

Isolation of Viruses from Mosquitoes (Diptera: Culicidae) Collected in the Amazon Basin Region of Peru

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ABSTRACT As part of a comprehensive study on the ecology of arthropod-borne viruses in the Amazon Basin region of Peru, we assayed 539,694 mosquitoes captured in Loreto Department, Peru, for arboviruses. Mosquitoes were captured either by dry ice-baited miniature light traps or with aspirators while mosquitoes were landing on human collectors, identified to species, and later tested on Vero cells for virus. In total, 164 virus isolations were made and included members of the *Alphavirus* (eastern equine encephalomyelitis, Trocara, Una, Venezuelan equine encephalomyelitis, and western equine encephalomyelitis viruses), *Flavivirus* (Ilheus and St. Louis encephalitis), and *Orthobunyavirus* (Caraparu, Itaqui, Mirim, Murutucu, and Wyeomyia viruses) genera. In addition, several viruses distinct from the above-mentioned genera were identified to the serogroup level. Eastern equine encephalomyelitis virus was associated primarily with *Culex pedroi* Sirivanakarn & Belkin, whereas Venezuelan equine encephalomyelitis virus was associated primarily with *Culex gnatos* Sallum, Huchings & Ferreira. Most isolations of Ilheus virus were made from *Psorophora ferox* (Von Humboldt). Although species of the *Culex* subgenus *Melanoconion* accounted for only 45% of the mosquitoes collected, 85% of the virus isolations were made from this subgenus. Knowledge of the viruses that are being transmitted in the Amazon Basin region of Peru will enable the development of more effective diagnostic assays, more efficient and rapid diagnoses of clinical illnesses caused by these pathogens, risk analysis for military/civilian operations, and development of potential disease control measures.

KEY WORDS virus, isolation, mosquitoes, Peru

THE EPIDEMIOLOGY OF MOSQUITO-BORNE diseases in the Amazon Basin is poorly understood, and the impact that these diseases have on local and immigrant populations remains largely unknown. Although several arboviruses associated with human disease have been identified in this region (e.g., dengue, yellow fever, Venezuelan equine encephalomyelitis [VEE],

Mayaro, and Oropouche), most cases of febrile disease remain undiagnosed, and little is known about their potential vectors (Hayes et al. 1996; Watts et al. 1997a, b, 1998a, b; Tesh et al. 1999; D.M.W., unpublished data). It is often difficult to isolate viruses from febrile patients, and diagnostic reagents are often not available for many of the viruses potentially circulating in

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this region. In an attempt to identify viruses that are currently circulating in the Amazon Basin region of Peru and to determine the potential vector associations for these viruses, we captured mosquitoes in dry ice-baited miniature light traps and as they came to human collectors and processed them for virus isolation. This study is part of a vector ecology/arboviral research program designed to evaluate arboviral disease threats in this region (Jones et al. 2004). This area was selected because human cases of VEE and large numbers of fevers of unknown origin were reported previously (Watts et al. 1997a, 1998a, b).

Materials and Methods

Study Site. Mosquitoes were collected at several sites near Iquitos, Loreto Department, in the Amazon Basin in northeastern Peru. Iquitos (approximate population 300,000) is ≈ 125 m above sea level and is bordered by the Amazon, Itaya, and Nanay rivers ($3^{\circ} 51' S$, $73^{\circ} 13' W$). Study sites consisted of the village of Puerto Almendras, ≈ 20 km west-southwest of Iquitos; a forested area ≈ 0.4 km from the village; and three Peruvian military bases, one in Iquitos and two sites ≈ 35 and 40 km west-southwest of Iquitos, respectively; and the Amazon Center for Environmental Education and Research (ACEER) ($3^{\circ} 15' S$, $72^{\circ} 54' W$) located on the Napo River ≈ 160 km north of Iquitos. Dogs and chickens were the most common peridomestic animals. Feral animals (i.e., squirrel monkeys [*Saimiri* spp.], woolly monkeys [*Lagothrix* spp.], bandicoots [*Nassua* spp.], macaws [*Ara* spp.], and parrots [*Amazona* spp.]) were kept as pets by some of the local residents in Puerto Almendras. The local population includes hunters and gatherers, who have reduced most of the wild game and large rodents to low population levels. Mammals commonly associated with forested areas surrounding Puerto Almendras include rodents (*Proechimys* spp., *Oryzomys* spp., and *Neacomys* spp.), marsupials (*Philander* spp., *Metachirus* spp., and *Marmosops* spp.), and sloths (*Choloepus hoffmanni* Peters and *Bradypus* spp.) (A. Gozalo, personal communication). Most mosquito collections were made at a forested site ≈ 0.4 km from Puerto Almendras that is composed of secondary growth trees (the oldest estimated to be ≈ 45 yr old) and managed as the Arboretum "El Huayo," as part of the Botanic Garden at the Centro de Investigación y Enseñanza Forestal Puerto Almendra of the Faculty of Forestry Engineering, Universidad Nacional de la Amazonía Peruana. This study area, located at an altitude of 115 m above sea level, consists of numerous deciduous trees, including *Cedrelinga catanaeformis* Durke, *Caryocar glabrum* (Aubl.), *Bertholetia excelsa* H.B.K., and *Parckia* spp., with a canopy that extended to ≈ 20 – 30 m in height. In addition, there were numerous epiphytes belonging to the bromeliaceae and orchidaceae subfamilies. Ground cover consisted of abundant leaf litter and moderate undergrowth due to extensive canopy shade.

