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Electron microscopic and antigenic studies of uncharacterized viruses. II. Evidence suggesting the placement of viruses in the family *Bunyaviridae*

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Summary. This is the second of three papers describing the use of electron microscopy and antigenic analyses intended to characterize and place in taxa more than 60 previously unclassified viruses. The first paper of the series describes the viruses we classified as provisional members of the families *Arenaviridae*, *Paramyxoviridae*, or *Poxviridae*; another paper, published separately, discusses the *Rhabdoviridae*. In this paper we report that electron microscopy provided sufficient evidence to place 17 of these viruses (Belem, Erve, Estero Real, Mojui dos Campos, Nyando, Odrenisrou, Okola, Pacora, Para, Santarem, Tanga, Telok Forest, Termeil, Thiafora, Thottapalayam, Wanowrie, and Yacaaba) in the family *Bunyaviridae* and to support the observations of others that Yogue and Kasokero viruses are members of this virus family. Subsequent antigenic studies allowed us to place some of these viruses in recognized antigenic groups and to establish new antigenic groups for others.

Introduction

In a previous paper, we introduced our attempts to place in taxa more than 60 uncharacterized and previously unclassified viruses isolated from a wide variety of vertebrates and invertebrates [16]. The viruses were accumulated over many

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years by arbovirologists investigating the ecology and epidemiologic importance of arboviruses. Because information about virus characterization was largely unavailable, taxonomic placement was not possible. Using electron microscopy, we provisionally placed viruses in taxa based on morphologic and morphogenetic characteristics. Antigenic relationships detected between these viruses and those of recognized serogroups provided additional information for further taxonomic refinement. This paper reports results of serologic tests with viruses that appear by electron microscopy to be members of the family *Bunyaviridae*.

Materials and methods

Information concerning the biologic and geographic sources of viruses described in this paper are presented in Table 1. For additional information, refer to the *International Catalogue of Arboviruses* [1]. Details of preparation of working seed viruses, passage in cell cultures and in mice, methods of electron microscopy, antibody preparation, tests for chemical and physical sensitivity, and serologic tests are presented in the first paper of this series [16]. Antibody preparations used are listed in Table 2.

We adapted an in situ enzyme-linked immunosorbent assay (EIA) [6] to confirm reactivity between antigens detected by indirect fluorescent antibody (IFA) but not confirmed by other tests. Cells predetermined to be susceptible to a given virus were grown in flat-bottomed wells of sterile 96-well plates (Costar, Cambridge, MA)*. Monolayer cultures were infected with about 1,000 plaque-forming units or 1,000 suckling mouse intracranial lethal $doses_{50}$. When approximately 25% of the cells showed cytopathic effects (CPE), the medium was decanted and replaced with 3% formalin in phosphate buffered saline (PBS). Plates were stored at room temperature overnight and then at 4 °C until used for EIA. For these tests, 50 µl of one of a series of twofold dilutions of ascitic fluid from mice hyperimmunized with a given virus and from control mice (normal mouse ascitic fluid) were added to separate wells, and the plates were incubated at 37 °C for 1 h. The plates were washed (in PBS containing 0.05% Tween 20) 10 times using a mechanical plate washer (Titertek Microplate Washer 120, Flow Laboratories, Irvine, Scotland). Then 50 µl of a commercially available goat anti-mouse immunoglobulin G conjugated to peroxidase was added to each well; the plates were incubated at 37 °C for 1 h and again washed 10 times. Fifty µl of substrate [ABTS; 2,2′-azino-di-(3-ethylbenzthiazoline sulfonate); Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD] was added to each well. The plates were incubated at room temperature for 5 to 10 min, and the optical density was determined and recorded mechanically (Titertek Multiskan MC, Eflab Oy, Helsinki, Finland). Ratios were calculated for the optical densities of wells to which antibody had been added and those to which normal mouse ascitic fluid had been added. Ratios that exceeded 2.0 were considered positive. In a second EIA, wells in alternating rows of infected and uninfected cells were reacted with hyperimmune mouse ascitic fluid, rather than reacting infected cells with hyperimmune and control ascitic fluids. Both tests gave essentially the same results.

Yogue and Kasokero viruses were included in these investigations because they have been shown to be antigenically related to each other, and electron microscopic observations indicated that Yogue virus is a member of the family *Bunyaviridae* [8]. However, they had

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* Use of trade names and commercial sources is for identification only and does not imply endorsement by the Pasteur Institute, Public Health Service, US Department of Health and Human Services, or Yale University.

Table 1. Strain, source, location, and date of original isolation of viruses shown to be members of the family *Bunyaviridae*

Virus	Strain	Source[a]	Location	Date of original isolation
Belem	BeAn 141106	*Pyriglena leucoptera* (bird)	Brazil	05/30/68
Erve	Brest/An 221	*Crocidura russala* (shrew)	France	05/05/82
Estero Real	K 329	*Ornithodoros tadaridae* (tick)	Cuba	04/29/80
Kasokero	UgZ 52969	*Rousettus aegyptiacus* (bat)	Uganda	08/??/77
Mojui dos Campos	BeAn 276121	(bat)	Brazil	01/09/76
Nyando	MP 401	*Anopheles funestus*	Kenya	12/16/59
Odrenisrou	DakArA 1131	*Culex albiventris*	Côte d'Ivoire	10/29/80
Okola	YM 50/64	*Eretmapodites chrysogaster*	Cameroon	06/02/64
Pacora	J 19	*Culex dunni*	Panama	10/16/58
Para	BeAn 280577	(sentinel mouse)	Brazil	06/06/75
Santarem	BeAn 238758	*Oryzomys* sp. (rat)	Brazil	12/06/73
Tanga	MP 1329	*Anopheles funestus*	Tanzania	03/12/62
Telok Forest	P72–4	*Macaca nemestrina* (monkey)	Malaysia	01/11/72
Termeil	BP 8090	*Aedes camptorhynchus*	Australia	07/25/72
Thiafora	DakAnD 11411	*Crocidura* species (shrew)	Senegal	02/26/71
Thottapalayam	VRC-66412	*Suncus murinus* (shrew)	India	01/01/65
Wanowrie	IG-700	*Hyalomma marginatum isaaci* (tick)	India	12/29/54
Yacaaba	NB 6028	*Aedes vigilax*	Australia	05/08/70
Yogue	DakAnD 5634	*Rousettus aegyptiacus* (bat)	Senegal	06/19/68

