

## CHARACTERIZATION OF EIGHT NEW PHLEBOTOMUS FEVER SEROGROUP ARBOVIRUSES (BUNYAVIRIDAE: *PHLEBOVIRUS*) FROM THE AMAZON REGION OF BRAZIL\*

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**Abstract.** Eight new members of the phlebotomus fever arbovirus serogroup (family Bunyaviridae; genus *Phlebovirus*) from the Amazon region of Brazil are described. One serotype was recovered from a febrile patient, three from small wild animals and four from sand flies. A small serum survey carried out with the human isolate, Alenquer virus, suggests that it rarely infects man. Complement-fixation and plaque reduction neutralization tests were done, comparing the eight new viruses with other members of the phlebotomus fever serogroup. A close antigenic relationship was demonstrated between one of the new agents (Belterra) and Rift Valley fever virus. This finding is of considerable interest and deserves further investigation. Addition of these eight new viruses to the genus *Phlebovirus* brings to 14 the number of serotypes known to occur in the Amazon region and to 36 the total number reported worldwide. More detailed clinical and epidemiological studies should be conducted in Amazonia in order to define the public health impact caused by phleboviruses.

During the early 1970s construction began on an extensive network of highways across vast areas of virgin tropical forest in the Amazon region of Brazil. As these highways were completed, colonists began to settle in accessible land adjacent to the new roads. Most of the settlers were from outside the region, and became engaged in agricultural activities. Because of their presumed non-immune status and their close contact with the forest, it was important to determine which local pathogens, including arboviruses, were potentially hazardous to their health. Consequently, studies were conducted at numerous sites along the highways to determine the possible occurrence of arboviruses and to assess their public health importance. As a result of these investigations, a number of arboviruses (some known and others new to science) were isolated. Among the new viruses were eight members of the phlebotomus fe-

ver serogroup (family Bunyaviridae; genus *Phlebovirus*). The purpose of this report is to characterize the eight new phlebovirus serotypes, to describe their antigenic relationship to other members of the phlebotomus fever group and to discuss their potential role in human disease.

### MATERIALS AND METHODS

#### *Viruses*

The 36 phleboviruses used in this study are listed in Table 1. The eight new Brazilian serotypes are Urucuri, Itaituba, Alenquer, Turuna, Belterra, Joa, Oriximina and Munguba. The circumstances of their isolation are given in Table 2. The approximate locality in which they were recovered is shown in Figure 1.

Stocks of 35 of the viruses were prepared from infected mouse brain or Vero cells. These stocks were used in plaque reduction neutralization tests and to inoculate newborn mice for preparation of antigens. We were unable to work with Rift Valley fever (RVF) virus, since it is a restricted agent in the United States. However, Dr. C. J. Peters, U.S. Army Medical Research Institute of Infectious Diseases, Frederick, Maryland, kindly performed neutralization tests with this agent and

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TABLE 1

*Viruses used in complement-fixation and neutralization tests*

Virus serotype	Strain
Aguacate	VP-175A
Alenquer	Be H 301101
Anhanga	Be An 46852
Arumowot	Ar 1284-64
Belterra	Be An 356637
Buenaventura	Co Ar 3319
Bujaru	Be An 47693
Cacao	VP-437R
Caimito	VP-488A
Candiru	Be H 22511
Chagres	JW 10
Chilibre	VP-118D
Frijoles	VP-161A
Gabek Forest	Sud An 754-61
Gordil	Dak An B 496d
Icoaraci	Be An 24262
ISS. Phl. 18*	ISS. Phl. 18
Itaituba	Be An 213452
Itaporanga	Original
Joa	Be Ar 371637
Karimabad	I-58
Munguba	Be Ar 389707
Naples (Sandfly fever)	Naples
Nique	Nique-9C
Oriximina	Be An 385309
Pacui	Be An 27326
Punta Toro	D-40210 A
Rift Valley fever	Entebbe
Rio Grande	TBM4-719
Saint Floris	DAK An B 512
Salehabad	I-81
Sicilian (Sandfly fever)	Sicilian
Tehran	I-47
Toscana	ISS. Phl. 3
Turuna	Be Ar 352492
Urucuri	Be An 100049

\* ISS. Phl. 18 was supplied by Dr. Paola Verani, Istituto Superiore di Sanita, Rome. Its inclusion here is not intended to constitute priority of publication.

provided us with RVF immune serum and inactivated antigen.