Mosquito Collections. Adult mosquitoes were collected on 27 field trips from August 1996 through

October 2001. Field trips consisted of three to six, 24-h collections over a 4- to 16-d period. Adult mosquitoes were collected using dry ice-baited miniature light traps operated for 12-h periods from 0600 to 1800 and 1800 to 0600 hours. Three to five light traps were set daily at 1.5 and 10 m above the forest floor. In addition to the dry ice-baited light traps, human volunteers exposed the lower half of their legs and used aspirators to collect mosquitoes as they landed to blood feed. The use of humans for collecting mosquitoes was carried out under an approved United States Army Medical Research Institute for Infectious Diseases (USAMRIID) human use protocol number A-7421. Volunteers who were involved in the human landing collections wore hooded, screened jackets to prevent mosquitoes and other biting flies from feeding on the upper parts of the body. At the end of each 12-h collection period, the mosquitoes were placed in humidified coolers and transported to a central laboratory in Iquitos where they were immobilized by chilling and identified over wet ice or were anesthetized with triethylamine and identified at room temperature according to Lane (1953), Pratt (1953), Guedes and Souza (1964), Bram (1967), Pecor et al. (1992), and Sallum and Forattini (1996). Mosquito species found are listed in Pecor et al. (2000), and preliminary mosquito bionomic findings are presented in Jones et al. (2004). After identification, the mosquitoes were pooled (25–50 specimens) according to species, placed in sterile 1.5-ml cryovials, and then maintained on dry ice or at $-70^{\circ}C$ until assayed for virus. Voucher specimens were deposited in the Walter Reed Biosystematics Unit, Smithsonian Institution, Washington, DC, where our field mosquito identifications were confirmed (Pecor et al. 2000).

Virus Isolation. Each mosquito pool was triturated in 2 ml of diluent (10% heat-inactivated fetal bovine serum in Medium 199 with Earle's salts, $NaHCO_3$ and penicillin [100 U/ml], streptomycin [100 $\mu g/ml$], and nystatin [100 U/ml]). The suspensions were clarified by centrifugation ($3,000 \times g$ for 10 min) and tested for virus by plaque assay on African green monkey kidney (Vero) cell monolayers. A 0.1-ml aliquot of each original mosquito suspension and a 1:100 dilution of these suspensions were inoculated into duplicate wells of Vero cell monolayers. A second overlay, containing neutral red stain, was added 2 or 6 d later. If plaques were observed, the agar was removed, and the cells were washed with fresh diluent, and the resulting viral suspensions were aliquoted into three cryovials and frozen at $-70^{\circ}C$. An aliquot of each suspension was inoculated onto confluent monolayers of Vero cells grown in T-25 culture flasks with 5 ml of liquid cell culture medium and observed daily for evidence of cytopathology. Cell cultures showing cytopathic effects were frozen at $-70^{\circ}C$ until they were thawed, the suspension clarified by centrifugation at $3,000 \times g$ for 5 min, and then stored as 0.5-ml aliquots at $-70^{\circ}C$ for later virus identification studies.