[a] Source was mosquitoes unless otherwise indicated

Table 2. Antibody preparations[a] with which apparent bunyaviruses were tested[b] by indirect immunofluorescence for virus identification

Grouping fluids: Groups Bunyamwera, California, Capim, Guama, Patois, Phlebotomus fever, Simbu. NIH Polyvalent fluids: 1 (Bahig, Burg el Arab, EgAn 1398-61, Matruh, Matariya, Tete), 2 (Alajuela, Belem, Gamboa, Jurona, Minatitlan), 3 (Bakau, Ketapang, Koongol, Mapputta, Maprik, Trubanaman, Wongal), 4 (Grand Arbaud, Nyamanini, Thogoto, Uukuniemi), 5 (Hughes, Lone Star, Matucare, Sawgrass, Soldado), 10 (Dera Ghazi Khan, Dhori, Upolu, Wanowrie), 11 (Anopheles A, Lukuni, Tacaiuma, CoAr 1071, CoAr 3624, Anopheles B, Boraceia, M'Poko, Turlock, Umbre), 12 (Bobia, Olifantsvlei, Okolo, Tataguine, Witwatersrand), 13 (Bwamba, Eretmapodites 147, Kamese, Mossuril, Nyando, Pongola), 14 (Bhanja, Congo, Dugbe, Ganjam, Hazara), 16 (Bandia, Johnston Atoll, Kaisodi, Lanjan, Quaranfil, Qalyub, Silverwater).
Bunyaviridae: Bunyavirus (Anopheles A) Las Maloyas, Virgin River, (Bunyamwera) Anhembi, Birao, Calovo, Lokern, Main Drain, Northway, Playas, Santa Rosa, Shokwe, Tlacotalpan, (Group C) Gumbo Limbo, Restan, Vinces, (California) Inkoo, Jamestown Canyon, Jerry Slough, Keystone, La Crosse, San Angelo, snowshoe hare, (Capim) Benevides, Juan Diaz, Moriche, (Gamboa) Pueblo Viejo, San Juan (strain 75 V-2374), (Guama) Ananindeua, Cananeia, Guaratuba, Itimirim, Mahogany Hammock, (Minatitlan) Palestina, (Patois) Abras, Pahayokee, (Simbu) Aino, Douglas, Inini, Kaikalur, Mermet, Nola, Peaton, Sabo, Sango, Shamonda, Shuni, Thimiri, Tinaroo, (Turlock) Lednice, (ungrouped) Kaeng Khoi; *Nairovirus* (Bhanja) Kismayo, (Hughes) Punta Salinas, (Sakhalin) Avalon, Clo Mor, Sakhalin, Taggert, (Upolu) Aransas Bay; *Uukuvirus* Argas 461, Manawa, Zaliv Terpeniya; *Phlebovirus* (Phlebotomus fever) Chilibre, Gordil, Rift Valley fever, Rio Grande, Toscana; *Hantavirus* (Hantaan) Hantaan, Prospect Hill, Puumala, Seoul; (bunyavirus-like) Antequera, Barranqueras, Belmont, Bobaya, Enseada, Erve, Keterah, Kowanyama, Nyando, Resistencia, Sunday Canyon, Yogue.
Others: Aride, Batken, Belem, Estero Real, Jos, Mojui dos Campos, Nyamanini (strain EgAr 1304), Odrenisrou, Okola, Oyo, Para, Santarem, Sebokele, Sembalam, Slovakia, Somone, Tanga, Telok Forest, Tembe, Termeil, Thiafora, Thottapalayam, Toure, Venkatapuram, Wongorr, Yacaaba

[a] Unless otherwise indicated, antibody to the prototype virus was used

[b] Uncharacterized viruses were tested for reactivity with all antibodies listed in this table. Many also were tested for reactivity with antibodies to other viruses, including murine viruses, but none reacted.

not been shown to be antigenic relatives of other viruses of the family. We examined both viruses by electron microscopy to determine relationships between them and viruses in recognized serogroups.