*Antigens*

The RVF antigen used in complement-fixation (CF) tests was infected mouse liver which had been beta-propiolactone-inactivated and sucrose acetone-extracted.<sup>1</sup> The complement-fixing antigens used for Chilibre and Cacao viruses were prepared from infected Vero cells.<sup>2</sup> The remaining 33 viral antigens were made from infected newborn mouse brain. These were prepared as 10% crude brain suspensions in veronal-buffered saline, or by the sucrose acetone extraction method.<sup>1</sup>

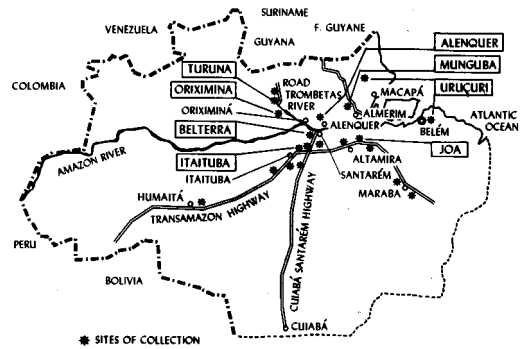


FIGURE 1. Map of the Amazon region of Brazil, showing study sites and localities where the eight new phleboviruses were isolated.

*Immune reagents*

The RVF antiserum was made in a sheep and was supplied to us by Dr. C. J. Peters. Specific hyperimmune ascitic fluids against each of the other 35 viruses were prepared in mice. Infected BHK-21 cells were used as the immunizing antigen for Cacao virus. Ten percent suspensions of infected mouse brain in phosphate-buffered saline, pH 7.2, were the antigens used to prepare the remaining immune reagents. The immunization schedule for the mice consisted of four intraperitoneal injections given at weekly intervals. Immunizing antigens were mixed with equal volumes of Freund's complete adjuvant just prior to inoculation. Sarcoma 180 cells were also given intraperitoneally with the final injection in order to induce ascites formation.

*Complement-fixation tests*

Complement fixation (CF) tests were done according to a microtechnique modified from Fulton and Dumbell,<sup>3</sup> using 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+.

*Neutralization tests*

Plaque reduction neutralization tests (PRNT) were performed in microplate cultures of Vero cells, using a fixed virus inoculum (40-150 plaque-forming units) against varying antiserum dilutions. After heat inactivation (56°C for 30 min), antisera were prepared in twofold serial dilutions (begin-

TABLE 2  
Source, date and geographical area of isolation of eight new phleboviruses from Brazil

Virus serotype	Strain	Source	Date of isolation	Geographical area
Urucuri*	Be An 100049	<i>Proechimys guyannensis</i> (blood)†	Apr 19, 1966	Utinga forest, Belém
Itaituba	Be An 213452	<i>Didelphis m. marsupialis</i> (blood)	Dec 7, 1971	Tapacurazinho stream, Itaituba
Alenquer	Be H 301101	Human blood	May 31, 1976	Ramal das Pias Alenquer
Turuna‡	Be Ar 352492	<i>Lutzomyia</i> sp. (human bait)	Jul 20-26, 1978	Km 4, Cachoeira Porteira, Oriximina
Belterra	Be An 356637	<i>Proechimys longicaudatus</i> (pooled viscera)	Sep 22, 1978	Belterra, Santarem
Joa	Be Ar 371637	<i>Lutzomyia</i> spp. (tree buttress collection)	Mar 29, 1979	Joa, Altamira
Oriximina	Be Ar 385309	<i>Lutzomyia</i> spp. (light trap)	Jun 26-30, 1980	Saracazinho, Porto Trombetas, Oriximina
Munguba	Be Ar 389707	<i>Lutzomyia umbratilis</i> (tree buttress collection)	Sep 20, 1980	Monte Dourado, Jari Almerim

\* Six additional strains of Urucuri virus were isolated from *Proechimys* captured in Utinga forest (4), at Serra do Navio, Amapa (1), and at Porto Trombetas (1).