Virus Identification. Viral isolates were initially screened by an immunofluorescence assay (IFA) for reactivity against *Alphavirus* and *Flavivirus* genus-

specific monoclonal antibodies (USAMRIID) as well as a number of different *Orthobunyavirus* group-specific (Group C, Guama group, and Bunyamwera serogroup) mouse hyperimmune ascitic fluids (National Institute of Allergy and Infectious Diseases, Bethesda MD). Follow-up IFA tests were performed using available antisera to complex or virus-specific members according to standard procedures (Lennette and Schmidt 1964). Briefly, selected optimized reference antibodies were diluted in 10 mM phosphate-buffered saline, pH 7.4, and 25 μ l/well was added, in duplicate, to each slide. A fluorescein isothiocyanate-labeled goat anti-mouse IgG, whole molecule conjugate (catalog no. 1211-0081, Cappel, West Chester, PA) was used at 4 μ g/well to detect bound antibody. Ascitic fluid from uninfected mice was used as a negative control.

The identification of viral isolates as members of the genera *Flavivirus* and *Alphavirus* was confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR) and virus sequencing. Aliquots of viral stock was added to 750 μ l of TRIZOL LS (Invitrogen, Carlsbad, CA) or 560 μ l of AVL buffer (QIAGEN, Valencia, CA), and the RNA was extracted according to the manufacturer's instructions. The resulting RNA pellet was suspended in 25 μ l of RNAase-free H₂O. First-strand cDNA was synthesized with a SuperScript II system and primed with either an oligo(dT) primer or random hexamers (Invitrogen). These cDNAs served as templates in subsequent PCR reactions containing virus-specific oligonucleotide primers. PCR amplifications were typically conducted in a PerkinElmer 9600 thermocycler in a 50- μ l volume that contained 45 μ l of high-fidelity PCR supermix (Invitrogen) and 20 pmol of each primer. PCR products were purified using a QIAquick PCR purification kit (QIAGEN) and added directly to the sequencing reaction. Sequencing was performed with a fluorescent-labeled dideoxynucleotide termination method using BigDye (Applied Biosystems, Foster City, CA) on an Applied Biosystem 3100 ABI PRISM automated DNA sequencer. Lasergene software (DNASTAR, Madison, WI) was used to analyze the sequences.

Viruses belonging to other genera (not *Alphavirus* or *Flavivirus*) were identified mainly by complement fixation (CF) and hemagglutination inhibition (HI) tests. Viruses were assigned to serogroups based on their CF reaction with broad group-reactive immune sera. Typing of individual strains to species level was accomplished by CF and/or HI tests, by using specific immune sera.

CF tests were done by a microtechnique (Beatty et al. 1989) using two units of complement and primary incubation overnight at 4°C. Positive reactions were recorded as the serum patterns giving 3+ or 4+ fixation of complement (scale of 0–4) with the unknown antigen.

HI tests were done according to methods described by Shope (1963) using antigens treated by acetone (cell culture fluids) or by sucrose-acetone extraction (suckling mouse brain). HI tests were performed us-

ing a constant amount of antigen (4 units) and a 1:200 dilution of goose erythrocytes.

Results and Discussion

We assayed a total of 539,694 mosquitoes and detected virus in 164 of >15,000 pools (Tables 1 and 2). Viruses isolated included members of the *Alphavirus* (eastern equine encephalomyelitis [EEE], Trocara, Una, VEE, and western equine encephalomyelitis [WEE] viruses), *Flavivirus* (Ilheus and St. Louis encephalitis [SLE] viruses), and *Orthobunyavirus* (Carpaparú, Murutucu, Itaquí, Mirim, and Wyeomyia viruses) genera. In addition, several viruses distinct from the above were identified to the serogroup level.

Alphaviruses

Eastern Equine Encephalomyelitis Virus. Both the Brazil-Peru and the Panama-Argentina subtypes of EEEV (Weaver et al. 1994, Brault et al. 1999) were isolated. In fact, both subtypes were isolated from consecutive pools of *Culex pedroí* Sirivanakarn & Belkin collected in the same trap, indicating that both subtypes were circulating concurrently in the same location. There was a strong association between *Cx. pedroí* and EEEV (Table 1). Nearly all (87%, 34 of 39) of the isolations of EEEV came from this single species, despite this species accounting for only 17% of all the mosquito specimens tested. Also, in a study conducted to evaluate molecular diagnostic assays under field conditions in the same forested area near Puerto Almendras, EEEV was detected in five additional pools of *Cx. pedroí*, including both viral subtypes (O'Guinn et al. 2004). In addition, laboratory studies at USAMRIID indicated that *Cx. pedroí* was an efficient laboratory vector of EEEV (M.J.T., unpublished data). Collectively, these data implicate *Cx. pedroí* as the principal enzootic vector of EEEV in this region. In addition to *Cx. pedroí*, EEEV also was isolated from *Culex gnomatos* Sallum, Huchings & Ferreira, *Ochlerotatus fulvus* (Wiedemann), and *Psorophora albigena* (Peryassu). In studies conducted in Peru during the 1970s, EEEV also was isolated from sentinel hamsters in study sites located near the ones used in the current study (Scherer et al. 1975).