Results

Belem, Estero Real, Kasokero, Mojui dos Campos, Odrenisrou, Okola, Para, Santarem, Tanga, Telok Forest, Termeil, Thiafora, Thottapalayam, Wanowrie, Yacaaba, and Yogue had morphologic and morphogenetic characteristics in common with viruses of the family *Bunyaviridae* [11] (Figs. 1 and 2). These characteristics included shape (spherical or oval) and size (Estero Real virus is 65–205 nm in diameter, Thottapalayam virus is 95–185 nm in diameter; the

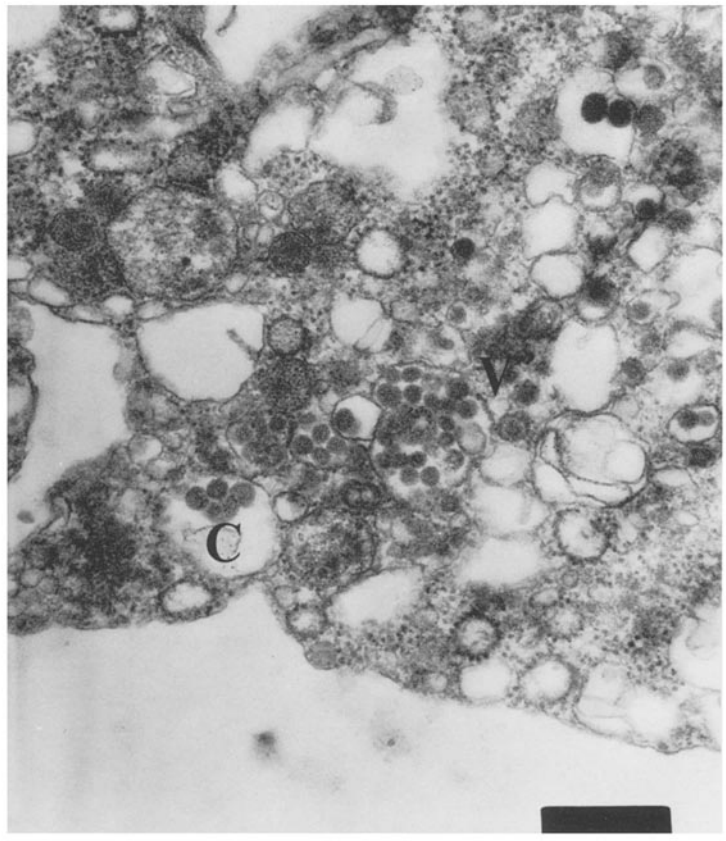

Fig. 1. Electron micrograph of a Vero cell 2 days after infection with Mojui dos Campos virus (strain BeAn 276121), showing virions (*V*) in cytoplasmic vacuoles (*C*) (× 46,500)

other viruses range in diameter from 78–108 nm; mean 86 nm). Enveloped virions were seen in extracellular spaces, singly or in groups, or within vacuoles and cisternae of the endoplasmic reticulum or the Golgi apparatus. Virion assembly was completed by budding from the Golgi apparatus and the endoplasmic reticulum; no precursors were observed before the budding process.

These 16 viruses were sensitive to sodium deoxycholate; they lost at least 2 $\log_{10}$ of infectivity after treatment. Subsequently, they were tested by one or more tests [IFA, HI, complement fixation (CF), and neutralization] with antibodies to viruses of the family *Bunyaviridae*. Santarem, Wanowrie, and Yacaaba viruses did not react by IFA with grouping fluids or antibodies to any of the known members of the family. Whereas antigenic studies confirmed that Kasokero and Yogue viruses are related to each other (data not shown), no relationships were detected between either of them and other viruses of the family *Bunyaviridae*. Cells infected with Pacora virus reacted by IFA with antibody to Pacora (homologous titer 40), Bobaya (10), and Douglas (10) viruses.

Mojui dos Campos virus reacted strongly by IFA with antibody to Nyando

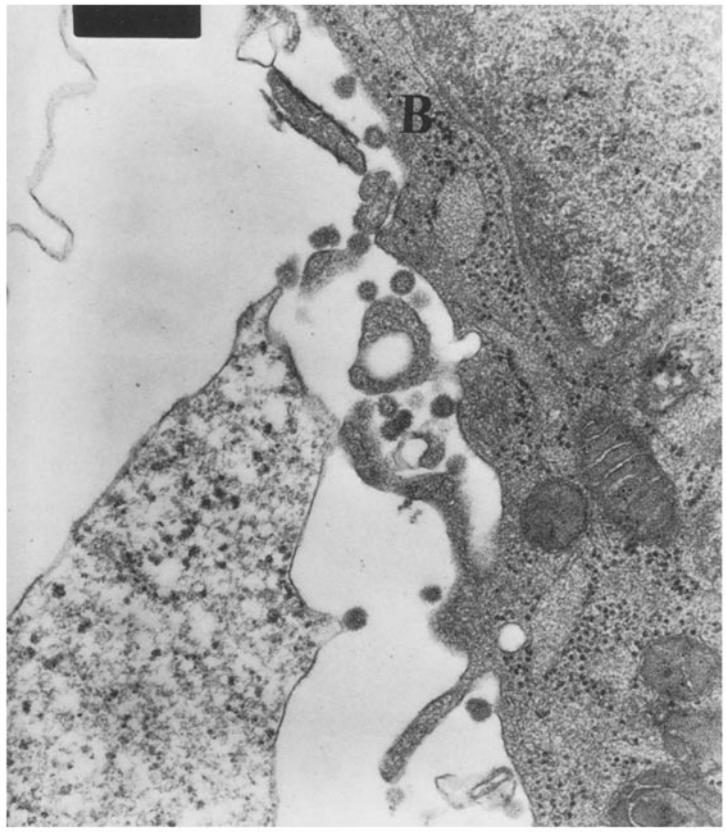

Fig. 2. Electron micrograph of a Vero cell 3 days after infection with Telok Forest virus (strain P 72-4), showing virion budding (*B*) (× 46,500)

(Nyando antigenic group) and San Angelo (California antigenic group) viruses (Table 3). These reactions were confirmed by CF tests, but neutralization tests with other Nyando and California antigenic group viruses (Eretmapodites 147 and La Crosse, showshoe hare, and Jamestown Canyon) did not provide evidence of antigenic relationships and did not extend these findings. However, cells infected with Nyando virus reacted with antibody to Jamestown Canyon and Keystone viruses of the California antigenic group and with Tinaroo of the Simbu antigenic group; antibody to Nyando virus did not react by IFA with cells infected with San Angelo or Keystone viruses. Hemagglutinins of Nyando, Jamestown Canyon, La Crosse, and snowshoe hare viruses were not inhibited by antibody to Eretmapodites 147, Mojui dos Campos, or Nyando viruses; homologous hemagglutination-inhibition (HI) titers were satisfactory.

