VENEZUELAN EQUINE ENCEPHALITIS FEBRILE CASES AMONG HUMANS IN THE PERUVIAN AMAZON RIVER REGION

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Abstract. A survey was conducted from October 1, 1993 to June 30, 1995 to determine the arboviral etiologies of febrile illnesses in the city of Iquitos in the Amazon River Basin of Peru. The study subjects were patients who were enrolled at medical care clinics in or their homes by Peruvian Ministry of Health (MOH) workers as part of the passive and active disease surveillance program of the MOH. The clinical criterion for enrollment was the diagnosis of a suspected viral-associated, acute, undifferentiated febrile illness of ≤ 5 days duration. A total of 598 patients were enrolled in the study. Demographic information, medical history, clinical data, and blood samples were obtained from each patient. The more common clinical features were fever, headache, myalgia, arthralgia, retro-ocular pain, and chills. Sera were tested for virus by the newborn mouse and cell culture assays. Viral isolates were identified initially by immunofluorescence using polyclonal antibody. An ELISA using viral-specific monoclonal antibodies and nucleotide sequence analysis were used to determine the specific variety of the viruses. In addition, thin and thick blood smears were observed for malaria parasites. Venezuelan equine encephalitis (VEE) virus subtype I, variety ID virus was isolated from 10 cases, including three cases in October, November, and December 1993, five cases in January and February 1994, and two cases in June 1995. The ELISA for IgM and IgG antibody indicated that VEE virus was the cause of an additional four confirmed and four presumptive cases, including five from January through March 1994 and three in August 1994. Sixteen cases were positive for malaria. The 18 cases of VEE occurred among military recruits (n = 7), agriculture workers (n = 3), students (n = 3), and general laborers (n = 5). These data indicated that an enzootic strain of VEE virus was the cause of at least 3% (18 of 598) of the cases of febrile illnesses studied in the city of Iquitos in the Amazon Basin region of Peru.

Several arboviruses, including dengue (DEN), yellow fever (YF), Oropouche (ORO), and Venezuelan equine encephalitis (VEE), have been reported as the cause of febrile illnesses in Peru.1-4 Outbreaks of dengue and ORO virus fever were recorded in 1991 and 1992 in the city of Iquitos in the Amazon Basin region.1-5 An estimated 150,000 persons were affected during the outbreak of dengue. While estimates of ORO virus fever cases were not determined, this disease has been recognized as a cause of major epidemics in Brazil during the past three decades, and more recently, as the cause of an outbreak in Panama6 and in Peru (Watts DM, 1994, unpublished data). Epizootics of VEE virus subtype I, variety AB that occurred before and during 1973 along the Pacific coastal plains of Peru affected thousands of horses and humans.4

Venezuelan equine encephalitis virus subtype I, variety ID, Eastern equine encephalitis (EEE) virus, St. Louis encephalitis (SLE) virus, group C, and Guama group arboviruses were isolated from mosquitoes and/or sentinel hamsters during the early to mid 1970s in Yurimaguas and/or Iquitos.6-7 Serologic evidence of VEE virus infection was detected among humans and horses in Iquitos, Pucallpa, and Yurimaguas, but symptomatic infection was not reported for this or any of the other viruses during these studies.6 In 1994, VEE ID virus was associated with cases of febrile illness at a remote military outpost near Pantoja, Peru, approximately 380 km northwest of Iquitos.8 The objective of this study was to further investigate the possible role of arboviruses as the cause of febrile illnesses in 1993, 1994, and 1995 among residents in Iquitos. Observations included in this report are limited to VEE virus; results for Oropouche and dengue fever will be presented in a separate report on completion of the study.

SUBJECTS, MATERIALS, AND METHODS

Description of the study site. The study site was located in Iquitos, a city with approximately 300,000 people in the Amazon River Basin of the Department of Loreto, Peru. Iquitos is located 120 meters above sea level (73.2°W, 3.7°S) on the bank of the Amazon River. The major occupations are housekeeping, teaching, military work, agriculture, fishing, business merchants, and tourism. The climate in the region is tropical with an average temperature of 27.5°C and an average mean annual precipitation of 2.7 meters.

Study subjects. The study subjects were patients who presented with a clinical diagnosis of an acute, undifferentiated, febrile illness either in their homes or at civilian or military outpatient clinics in Iquitos, from October 1, 1993 to June 30, 1995. An active surveillance program was used in collaboration with Peruvian Ministry of Health workers to locate patients in their homes. The criteria for enrollment was a fever of 38°C or higher of not more than five days duration, accompanied by at least a headache, myalgia, and other non-specific signs and symptoms at the time of enrollment or within the past 10 days. A standardized questionnaire was used to obtain demographic data, medical history, and clinical features for each patient. A blood sample was also obtained from each patient during the acute phase of illness, and when possible, convalescence samples were obtained approximately 2–4 weeks later for viral isolation and/or serologic studies.