Venezuelan Equine Encephalomyelitis Virus. The VEEV isolations included subtype ID, an enzootic subtype previously found in this region (Scherer and Chin 1983), and subtype IIIC (Aguilar et al. 2004). Nearly all of the VEEV isolates belonged to subtype IIIC. However, an isolate from *Culex ocososa* Dyar & Knab and one of the two isolates from the unidentified *Cx. (Mel.)* spp. were subtype ID VEEV. VEEV was isolated from four distinct species (Table 1), all of which belong to the subgenus *Culex (Melanoconion)*. The 14 isolates from *Cx. gnomatos* indicated that it may be the principal enzootic vector of VEEV in this region. In the initial collections, we did not separate *Culex vomerifer* Komp from *Cx. gnomatos*, and the VEEV isolations from the *Cx. vomerifer/gnomatos* pools may represent isolations from *Cx. gnomatos*, be-

Table 1. Relationship between mosquito species and viruses isolated from mosquitoes captured in the Amazon Basin region of Peru

Virus mosquito species ^a	No. isolates ^b	% ^c
Family Togaviridae, genus <i>Alphavirus</i> (71)		
EASTERN EQUINE ENCEPHALOMYELITIS - 39		
<i>Cx. (Mel.) pedroi</i>	34	87
<i>Cx. (Mel.) gnomatos</i>	1	3
<i>Cx. (Mel.) spp.</i>	2	5
<i>Oc. fulvus</i>	1	3
<i>Ps. albigena</i>	1	3
TROCARA - 1		
<i>Oc. serratus</i>	1	100
UNA - 5		
<i>Oc. fulvus</i>	1	20
<i>Ps. ferox</i>	3	60
<i>Ps. albigena</i>	1	20
VENEZUELAN EQUINE ENCEPHALOMYELITIS^d - 25		
<i>Cx. (Mel.) gnomatos</i>	14	56
<i>Cx. (Mel.) vomerifer/gnomatos</i>	3	12
<i>Cx. (Mel.) ocosa</i>	1	4
<i>Cx. (Mel.) pedroi</i>	4	16
<i>Culex (Mel.) spissipes</i> (Theobald)	1	4
<i>Cx. (Mel.) spp. (other)</i>	2	8
WESTERN EQUINE ENCEPHALITIS VIRUSES - 1		
<i>Aedes (Och.) hastatus</i> (Dyar)	1	100
Family Flaviviridae, genus <i>Flavivirus</i> (5)		
ILHEUS - 2		
<i>Ps. ferox</i>	2	100
ST. LOUIS ENCEPHALITIS - 3		
<i>Cx. (Cux.) spp.^e</i>	3	100
Family Bunyaviridae, genus <i>Orthobunyavirus</i> (69)		
Bunyamwera Group (7)		
WYEOMYIA - 1		
<i>Limatus flavisetosus</i> De Oliveira Castro	1	100
Unidentified Bunyamwera Group - 6		
<i>Li. flavisetosus</i>	2	29 ^f
<i>Oc. fulvus</i>	1	14 ^f
<i>Ps. albigena</i>	1	14 ^f
<i>Ps. ferox</i>	1	14 ^f
<i>Aedeomyia squamipennis</i> (Lynch Arribalzaga)	1	14 ^f
Group C - 42		
CARAPARU - 12		
<i>Cx. (Mel.) gnomatos</i>	4	33
<i>Cx. (Mel.) vomerifer</i>	7	58
<i>Cx. (Mel.) vomerifer/gnomatos</i>	1	8
ITAQUI - 3		
<i>Cx. (Mel.) vomerifer</i>	2	67
<i>Cx. (Mel.) vomerifer/gnomatos</i>	1	33
ITAQUI/CARAPARU - 4		
<i>Cx. (Mel.) vomerifer</i>	3	75
<i>Cx. (Mel.) pedroi</i>	1	25
MURUTUCU - 3		
<i>Cx. (Mel.) ocosa</i>	2	67
<i>Cx. (Mel.) vomerifer/gnomatos</i>	1	33
Unidentified Group C - 20		
<i>Cx. (Cux.) spp.</i>	1	2 ^f
<i>Cx. (Mel.) gnomatos</i>	1	12 ^f
<i>Cx. (Mel.) ocosa</i>	0	5 ^f
<i>Cx. (Mel.) pedroi</i>	2	7 ^f
<i>Cx. (Mel.) vomerifer</i>	6	45 ^f
<i>Cx. (Mel.) vomerifer/gnomatos</i>	1	7 ^f
<i>Cx. (Mel.) spp.</i>	8	19 ^f
<i>Limatus durhami</i> Theobald	1	2 ^f
Guama Group (17)		
MIRIM - 2		
<i>Cx. (Mel.) pedroi</i>	1	50
<i>Cx. (Mel.) spp.</i>	1	50
Unidentified Guama Group - 15		
<i>Cx. (Mel.) gnomatos</i>	5	29 ^f
<i>Cx. (Mel.) ocosa</i>	1	6 ^f
<i>Cx. (Mel.) pedroi</i>	0	0 ^f
<i>Cx. (Mel.) vomerifer</i>	7	41 ^f
<i>Cx. (Mel.) spp.</i>	2	18 ^f