Termeil virus reacted weakly by IFA with antibody to Inkoo and Keystone viruses (Table 4) and by IFA and CF with antibody to Jamestown Canyon virus. Termeil virus did not react by IFA with antibody to San Angelo, La Crosse, or snowshoe hare viruses, nor by CF with Inkoo or Keystone viruses, and it was not neutralized by antibody to Inkoo, Lumbo, or Keystone viruses.

Table 3. Antigenic relationships of Mojui dos Campos, Eretmapodites 147, Nyando, San Angelo, and other California serogroup viruses by indirect immunofluorescence (*IFA*), complement-fixation (*CF*), and neutralization (*N*) tests

Virus	Titer[a] of antibody to virus											
	MDC			ERET			NDO			SA		
	IFA	CF	N	IFA	CF	N	IFA	CF	N	IFA	CF	N
Mojui dos Campos	*160*	*64*	*160*	—	—	—	160	64	—	80	32	—
Eret. 147	40	—	—	*20*	*32*	*40*	640	1,024	—	—	—	—
Nyando	40	—	—	20	8	—	*640*	*1,024*	*640*	20	8	—
San Angelo	—	—	—	—	—	—	—	—	—	*80*	*640*	*640*

[a] — signifies <10 by IFA, <8 by CF, <20 by N

Table 4. Comparison of Termeil virus with California serogroup viruses by indirect immunofluorescence (*IFA*) and complement-fixation (*CF*) tests

Virus	Titer[a] of antibody to virus										
	TER		INK		JC		KEY		SA	LAC	SSH
	IFA	CF	IFA	CF	IFA	CF	IFA	CF	IFA	IFA	IFA
Termeil	*640*	*1024*	40	—	20	16	20	—	—	—	—
Inkoo	10	—	*640*	*512*	320	nt	640	nt	160	80	20
Jamestown Canyon	—	—	—	nt	*1280*	*512*	—	nt	—	—	—
Keystone	—	—	320	nt	160	nt	*640*	*512*	80	40	—
San Angelo	—	—	—	nt	20	nt	80	nt	*80*	20	—
La Crosse	—	—	320	nt	640	nt	640	nt	320	*320*	20
snowshoe hare	—	—	640	nt	640	nt	320	nt	640	320	*40*

[a] — signifies <8 CF, <10 IFA
nt Not tested

Antibody to Termeil (homologous titer $\geqslant$ 6,400) reacted in EIA with San Angelo (200), Jamestown Canyon (400), and Inkoo (200) viruses but not with Keystone virus (data not shown). We could not produce a hemagglutinin for Termeil virus, but antibody to that virus did not inhibit hemagglutinins of Jamestown Canyon, La Crosse, Tahyna, or snowshoe hare viruses (data not shown).

Para virus reacted by IFA with antibodies to the Simbu antigenic group viruses Kaikalur, Aino, Sango, and Shuni but not with a polyvalent (grouping) antibody prepared with many Simbu antigenic group viruses (Table 5). In addition, Para antigen reacted with antibody to Belem virus and with poly-

Table 5. Para and Kaikalur viruses compared with certain bunyaviruses by indirect immunofluorescence tests

Virus	Titer of antibody to virus							
	PARA	KAI	AINO	SAN	SHU	Group Simbu	Polyvalent 2[a]	BLM
Para	*80*	160	20	10	20	—[b]	20	20
Kaikalur	—	*5,120*	160	320	320	320	—	—
Aino	—	2,560	*5,120*	1,280	320	320	—	—
Sango	—	20	—	*320*	—	160	—	—
Shuni	—	1,280	1,280	1,280	*2,560*	640	—	—
Belem	—	—	—	—	—	—	160	*160*

[a] For constituent antibodies, see Table 2
[b] <10

Table 6. Comparison of Telok Forest, Bakau, Ketapang, Koongol[a], Nola and five other bunyaviruses by indirect immunofluorescence

Virus	TF	BAK	Poly 3[b]	KET	KOO	WOL	CVO	NOLA	ANU	CNA	Group Simbu	MAN
Telok Forest	*160*	160	20	10	40	—[c]	10	40	10	10	—	—
Bakau	—	*320*	160	40	—	—	—	40	—	—	—	—
Ketapang	—	160	20	*40*	—	—	—	40	—	—	—	—
Koongol	—	—	40	—	—	—	—	—	—	—	—	—
Wongal	—	—	20	—	20	*10*	—	—	—	—	—	—
Calovo	—	—	—	—	—	—	*160*	—	—	—	—	—
Nola	40	40	10	—	—	—	—	*320*	—	—	—	—
Ananindeua	—	—	10	—	—	—	—	—	*640*	—	—	10
Cananeia	—	—	—	—	—	—	—	—	—	*80*	—	—
Manzanilla	—	—	—	—	—	—	—	—	—	—	80	*320*

