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Bushbush, Ieri and Lukuni Viruses, Three Unrelated New Agents Isolated from Trinidadian Forest Mosquitoes.* (32009)

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During arbovirus studies carried out in the forests of eastern Trinidad from 1954 to 1964 a number of new agents were encountered, 10 of which have now been formally described(1-10). The present paper describes a further 3 new virus agents, antigenically unrelated, which are represented by 7 strains isolated exclusively from Trinidadian forest mosquitoes.

It is proposed to call these 3 viruses Bushbush, Ieri and Lukuni. The name Bushbush is derived from Bush Bush forest, a swamp island in the Nariva swamp, eastern Trinidad. Ieri, meaning "land of the humingbird," is an Amerindian name for Trinidad. Lukuni, the Amerindian word for people, refers to the inhabited forest camp site where the virus was first encountered.

Materials and methods. The methods used at this laboratory for capturing mosquitoes and processing them for virus isolation have been described previously(11). The techniques used for preparing viral antigens and carrying out hemagglutination-inhibition (HI) tests were similar to those of Clarke and Casals(12). Complement-fixation (CF) tests were done according to the method of Kerr(13). The neutralization (N) test was done by a method described previously(4).

Viruses were tested for sensitivity to sodium desoxycholate (DCA) by the method of Theiler(14). Titrations were done in 2-day-old mice inoculated intracerebrally (i.c.) with 0.02 ml of virus suspension. Titration endpoints were calculated by the method of Reed and Muench(15).

Primary hamster kidney cell cultures were grown and maintained as described by Kissling(16).

Results. The 7 isolates consist of 1 strain of Bushbush virus, 3 of Ieri virus and 3 of Lukuni virus. Table I provides pertinent data about their mosquito sources. In the following account, further details of isolation and information about identification and characterization are given separately for each virus.

1. Bushbush virus. Isolation. The single strain of this virus, TRVL 26668, was recovered from *Culex amazonensis* caught in Bush Bush forest (Table I). The mosquitoes were held overnight at ambient temperatures, sorted and stored at approximately −55°C until inoculated into mice 6 days later. Two-day-old (infant) mice inoculated i.c. with a portion of the mosquito suspension first showed signs of illness on day 8 postinoculation (p.i.). The virus was adapted relatively slowly, and not until the 4th brain passage did it kill all infant mice in the group, with deaths occurring on days 4-9 p.i. In January

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TABLE I. Strains of Bushbush, Ieri and Lukuni Viruses Isolated in Trinidad.

Virus		Mosquito source				
Name	TRVL strain No.	Species	No. in pool	Forest where collected	Date collected	Method of collection
Bushbush	26668*	*Culex (Eubonnea) amazonensis* (Lutz)	792	Bush Bush	6/10/59	Human bait
Ieri	8762*	*Psorophora (Janthinosoma) albipes* (Theobald)	137	Melajo	28/ 7/55	"
	8853	*Psorophora (Janthinosoma) ferox* (Humboldt)	186	"	4/ 8/55	"
	9156	*Psorophora (Janthinosoma) ferox* (Humboldt)	396	"	22–23/ 8/55	"
Lukuni	8923	*Aedes (Ochlerotatus) scapularis* (Rondani)	166	Melajo	10/ 8/55	"
	10076*	*Idem*	9	"	14/10/55	"
	20989	"	108	Rio Grande	28–29/ 7/58	"

* Prototype strain.

1960, an unsuccessful attempt was made to reisolate the virus from the portion of the original mosquito suspension that had been stored frozen. The virus was filtered through a bacteria-tight Seitz-EK pad.

Identification. TRVL 26668 virus was shown to be sensitive to DCA. The amount of virus as determined by the control titration was $10^{5.6}$ LD_{50} per 0.02 ml, while the amount after exposure to DCA was $\leq 10^{2.5}$ LD_{50} per 0.02 ml.