Table 1. Continued

Virus mosquito species ^a	No. isolates ^b	% ^c
Not yet identified (22)		
<i>Cx. (Mel.) gnomatos</i>	4	18
<i>Cx. (Mel.) pedroi</i>	5	23
<i>Culex (Mel.) portesi</i> Senevet and Abonnenc	2	9
<i>Cx. (Mel.) vomerifer</i>	5	23
<i>Cx. (Mel.) spp.</i>	4	18
<i>Oc. fulvus</i>	1	5
<i>Wyeomyia (Wyo.) spp.</i>	1	5

^a Number of each species tested is provided in Table 2.

^b The number following each genus (in parentheses) is the total number of isolations of that viral genus, the number following each virus (after the hyphen) is the total number of isolations of that virus, and the number following each mosquito species is the number of isolations of that specific virus from that species.

^c Percentage of virus isolations of that virus that came from that species.

^d These viruses consisted almost entirely of subtype IIC VEEV. However, the isolate from *Cx. ocosa* and one of the two isolates from "*Cx. (Mel.) spp. other*" were subtype ID VEEV.

^e *Culex (Cux.) spp.*, all *Cx. (Cux.)* species combined. These consisted almost entirely of *Cx. coronator*, *Culex declarator* Dyar & Knab, and *Culex mollis* Dyar & Knab.

^f For the members of the three serogroups in the genus *Orthobunyavirus*, the percentage listed for each species in the "unidentified" category is the percentage of isolations of all members of that serogroup from that mosquito species, regardless of whether the virus had been completely identified.

cause VEEV was not isolated from *Cx. vomerifer* after the two species were tested separately. When the two species were tested separately, VEEV was significantly ($\chi^2 = 16.1$, $df = 1$, $P < 0.001$) more closely associated with *Cx. gnomatos* than with *Cx. vomerifer*, minimum field infection rates (MFIRs) of 8.6 and $<0.5/10,000$, respectively. In vector competence studies conducted at USAMRIID, the combined *Cx. vomerifer/Cx. gnomatos* were efficient vectors of subtypes IAB, IC, ID, and IE of VEEV, with infection rates $\geq 83\%$ and dissemination rates $\geq 50\%$ for each of the four subtypes (Turell et al. 2000). However in later studies, *Cx. gnomatos* was significantly more susceptible to infection and dissemination with subtype IIC VEEV than was *Cx. vomerifer* (M.J.T., unpublished data). The field isolation rates and laboratory vector competence studies indicated that *Cx. gnomatos* is the principal vector of this subtype in the Iquitos area.

Flaviviruses

Ilheus Virus. Both of the isolations of Ilheus virus were made from *Psorophora ferox* (Von Humboldt). However, in a separate study, we isolated Ilheus virus from *Culex coronator* Dyar & Knab (M.J.T., unpublished data). *Ps. ferox* is an avid daytime biter and therefore poses a threat to daytime forest workers. Ilheus virus may be more common than reported in human populations, because infections with this virus may produce serological results that may be confused with dengue infections.