The study was explained in Spanish to the patients and written informed consent was obtained from all adults and from parents or legal guardians of minors who volunteered...
to participate in the study. The study protocol was reviewed and approved by the Naval Medical Research Institute's Committee for the Protection of Human Subjects and the Peruvian Navy Surgeon General's Ethical Committee.

**Viruses isolation assays.** Sera from febrile cases were diluted 1:5 in Earle’s based minimal essential medium supplemented with 5% fetal calf serum, 200 μg/ml of streptomycin, and 200 units/ml of penicillin. An aliquot of each diluted serum was tested for virus by intracerebral inoculation of newborn mouse, and by the inoculation of Vero and C6/36 cell culture as described previously.5,10

Aliquots of the original sera that yielded suspected viral isolates were reinoculated intracerebrally into 1–3-day-old outbred mice in an attempt to reisolate the viruses.

The experiments reported herein were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council, DHHS, Publication No. (NIH) 86-23 (1985). The maintenance and care of newborn mice was in compliance with the U.S. Naval Medical Research Institute and Institutional policy guidelines for the humane use of laboratory animals.

**Identification and subtyping of viral isolates.** The reference viruses used for preliminary identification of viral isolates by the standard indirect immunofluorescence (IFA) technique included VEE virus subtype IB (6921), Mayaro (MAY) (TR467), DEN-3 (H87), and ORO (TR9760) (American Type Culture Collection, Rockville, MD). Reference mouse hyperimmune ascitic fluids (MAF), alphavirus and Simbu virus groups reagents, ORO virus, and normal MAF used to perform the IFA were obtained from the National Institute of Allergy and Infectious Diseases (Bethesda, MD). A flavivirus group-specific monoclonal antibody (MAb-4G-2) was also used.11 The MAF and MAB were used in the IFA at a 1:50 and/or 1:100 dilution. Viral antigens for the isolates and reference viruses were prepared from infected LLE-MK2 and Vero cell cultures according to standard procedures. Aliquots of 20 μl of phosphate-buffered saline–washed cells from the infected cultures were spotted onto the ringed areas of standard microscope slides. Cell suspensions were dried at room temperature, submerged in chilled acetone for 15 min, and then stored at −70°C until tested for viral antigen by the IFA according to standard procedures.

**Enzyme-linked immunosorbent assay.** The reference VEE viruses and the panel of MAbs used to identify and subtype the viral isolates by an ELISA included alphavirus genus MAF, all varieties of VEE virus subtype I, a subtype II virus, EEE virus, WEE virus, and Flavivirus genus MAB.12–18 Viral antigens were prepared from infected Vero cells and purified by polyethylene glycol precipitation and ultracentrifugation, and used in an ELISA for epitope mapping of one of the viral isolates as described previously.15

**Nucleotide sequencing.** Nucleotide sequencing and sequence analyses were performed as described previously.9 Briefly, RNA was extracted from viral isolates (first Vero cell passage) using Trizol-LS according to the manufacturer’s (Life Technologies, Inc., Gaithersburg, MD) recommended procedure. A 554-nucleotide sequence at the 3’ end of the VEE virus nonstructural protein 4 (ns4) gene was amplified by the reverse transcription–polymerase chain reaction (RT-PCR) technique using VEE virus-specific primers. The 518 nucleotides between the PCR primers were sequenced directly using the PRISM dye deoxy terminator kit and a model 373A automated sequencer (Applied Biosystems Division, Perkin Elmer Corp., Foster City, CA). Since only a 177 nucleotide fragment at the 3’ end of the ns4 gene had been sequenced for many strains of VEE virus,19 this sequence was used for subsequent analyses. Nucleotide sequences were aligned with those of other VEE virus strains, including IAB-TrD, IC-P676, IC-SH3, IC-SH5, ID-3880, ID-83U434, ID-V-209A, ID-75D143, ID-DE15191, ID-DE15193, II-Fe3-7c, IE-68U201, and IIC-71D125220–24 using Pileup (Genetics Computer Group, Inc., Madison, WI), and phylogenetic analysis was performed by the maximum parsimony algorithm implemented by phylogenetic analysis using parsimony.25

**Serologic assays.** Confluent monolayers of LLE-MK2 or Vero cells were infected with DEN-1, Hawaii 1944; ORO, Peru 1992; VEE subtype IB (6921); MAY (TR467); and EEE viruses to produce antigens to test sera for antibody as described previously.26,27 An IgM capture ELISA using goat anti-human IgM antibody (Tago, Camarillo, CA) bound to 96-well microtiter plates, and tissue culture supernatant antigens were used to test for IgM antibody.28 A direct ELISA using viral infected or uninfected cell lysates bound to 96-well microtiter plates was used to test sera for IgG antibody.29 The antibody titters were expressed as the reciprocal of the highest dilution yielding a positive value.

