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# TROCARA VIRUS: A NEWLY RECOGNIZED *ALPHAVIRUS* (TOGAVIRIDAE) ISOLATED FROM MOSQUITOES IN THE AMAZON BASIN

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*Abstract.* This report describes Trocara virus, a newly recognized member of the genus *Alphavirus*, that has been isolated from *Aedes serratus* mosquitoes collected at two widely separated sites in the Amazon Basin. Biological, antigenic and genetic characteristics of the new virus are given. Results of these studies indicate that Trocara virus is the first member of a newly discovered antigenic complex within the family Togaviridae genus *Alphavirus*. The public health and veterinary importance of Trocara virus is still unknown.

## INTRODUCTION

The genus *Alphavirus* (family Togaviridae) consists of a group of enveloped, single-stranded, positive-sense RNA viruses, which are mostly mosquito-borne and nearly worldwide in their distribution.[1,2] At present, 25 distinct virus species are recognized within the *Alphavirus* genus;[1] based on their antigenic relationships, these 25 viruses are further subdivided into 7 antigenic complexes.[1–3] Many of the alphaviruses are significant pathogens for humans and domestic animals.[4] This communication describes a newly recognized alphavirus, designated Trocara virus, which was isolated from *Aedes serratus* mosquitoes collected at two widely separated sites in the Amazon Basin in northern Brazil and northeastern Peru.

## MATERIALS AND METHODS

**Circumstances of virus isolations.** The prototype strain of Trocara virus, BeAr 422431, was originally isolated at the Instituto Evandro Chagas (IEC) from newborn mice that had been inoculated intracerebrally with a homogenate prepared from a pool of *Aedes serratus* female mosquitoes collected at Tucurui, Para State, Brazil in 1984. Tucurui (3°45′S, 49°41′W) is located at an elevation of about 40 m above sea level in the Tocantins River Basin and is the site of a large dam and hydroelectric plant. The mosquito collection yielding the original Trocara virus isolate was made during a long-term study, conducted by the IEC between 1983 and 1989, on the ecological effects of dam construction and impoundment on the surrounding region.[5]

A second strain of Trocara virus, PE 70009, was isolated at the Army Medical Research Institute of Infectious Diseases (USAMRIID) from a Vero cell culture which was inoculated with a pool of *Ae. serratus* females collected in a humid tropical forest near Iquitos, Loreto Department, Peru (3°7′S, 73°2′W) in 1997.

Both viruses subsequently were submitted to the Arbovirus Reference Center at the University of Texas Medical Branch (UTMB), where virus identification and characterization were done.

**Other alphaviruses used.** The following alphaviruses[6] were used to prepare antigens and immune reagents for the serologic studies: Aura (BeAr 10315 strain), Mayaro (TRVL 4675 strain), eastern equine encephalitis (Ten Broeck strain), western equine encephalitis (McMillan strain), Venezuelan equine encephalitis (TC-83 strain), Mucambo (BeAn 8 strain), Pixuna (BeAr 35645 strain), Una (BeAr 13136 strain), chikungunya (Ross strain), Highlands J (B230), Ross River (T48 strain), Semliki Forest (original), Sindbis (EgAr 339 strain), Barmah Forest (BH 2193) and Ndumu (SA Ar 2204).

**Serologic tests.** Complement fixation (CF) tests were performed using a microtechnique,[7] with two full units of guinea pig complement. Titers were recorded as the highest dilutions giving 3+ or 4+ fixation of complement on a scale of 0 to 4+. Hemagglutination-inhibition (HI) tests were performed using a microtechnique, as described before;[7] immune sera and ascitic fluids were acetone-extracted. Trocara antigens were tested at pH 6.0; all of the other antigens were tested at pH 6.2.

