Relationships of Anopheles A Group Arboviruses (37344)

CHARLES H. CALISHER, DONNA R. SASSO, KATHRYN S. C. MANESS, VLADIMIR N. GHEORGHIU,[1] AND ROBERT E. SHOPE[2]
(C. C. Shepard)

Arbovirus Unit, Virology Section, Laboratory Program, Center for Disease Control, Atlanta, Georgia 30333

Anopheles A virus was isolated in 1940 from a pool of *Anopheles boliviensis* mosquitoes collected in Colombia (1). Lukuni virus was isolated from a pool of *Aedes scapularis* mosquitoes collected in Trinidad (2). These virues were shown to be related antigenically (3) and compose the Anopheles A group. Until recently they were the only known members of this particular group of arboviruses.

However, six recently isolated viruses are antigenically related to members of the Anopheles A group (4–7). This paper presents the results of cross-testing all eight viruses and proposes a classification scheme for the newly enlarged group. Hemagglutination-inhibition (HI), complement-fixation (CF), neutralization (NT), and precipitin tests were performed in the process of antigenic characterization. No attempt was made to determine epidemiologic or epizoologic significance of these viruses.

Materials and Methods. The viruses used were prototype viruses Anopheles A (Original), suckling mouse passage (SMP) 6; Tacaiuma (BeAn 73), SMP 12; Lukuni (Tr 10076), SMP 3; CoAr 3627, SMP 1; CoAr 1071, SMP 6; CoAr 3524, SMP 6; and SpAr 2317, SMP 4. They were all obtained from the Yale Arbovirus Research Unit (YARU). Besides these seed viruses, Dr. Nick Karabatsos of YARU supplied homologous hyperimmune mouse ascitic fluids (HIAF) to all but Anopheles A virus. HIAF prepared against the latter was obtained from the Research Resources Branch, NIH, Bethesda, MD.

Strain Col An 57389 (SMP 5) was originally isolated in suckling mice from the undiluted serum of an eight-year-old donkey (*Equus assinus*), bled July 17, 1969 in Guacoche, 30 km northwest of Valledupar, Colombia. Hyperimmune mouse ascitic fluid to this virus was produced at CDC. After one passage of each strain in 2- to 4-day-old suckling mice inoculated intracranially (ic) aliquots of infected brain were either stored as 20% stock suspensions in 0.4% bovine albumin-phosphate buffered saline (pH 7.8–8.0) for use in neutralization or titration tests or were extracted with sucrose–acetone for use in HI and CF tests (8). Since satisfactory hemagglutinins were not produced by the standard sucrose–acetone method, an additional passage of stock virus was made, and trypsin-treated, sonicated, sucrose–acetone antigens produced (9). HI tests were performed by the microtiter technique (8) and CF tests by the $LBCF_{50}$ technique (Microtiter modification) (10).

Constant virus (approximately 100 plaque-forming units) diluted serum neutralization tests were performed using BHK-21 cells. One-tenth milliliter of serum–virus mixture, incubated at 4° for 45–60 min, was allowed to adsorb for one hour at 35° onto cells grown to a monolayer in 1 oz. prescription bottles. Then the virus-infected cells were overlayed with 4 ml of medium and incubated at 35°. Incubation of the overlayed virus-serum mixtures at 37° resulted in loss of virus titer.

After five days at 35°, a second overlay containing 1:30,000 neutral red was added

[1] Formerly: W.H.O. Fellow from Romania, CDC, Atlanta, Georgia 30333.

[2] Director, Yale Arbovirus Research Unit, Yale University College of Medicine, New Haven, CT 06510.

TABLE I. Titers of Eight Anopheles A Group Virus Isolates in Mice and Cell Cultures.

Virus	Host: Mice[a] (average survival time)				Cell cultures[b,c]		
	$SMicLD_{50}$	$SMipLD_{50}$	$WMicLD_{50}$	$WMipLD_{50}$	BHK-21	$LLCMK_2$	VERO
Anopheles A	8.2 (4)	5.2 (8)	8.3 (6)	≤3.0	7.8	5.0	>8.0
Tacaiuma	8.7 (8)	8.2 (8)	5.7 (10)	≤3.0	7.6	3.0	7.0
Lukuni	7.8 (5)	≤3.2	6.9 (8)	≤3.0	6.3	<2.0	7.1
CoAr 3627	6.3 (13)	5.7 (13)	≤3.0	≤3.0	>6.3	<2.0	<2.0
CoAr 1071	6.7 (15)	6.0 (18)	≤3.0	≤3.0	5.9	<2.0	<2.0
CoAr 3624	7.3 (8)	≤3.2	≤3.0	≤3.0	4.9	<2.0	5.3
SPAr 2317	8.2 (8)	7.7 (9)	6.2 (11)	≤3.0	7.0	2.0	6.3
ColAn 57389	7.2 (5)	≤3.2	5.9 (8)	≤3.0	5.1	<2.0	5.9

