UV light.

Statistical analysis. All data was double-data entered into MS Access (Microsoft Inc., Redmond, WA). Data was imported into SAS v 9.1 (SAS, Cary, NC), which was used for all statistical analyses.

RESULTS

From December 2006 through December 2009, 9,997 patients were enrolled (Figure 3). All patients enrolled met the criteria of fever (> 38.0°C). Among the 9,997 patients, 89.3% had a complete questionnaire. A serum sample, nose and/or throat swab, and blood culture sample was available from 99.8%, 99.7%, and 98.2%, respectively.

The median age of enrolled patients was 13 years of age (interquartile range [IQR] 6–28) years of age (Table 1) and 53.9% were male. Patients reported both recent travel to other provinces or countries (7.9%) and travel into forested areas (7.1%). Study subject occupation was recorded, however students and children accounted for 63.5% of enrolled study subjects. Among study subjects reporting a specific occupation, grain farmers were most common (31.3%). Contact with cattle (8.3%), chickens (7.7%), and dogs (7.0%) were the most common reported animal exposures. Prior medication usage was reported by 36.6% of enrolled subjects. The most common symptoms reported by enrolled patients included headache (69.5%), cough (57.5%), sore throat (46.6%), chills (48.1%), and malaise (46.8%) (Table 1). Among enrolled participants, 65.7% presented with signs and symptoms characteristic of influenza-like illness, 17.1% with gastroenteritis, and 1.4% with jaundice. Close contact with an individual who had similar symptoms was reported among 9.0% of participants. Of the enrolled patients, 10.1% were hospitalized or referred for hospitalization.

Laboratory testing was performed for agents believed to be endemic to the region in addition to a variety of emerging pathogens (Table 2). Of enrolled patients, 19.9% (N = 1,983) were PCR positive for influenza. Dengue virus was considered the probable cause of illness (as defined by only a positive IgM result) in 842 enrollees (8.4%) and confirmed (as defined by a positive PCR result) in 883 cases (8.9%). All four serotypes of dengue were detected during the study period. Malaria was identified among 7.2% (N = 716) of patients, with Plasmodium falciparum identified in 30.2% of malaria cases. In addition, a total of 19 mixed infections of Plasmodium vivax and P. falciparum were identified. Acute leptospirosis was serologically identified in 20.8% of study subjects. Hepatitis A IgM testing was performed on the first 3,243 patients enrolled, 56 (1.7%) of the patients were positive. Hepatitis B surface antigen testing was done on the first 4,596 enrollees, and 261 (5.8%) were positive. Follow-up on testing of HBsAG-positive samples identified eight samples that were also positive for IgM. A hepatitis E IgM response was detected among 11.1% of participants. The PCR testing of 517 serum samples that were positive or equivocal for Hepatitis E IgM identified five samples positive for hepatitis E nucleic acid.

Serological testing of paired samples was conducted for antibodies against chikungunya, hantavirus, and rickettsiae (scrub typhus group, typhus group, and spotted fever group) (Table 3). Testing of chikungunya IgG among the first 753 patients enrolled in 2007 identified a previous exposure in 16.3%. Chikungunya IgM was identified among 0.1% (2 of 1,438) of enrolled patients and PCR of patients presenting with fever and joint pain identified three cases out of 571 samples tested.

Hantavirus IgG was detected in convalescent specimens of 8.1% of patients tested. Paired testing of acute and convalescent samples identified 71 patients that had evidence of an acute hantavirus infection. No IgM-positive or PCR-positive cases of hantaviral infection were identified by IgM or molecular analysis.

Testing for scrub typhus group, typhus group, and spotted fever group rickettsiae-specific IgG in convalescent specimens identified seroprevalences of 7.0%, 13.4%, and 11.6%, respectively (Table 3). From positive convalescent samples, testing of paired samples to identify acute cases identified 35, 56, and 21 cases of scrub typhus, typhus, and spotted fever, respectively.
Testing acute serum samples (seroconversion-positive samples) by qPCR assays confirmed two cases of scrub typhus and one case of murine typhus. No species of the spotted fever group were identified by qPCR.

Blood culture testing yielded a bacterial isolate in 6.3% (642 of 9,821) of study subjects (Table 4). The most common isolates were non-pathogenic skin flora (e.g., coagulase-negative Staphylococcus, micrococcus sp., bacillus sp. (non-anthracis); however, a number of known pathogens were also identified including Salmonella typhi (57), Staphylococcus aureus (4), Burkholderia pseudomallei (2), group C Streptococcus (1), and Moraxella catarrhalis (6) (Table 4).