**Antigens and immune reagents.** Viral antigens used for CF and HI tests were prepared as follows: Trocara (strain PE-70009), Una and chikungunya were prepared from infected cultures of C6/36 cells; Barmah Forest and Ndumu antigens were prepared from infected Vero cells. All other alphavirus antigens, including Trocara strain Be Ar 422431, were prepared from infected newborn-mouse brain which had been extracted by the acetone method.[7]

An immune serum to Trocara virus strain PE-70009 was prepared in adult hamsters. These animals were given a single intraperitoneal injection of infected Vero cells, and their serum was collected 30 days later. For all other alphaviruses, including Trocara strain BeAr 422431, hyperimmune mouse ascitic fluids (HMAF) were prepared. The adult mouse immunization schedule was four weekly intraperitoneal injections of a 10% crude suspension of infected suckling mouse brains in phosphate-buffered saline mixed with Freund's adjuvant, as described previously.[8] Sarcoma 180 cells were given intraperitoneally with the final injection to induce ascites.

**Virus preparation for RNA extraction.** Virus strains BeAr 422431 and PE 70009 were diluted and grown on BHK-21 cells at low multiplicity of infection. After approximately 75% of the cells exhibited cytopathic effects (CPE),

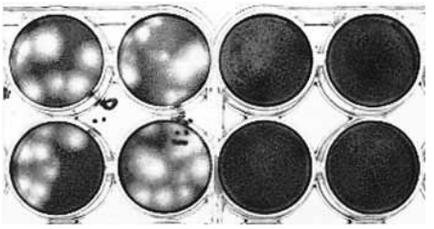

FIGURE 1. Plaques of Trocara virus (strain PE-70009) measuring 3–6 mm in diameter, as seen on the third day after inoculation in BHK-21 cells. The four wells on right side of figure are uninoculated controls.

the virus present in the supernatant was concentrated by polyethylene glycol (PEG) precipitation.[9] The virus pellet was resuspended in a 150 μl buffer consisting of 10 mM Tris, pH 8.0, 1 mM EDTA and 100 mM NaCl. Trizol LS (GibcoBRL, Bethesda, MD) was added, and RNA extraction was performed according to the manufacturer's protocol.

**RNA extraction and reverse transcription PCR.** RNA was extracted from one half of each virus/Trizol suspension according to manufacturer's protocols, as described previously.[10] cDNAs were synthesized from the RNA using a poly(T) oligonucleotide primer ($T_{25}$V-Mlu; 5′-TTACGAATTCACGCGT$_{25}$V-3′). The polymerase chain reaction (PCR) amplification was performed on the cDNA using the poly(T) primer and a forward primer designated α10247 (5′-TACCCNTTYATGTGGGG-3′) that corresponds to a conserved alphavirus sequence near the N-terminus of the E1 glycoprotein gene. Amplification of the carboxy portion of the E1 gene and the 3′ non-coding region utilized the following parameters: 30 cycles of denaturation at 95°C for 30 sec, primer annealing at 49°C for 30 sec, and extension at 72°C for 3 min. A 10 minute final extension was used to insure complete product synthesis.

**Sequencing/genetic analysis.** The PCR products of approximately 1.1 kb were isolated from 1% agarose gels. The cleaned DNA fragments were either sequenced directly or cloned into pBluescript II SK (Strategene, LaJolla, CA) that had been linearized with SmaI. SmaI restriction enzyme was included in the ligation reactions to reduce the re-ligation of the vector to itself. White bacterial colonies were screened for plasmids containing inserts of the correct size. Selected clones (at least two per strain) were sequenced using plasmid specific T7 promoter and m13 reverse primers. PCR amplicons were sequenced directly with α 10247A and α 10720 (+) (5′-TAACAGCGGGAAATCGTTGC-3′) primers. Sequencing was performed using an Applied Biosystems (Foster City, CA) Prism 377 sequencer and automated DNA sequencing kit. The nucleotide sequences were aligned with those of other alphaviruses[11,12] (Powers AM, unpublished data) using the PILEUP program in the Wisconsin Package (Genetics Computer Group), and pairwise comparisons analyzed using the GAP program.[13] The sequences have been deposited into Genbank under accession numbers AF252264 and AF252265.