† Sample positive or method of collection.

‡ Two additional isolates of Turuna virus were made from sand flies at Porto Trombetas.

ning at 1:10) in phosphate-buffered saline, pH 7.2, containing 0.5% gelatin. An appropriate amount of virus was then added to each dilution. Antiserum-virus mixtures were incubated overnight at 5°C prior to inoculation. Two microplate wells were inoculated with each antiserum dilution. The highest antiserum dilution producing  $\geq 90\%$  plaque inhibition was recorded as the endpoint.

Mouse neutralization tests (MNT) were done in suckling mice, using a final serum dilution of 1:4. Serum-virus mixtures were incubated for 1 hour at 37°C and then were inoculated intracerebrally.

#### Hemagglutination-inhibition tests

Hemagglutination-inhibition (HI) tests were performed as described by Clarke and Casals,<sup>1</sup> using a microtechnique; serum or plasma was acetone extracted.

#### RESULTS

Table 3 summarizes results of cross-CF tests between Urucuri, Itaituba, Alenquer, Turuna, Belterra, Joa, Munguba, Oriximina and the other known phleboviruses. In constructing this table, an attempt was made to group the viruses ac-

cording to their closest antigenic relatives. By CF test, each of the eight new Brazilian viruses is antigenically related to one or more of the existing phlebovirus serotypes. In fact, some of these agents are indistinguishable by CF test.

In an attempt to differentiate these antigenically related phleboviruses, PRNT were done with 20 selected viruses and antisera. Results are summarized in Tables 4 and 5. By PRNT, Munguba and Bujaru viruses were clearly separable. Likewise, RVF, Belterra and Icoaraci viruses were distinct. Joa and Frijoles viruses were also differentiated by this method.

The antigenic relationship between Oriximina, Itaituba, Alenquer, Nique, Candiru, Turuna, Punta Toro and Buenaventura is more complex than the others. By CF test, some of these agents are indistinguishable (Table 3). Most of them are also related by PRNT (Tables 4 and 5); however, a fourfold or greater difference in neutralizing antibody titers was demonstrated between the homologous and heterologous viruses, indicating that each of these agents represents a distinct phlebovirus serotype.

Toscana, Tehran and Naples viruses were also differentiated by PRNT, as were Salehabad and ISS. Phl. 18 viruses (Table 5).

The eight new Brazilian phleboviruses were initially isolated in newborn mice. All isolates killed suckling mice when inoculated intracerebrally; average survival time at the fourth passage level varied from 3.0 to 6.8 days. With the exception of Turuna virus which rarely killed, each of the new viruses was also lethal by the intraperitoneal route. In weanling mice, however, only Itaituba virus was lethal by the intracerebral route; none of the viruses killed weanling mice when injected intraperitoneally. A hemagglutinin active against goose erythrocytes was obtained from mouse brains infected with Urucuri, Joa and Belterra viruses after treatment with sucrose-acetone, but identical procedures failed to demonstrate a hemagglutinin for the other five new serotypes.

During our field studies, a total of seven strains of Urucuri virus and three of Turuna were recovered, whereas only a single isolation of each of the other six agents was made (Table 2). All of the Urucuri recoveries were made from spiny rats of the genus *Proechimys*. A variety of animal sera from the Utinga forest area were screened by HI test against Urucuri antigen. The results are shown in Table 6. The highest prevalence of antibodies was found in rodents (*Proechimys*) and edentates, although the sample of the latter was rather small. These serological results, as well as the seven recoveries of Urucuri virus from *Proechimys*, indicate that spiny rats play a role in the ecology of this agent.