[a] Titers given as reciprocal of $\log_{10}$/ml of undiluted inoculum.
[b] Titers given as reciprocal of $\log_{10}$ PFU/ml of undiluted inoculum.
[c] No plaques were observed in Pekin duck embryo cells inoculated with any of these viruses.

and the cultures reincubated at 35° for one week. Cultures were examined daily, and if plaques did not appear during that week-long period, the cultures were placed at ambient temperatures (26–28°) for as long as an additional 18 days. A 90% plaque reduction was considered significant in these tests.

For precipitin-in-agar tests, we used a method published previously (11). Briefly, SM were infected by ic inoculation with one or another of the eight viruses. When the mice presented typical signs of encephalitis or another characteristic illness, they were sacrificed, their brains removed by aspiration, and the crude undiluted brei used as an antigen. The same HIAF's as used in all other tests were distributed in wells opposed to one or another of these crude antigens. After 24–48 hr of incubation at 4°, the results were read and recorded as to arc intensity and pattern.

Results and Discussion. Preliminary comparison of the eight strains for determination of host spectrum and pathogenicity gave the results summarized in Table I. Higher LD_{50} titers were obtained when suckling mice were inoculated intracranially than intraperitoneally. By either route, the LD_{50} titers obtained in weaned mice were, with the exception of Anopheles A, less than in sucklings inoculated similarly. Deaths were not observed in weaned mice inoculated intraperitoneally.

With two exceptions (CoAr 3627 and CoAr 1071), Vero cells were as susceptible as the cell line of choice, BHK-21. The latter supported the replication of all eight strains with the production of sufficient virus titers to permit *in vitro* neutralization tests. Plaques in $LLCMK_2$ cells were observed after inocula-

TABLE II. Results of Cross-Testing Eight Anopheles A Group Viruses by Complement Fixation.

Virus	Reciprocal antibody titer: AnA	CoAr 3624	LUK	ColAn 57389	TCM	CoAr 3627	CoAr 1071	SPAr 2317
Anopheles A	64	≥512	≥512	128	8	8	<8	8
CoAr 3624	64	*≥512*	128	128	8	8	8	8
Lukuni	<8	8	*≥512*	64	8	8	<8	8
ColAn 57389	8	16	≥512	*≥512*	8	8	<8	8
Tacaiuma	8	<8	8	8	*128*	≥512	16	32
CoAr 3627	8	<8	≥512	8	≥512	*≥512*	≥512	256
CoAr 1071	8	<8	8	8	16	≥512	*≥512*	8
SPAr 2317	8	8	16	16	64	256	16	*256*

TABLE III. Results of Cross-Testing Eight Anopheles A Group Viruses by Precipitin in Agar.[a]

Virus	Hyperimmune mouse ascitic fluid to: AnA	CoAr 3624	LUK	ColAn 57389	TCM	CoAr 3627	CoAr 1071	SPAr 2317
Anopheles A	3	3	0	2	0	0	0	0
CoAr 3624	3	2	0	1	0	0	0	0
Lukuni	0	0	4	3	0	2	0	0
ColAn 57389	0	0	0	4	0	0	0	0
Tacaiuma	0	0	0	0	4	1	0	3
CoAr 3627	0	0	0	0	0	0	0	0
CoAr 1071	0	0	0	0	0	0	0	0
SPAr 2317	0	0	0	0	2	0	0	2

[a] Precipitin results recorded on a 0 to 4+ scale, depending upon arc intensity; 0 = no visible reaction, 1+ = minimal specific reaction, 2+ = weak to moderate, 3+ = strong, 4+ = maximum intensity and narrow precipitin arc.

tion with only 3 of the 8 strains and no plaques were formed in duck embryo cells.

Plaques appeared 6–25 days after inoculation. With few exceptions, plaque morphology was poorly delineated; however, with experience it was possible to distinguish individual plaques well enough to count them.

Trypsin-treated, sonicated antigens failed to provide useable hemagglutinins for six of the eight viruses; Tacaiuma and ColAn 57389 were the exceptions.