Attempts to prepare a hemagglutinin from infected infant mouse brain by sucrose-acetone and acetone-ether extraction were unsuccessful. A TRVL 26668 hyperimmune mouse serum failed to inhibit hemagglutination by the following viruses: eastern equine encephalitis (EEE), western equine encephalitis (WEE), Venezuelan equine encephalitis (VEE) and Mayaro in group A; dengue 2, Ilhéus, St. Louis encephalitis (SLE) and yellow fever in group B; and Caraparu-like, Marituba and Oriboca in group C (strain numbers of these viruses are given in Table II).

In CF tests, a TRVL 26668 antigen prepared by acetone-ether extraction of infected infant mouse brain did not react with immune reagents prepared from 36 other viruses but did react in its homologous system (Table II).

Additional HI studies with a TRVL 26668 mouse antiserum were carried out by Dr. Robert E. Shope at the Belém Virus Laboratory, Brazil. These studies showed that TRVL 26668 virus was closely related to Belém virus isolate BE An-20076 (now provisionally regarded as another strain of Bushbush virus) and less closely related to Belém

TABLE II. Viruses Used to Prepare Immune Reagents with Which Bushbush (TRVL 26668), Ieri (TRVL 8762) and Lukuni (TRVL 10076) Antigens Were Compared in CF Tests in Trinidad.*

Virus	Tested with		
	Bushbush	Ieri	Lukuni
Group A			
Chikungunya (Ross S-27)		X	X
EEE (TRVL 24443)	X	X	X
WEE (TRVL 25717)	X	X	X
VEE (TRVL 28215)	X	X	X
Sindbis (Egypt Ar-339)	X	X	X
Semliki Forest (Smithburn)		X	X
Group B			
Bat salivary gland (Rio Bravo M-64)		X	X
Dengue 2 (TRVL 1751)		X	X
Ilheus (Brazil: Laemmert & Hughes)		X	X
Japanese B (Nakayama)		X	X
Ntaya (Smithburn & Haddow)		X	X
Russian SSE (Parker strain)		X	X
Spondweni (SA Ar-94)		X	X
St. Louis (TRVL 9464)	X	X	X
Makonde (Haddow)		X	X
West Nile (Egypt 101)		X	X
Yellow fever (TRVL 2942)		X	X
Zika (MR-766)		X	X
Group C			
Apeu (Belem An-848)	X	X	X
Marituba (Belem An-15)	X	X	X
Oriboca (Belem An-17)	X	X	X
Caraparu-like (TRVL 34053)	X	X	X
Itaqui (Belem An-12797)	X	X	X
Murutucu (Belem An-974)	X		
Nepuyo (TRVL 18462)	X	X	X

TABLE II (continued)

Virus	Tested with Bush-bush	Ieri	Lukuni
Guama group			
Catu (Belem H-151)	X	X	X
Guama (Belem H-5056)	X	X	X
Bimiti (TRVL 8362)	X	X	X
Bunyamwera group			
Kairi (TRVL 8900)	X	X	X
Cache Valley (TRVL 20659)	X	X	X
Bunyamwera (Smithburn)	X		X
Wyeomyia (TRVL 8349)	X	X	X
Guaroa (Colombia H-35211)	X		X
California complex			
Calif. enceph. (BFS-283)			X
Melao (TRVL 9375)	X	X	X
Other viruses			
Anopheles A (Roca-Garcia)	X	X	
Bwamba (M-459)	X	X	X
Naples phlebotomus fever (Sabin)		X	X
Simbu (SA Ar-53)	X	X	X
Manzanilla (TRVL 3587)	X	X	X
Oropouche (TRVL 9760)	X	X	X
Tacaribe (TRVL 11573)	X	X	X
Turlock (Lennette 781-19)	X	X	X
Aruac (TRVL 9223)	X	X	X
Chenuda (Egypt Ar-1152)		X	X
Quaranfil (Egypt Ar-1095)		X	X
Rift Valley fever (Lunyo)	X	X	X
Tacaiuma (Belem An-73)	X	X	X
Triniti (TRVL 7994)	X	X	X
Wad Medani (Egypt Ar-492)		X	X
Mengo	X	X	X
Mouse encephalomyelitis (GD VII)	X	X	X

* The 3 agents described in this paper were each tested with one another. No reactions occurred except in the case of the homologous systems: Bushbush, 32/32; Ieri, 128/32; Lukuni, 512/128 (reciprocal of serum titer/reciprocal of antigen titer).

isolate BE An-10615 (provisionally named Guajara)(17). These viruses, along with a third Belém virus, Capim, constitute the Capim complex(18). It should be noted that Shope has succeeded in preparing a hemagglutinin for BE An-20076 virus from brain of infant mice by sucrose-acetone extraction (17).