Alenquer virus was recovered from the blood of a 27-year-old male forest worker. This individual had fever, headache and myalgia of 2 days' duration. His recovery was uneventful, and hospitalization was not required. A seroconversion to Alenquer virus was demonstrated by MNT on acute and convalescent sera from the patient.

In order to get some indication of the frequency of human infection with Alenquer virus in the Amazon region, sera from 227 selected residents of the region were screened by MNT against the virus. Only one of the specimens neutralized Alenquer virus, suggesting that the frequency of human infection with this agent is low.

#### DISCUSSION

Results of our study (Tables 3-5) indicate that Urucuri, Alenquer, Itaituba, Turuna, Belterra, Joa, Munguba and Oriximina viruses represent new serotypes in the phlebotomus fever group. The addition of these eight agents brings the total

number of serotypes in the group to 36. The antigenic relationship among the 36 viruses varies considerably. For example, Sicilian sandfly fever virus is unrelated to any of the known phleboviruses by CF test. Its inclusion in the genus is based on a weak relationship demonstrated by HI and fluorescent antibody tests.<sup>4,5</sup> Other viruses, such as Naples, Tehran and Toscana, are indistinguishable by CF test but are distinct by neutralization method (Tables 3 and 5). Still others, such as Itaituba, Nique, Candiru and Oriximina are closely related by both CF and PRNT (Tables 3 and 4). This complex antigenic relationship observed among phleboviruses may be related to their segmented genome, since the CF and neutralization determinants on the virion are thought to be coded for by different genes.<sup>6</sup>

The antigenic relationship demonstrated between RVF, Belterra and Icoaraci viruses (Tables 3 and 4) is very interesting. This relationship has subsequently been confirmed by HI test, immunofluorescence and enzyme-linked immunosorbent assay (Dr. J. M. Meegan, Yale Arbovirus Research Unit, personal communication). The close antigenic relationship between these three agents raises an important question of whether immunity to one would provide cross-protection against either of the other two. The answer to this question is relevant, since there is currently considerable interest in developing a safe and effective vaccine against RVF virus. It is possible that Belterra virus could be used in domestic animals as a vaccine, or that a non-pathogenic but immunogenic recombinant could be formed from segments of RVF and Belterra viruses. Further study in this area would seem appropriate.

Results of the CF test (Table 3) indicate the existence of seven antigenic complexes within the phlebotomus fever serogroup. These are as follows: 1) a Bujaru complex consisting of Bujaru, Aguacate and Munguba viruses; 2) a Rift Valley fever complex comprised of RVF, Belterra and Icoaraci viruses; 3) a Candiru complex consisting of Candiru, Itaituba, Alenquer, Nique, Turuna, Oriximina, Punta Toro and Buenaventura viruses; 4) a Naples complex comprised of Naples, Tehran, Toscana and Karimabad viruses; 5) a Frijoles complex consisting of Frijoles and Joa viruses; 6) a Salehabad complex comprised of Salehabad and ISS. Phl. 18 viruses; and 7) a Chilibre complex consisting of Chilibre and Cacao viruses. Undoubtedly, additional members will be added to this list as new phleboviruses are isolated.

TABLE 3  
Results of complement fixation tests with 36 phlebotomus fever group virus serotypes