St. Louis Encephalitis Virus. All three of the SLEV isolations were made from *Cx. (Culex)* mosquitoes, most likely *Cx. coronator*, the most commonly col-

Table 2. Associations between mosquito species and viruses isolated from the Amazon Basin region of Peru

Mosquito species ^a	No. tested	No. isolates	Viruses isolated (no. of isolations)	MFIR ^b
<i>Aedeomyia squamipennis</i>	2,006	1	BUN (1)	5.0 (~0, 14.8)
<i>Coquillettidia</i> spp.	6,359	0		<1 (0.0, 6.0)
<i>Culex (Cux.)</i> spp. ^c	64,145	4	SLE (3), GpC (1)	0.6 (~0, 1.2)
<i>Cx. (Mel.) adamesi</i>	1,661	0		<6 (0.0, 17.9)
<i>Cx. (Mel.) dunni</i>	1,938	0		<5 (0.0, 15.0)
<i>Cx. (Mel.) gnomatos</i>	16,264	29	EEE (1), VEE (14), CAR (4), GpC (1), GUA (5), N.I. (4)	17.8 (11.0, 23.8)
<i>Cx. (Mel.) vomerifer/gnomatos</i>	12,600	7	VEE (3), CAR (1), ITQ (1), MUR (1), GpC (1)	5.6 (1.4, 9.7)
<i>Cx. (Mel.) vomerifer</i>	21,336	30	CAR (7), ITQ (2), ITQ/CAR (3), GpC (6), GUA (7), N.I. (5)	14.3 (9.0, 19.1)
<i>Cx. (Mel.) ocosa</i>	6,870	4	VEE (1), MUR (2), GUA (1)	5.9 (0.1, 11.5)
<i>Cx. (Mel.) pedroi</i>	91,583	47	EEE (34), VEE (4), ITQ/CAR (1), GpC (2), MIR (1), N.I. (5)	5.1 (3.7, 6.6)
<i>Cx. (Mel.) portesi</i>	6,204	2	N.I. (2)	3.2 (~0, 7.7)
<i>Cx. (Mel.) spissipes</i>	17,699	1	VEE (1)	0.6 (~0, 1.7)
<i>Cx. (Mel.) theobaldi</i>	5,649	0		<2 (0.0, 6.7)
<i>Cx. (Mel.)</i> spp.	63,249	19	EEE (2), VEE (2), GpC (8), MIR (1), GUA (2), N.I. (4)	3.0 (1.7, 4.4)
<i>Limatus flavisetosus</i>	2,992	3	WYE (1), BUN (2)	10.1 (~0, 21.3)
<i>Lt. durhamii</i>	2,017	1	GpC (1)	5.0 (~0, 14.6)
<i>Mansonia indubitans/titillans</i>	29,990	0		<1 (0.0, 1.3)
<i>Ochlerotatus fulvus</i>	40,064	4	VEE (1), UNA (1), BUN (1), N.I. (1)	1.0 (~0, 2.0)
<i>Oc. hastatus</i>	1,640	1	WEE (1)	6.1 (~0, 17.9)
<i>Oc. serratus</i>	41,606	1	TRO (1)	0.2 (~0, 0.7)
<i>Psorophora albigena</i>	70,135	3	EEE (1), UNA (1), BUN (1)	0.4 (~0, 0.9)
<i>Ps. ferox</i>	9,214	6	UNA (3), ILH (2), BUN (1)	6.6 (1.3, 11.7)
<i>Uranotaenia</i> spp.	1,979	0		<5 (0.0, 15.0)
<i>Wyeomyia</i> spp.	4,135	1	BUN (1)	2.4 (~0, 7.2)
Other species (>40 additional species) ^a	17,486	0		<1 (0.0, 2.2)
All species	539,694	164		3.0 (2.6, 3.5)

BUN, unidentified Bunyamwera Group virus; CAR, Caraparu virus; EEE, eastern equine encephalomyelitis virus; GpC, unidentified Group C virus; GUA, unidentified Guama Group virus; ILH, Ilheus virus; ITQ, Itaquí virus; MIR, Mirim virus; MUR, Murutucu virus; N.I., not identified; TRO, Trocara virus; UNA, Una virus; VEE, Venezuelan equine encephalomyelitis virus; WEE, western equine encephalomyelitis virus; WYE, Wyeomyia virus.

^a See Pecor et al. (2000) for a complete listing of all species collected.

^b MFIR, per 10,000 specimens tested (95% confidence interval).

^c *Cx. (Cux.)* spp., all *Cx. (Cux.)* species combined. These consisted almost entirely of *Cx. coronator*, *Cx. declarator*, and *Cx. mollis*.

lected *Culex* spp. In addition, laboratory studies (M.J.T., unpublished data) indicated that *Cx. coronator* was susceptible to oral infection with this virus. However, with low levels of infection, the principal vector could not be identified.