**Etiology diagnostic criteria.** The viral etiology of cases of acute febrile illness was based on the isolation of virus and/or a four-fold or greater increase in IgM and/or IgG antibody titer between the acute phase of illness sera and convalescence samples. A presumptive diagnosis was the detection of IgM antibody at a 1:200 dilution or greater in sera obtained from cases only during the acute phase of illness.

**Malaria.** All patients were tested for malaria by microscopic examination of thin and thick blood smears.

**RESULTS**

A total of 598 cases of febrile illness ranging in age from one to more than 79 years (mean age = 28 years) were enrolled in Iquitos from October 1, 1993 through June 30, 1995. The number of cases studied ranged from seven to 54 per month (mean = 30 cases). The 225 female cases ranged in age from one to 79 years (mean = 29 years), and the 373 male cases ranged in age from one to 76 years (mean = 26 years). The more common clinical manifestations were fever, headache, myalgia, arthralgia, chills, ocular pain, nausea and vomiting.

Virus was isolated from 10 patients ranging in age from six to 43 years, and isolates were obtained more frequently from males (n = 8) than females (n = 2). Clinical features of the cases were fever, chills, headache, myalgia, arthralgia, lower back pain, ocular pain, nausea/vomiting, and diarrhea.

Viral isolates were obtained from one case each in October, November, and December 1993, from five cases in January and February 1994, and from two cases in June 1995. The cases included military personnel (n = 5), students (n = 1), agriculture workers (n = 1), and general laborers (n = 3). The cases were distributed throughout the Iquitos area with
corresponding to a 554-nucleotide region at the 3’ end of the ns4 gene was amplified by RT-PCR, and the region between the PCR primers was sequenced. Alignment of the sequences indicated that eight of the 10 isolates were identical to one another and that the other two isolates differed by one and two nucleotides, respectively. A 177-nucleotide sequence of this ns4 sequence, corresponding to nucleotides 7327 to 7503 of the IAB-TrD isolate, was also aligned with the homologous sequences of other VEE virus subtypes. Phylogenetic analysis of the aligned sequences by the maximum parsimony method indicated that the isolates were most closely related to VEE subtype ID virus isolated in 1961 in Panama (Figure 1).

In addition to the VEE cases diagnosed by virus isolation, there were four serologically confirmed and four probable cases based on the detection of VEE virus-specific IgM antibody. Five of these cases occurred from January through March 1994, and three in August 1994 (Table 3). The clinical manifestations of the serologically diagnosed cases were similar to those described above for the cases diagnosed by virus isolations. An additional 16 cases were positive for malaria parasites by microscopic examination of blood smears. Overall, the 18 VEE cases occurred among military recruits (n = 7), agriculture workers (n = 3), students (n = 3), and general laborers (n = 5).

**DISCUSSION**

This study indicated that the enzootic subtype/variety VEE ID virus was the cause of 18 of 598 human cases of febrile illness in 1993, 1994, and 1995 in the urban community of Iquitos, Peru. These data suggested that VEE ID virus was endemic in the area; however, our isolates differed genetically from the ID viruses isolated during the 1970s from sentinel hamsters and/or mosquitoes near Iquitos.30 These data suggested that mutational changes may have occurred in the VEE viruses or that a different genotype was introduced into the area.

Our data that supported VEE virus as the cause of cases included the isolation of the virus from 10 patients, and the reisolation of the virus from the sera of seven patients. The clinical manifestations were consistent with those previously

### Table 1

Morbidity among mice over a 14-day observation period for each viral isolate in relation to the day of illness that blood samples were obtained from the patients