**Transmission electron microscopy.** Infected monolayers of Vero and C6/36 cells were fixed, in 1.25% formaldehyde, 2.5% glutaraldehyde, 0.03% trinitrophenol and 0.03% $CaCl_2$ in 0.05 M cacodylate buffer pH 7.3, and washed in 0.1 M cacodylate buffer.[14] Cells were scraped from the plastic, pelleted in buffer, postfixed in 1% $OsO_4$ in 0.1 M cacodylate buffer, *en bloc* stained with 1% uranyl acetate in 0.1 M maleate buffer pH 5.2, dehydrated in ethanol and embedded in Poly/Bed 812 (Polysciences, Warrington, PA). Ultrathin sections were cut on Reichert Ultracut S ultramicrotome, stained with 2% aqueous uranyl acetate and 0.4% lead citrate, and examined in a Philips 201 electron microscope at 60 kV.

TABLE 1

Hemagglutination-inhibition tests with Trocara virus (strain BeAr 422431) and other alphavirus antigens and hyperimmune mouse ascitic fluids (MIAF)

| MIAF | Trocara antigen Ht/Ho* | Antigen | Trocara MIAF Ht/Ho* |
|---|---|---|---|
| Trocara | 20,480 | Trocara | 20,480 |
| VEE | 40/≥2,560 | VEE | 80/≥2,560 |
| Mucambo | <20/640 | Mucambo | 40/640 |
| Pixuna | 20/≥2,560 | Pixuna | 20/>2,560 |
| EEE | 80/≥2,560 | EEE | 160/≥2,560 |
| WEE | 40/2,560 | WEE | 320/2,560 |
| Aura | 80/≥2,560 | Aura | 80/≥2,560 |
| Mayaro | 40/≥2,560 | Mayaro | 80/≥2,560 |
| Una | 40/≥2,560 | Una | 80/≥2,560 |
| Semliki Forest | 40/≥2,560 | Semliki Forest | 160/>2,560 |
| Sindibis | 80/≥2,560 | Sindibis | 80/≥2,560 |
| Chikungunya | <20/≥2,560 | Chikungunya | 80/≥2,560 |
| Highlands J | <20/≥2,560 | Highlands J | 80/≥2,560 |
| Ross River | 40/≥1,280 | Ross River | 40/>1,280 |
| Barmah Forest | <10/640 | Barmah Forest | 40/640 |
| Ndumu | <10/≥640 | Ndumu | 40/≥640 |

* Reciprocal of heterologous titer/reciprocal of homologous titer. VEE = Venezuelan equine encephalitis; EEE = eastern equine encephalitis; WEE = western equine encephalitis.

**Animal use.** The maintenance and care of experimental animals used in this research complied with the United States National Institutes of Health guidelines for the humane use of laboratory animals.

## RESULTS

**Biological characteristics.** The prototype strain of Trocara virus (BeAr 422431) was originally isolated by intracerebral inoculation of newborn mice. On subsequent intracerebral passages, newborn mice inoculated with this strain began to die on day 7 post-inoculation (PI). In contrast, adult Swiss mice inoculated intraperitoneally developed antibodies and survived. Trocara strain PE-70009 was initially isolated in Vero cell culture. This virus did not kill baby mice on the first intracerebral inoculation; three intracerebral blind passages were required before it began to kill newborn mice irregularly, beginning on the eleventh day PI. Adult Syrian hamsters inoculated intraperitoneally with strain PE-70009 developed antibodies and survived infection.

Both Trocara virus strains (BeAr 422431 and PE-70009) produced CPE in Vero and BHK-21 cells maintained at 37°C, and in *Aedes albopictus* cells (C6/36 clone) at 27°C. In all three cell lines, CPE developed 2–4 days PI, depending upon the titer of the virus inoculum. Both Trocara strains produced plaques in Vero and BHK-21 cells under agar. On day 3, Trocara virus plaques in BHK-21 cells were 3–6 mm in diameter, fuzzy in appearance and slightly irregular in shape (Figure 1).