Orthobunyaviruses

We made 69 isolations of viruses belonging to the genus *Orthobunyavirus*. These included members of the Guama, Bunyamwera, and Group C serogroups (Tables 1 and 2). Although VEEV was significantly more closely associated with *Cx. gnomatos* than with *Cx. vomerifer*, the reverse was true with Group C Bunyaviruses. For this serogroup, MFIRs for *Cx. gnomatos* were significantly lower ($\chi^2 = 4.1$, $df = 1$, $P = 0.04$) than for *Cx. vomerifer*, 3.1 and 8.9/10,000, respectively. However, there was essentially no difference in the infection rate for these two species for members of the Guama serogroup, MFIRs of 3.1 and 3.3/10,000, respectively. Several Group C and Guama group viruses (identified to species complex) were detected by Scherer et al. (1975) in sentinel hamsters and mosquitoes (not identified) captured near our study site.

Caraparu. We isolated Caraparu virus from 12 pools of *Culex (Mel.)* mosquitoes: *Cx. gnomatos* (four) and *Cx. vomerifer* (eight). This virus has been associated

with febrile disease in humans (Causey et al. 1961, Iversson et al. 1987, Coimbra et al. 1993). Essential to risk assessment, vector competence studies would help to identify the most probable vector of Caraparu virus.

Seasonal Activity. Although viruses were isolated during each month that mosquitoes were captured, isolation rates were highest during January–February and August–October (Fig. 1A and B). There was no association between the number of mosquitoes captured and MFIR (data not shown). For most viruses detected, sample sizes were too small to provide an accurate assessment of seasonal activity. However, peak EEEV activity was closely associated with the January–February and August–September peaks of viral activity. Despite the collection of >10,000 *Cx. pedroi* from March through July, we did not detect any EEEV during this time period (Fig. 1A). Based on the isolation rate during the rest of the year, we would have expected 4.4 isolations from these mosquitoes. In contrast to our results with EEEV, members of the genus *Orthobunyavirus* were isolated more commonly during August–October, with a secondary peak in February (Fig. 1B).

The MFIRs for VEEV and EEEV remained relatively constant by year of mosquito collection (Fig. 2). MFIR rates for all viruses combined were higher in July 1997–June 1998 and in July 1999–June 2000 than

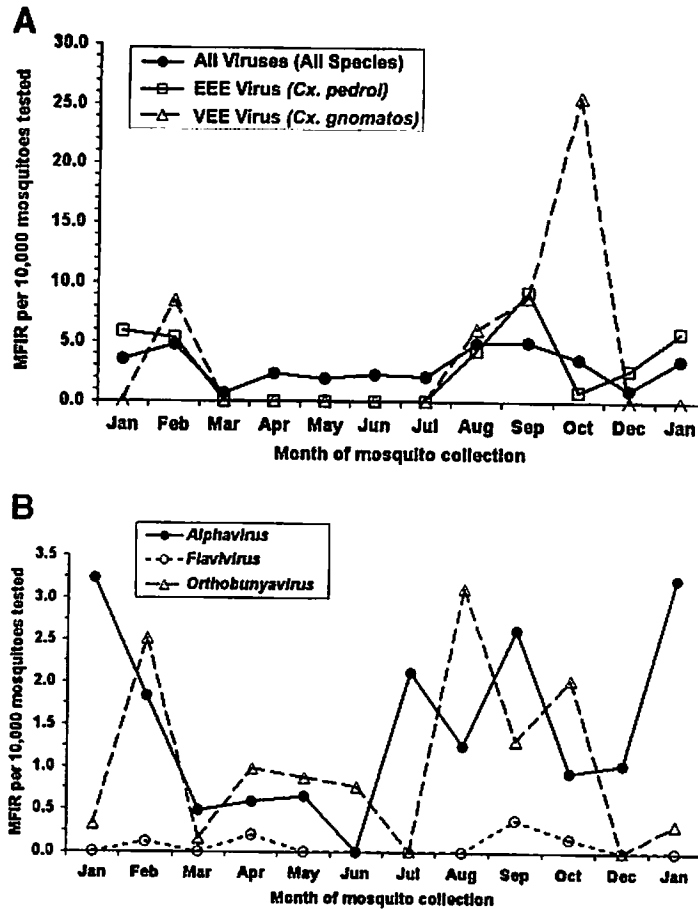


Fig. 1. MFIR for all viruses isolated for all mosquitoes captured, MFIR for subtype IIIC VEEV in *Cx. (Mel.) gnomatos*, and MFIR for EEEV in *Cx. pedroi* captured by month of collection (A). MFIRs for all Alpha-, Flavi-, and Bunyaviruses isolated for all mosquitoes captured by month of collection (B).