[a] NIH Polyvalent 3 (*Poly 3*) antibody had a titer of 40 when tested with cells infected with Koongol virus; antibody to Koongol virus had titers of 40 with Telok Forest and 20 with Wongal viruses; antibody to Koongol virus had a titer <10 when tested with cells infected with Koongol virus
[b] For constituent antibodies of this polyvalent preparation, see Table 2
[c] <10.

valent 2, which contains antibody to Belem virus. Antibody to Para virus did not react with Kaikalur, Aino, Sango, Shuni, or Belem viruses by IFA. Complement-fixation tests with Para and Kaikalur antigens and antibodies, and with polyvalent Simbu antibody, gave only minimal reactions (antibody to Kaikalur virus $8/\geqslant 1{,}024$ with Para antigen, polyvalent Simbu 8 with Para

Table 7. Comparison of Telok Forest virus with Bakau, Ketapang, Koongol, and Nola virus by complement-fixation tests

Antigen	Titer of antibody to virus					
	TF	BAK	KET	KOO	NOLA	Polyvalent 3[a]
Telok Forest	*256*	16	—[b]	—	16	8
Bakau	—	*⩾1,024*	16	—	8	256
Ketapang	—	512	*32*	—	8	128
Koongol	—	—	8	*32*	—	32
Nola	—	8	—	—	*⩾1,024*	—

[a] For constituent antibodies, see Table 2
[b] <8

Table 8. Comparison of Estero Real virus with Patois serogroup viruses by indirect immunofluorescence (*IFA*) and complement-fixation (*CF*) tests

Virus	Titer of antibody to virus								
	ER		ABR		BAB	PAH	PAT	ZEG	SR
	IFA	CF	IFA	CF[a]	IFA	IFA	IFA	IFA	IFA
Estero Real	*320*	*1,024*	40	32	—[b]	—	—	—	—
Abras	—	—	*640*	*1,024*	10	40	10	40	—
Babahoyo	—	—	320	nt	*80*	40	160	80	80
Pahayokee	—	—	80	nt	20	40	320	40	80
Patois	—	—	40	nt	20	80	*160*	80	80
Zegla	—	32	40	nt	10	40	80	*160*	80
Shark River	—	—	10	nt	20	80	80	80	*160*

[a] Estero Real virus did not react by CF with antibodies to Babahoyo, Pahayokee, Patois, Zegla, or Shark River viruses
[b] <8
nt Not tested

antigen) or were not reactive (antibody to Para virus < 8/64 with Kaikalur antigen), whereas polyvalent Simbu antibody had a titer of ⩾ 1,024 with Kaikalur antigen.

Telok Forest virus was shown by IFA to be related to Bakau virus and to other bunyaviruses (Table 6). Except for the reaction with antibody to Nola virus, Telok Forest reactivity with other viruses shown by IFA was not confirmed by CF tests (Table 7). Antibody to Nola virus (homologous titer 3,200) reacted by EIA with Telok Forest (3,200), Bakau (1,600), and Koongol (400)

Table 9. Comparison of Tanga and Okola viruses by indirect immunofluorescence (*IFA*) and complement-fixation (*CF*) tests

Virus	Titer of antibody to virus			
	TAN		OKO	
	IFA	CF	IFA	CF
Tanga	*320*	*256*	40	<8
Okola	20	<8	*320*	*128*

viruses (data not shown). Polyvalent 3 antibody, which contains antibodies to Bakau, Ketapang, Koongol, Maputta, Maprik, Trubanaman, and Wongal, had a CF titer of 8 with antigen of Telok Forest. Low titer CF reactions also were detected between Bakau, Ketapang, and Nola viruses and between Ketapang and Koongol viruses.

Estero Real virus reacted by IFA and CF with antibody to Abras virus of the Patois antigenic group (Table 8). Estero Real virus did not react with antibodies to any of the other five Patois group viruses and antibody to Estero Real virus did not react in IFA tests with any of the six Patois group viruses. However, the antigenic relationship of Estero Real virus to Zegla virus (related to Abras virus) was shown by CF (Table 8). These tests were repeated with these and other reagents; similar results were obtained.

Tanga and Okola reacted with each other by IFA but not CF (Table 9), and Tanga but not Okola virus reacted by IFA at minimal (10) or low (20 to 40) titer with antibodies to the bunyaviruses Anhembi, Birao, Cache Valley, Northway, Tensaw, Tlacotalpan, and Santa Rosa. Antibody to Tanga and Okola viruses did not react by IFA with antigens of these viruses. Furthermore, Okola but not Tanga virus reacted by IFA with polyvalent 12, which contains antibody to Okola virus; Tanga but not Okola virus reacted by IFA with a Bunyamwera grouping fluid. These low-level reactions may be additional evidence of antigen sharing among the bunyaviruses. A hemagglutinin, prepared from supernatant fluid of Vero cells infected with Okola virus by PEG precipitation and acetone extraction, reacted by HI with antibodies to Okala (80) and Tanga (10) viruses and with polyvalent 12 but not with antibody to grouping fluids Bunyamwera, Patois, Capim, Guama, California, Simbu or Group C or with antibody to Bwamba, Turlock, Tete, Tataguine, or Witwatersrand viruses; the hemagglutinin had a titer of 160 at optimal pH 5.7.