ANTIGEN	Antiserum															
	AGU	BUJ	MUN	RVF	BTA	ICO	CHG	GOR	RG	ITA	ALE	NIQ	CDU	TUA	ORK	PT
Aguacate	<u>256</u> 128	0	0	—	0	0	0	0	0	0	0	0	0	0	0	0
Bujaru	<u>8</u> 32	<u>256</u> 128	<u>256</u> 128	—	0	0	0	0	0	0	0	0	0	0	0	0
Munguba	<u>16</u> 128	<u>128</u> 8	<u>256</u> 128	0	0	0	<u>8</u> 8	0	0	0	0	0	0	0	0	0
Rift Valley fever	0	0	<u>8</u> 16	<u>128</u> 256	<u>512</u> 256	<u>64</u> 128	<u>8</u> 16	<u>8</u> 8	<u>8</u> 8	<u>32</u> 32	0	0	0	0	0	0
Belterra	0	0	0	0	<u>2560</u> 640	<u>512</u> 512	0	0	0	0	0	0	0	0	0	<u>32</u> 32
Icoaraci	0	0	0	0	<u>512</u> 512	<u>1280</u> 640	0	0	0	0	0	0	0	0	0	0
Chagres	0	0	0	—	0	0	<u>256</u> 128	0	0	0	0	0	0	0	0	0
Gordil	0	0	0	—	0	0	<u>256</u> 32	0	0	0	0	0	0	0	0	0
Rio Grande	0	0	0	—	0	0	0	<u>256</u> 8	0	0	0	0	0	0	0	0
Itaituba	0	0	0	—	0	0	0	0	0	<u>256</u> 128	0	<u>16</u> 128	<u>256</u> 128	<u>128</u> 32	<u>64</u> 32	0
Alenquer	0	0	0	—	0	0	0	0	0	0	<u>256</u> 32	<u>256</u> 8	0	<u>64</u> 4	0	0
Nique	0	0	0	—	0	0	0	0	0	<u>32</u> 8	<u>64</u> 8	<u>256</u> 8	<u>8</u> 4	<u>256</u> 8	<u>16</u> 8	0
Candiru	0	0	0	—	0	0	0	0	0	<u>256</u> 128	0	<u>16</u> 128	<u>256</u> 128	<u>64</u> 128	<u>64</u> 128	0
Turuna	0	0	0	—	0	0	0	0	0	<u>64</u> 32	<u>32</u> 32	<u>128</u> 32	<u>64</u> 32	<u>256</u> 32	<u>8</u> 8	<u>16</u> 8
Oriximina	0	0	0	0	0	0	0	0	0	<u>128</u> 128	<u>16</u> 128	<u>128</u> 128	<u>256</u> 128	<u>64</u> 128	<u>256</u> 128	<u>16</u> 32
Punta Toro	0	0	0	—	0	0	0	0	0	0	0	<u>32</u> 128	0	<u>16</u> 128	0	<u>256</u> 128
Buenaventura	0	0	0	—	0	0	0	0	0	0	0	0	0	<u>8</u> 32	0	<u>128</u> 128
Toscana	0	0	0	—	<u>16</u> 8	0	0	0	0	0	0	0	0	0	0	0
Tehran	0	0	0	—	0	0	0	0	0	0	0	0	0	0	0	0
Naples	0	0	0	—	0	0	0	0	0	0	0	0	0	0	0	0
Karimabad	0	0	0	—	0	0	0	0	0	0	0	0	0	0	0	0
Urucuri	0	0	0	—	<u>8</u> 8	0	0	0	0	0	0	0	<u>8</u> 8	0	0	0
Pacui	0	0	0	—	<u>8</u> 32	0	0	0	0	0	0	0	0	0	0	0
Itaporanga	0	0	0	—	<u>8</u> 8	0	0	0	0	0	0	0	0	0	0	0
Sicilian	0	0	0	—	0	0	0	0	0	0	0	0	0	0	0	0
Anhanga	0	0	0	—	0	0	0	0	0	0	0	0	0	0	0	0
Frijoles	0	0	0	—	0	0	0	0	0	0	0	0	0	0	0	0
Joa	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cacao	0	0	0	—	0	0	0	0	0	0	0	0	0	0	0	0
Chilibre	0	0	0	—	0	0	0	0	0	0	0	0	0	0	0	0
Caimito	0	0	0	—	0	0	0	0	0	0	0	0	0	0	0	0
Saint Floris	0	0	0	—	0	0	0	0	0	0	0	0	0	0	0	0
Arumowot	0	0	0	—	0	0	0	0	0	0	0	0	0	0	0	0
Gabek Forest	0	0	0	—	0	0	0	0	0	0	0	0	0	0	0	0
Salehabad	0	0	0	—	0	0	0	0	0	0	0	0	0	0	0	0
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\* Reciprocal of highest antiserum dilution/highest antigen dilution. 0 = <4/4