**Ultrastructure of Trocara virus in cell culture.** Virions

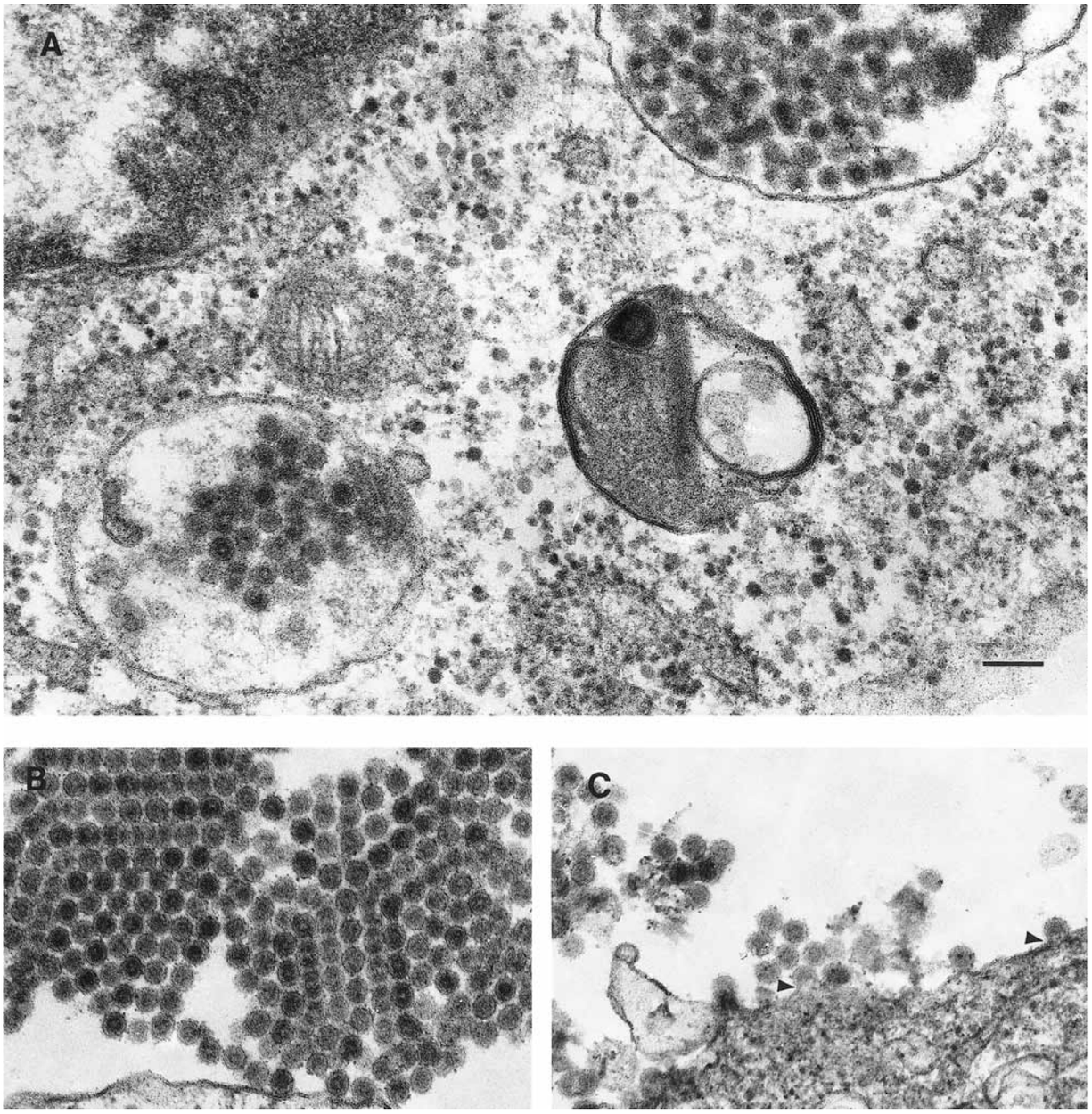


FIGURE 2. Ultrastructure of Trocara virus (strain PE-70009) in vertebrate and mosquito cell cultures. Bar = 100 nm. Magnification is the same in each plate. **A.** Virions within cytoplasmic vacuoles of infected cells of the C6/36 clone of *Aedes albopictus* cell line. **B.** Trocara virions forming pseudo-crystalline arrays at the surface of infected C6/36 cells. **C.** Virions budding from the surface of Vero cells (arrowheads).