Odrenisrou virus is a member of the phlebotomus fever antigenic group. It is most closely related to Arumowot virus by IFA and CF tests (Table 10). RB Tesh (unpubl. data, 1988, Yale Arbovirus Research Unit, New Haven, CT) tested Odrenisrou virus and antibodies to 38 registered phleboviruses, including Rift Valley fever virus, and to six unregistered phleboviruses by serum dilution-

Table 10. Comparison of Odrenisrou virus with certain phleboviruses by indirect immunofluorescence (*IFA*) and complement-fixation (*CF*) tests

Virus	Titer of antibody to virus											
	ODR		AMT		CHG		BUJ		ICO		Group Phlebotomus fever[a]	
	IFA	CF	IFA	CF	IFA	CF	IFA	CF	IFA	CF	IFA	CF
Odrenisrou	*160*	*512*	160	64	40	*16*	40	32	80	32	80	32
Arumowot	160	32	*640*	*1,024*								
Chagres	20	8			*320*	*1,024*						
Bujaru	<10	8					*320*	*1,024*				
Icoaraci	<10	8							*640*	*1,024*		

Blanks indicate not tested
[a] For constituent antibodies, see Table 2

Table 11. Comparison of Thiafora and Erve viruses by indirect immunofluorescence (*IFA*) and complement-fixation (*CF*) tests

Virus	Titer of antibody to virus				
	THF		ERVE		Polyvalent 14[a]
	IFA	CF	IFA	CF	IFA
Thiafora	*160*	*64*	40	<8	10
Erve	80	16	*160*	*128*	10

[a] For constituent antibodies, see Table 2

plaque reduction neutralization tests. Only antibody to Arumowot virus (homologous titer 5,120) neutralized Odrenisrou virus (80), antibody to Odrenisrou virus (homologous titer 640) did not neutralize Arumowot virus (< 10).

Thiafora and Erve viruses are related to each other, as shown by IFA and CF tests, and, at a dilution considered minimally positive (10), both reacted by IFA with a polyvalent reagent that contains antibody to Crimean-Congo hemorrhagic fever viruses (Table 11). Neither virus reacted by IFA with antibodies to the viruses with which the polyvalent reagent was prepared (Crimean-Congo hemorrhagic fever, Hazara, Ganjam, Dugbe, and Bhanja). Furthermore, Thiafora virus was not reactive in EIA tests with monoclonal antibodies to nucleocapsids or glycoproteins of Crimean-Congo hemorrhagic fever virus or in immunoprecipitation assays with Crimean-Congo hemorrhagic fever group viruses (JF Saluzzo, pers. comm., 1988).

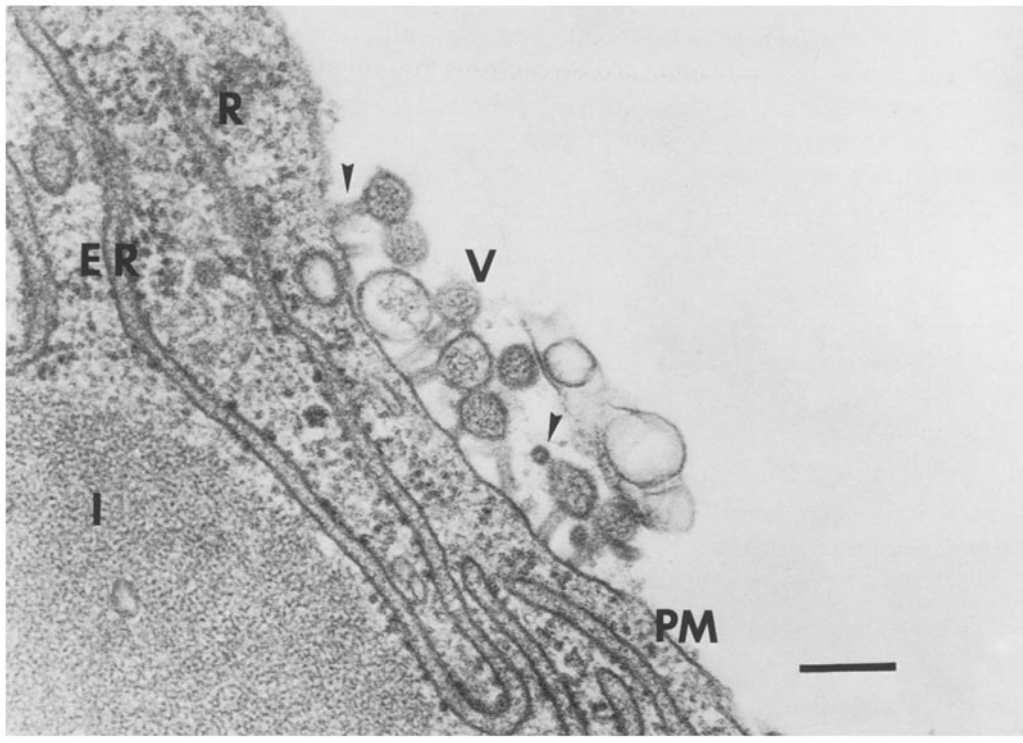

Fig. 3. Electron micrograph of Vero cell 9 days after infection with Thottapalayam virus (strain VRC-66412), showing virions (*V*) budding from the plasma membrane (*PM*). Many virions have a tail-like structure (▼), and the cell contains a large granulous inclusion body (*I*); ribosomes (*R*), endoplasmic reticulum (*ER*). Bar = 200 nm