Biological properties. In a single experiment, primary hamster kidney cell cultures inoculated with Bushbush virus did not support growth of the virus and no cytopathic effect was observed.

The amount of virus in a 20% infant mouse brain suspension of Bushbush virus in bovalbumin diluent (10th brain passage) was shown to be $10^{5.6}$ LD_{50} per 0.02 ml in 2-day-old mice inoculated i.c. and $10^{5.0}$ LD_{50} per 0.03 ml in 2-day-old mice inoculated intraperitoneally (i.p.). This virus preparation did not produce illness or death in weanling (28- to 35-day-old) mice inoculated i.c. or i.p.

At the Rockefeller Foundation Virus Laboratories in New York, Bushbush virus was readily maintained for several passages in both *Aedes aegypti* and *Culex quinquefasciatus* by serial mosquito inoculations of mosquito salivary glands(19).

2. Ieri virus. Isolation. The 3 strains of Ieri virus were isolated from *Psorophora* spp. (Table I) collected at the Melajo forest tree station(11) while lumberers were working in the forest. The 3 lots of mosquitoes were held overnight at 4°C, identified the next morning and inoculated into mice either immediately or after 24 hours' storage in a CO_2-ice chest.

In the case of the prototype strain, TRVL 8762, 2-day-old (infant) mice inoculated i.c. with a portion of the mosquito suspension first showed signs of illness on days 13-15 p.i. After 3 brain-to-brain passages, however, all infant mice sickened in 3-4 days p.i. The isolations of strains TRVL 8853 and TRVL 9156 followed a similar pattern. TRVL 8762 virus was filtered through a bacteria-tight Seitz-EK pad.

The 3 strains were shown to be indistinguishable by CF test in studies undertaken by Dr. Edward Belle at this laboratory.

In 1959 unsuccessful attempts were made to reisolate the 3 strains from portions of the original mosquito suspensions that had been stored frozen.

Identification. The prototype strain of Ieri virus was shown to be sensitive to DCA. The amount of virus as determined by the control titration was $10^{4.1}$ LD_{50} per 0.02 ml, while the amount after exposure to DCA was $\leq 10^{2.5}$ LD_{50} per 0.02 ml.

Attempts to prepare a hemagglutinin from TRVL 8762 virus by sucrose-acetone and acetone-ether extraction of infected infant mouse brain were unsuccessful. A TRVL 8762 hyperimmune mouse serum failed to inhibit hemagglutination by the following viruses: EEE, WEE, VEE and Mayaro in group A; dengue 2, Ilhéus, SLE and yellow fever in group B; and Caraparu-like and Ori-

boca in group C (strain numbers are given in Table II).

In CF tests done in Trinidad, a TRVL 8762 antigen prepared by acetone-ether extraction of infected infant mouse brain did not react with immune reagents prepared from 50 other viruses but did react in its homologous system (Table II).

In further studies carried out at the Rockefeller Foundation Virus Laboratories in New York, weak cross-reactions were demonstrated in a CF test with TRVL 8762 virus and 2 other Trinidadian viruses, Triniti and Aruac(20). It was not possible to confirm these cross-reactions or to show a relationship among the 3 viruses in N test at this laboratory or in studies done by one of the authors at the Yale Arbovirus Research Unit, New Haven.

Biological properties. Seven-day-old chick embryos were inoculated with the 3 strains of Ieri virus by the yolk sac, allantoic and amniotic routes. On day 4 p.i. yolk sac material and allantoic and amniotic fluids were harvested and inoculated i.c. into infant mice; on the same day, passages were made to additional embryos, from which material was likewise harvested on day 4 p.i. When 1st passage yolk sac material was inoculated into mice, TRVL 9156 virus could not be detected, results were inconclusive for TRVL 8762 virus because of nonspecific mouse deaths, but TRVL 8853 virus was recovered. However, none of the viruses could be detected in mice inoculated with 2nd passage yolk sac material or with allantoic or amniotic fluids of either passage.