45 nm in diameter were found both in C6/36 cells and Vero cells (Figure 2). They had a dense core about 30 nm in diameter, surrounded by electron-lucent and electron-dense layers comprising an envelope about 7 nm thick. In C6/36 cells, virions were located inside intracytoplasmic vacuoles (Figure 2A) and at the cell surface. Virions were abundant at the cell surface and formed pseudo-crystalline arrays (Figure 2B). In Vero cells, virions of strain BeAr 422431 were found budding from the cell surface (Figure 2C).

**Antigenic relationships with other alphaviruses.** In CF tests, a Vero cell antigen of Trocara strain PE-70009 and a mouse brain antigen of the protype strain Be Ar 422431 both had titers of 1:32 against the PE-70009 hamster immune serum and 1:128 with BeAr 422431 HMAF, but both antigens failed to react with any of the other 15 specific alphavirus HMAF or with an alphavirus group reference reagent.[15]

Table 1 shows the results of HI tests with Trocara virus strain BeAr 422431 antigen and HMAF and 15 other alphavirus antigens and HMAF. In HI tests, the two Trocara antigens (PE-70009 and Be Ar 422431) gave identical results (both 1:20,480) with the Be Ar 422431 HMAF and 1:1,280 with PE-70009 hamster immune serum. Using this higher titered Trocara HMAF, weak cross-reactions were ob-

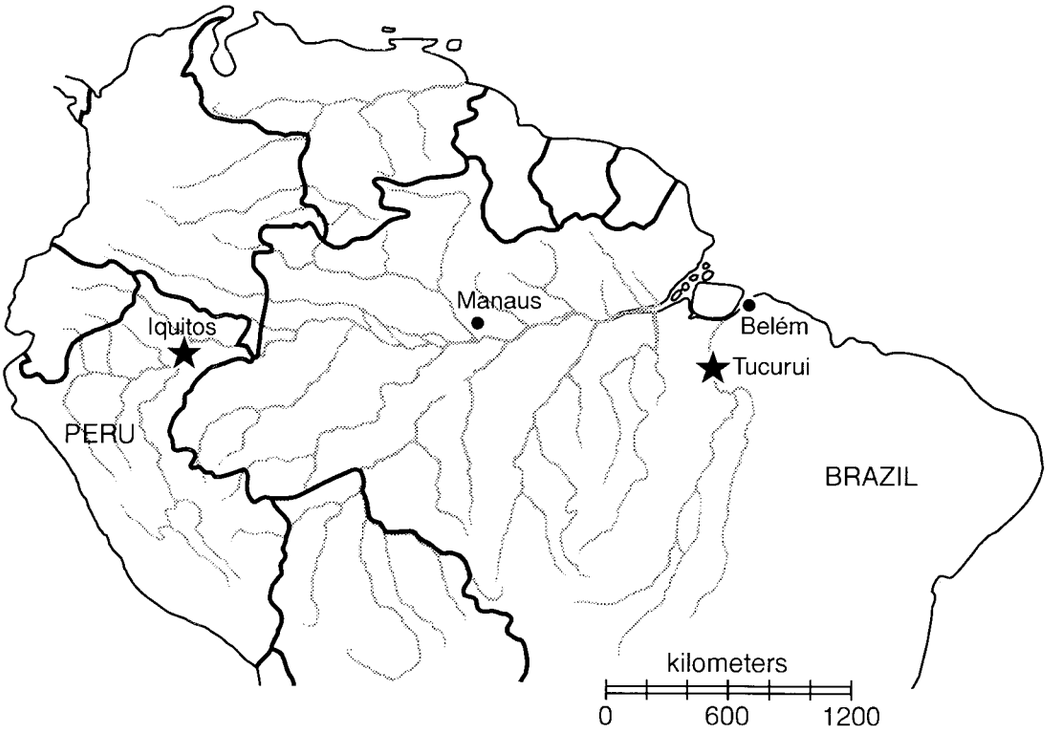


FIGURE 3. Map of the Amazon River Basin showing the locations of Tucurui and Iquitos, and the approximate localities where mosquitoes yielding the two isolates of Trocara virus were collected in 1984 and 1997, respectively.

served with most of the other alphaviruses in HI tests. However Trocara virus was antigenically distinct from the other viruses tested.