To identify active co-infections, we limited analysis to patients who were positive by a combination of PCR, blood culture, and/or malaria microscopy. There were 94 (1.0%) patients whose specimens were positive by two or more of these tests (Table 5). Influenza was identified with confirmed dengue in 55 cases, with malaria in 8 cases, and an isolate from blood culture in 10 cases. Dengue was identified with malaria in 15 cases and a blood culture isolate in 4 cases (1 B. pseudomallei/dengue-2, 2 S. typhi/dengue-4, and 1 S. typhi/dengue-2). In one patient, influenza was identified with Salmonella cholerasuis and dengue-4.

**DISCUSSION**

The purpose of this study was to identify the infectious etiologies of AFI among patients 2 years of age and older.

### Table 1
Demographics of enrolled patients from Dec. 2006 to Dec. 2009

| Demographic and clinical information | n | % | Temperature, mean (SD) | n | % |
|-------------------------------------|---|---|------------------------|---|---|
| Patients enrolled                    | 9,997 | Temperature, mean (SD) | 39 (0.6) | |
| Age (years), mean (SD)              | 19.6 (16.9) | Malaise | 4,675 | 46.8 |
| Age (years), median (SD)            | 13.0 (16.9) | Chills | 4,807 | 48.1 |
| Gender                               |  | Muscle aches | 2,319 | 23.2 |
| Male                                 | 5,392 | Rash | 197 | 2.0 |
| Female                               | 4,601 | Lesion | 96 | 1.0 |
| Medication                           | 3,627 | Joint pain | 1,894 | 19.0 |
| Travel history                       |  | Headache | 6,947 | 69.5 |
| Province/country                     | 793 | Seizure | 83 | 0.8 |
| Jungle                               | 708 | Sore throat | 4,655 | 46.6 |
| Occupation                           |  | Cough | 5,748 | 57.5 |
| Cattle/sheep farmer                  | 9 | Shortness of breath | 964 | 9.7 |
| Grain farmer                         | 3,133 | Nausea | 876 | 8.8 |
| Poultry farmer                       | 18 | Vomiting | 1,332 | 13.4 |
| Driver                               | 16 | Abdominal cramp | 2,412 | 24.2 |
| Factory worker                       | 387 | Diarrhea | 580 | 5.8 |
| Fisherman                            | 17 | Bloody stool | 66 | 0.7 |
| Office worker                        | 50 | Bloody urine | 20 | 0.2 |
| Other                                | 6,347 | Bleeding | 25 | 0.3 |
| Exposure history                     |  | Jaundice | 132 | 1.4 |
| Cats                                 | 278 | Person same symptom | 893 | 9.0 |
| Dogs                                 | 700 | Patient disposition |  | |
| Cattle                               | 824 | Admitted at hospital | 990 | 9.9 |
| Horses                               | 5 | Outpatient follow-up | 8,975 | 89.9 |
| Goat/sheep                           | 5 | Referred to hospital | 15 | 0.2 |
| Pigs                                 | 81 |  |  | |
| Monkeys                              | 7 |  |  | |
| Rodents                              | 8 |  |  | |
| Chickens                             | 765 |  |  | |
| Ducks                                | 186 |  |  | |
TABLE 2
Distribution of pathogens identified among enrolled patients from Dec. 2006 to Dec. 2009

| Pathogens Tested cases | Positive (N) | Positive (%) |
|------------------------|--------------|--------------|
| Influenza viruses      | 9,968        | 1,983        | 19.9         |
| H1N1                   | 224          | 11.3         |
| H3N2                   | 827          | 41.7         |
| H5N1                   | 1            | < 0.1        |
| Pandemic H1N1          | 286          | 14.4         |
| Influenza B            | 627          | 31.6         |
| Influenza A, untyped   | 18           | 0.9          |
| H1N1 and influenza B   | 1            | < 0.1        |
| H3N2 and influenza B   | 3            | 0.2          |
| Pandemic H1N1 and H3N2 | 4            | 0.2          |
| Malaria                | 9,954        | 716          | 7.2          |
| P. falciparum          | 216          | 30.2         |
| P. vivax               | 481          | 67.2         |
| P. falciparum and P. vivax | 19        | 2.6          |
| Dengue                 | 9,975        | 842          | 8.6          |
| IgM positive only (probable dengue) | 883     |              |
| PCR positive (confirmed dengue) | 83        | 9.4          |
| Serotype-1-1           | 398          | 45.1         |
| Serotype-2             | 108          | 12.2         |
| Serotype-3             | 294          | 33.3         |
| Blood-borne pathogen   | 9,821        | 629          | 6.3          |
| Leptospirosis IgM      | 9,975        | 2,076        | 20.8         |
| Hepatitis A IgM        | 3,243        | 58           | 1.7          |
| Hepatitis B sAG        | 4,596        | 267          | 5.8          |
| Hepatitis B IgM        | 267          | 8            | 3.0          |
| Hepatitis E IgM        | 9,353        | 1038         | 11.1         |
| Hepatitis E PCR        | 517          | 5            | 1.0          |