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Blood sample day of illness</th>
<th>No. of sick microtiter inoculated</th>
<th>Reisolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>IQT 0988</td>
<td>1</td>
<td>3/12</td>
<td>No</td>
</tr>
<tr>
<td>IQT 1015</td>
<td>1</td>
<td>2/16</td>
<td>Yes</td>
</tr>
<tr>
<td>IQT 1026</td>
<td>1</td>
<td>1/11</td>
<td>Yes</td>
</tr>
<tr>
<td>IQT 1081</td>
<td>1</td>
<td>2/10</td>
<td>No</td>
</tr>
<tr>
<td>IQT 1085</td>
<td>3</td>
<td>11/11</td>
<td>Yes</td>
</tr>
<tr>
<td>IQT 1098</td>
<td>2</td>
<td>9/09</td>
<td>Yes</td>
</tr>
<tr>
<td>IQT 1101</td>
<td>4</td>
<td>1/11</td>
<td>No</td>
</tr>
<tr>
<td>IQT 1120</td>
<td>2</td>
<td>6/07</td>
<td>Yes</td>
</tr>
<tr>
<td>IQT 1724</td>
<td>2</td>
<td>9/09</td>
<td>Yes</td>
</tr>
<tr>
<td>IQT 1735</td>
<td>3</td>
<td>11/11</td>
<td>Yes</td>
</tr>
</tbody>
</table>

* Isolates produced a cytopathic effect in Vero cells within 30–48 hr postinoculation.

no obvious clustering or relationship among individual cases.

All of the viral isolates were obtained from sera collected four days or less following the onset of illness (Table 1). A cytopathic effect was observed in cell culture 30–48 hr postinoculation, and mice became ill on days 2 through 5 following primary inoculation. Virus was reisolated from seven of the 10 original patient sera by the newborn mouse and Vero cell culture assays.

Initially, all 10 viral isolates were identified as alphaviruses by the IFA technique. Reactivity by the isolates was demonstrated to the alphavirus grouping MAF, but not to the Flavivirus, or Simbu and ORO viral grouping MAFs. Epi
tope mapping using viral-specific MAbs showed that isolate #1101 and reference VEE viruses reacted with the alphavirus- and VEE virus-specific MAbs, but not with the EEE virus, WEE virus, or flavivirus MAbs (Table 2).

Further analysis using additional VEE viral-specific MAbs showed that the pattern of reactivity of the isolate was similar to that of three reference VEE subtype I, variety D viruses (83U434, 83U18, and 59001) originally isolated in Venezuela and Colombia (Table 2).

A portion of the genomes of eight of the 10 viral isolates corresponding to a 554-nucleotide region at the 3’ end of the ns4 sequence 7327 to 7503 of the IAB-TrD isolate was also aligned with the homologous sequences of other VEE virus subtypes, and showed that the pattern of reactivity of the isolate was similar to those described above for the cases diagnosed by virus isolations. An additional 16 cases were positive for malaria parasites by microscopic examination of blood smears. Overall, the 18 VEE cases occurred among military recruits (n = 7), agriculture workers (n = 3), students (n = 3), and general laborers (n = 5).

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### Table 2

Identification of the Peruvian isolate IQT1101 as Venezuelan equine encephalitis (VEE) virus subtype ID by an epitope mapping enzyme-linked immunosorbent assay

<table>
<thead>
<tr>
<th>VEE viruses subtype/virus</th>
<th>VEE monoclonal antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5B4D-6*</td>
</tr>
<tr>
<td></td>
<td>TC83 only</td>
</tr>
<tr>
<td></td>
<td>1A2B-10/ALL VEE</td>
</tr>
<tr>
<td></td>
<td>Exc. TC-83 and subtype 6</td>
</tr>
<tr>
<td></td>
<td>1A3A-9/ALL I</td>
</tr>
<tr>
<td></td>
<td>3B4C-4/IAB, IC, ID, II</td>
</tr>
<tr>
<td></td>
<td>1A4D-1/ID, IAB, IC, ID</td>
</tr>
<tr>
<td></td>
<td>1A1B-9/ID, IE, II</td>
</tr>
<tr>
<td></td>
<td>7A3A-4/ID, Variant</td>
</tr>
</tbody>
</table>

* Monoclonal antibody designation/reactivity profile.
† ND = not done.
FIGURE 1. Phylogenetic tree showing the relationship of Venezuelan equine encephalitis (VEE) viruses isolated in Iquitos, Peru to previously described VEE virus isolates.