The amount of virus in a 20% infant mouse brain suspension of TRVL 8762 virus in bovalbumin diluent (10th mouse brain passage) was shown to be $10^{3.6}$ LD_{50} per 0.02 ml in 2-day-old mice inoculated i.c. This virus preparation did not produce illness or death in 2-day-old mice inoculated i.p. or in weanling (28- to 35-day-old) mice inoculated i.c. or i.p.

At the Rockefeller Foundation Virus Laboratories, TRVL 8762 virus was readily maintained for several passages in both *Aedes aegypti* and *Culex quinquefasciatus* by serial mosquito inoculations of mosquito salivary glands(19).

3. Lukuni virus. Isolation. The 3 strains of Lukuni virus were each isolated from *Aedes scapularis*. TRVL 10076, the prototype strain, was recovered from mosquitoes collected in the Melajo forest during epidemiological investigations which followed on the isolation of Oropouche virus from serum of a febrile patient in September 1955(3). The point of collection was close to the camp site of a charcoal burner and about 0.25 mile from the tree station(11). The mosquitoes were held overnight at 4°C, and the next morning a portion of the mosquito suspension was inoculated i.c. into a group of 2-day-old (infant) mice and a group of weanling (28- to 35-day-old) mice. Virus was recovered only from the infant mice. At the 3rd infant mouse brain passage, however, the virus killed infant mice in 4 days and in weanling mice produced illness in 5-6 days and death in 7-13 days. Similar isolation patterns were observed with strains TRVL 8923 and TRVL 20989. TRVL 10076 virus was filtered through a bacteria-tight Seitz-EK pad.

The antigenic relationship of the 3 strains was discovered initially in the course of CF studies with a large number of unidentified isolates from Trinidadian forest mosquitoes. Further studies showed that the 3 were indistinguishable in N test.

In May 1959, an unsuccessful attempt was made to reisolate TRVL 10076 virus from the portion of the original mosquito suspension that had been stored frozen since October 1955. In July 1959, TRVL 20989 virus was reisolated from the original specimen. No reisolation attempt was made on specimen TRVL 8923, which was misplaced.

A fourth strain of Lukuni virus has been isolated in Brazil from *Anopheles (Stethomyia) nimbus* collected at Km 94 on the Belém-Brasilia highway(21).

Indentification. TRVL 10076 virus was shown to be sensitive to DCA. The amount of virus as determined by the control titration was $10^{4.8}$ LD_{50} per 0.02 ml, while the amount after exposure to DCA was $\leq 10^{1.9}$ LD_{50} per 0.02 ml.

Attempts to prepare a hemagglutinin from TRVL 10076 virus by sucrose-acetone and acetone-ether extraction of infected infant mouse brain were unsuccessful. A TRVL 10076 hy-

TABLE III. CF Tests with Lukuni and Anopheles A Antigens and Mouse Hyperimmune Ascitic Fluids.

Antigen	Ascitic fluid		
	Lukuni	Anopheles A	Bwamba (control)
Lukuni	128/512*	32/256	0/0
Anopheles A	16/128	256/1024	0/0
Bwamba (control)	0/0	0/0	32/128

* Reciprocal of antigen titer/reciprocal of ascitic fluid titer.

perimmune mouse serum failed to inhibit hemagglutination by the following viruses: EEE, WEE, VEE and Mayaro in group A; dengue 2, Ilhéus, SLE and yellow fever in group B; and Caraparu-like and Oriboca in group C (strain numbers are given in Table II).

In CF tests done in Trinidad, a TRVL 10076 antigen prepared by acetone-ether extraction of infected infant mouse brain did not react with immune reagents prepared from 52 other viruses but did react in its homologous system (Table II). Later Dr. Loring Whitman, at the Rockefeller Foundation Virus Laboratories in New York, found and confirmed in repeated CF tests a significant degree of cross-reactivity with Anopheles A virus(22). Results of a typical test are shown in Table III.