TABLE 3
Pathogens identified from testing of acute and convalescent serum samples

| Pathogens Tested cases | Positive (N) | Positive (%) |
|------------------------|--------------|--------------|
| Chikungunya IgG (convalescent) | 753        | 123          | 16.3         |
| Chikungunya IgM        | 1438         | 2            | 0.1          |
| Chikungunya PCR        | 571          | 3            | 0.5          |
| Hantavirus IgG (convalescent) | 5675      | 459          | 8.1          |
| Hantavirus 4× IgG rise and/or seroconverter | 459 | 71 | 15.5 |
| Hantavirus PCR         | 79           | 0            | 0.0          |
| Scrub typhus group IgG (convalescent) | 1906 | 133         | 7.0          |
| Scrub typhus group-specific IgG 4-fold rise in titer or seroconversion | 133 | 35 | 26.3 |
| Orientia tsutsugamushi-specific qPCR | 67 | 2 | 3.0 |
| Typhus group IgG (convalescent) | 1946 | 261 | 13.4 |
| Typhus group-specific IgG 4-fold rise in titer or seroconversion | 261 | 56 | 21.5 |
| R. typhi-specific qPCR | 83 | 1 | 1.2 |
| Spotted fever group IgG (convalescent) | 1263 | 146 | 11.6 |
| Spotted fever group-specific IgG 4-fold rise in titer or seroconversion | 146 | 21 | 14.4 |
| Tick-borne rickettsiae-specific qPCR | 37 | 0 | 0.0 |

FEBRILE SURVEILLANCE IN CAMBODIA

presenting for treatment at clinics in south-central Cambodia. This is the first comprehensive study to identify etiologies of AFI in the general population of Cambodia.

Our study enrolled nearly 10,000 patients over a 3-year period. During this time period, there was a seasonality to enrollment that corresponded to the rainy season, which roughly corresponds with the influenza season in Cambodia peaking in August–October of each year.

In this study, we identified influenza, Leptospira, dengue, hepatitis E, and malaria as the most frequently identified pathogens associated with AFI among Cambodians. Previous regional studies had identified similar etiologies among patients with AFI with some variation of etiologies and distribution that could be based on the sampled population or the pathogen testing schemes. However, we identified that many of the acute serological responses to Leptospira and hepatitis E were associated with co-detection of other organisms (data not shown).

A large number of study subjects were classified chronic hepatitis B cases; additional testing revealed that only a small number of those individuals presented with an AFI caused by hepatitis B. A recent study of hepatitis B among blood donors found a similar percentage of HBsAG-positive volunteers in Cambodia.

Despite a lack of jaundice reported among the study population, a high percentage of patients showed an acute serological response to hepatitis E. Of the 175 patients presenting with clinical jaundice, only 29 (16.6%) were identified to have a positive hepatitis E IgM response. This represents a diagnostic challenge that may be caused by a long-term IgM response or continuous exposure in the environment to hepatitis E virus.

A large number of patients were positive for an acute serological response to Leptospira. A recent study focusing on hospitalized patients in Cambodia found a similar prevalence of acute or prior Leptospira infection, with approximately half of the patients presenting with an acute infection. Although leptospirosis has been documented to be endemic in the region, the more definitive microscopic agglutination testing (MAT) was not performed on these samples. In addition, a recent study showed poor diagnostic accuracy of the Leptospira IgM PanBio ELISA compared with MAT testing. Further laboratory studies and analysis is needed to understand the contribution of Leptospira to AFI in Cambodia.

Almost 1% of enrollees tested had results suggesting an acute hantavirus infection. However, PCR testing failed to identify Hantaan, Seoul, Puumala, or Dobrava strains among any study subjects. This may reflect genetic differences of Hantavirus strains in Cambodia that need to be further defined. Alternatively, the serology results may be from a cross-reaction from a similar yet previously unidentified pathogen. Previous studies conducted in Cambodia have identified the presence of Hantaan, Seoul, Puumala, or Dobrava strains among any study subjects. This may reflect genetic differences of Hantavirus strains in Cambodia that need to be further defined.
identified a number of unique co-infections such as Dengue and B. pseudomallei and S. typhi. The co-infections with malaria may represent asymptomatic infections among our study population. Currently, our group has several malaria-specific studies underway, accessing prevalence, incidence, and drug resistance. Our study identified over 50 co-infected cases and concurrent outbreaks. Further studies are required to better understand the clinical outcome of patients who have tested positive for multiple pathogens.