While all of the 8 strains reacted in a CF system (Table II), cross reactivity was extensive and relationships ill-defined. Therefore, this test was of little use in discriminating between group and type. The value of the CF test for the Anopheles A viruses obviously lies in its utilization as a grouping test. By CF, Anopheles A and CoAr 3624 are antigenically identical, Lukuni and CoAr 1071 are distantly related, and Lukuni and CoAr 3627 are related closely only one way. Nevertheless all 8 strains react strongly with one or more of the seven others. Using a CF antigen prepared from infected mouse serum, essentially similar results are obtained.

Precipitin testing by double diffusion in agar gave equally inconclusive results (Table III). They supported the CF findings, but the test appeared to be so specific that sensitivity was compromised. No homologous reactions were seen with CoAr 3627 and CoAr 1071.

It was only after using the neutralization test in BHK-21 cells that relationships between the 8 strains became clear (Table IV).

TABLE IV. Results of Cross-Testing Eight Anopheles A Group Viruses by Neutralization in BHK-21 Cells.

Virus	Day plaques observed	HIAF titer (90% plaque reduction) AnA	CoAr 3624	LUK	ColAn 57389	TCM	CoAr 3627	CoAr 1071	SPAr 2317
Anopheles A	6	*40*							
CoAr 3624	25		*20*						
Lukuni	6			*320*					
ColAn 57389	6				*>320*				
Tacaiuma	7					*160*	10		40
CoAr 3627	18					10	*80*	*20*	
CoAr 1071	25					10	40	*20*	
SPAr 2317	8					80	10		*80*

TABLE V. Proposed Classification of Anopheles A Group.

Group	Complex	Virus	Subtype
Anopheles A	Anopheles A	Anopheles A	
		CoAr 3624	
		Lukuni	
		ColAn 57389	
	Tacaiuma	Tacaiuma	SPAr 2317
		CoAr 1071	CoAr 3627

By determining endpoints based upon 90% plaque reduction, six of the eight strains were shown to be distinct. To some extent, these results confirmed the CF results, but minor group reactions were reduced. Further, SPAr 2317 was shown to be a variant of Tacaiuma, confirming the findings of Souza-Lopez (6) while CoAr 3527 and CoAr 1971 appear to be subtypes of each other. Neutralization tests in suckling mice, performed at YARU, gave similar results.

From the results presented here, a provisional classification was devised (Table V). Mainly on the basis of neutralization tests, Anopheles A, CoAr 3624, Lukuni, and ColAn 57389 were assigned to the Anopheles A Complex, and Tacaiuma and CoAr 1071 to the Tacaiuma Complex. This scheme is in line with the recommendations of the Subcommittee on Immunological Relationships Among Catalogued Arboviruses (SIRACA)[3], a subcommittee of the American Committee on Arthropod-borne Viruses.

An obvious problem in arbovirus nomenclature is whether or not to give a new name to antigenically distinguishable but very closely related isolates. Slight antigenic differences between two members of an arbovirus group may be associated with divergent epidemiologic characteristics. This is the case with California (Trivittatus) and California (LaCrosse), with Central European Encephalitis and Russian Spring–Summer Encephalitis, and with Venezuelan Equine Encephalitis type IB (ICA) and Venezuelan Equine Encephalitis type IE (Mena II). Until the parameters of arbovirus classification become more firmly grounded, it appears to be equally reasonable to assign a name as it is to retain the laboratory code. This scheme is proposed until such time as ecologic studies associate the viruses of the Anopheles A group with infections in man or lower vertebrates.

Holmes has shown that Anopheles A morphologically resembles certain Bunyamwera Supergroup viruses (12). Preliminary tests at CDC have shown that at least ColAn 57389 is a member of this Supergroup, as evidenced by repeatable HI titers of 1:10 with California (LaCrosse) HIAF and 1:20 with Tensaw HIAF. Further studies are needed to better define these relationships and the position of the Anopheles A group within the Bunyamwera Supergroup.

Summary. Anopheles A, Lukuni, and six serologically related viruses were partially characterized in mice and cell cultures and were cross-tested by a variety of serologic methods. Hemagglutinins were not successfully produced; complement-fixation (CF) demonstrated extensive cross reactivity and was, therefore, of little value in distinguishing between strains; precipitin testing by double diffusion in agar supported the CF test results but these tests were too specific to be useful in showing relationships. When neutralization tests were performed, the relationship between the eight strains became apparent. Based on the latter test, a provisional classification of the Anopheles A group was proposed.

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