In N tests done in Trinidad, TRVL 100076 virus was not neutralized by antisera prepared from the following viruses: Una (TRVL 29494), Cache Valley, Kairi, Wyeomyia, Melao, Aruac, Manzanilla, Nepuyo, Oropouche, Tacaribe, Triniti, Bushbush (TRVL 26668) and Ieri (TRVL 8762). Further N tests were performed at the Yale Arbovirus Research Unit in New Haven, using mouse hyperimmune ascitic fluids. With Anopheles A virus, the homologous immune fluid had a neutralizing index of 3.9 logs of virus while that of the Lukuni fluid was zero. With Lukuni virus, the homologous fluid had a neutralizing index of more than 4.0 logs of virus while that of the Anopheles A fluid was between 1 and 2 logs of virus. On the basis of these results, Lukuni virus is considered to be related to, although easily distinguishable from, Anopheles A virus.

Biological properties. In a single experiment, primary hamster kidney cell cultures inoculated with TRVL 10076 virus did not support growth of the virus and no cytopathic effect was observed.

Seven-day-old chick embryos were inoculated with TRVL 10076 virus by the yolk sac, allantoic and amniotic routes and passages were made from materials harvested on day 4 as described for Ieri virus. With 1st passage materials, results were inconclusive for the yolk sac suspension because of nonspecific deaths in the mice, and virus was recovered from the amniotic fluid but was not present in the allantoic fluid. No virus could be detected in 2nd passage materials.

The amount of virus in a 20% infant mouse brain suspension of TRVL 10076 virus in bovalbumin diluent (23rd mouse brain passage) was shown to be $10^{2.8}$ LD_{50} per 0.02 ml in 2-day-old mice inoculated i.c. and $\leq 10^{0.5}$ LD_{50} per 0.03 ml in 2-day-old mice inoculated i.p. The amount of virus in this preparation inoculated into weanling mice i.c. was $10^{2.6}$ LD_{50} per 0.03 ml. Weanling mice inoculated i.p. with 0.2 ml of a 10% suspension of the virus in physiological saline showed no signs of illness.

At the Rockefeller Foundation Virus Laboratories, TRVL 10076 virus was readily maintained for several passages in both *Aedes aegypti* and *Culex quinquefasciatus* by serial mosquito inoculations of mosquito salivary glands(19).

Serum survey. Sera from 10 members of the Trinidad laboratory field staff and 5 residents of Rio Claro, a small town in southeastern Trinidad, were tested with TRVL 10076 virus in N test. Sera from 3 of the field staff and 1 Rio Claro resident neutralized 209 LD_{50} or more of the virus in this test.

Discussion. Little is known of the ecology of Bushbush, Ieri and Lukuni viruses. They are thought to be arboviruses because of their sensitivity to DCA and their isolation from forest mosquitoes. Although thousands of specimens from humans, small mammals and birds in Trinidad have been tested for virus

in mice during the past 10 years, none of these 3 viruses have been recovered from these sources.

Satisfactory results with the N test have been obtained with Lukuni virus only, and it has not been possible to carry out serosurveys with Bushbush and Ieri viruses. Limited studies with Lukuni virus have demonstrated the presence of neutralizing antibody to this virus in the serum of Trinidadians. Many undiagnosed human fever cases occur in Trinidad and 1 or more of these 3 viruses may be responsible for some of the cases. The medical or other importance of the 3 viruses remains to be determined.

Summary. Seven virus strains, all isolated from forest mosquitoes collected in eastern Trinidad between 1955 and 1959, have been shown to represent 3 unrelated new viruses, designated Bushbush, Ieri and Lukuni. Neutralizing antibody to Lukuni virus has been demonstrated in human serum. No relationship has been found between either Bushbush or Ieri virus and recognized arboviruses, but Lukuni virus has been found to be related to Anopheles A virus in complement-fixation and neutralization tests. Some of the biological properties of the 3 viruses are described.

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A Plaque Method for Titration of Frog Viruses Using Starch Gel Overlay.* (32010)

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In the course of studies on the Lucké renal adenocarcinoma of the leopard frog (*Rana pipiens*) 3 laboratories have reported isolation of viruses from frog tissues. The development of plaquing methods using solid overlay permits the accurate quantitative study of these new agents.

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