The use of laboratory-based syndromic surveillance can alert regional officials and describe common infectious etiologies circulating in the region during a specific time period. Furthermore, laboratory-based syndromic surveillance can be used to help define symptoms and information that can be used for electronic-based surveillance programs and help to guide outbreak response and laboratory testing.

Testing for a number of pathogens in all samples as opposed to using a staged screening strategy allowed us to identify co-infections that may have otherwise been missed. The diversity of etiological agents, an inability to identify an etiology in the majority of enrolled patients, and the prevalence of multiple infections, demonstrate the complexity of diagnosis and treatment of medical care providers in this region of Cambodia.

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Authors’ addresses: Matthew R. Kasper, Department of Bacteriology, U.S. Naval Medical Research Unit 6, Lima, Peru, E-mail: matthew.kasper@med.navy.mil. Patrick Blair, Naval Health Research Center, San Diego, CA, E-mail: Patrick.blair@med.navy.mil. Sok Touch, Communicable Disease Control Department, Phnom Penh, Kingdom of Cambodia, E-mail: touch358@moh.gov.kh. Buth Sokhal, National Institute of Public Health, Phnom Penh, Kingdom of Cambodia, E-mail: buthsokhal@yahoo.com. Chadwick Yasuda, U.S. Naval Medical Research Unit Two, Phnom Penh, Kingdom of Cambodia.

Table 5

| Co-infection (n) | n | Notes |
|------------------|---|-------|
| Influenza/dengue | 55 | 55 are PCR positive for dengue |
| Influenza/malaria | 8 | |
| Influenza/blood culture | 10 | |
| Dengue/malaria | 15 | 15 are PCR positive for dengue |
| Dengue/blood culture | 1 | B. pseudomallei and dengue-2 |
| Dengue/blood culture | 2 | S. typhi and dengue-4 |
| Dengue/blood culture | 1 | S. typhi and dengue-2 |
| Malaria/blood culture | 1 | |
| Influenza/dengue/blood culture | 1 | S. cholerasuis and dengue-4 |

Table 4

Organisms isolated from 9821 patient blood cultures

| Organism | TOTAL | Organism | TOTAL |
|----------|-------|----------|-------|
| Pathogens: Achromobacter xylosoxidans | 1 | Potential pathogens: Acinetobacter baumannii/calcoaceticus | 3 |
| Actinomyces radiigae | 3 | | |
| Beta hemolytic streptococci group C | 1 | Bacillus sp. | 2 |
| Brevundimonas vesicularis | 7 | Chrysobacterium indologenes | 1 |
| Burkholderia cepacia | 1 | Mannheimia haemolytica/P. trehalosi | 3 |
| Burkholderia pseudomallei | 2 | Ochrobactrum anthropi | 1 |
| Edwardsiella tarda | 1 | Pseudomonas fluorescens | 1 |
| Escherichia coli | 12 | Pseudomonas oxytoca | 1 |
| Group D Salmonella sp. non-typhi | 1 | Pseudomonas paucimobilis | 6 |
| Gemella morbillorum | 2 | Pseudomonas stutzeri | 1 |
| Moraxella catarrhalis | 6 | | |
| Moraxella sp. non-catarrhalis | 3 | Likely contaminants: | |
| Neisseria sp. non-meningitidis | 2 | Aerococcus viridans | 1 |
| Pasteurella sp. | 1 | Arthrobacter sp. | 37 |
| Pseudomonas aeruginosa | 1 | Brevibacterium sp. | 1 |
| Ralstonia picketti | 1 | Cellulomonas sp. | 1 |
| Rhodococcus sp. | 15 | Cellulosimicrobium cellulans | 1 |
| Salmonella choleraesuis | 10 | Coagulase negative Staphylococcus | 203 |
| Salmonella Group D sp. non-typhi | 1 | Corynebacterium sp. | 16 |
| Salmonella ser. Paratyphi A | 6 | Leifsonia aquatica | 1 |
| Salmonella typhi | 57 | Microbacterium sp. | 35 |
| Sphingomonas paucimobilis | 6 | Micrococcus sp. | 20 |
| Staphylococcus aureus | 4 | | |
| Stenotrophomonas maltophilia | 2 | | |
| Streptococcus constellatus | 1 | | |
| Streptococcus mitis | 1 | | |
| Streptococcus pneumonia | 1 | | |
| Aspergillus sp. | 2 | | |
| Candida sp. other than albicans | 14 | | |
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