Electron microscopy of Thottapalayam virus repeatedly revealed spherical or oval virions with both a head (mean diameter, 120 nm; range, 95–185 nm) and either a stalk-like (mean length, 150 nm; range, 100–400 nm) or a tail-like (mean width, 32 nm; range, 30–35 nm) structure budding from the plasma membrane only, as shown in Fig. 3. At first, we considered this an artifact of virus replication in certain cell lines or evidence of a relatively unimportant variation of the termination process of budding. However, subsequent electron micrographs revealed that virus particles free from cell structures and from cell debris retain this structure. Also, large granular and filamentous inclusions (about 1,400 nm long and 700 nm wide) were observed in the cytoplasm of infected cells. Thottapalayam virus did not react by IFA with any of the antibody preparations with which it was tested. However, because the tail-like structure resembled those described for hantaviruses [14], we investigated whether Thottapalayam virus is related to hantaviruses. By IFA, Thottapalayam virus reacted with antibodies to many hantaviruses, and antibody to Thottapalayam virus reacted with cells infected wiht hantaviruses (JM Dalrymple, pers. comm., 1988; data not shown).

Discussion

Unexpected, if not unusual, cross-reactions obtained between some of these formerly unclassified viruses and members of recognized serogroups may have been due to either the presence of contaminating viruses or to natural reassortants. However, we consider such possibilities unlikely because plaques formed by these viruses were of uniform size and shape and electron microscopic observations did not reveal viruses that are apparent members of more than one virus family.

Santarem, Wanowrie, and Yacaaba viruses were not shown to be related to members of the family *Bunyaviridae* and therefore could not be placed in an antigenic group. Minimal (10) reactions by IFA between these three viruses and Pacora, the ungrouped Bobaya virus (from a bird in the Central African Republic [1]), Douglas virus (Simbu antigenic group) provide some evidence that Santarem, Wanowrie, and Yacaaba viruses are bunyaviruses and that both Pacora and Bobaya viruses are distantly related to Simbu antigenic group viruses.

Antigenic relationships between Mojui dos Campos virus and the bunyaviruses Nyando and San Angelo presaged the observation by electron microscopy that Mojui dos Campos virus is a member of the family *Bunyaviridae* (Table 3). Mojui dos Campos virus reacts in IFA tests with antibodies to Nyando virus, a human pathogen [1] and the prototype of the Nyando antigenic group, and with San Angelo virus, a member of the California group, and antibody to Mojui dos Campos virus reacts by IFA with Nyando virus; however, no further confirmation of these relationships was obtained by IFA with Eretmapodites 147, an unregistered virus related to Nyando virus, or with La Crosse, showshoe hare, or Jamestown Canyon virus by IFA. Because antigen of Mojui dos Campos reacted with antibodies to Nyando and San Angelo viruses, we suggest that Mojui dos Campos virus is a member of the genus *Bunyavirus*. Similar intergroup antigenic reactions were the basis on which Casals proposed establishment of the Bunyamwera Supergroup, now generally considered equivalent to the genus *Bunyavirus* [4, 11]. We could not place Mojui dos Campos in a particular antigenic group. It may be the prototype of a serogroup whose other members have not yet been isolated, or the virus may be a member of either the Nyando or California serogroup. In any case, Mojui dos Campos virus provides an antigenic link between viruses of these serogroups. Serologic surveys of a wide variety of vertebrates, collected in the area from which Mojui dos Campos virus comes, have provided no evidence that indicates its natural history. People in that area have not been tested to determine prevalence of antibody to Mojui dos Campos but, because Nyando is a human pathogen and San Angelo is related to human pathogens, such a serosurvey is indicated.

Weak reactions also were obtained between Termeil virus and Inkoo and Keystone (California group) viruses by IFA and Jamestown Canyon (California group) virus by IFA and CF (Table 4). We believe these reactions either indicate

very distant antigenic relationships that would place Termeil within the California group or, more likely, simply reflect the membership of Termeil in the genus *Bunyavirus*.

Para virus, isolated from a sentinel mouse in Brazil and from *Culex* (*Melanoconion*) *ocossa* mosquitoes collected in Argentina, appears to be a distant relative of Simbu group viruses but is sufficiently closely related to warrant provisional placement within that group. The Simbu group virus most closely related to Para virus appears to be Kaikalur virus, from *Cx. tritaeniorhynchus* mosquitoes in India, but Para virus is easily distinguished from Kaikalur virus. The significance of IFA reactions between Para virus and antibody to Belem virus and polyvalent 2 antibody [which contains antibody to Belem (a bunyavirus), Jurona (a vesiculovirus), Gamboa and Alajuela (Gamboa group bunyaviruses), and Minatitlan (Minatitlan group bunyavirus)], is not clear. Belem virus reportedly reacts by HI with antibody to Group C viruses (APA Travassos da Rosa, pers. comm., 1984), indicating that it is related to viruses of the genus *Bunyavirus*; however, it has recently been reported that Belem virus is a member of the genus *Nairovirus* (RE Shope, unpubl. data, 1988).