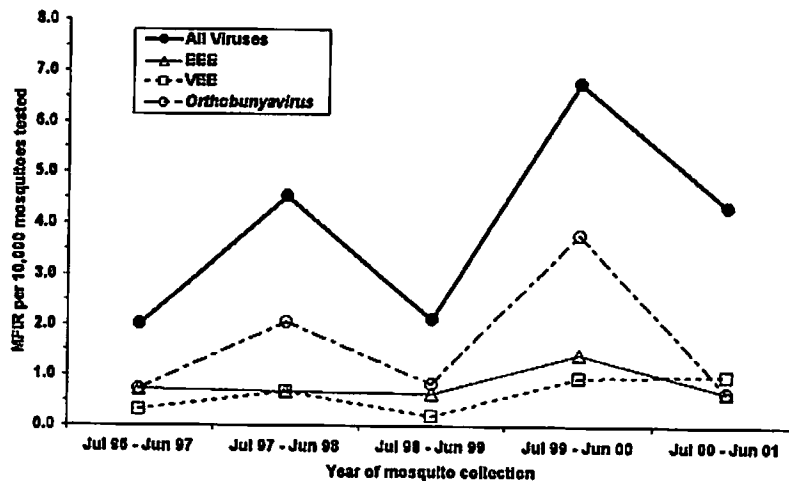


Fig. 2. MFIRs for all viruses isolated and separately for EEE, VEE, and members of the genus *Orthobunyavirus* for all mosquitoes captured by year of collection.

in the other 2 yr during which mosquitoes were collected. However, nearly all of this increase in virus activity could be accounted for by increased numbers of Bunyaviruses isolated during those 2 yr (Fig. 2).

Although a large number of mosquitoes were tested, virus was not isolated from >29,000 *Mansonia indubitans* Dyar & Shannon and *Mansonia titillans* (Walker). Likewise, MFIRs for *Oc. fulvus*, *Oc. serratus*, and *Ps. albigena* were less than one virus isolation per 10,000 specimens tested, and there was no consistent association between these mosquito species and any particular virus. Although *Cx. (Mel.)* spp. accounted for only 45% (245,053) of the specimens tested, 85% (139) of the virus isolations were made from these species, indicating the importance of this group of mosquitoes in virus transmission in the Amazon Basin region of Peru.

Baseline data on arboviruses circulating in various environments establishes potential health risks to human populations residing, visiting, or conducting various types of operations in endemic areas. Our study was the first time that many of the arboviruses were detected from this part of the Amazon Basin. Dengue virus, which was circulating in the urban environment of Iquitos (Phillips et al. 1992, Morrison et al. 2004), was not isolated during our study. This was primarily because of the very small sample of *Aedes aegypti* L., the principal vector of dengue virus, collected during these studies. Similarly, we did not detect Oropouche virus, a virus known to cause disease in this area (Watts et al. 1997a, b). Again, this probably was because we did not collect many *Culicoides* spp., the principal vectors of Oropouche virus, for assay. Although, we did not detect dengue virus during our study, we isolated Ilheus virus on several occasions, and Ilheus virus is serologically cross reactive with dengue virus in some assays. Therefore, there is the potential for people in the outlying villages infected with Ilheus virus to be misdiagnosed and for the initiation of inappropriate treatment/mosquito control.

Virus isolations are just one aspect of vector incrimination. In addition, knowledge of accurate vector identification, vector competence, biology and behavior, and health risk assessments must be used in the development of effective disease control strategies. It was not until part of the study was completed that *Cx. (Mel.) gnomatos* and *Cx. (Mel.) vomerifer* were recognized as distinct species. Yet, these species differed in the viruses that they carried and could potentially transmit. Also, because of the limited morphological characters to separate some species, there is the potential for the inclusion of one member of a species into the other group. Vector competence studies are needed to identify more accurately noncompetent and competent vectors. Similarly, good morphological keys for *Cx. (Cux.)* and *Cx. (Mel.)* spp. must be made available so that more accurate assessments of potential vectors can be made.

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