TABLE 3
Serologic evidence of Venezuelan equine encephalitis viral infection among febrile patients in Iquitos, Peru

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Sample days post acute/convalescence</th>
<th>Acute sera IgM</th>
<th>Acute sera IgG</th>
<th>Convalescence sera IgM</th>
<th>Convalescence sera IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>IQT 1049C</td>
<td>04/29</td>
<td>&lt;200</td>
<td>&lt;200</td>
<td>6,400‡</td>
<td>12,800</td>
</tr>
<tr>
<td>IQT 1225C</td>
<td>09/17</td>
<td>3,200</td>
<td>400</td>
<td>12,800</td>
<td>800</td>
</tr>
<tr>
<td>IQT 1117C</td>
<td>03/12</td>
<td>&lt;200</td>
<td>&lt;200</td>
<td>800</td>
<td>6,400</td>
</tr>
<tr>
<td>IQT 1341C</td>
<td>08/19</td>
<td>800</td>
<td>3,200</td>
<td>3,200</td>
<td>6,400</td>
</tr>
<tr>
<td>IQT 1073P</td>
<td>06/NS§</td>
<td>1,600</td>
<td>1,600</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>IQT 1338P</td>
<td>09/NS</td>
<td>800</td>
<td>200</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>IQT 1152P</td>
<td>05/NS</td>
<td>400</td>
<td>800</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>IQT 1351P</td>
<td>06/NS</td>
<td>400</td>
<td>800</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

* C = confirmed cases; P = probable cases.
† Days after onset of illness that acute samples were taken/convalescence were taken.
‡ Reciprocal of highest serum dilution positive by the ELISA.
§ No sample.

reported for cases of VEE. Stock viruses and/or isolates of VEE virus were not being used in the laboratory at the time the isolates were obtained from the sera. In addition, VEE viral infection was demonstrated by seroconversions for patients who provided paired sera and/or the detection of IgM antibody in sera of patients who provided only acute phase of illness sera. The incubation period of 24–72 hr following primary inoculation among newborn mice was consistent with that reported for VEE viral infection.

Antigenic analysis of one of the viral isolates (IQT1101) using a panel of VEE viral-specific MAbs indicated that it was similar to VEE subtype ID virus isolated from two sentinel hamster in 1983 in Venezuela, and from a sentinel hamster in 1970 in Colombia. Antigenic analysis was not performed on the other viral isolates, but selected nucleotide sequences at the 3' end of the ns4 gene of eight of the 10 isolates were identical to one another and the other two isolates differed by two changes, at most. The 10 sequences aligned most closely with the sequences of VEE ID virus...
isolated in 1961 in Panama. Subtype ID isolates from Panama cluster together (Panama genotype), but differ from the ID viruses isolated in Colombia and Venezuela or in Iquitos in the early 1970s (Colombia-Venezuela genotypes), suggesting a separate emergence of the Panama and Colombia-Venezuela genotypes, as previously described. The observation that the genotype of VEE virus isolated during this study was similar to the Panama VEE ID virus was the first recognition of the Panama genotype virus outside of Panama. An in-depth phylogenetic analysis of the relationship between Peruvian VEE ID viruses and those from elsewhere in South America and from Panama is presented elsewhere. Phylogenetic analyses have shown that in general, strains that type serologically as ID are very closely related to equine-virulent IC strains, but further studies are needed to determine whether the recent Peruvian isolates are virulent for horses.

Cases of VEE were diagnosed from October 1993 through March 1994, in August 1994, and in June 1995, with most occurring among male military recruits, students, and agriculture workers. The first two cases in 1993 occurred among military recruits who trained periodically near Quistococha, a rural community of approximately 500 people located about 5 km from the most urbanized sector of Iquitos. Cases were also diagnosed among military recruits at a training camp located about 2 km from the main urban center of Iquitos. Other cases among civilians were scattered, extending from the recruit training camp to most sectors of Iquitos. While our study was not designed to verify that cases of VEE began in Quistococha, VEE ID virus was isolated in this community during the 1970s from mosquitoes and sentinel hamsters. However, as mentioned above the viruses isolated during this study differed genetically from the 1970 isolates.

Overall, these data indicated that an enzootic strain of VEE virus was the cause of 3% (18 of 598) of the cases of febrile illnesses studied in the city of Iquitos in the Amazon Basin region of Peru. Serologic evidence of ORO (n = 16) and DEN (n = 24) viral infections was demonstrated for 40 additional cases of febrile illness (Watts DM, 1995, unpublished data). Clinical manifestations were similar to those described above for VEE cases, but the severity of illness appeared milder. The etiologies of the remaining 527 cases are unknown, except for the 16 cases positive for malaria. Clinically, the undiagnosed cases were similar to those of VEE, ORO, and DEN virus infections with the exception of a higher frequency of upper respiratory illness. The more recent isolations of Mayaro virus, a group C virus, and Guanarito virus and serologic evidence of a Sin Nombre-like viral infection among residents of Iquitos in 1995 (Watts DM and others, 1995, unpublished data) warrant further studies to consider these, as well as other infectious pathogens as the cause of human disease in the Amazon region of Peru.

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Disclaimer: The opinions and assertions contained herein are the private ones of the authors and are not to be construed as official at large.

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