TABLE 4  
Results of plaque reduction neutralization tests with selected phlebotomus fever group viruses

Virus	Antiserum										
	BUJ	MUN	RVF	BTA	ICO	ITA	ALE	NIQ	CDU	TUA	ORX
Bujaru	1,280*	160	—	—	—	—	—	—	—	—	—
Munguba	80	5,120	—	—	—	—	—	—	—	—	—
Rift Valley fever	<10	<10	320	640	40	<10	<10	<10	—	<10	<10
Belterra	—	—	<10	320	2,560	—	—	—	—	—	—
Icoaraci	—	—	<10	<10	10,240	—	—	—	—	—	—
Itaituba	—	—	<10	10	<10	5,120	<10	640	640	<10	320
Alenquer	—	—	—	—	—	<10	80	<10	<10	<10	<10
Nique	—	—	—	—	—	<10	<10	80	40	20	<10
Candiru	—	—	—	—	—	<10	<10	<10	1,280	10	<10
Turuna	—	—	—	—	—	<10	<10	40	320	1,280	40
Oriximina	—	—	—	—	—	160	20	160	320	20	160

\* Reciprocal of highest antiserum dilution producing  $\geq 90\%$  plaque inhibition. —, not tested

TABLE 5  
Results of plaque reduction neutralization tests with selected phlebotomus fever group viruses

Virus	Antiserum								
	PT	BUE	TOS	TEH	SFN	FRI	JOA	SAL	ISS
Punta Toro	1,280*	40	—	—	—	—	—	—	—
Buenaventura	20	40	—	—	—	—	—	—	—
Toscana	—	—	$\geq 5,120$	<10	40	—	—	—	—
Tehran	—	—	10	80	10	—	—	—	—
Naples	—	—	20	<10	320	—	—	—	—
Frijoles	—	—	—	—	—	5,120	1,280	—	—
Joa	—	—	—	—	—	320	10,240	—	—
Salehabad	—	—	—	—	—	—	—	320	<10
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\* Reciprocal of highest antiserum dilution producing  $\geq 90\%$  plaque inhibition. —, not tested.

The identification of eight new serotypes brings the total number of phleboviruses known to occur in the Amazon region of Brazil to 14. The large number of virus serotypes present in the region

TABLE 6  
Prevalence of hemagglutination-inhibiting antibodies to Urucuri virus in various wild animal sera from the Amazon region

Animal type	No. positive/total sera tested	% positive
Rodents		
<i>Proechimys</i>	144/1,253	11.5
Others	3/1,130	0.3
Marsupials	0/983	0
Primates	1/136	0.7
Edentates	1/8	12.5
Chiroptera	0/203	0
Ungulates	0/4	0
Birds	1/2,652	<0.1

may be related to its rich sand fly<sup>7</sup> and vertebrate fauna. Only two of the 14 viruses (Alenquer and Candiru) have been recovered from humans. The illness associated with Alenquer and Candiru virus infection is similar to the disease (pappataci or sandfly fever) produced by most other phleboviruses which infect humans.<sup>4, 8-10</sup> In view of the fact that Alenquer and Candiru viruses each have been recovered only once from man and that antibody rates to them among human residents of the Amazon region are low,<sup>4</sup> it appears that these two agents infrequently infect humans and thus are of little public health importance. Nonetheless, it is important to continue surveillance of human disease associated with these agents, especially among colonists moving into new geographic areas. In addition, more extensive serological surveys with all 14 phlebovirus serotypes should be done to properly assess their public health importance.

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