**Genetic characterization of Trocara strains BeAr 422431 and PE-70009.** DNA fragments of 1.1 kb were amplified by RT-PCR. The PCR products were either sequenced directly or were first cloned into a plasmid. Pairwise comparisons with other New World alphaviruses indicated that BeAr 422431 and PE-70009 were closely related to each other, sharing approximately 95% nucleotide sequence identity, but were distinct from other alphaviruses (Table 2). Viruses in the western equine encephalitis (WEE), eastern equine encephalitis (EEE), and Venezuelan equine encephalitis (VEE) serocomplexes were all equally divergent from BeAr 422431 and PE-70009, sharing about 55% nucleotide sequence identity. BeAr 422431 and PE-70009 were 97% identical at the amino acid level but, both showed between 47 and 54% amino acid divergence from the other alphaviruses.

## DISCUSSION

The two Trocara virus isolates were from *Ae. serratus* mosquitoes collected 13 years apart at sites about 2,500 km distant (Figure 3). *Aedes serratus* is a common forest-dwelling mosquito in the Neotropics with a wide geographic distribution in tropical South America, Central America and parts of the Caribbean.[16] The wide distribution of this mosquito suggests that the virus might also be present in other regions of tropical America. Since *Ae. serratus* is anthropophilic, the virus could be transmitted by bite to humans. At present, the public health importance of Trocara virus is unknown, but we plan to examine sera from persons residing

TABLE 2

Percent identity of the carboxy terminus of the E1 gene among Trocara virus isolates BeAr 422431 and PE 70009 and other alphaviruses*

| | BeAr 422431 | PE 70009 | WEE | Aura | VEE (IAB) | PIX | EEE | MAY | BF |
|---|---|---|---|---|---|---|---|---|---|
| BeAr 422431 | — | 97.3 | 49.7 | 51.7 | 52.7 | 53.6 | 48.5 | 49.1 | 46.1 |
| PE 70009 | 94.2 | — | 48.5 | 50.8 | 52.1 | 53.0 | 47.9 | 47.9 | 44.9 |
| WEE | 52.7 | 53.2 | — | 60.1 | 49.7 | 49.7 | 49.7 | 47.3 | 45.2 |
| Aura | 55.2 | 55.0 | 60.9 | — | 50.8 | 52.3 | 48.0 | 47.7 | 47.4 |
| VEE (IAB) | 56.0 | 55.5 | 55.1 | 54.7 | — | 81.7 | 62.9 | 53.3 | 49.4 |
| Pixuna | 54.6 | 54.4 | 53.9 | 55.3 | 74.0 | — | 60.5 | 52.7 | 50.3 |
| EEE | 55.9 | 55.8 | 52.8 | 53.2 | 61.6 | 60.0 | — | 53.6 | 52.7 |
| Mayaro | 53.9 | 53.1 | 53.6 | 53.7 | 55.8 | 54.9 | 57.7 | — | 55.4 |
| Barmah Forest | 51.3 | 51.2 | 51.1 | 53.0 | 54.2 | 54.0 | 57.0 | 57.6 | — |

* Upper diagonal displays percent amino acid identity; lower diagonal contains percent nucleotide identity. WEE = western equine encephalitis; VEE (IAB) = Venezuelan equine encephalitis, subtype IAB; EEE = eastern equine encephalitis; BF = Barmah Forest; MAY = Mayaro; PIX = Pixuna.

in forested areas around Iquitos for specific antibodies to the virus.

Antigenic and genetic analyses of Trocara virus demonstrate that it is distantly related to representatives of all of the known alphavirus antigenic complexes. These data indicate that Trocara is the first member of a newly discovered antigenic complex within the genus *Alphavirus.*

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