Both Tanjong Rabok virus, from a *Macaca nemestrina* (monkey) caught in Malaysia in 1968, and Telok Forest virus, from the same species in Malaysia in 1972, have been isolated only once. Telok Forest virus is registered as a member of the Tanjong Rabok serogroup; both were considered unclassified viruses but known to be antigenic relatives [1]. Tanjong Rabok virus was not available to us, but examination of Telok Forest virus by electron microscopy provided morphologic evidence that it is a member of the family *Bunyaviridae*, which led us to perform the serologic tests summarized in Tables 6 and 7. Telok Forest virus is related to members of the Bakau group, Bakau and Ketapang viruses, and all three are antigenic relatives of Nola virus, previously considered a distant member of the Simbu group [9]. Complement-fixation test results supported this perception (Table 7). We deduce from the above that the Bakau group includes Bakau, Ketapang, Nola, Tanjong Rabok, and Telok Forest, viruses. All but the latter have been isolated in Malaysia; Bakau virus from *Culex* (*Lophoceratomyia*) sp. mosquitoes and a wild monkey (*Macaca fascicularis*) in Malaysia and from *Argas abdussalami* ticks in Pakistan, Ketapang virus from *Cx.* (*Lophoceratomyia*) sp. mosquitoes in Malaysia, Tanjong Rabok and Telok Forest viruses from monkeys in Malaysia, and Nola virus has been isolated from *Cx. perfuscus* mosquitoes in Central African Republic. Minimal (8) reactivity of Koongol antigen with antibody to Ketapang was detected by CF but no reactivity was detected by IFA. Wongal and Telok Forest antigens reacted by IFA with antibody to Koongol, to which Wongal is related [5]. We attribute the weak reactions between viruses of the expanded Bakau group, and Koongol, Wongal, Calovo (Bunyamwera group) and Ananindeua and Cananeia viruses (Guama group) to intergroup antigenic reactivity typical of bunyaviruses [4].

The bunyavirus Estero Real poses ecologic questions difficult to explain.

As shown in Table 8, this virus is related by both IFA and CF in one direction to Abras virus, a member of the Patois group [2], and it is related by CF to Zegla virus, also a member of the Patois group. No serologic evidence suggested that Estero Real is related to any other bunyavirus. Other viruses in the Patois group are closely related by IFA and CF [2] and have been isolated in Ecuador, Panama, Guatemala, Honduras, Belize, Mexico, and south Florida from mosquitoes and rodents. Estero Real virus was isolated from *Ornithodoros tadaridae* ticks collected from leaves of a palm tree colonized by bats in Cuba [10]. Ushijima et al. found molecular evidence that Patois group viruses occur as natural recombinants [15]. Shope and Causey had shown that Group C bunyaviruses occur in pairs in sympatric situations [13]. Patois group bunyaviruses also may occur in pairs in a given area, and this pairing may result in sharing of gene products, as a result of genetic drift, reassortment between closely related serotypes, or mutation enhanced by selective pressure due to immune responses of the vertebrate host. Estero Real has no known corollary in the Americas. Perhaps its antigenic "mate" is yet to be isolated. The ecologic questions may be answered when field collections of viruses are studied with molecular probes and monoclonal antibodies, such as has been done with California group and other bunyaviruses [7, 12].

Odrenisrou virus is a newly recognized member of the phlebotomus fever group (family *Bunyaviridae*, genus *Phlebovirus*). It is closely related to but distinct from Arumowot virus, as shown by IFA, CF and neutralization tests. Isolates of Arumowot virus from Sudan (the prototype) and from South Africa are indistinguishable by neutralization; thus, Odrenisrou virus is not a geographic variant of Arumowot virus (RB Tesh, pers. comm., 1988).

Thiafora and Erve viruses, isolated from *Crocidura* sp. (shrews) in Senegal and Cameroon, and in France, respectively, constitute the Thiafora group, which may be within the genus *Nairovirus* (family *Bunyaviridae*). That, both reacted with a polyvalent antibody but not with antibodies to the viruses used to prepare that antibody may indicate that these viruses are related only distantly to one of the constituent viruses. Little is known of the natural history of Thiafora and Erve, but because other nairoviruses have been isolated from ticks Thiafora and Erve viruses probably will be isolated from ticks in the future.

Tanga and Okola viruses, from *Anopheles funestus* mosquitoes collected in Tanzania and *Eretmapodites chrysogaster* mosquitoes collected in Cameroon, form the Tanga serogroup. Inadequate information exists about the natural history of these bunyavirus-like viruses. However, Tanga virus has been isolated from the blood of a febrile person in Burkina Faso (JP Digoutte, unpubl. information, 1987).

Although definitive placement of Thottapalayam virus requires molecular studies of viral RNA, it is reasonable to provisionally place this virus in the genus *Hantavirus* because it is antigenically related to hantaviruses. This virus, isolated from the spleen of an apparently healthy shrew (*Suncus murinus*) collected near Vellore, Tamil Nadu, India [3], has not been isolated since; serologic

surveys for antibody in humans or other species have not been done, and no other information about this virus exists. Having such information might help to determine whether Thottapalayam virus causes hemorrhagic fever with renal syndrome-like or nephropathia epidemica-like illnesses in India.

There also is inadequate information about all these viruses with respect to their ability to replicate in arthropods and to be transmitted to vertebrates. In another study (H Zeller and CJ Mitchell, unpubl. data), it was found that Gomoka, Itupiranga, Kannamangalam, Mojui dos Campos, Odrenisrou, Okola, Para, Telok Forest, Tembe, and Yacaaba viruses replicate in C 6/36 and AP-61 mosquito cells in vitro and in *Toxorhynchites amboinensis* mosquitoes inoculated intrathoracically. They found no evidence of replication in mosquito cells or in *T. amboinensis* inoculated with Araguari, Jos, Kammavanpettai, Matucare, Nyamanini, Santarem, Sebokele, Thiafora, Thottapalayam, Toure, Yogue, or Yoka viruses. These results complement our serologic results and may help us to understand the complex ecologies of these viruses.

The electron microscopic, serologic, and other studies we report here take the first steps toward placing these viruses in taxonomic order. Scant information is available about the ability of these viruses to cause disease in people, livestock, or wildlife, and further studies are needed.

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