

Isolation of Ingwavuma Virus and Study of Ingwavuma Antibody Prevalence in the Chiangmai Valley

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PURPOSE: To define the role of Ingwavuma virus in infection of man and animals in the Chiangmai Valley.

BACKGROUND: A virus (BKM 705-70) was isolated from a pool of 10 *Culex vishnui* mosquitoes aspirated from a pig in the Chiangmai Valley on 11 May 1970. The virus was identified as a member of the Simbu group of arboviruses, Ingwavuma virus by the Department of Virus Diseases, WRAIR, and Dr. Robert E. Shope, Yale Arbovirus Research Unit. This strain represents the first isolation of a Simbu group virus in Thailand and the first isolation of Ingwavuma virus from a possible insect vector. Little is known of the role of Simbu group arboviruses and specifically Ingwavuma virus in human infection and disease. Further, potential vertebrate hosts of Ingwavuma virus, previously isolated only from birds in South Africa, India, and Egypt, remain unidentified. The isolation of Ingwavuma virus during the JEV study in the Chiangmai Valley during which sequential bleedings of Chiangmai villagers and schoolchildren and random bleedings from domestic animals were obtained, permitted a retrospective search for Ingwavuma antibody in human and domestic animals in the Valley.

This report describes the establishment and validity of a macro and micro plaque reduction neutralization test (PRNT) for Ingwavuma antibody and describes the prevalence of Ingwavuma antibody in the Valley based upon the micro-PRNT.

METHODS: Technique for isolation of viruses from mosquito pools has been described in the 1971 Annual Report. Technique for Ingwavuma macro and micro PRNT are identical to those described for JEV PRNTs in the 1971 Annual Report. LLC-MK-2 cells were utilized in both tests. In order to test the validity of a micro-PRNT as an antibody assay, 2 rabbit hyperimmune Ingwavuma antisera were assayed by the micro-PRNT and standard macro-PRNT. Serum A was undiluted BKM 705-70 antiserum and serum B an unknown dilution of serum A. Both sera were diluted to 1:10 and heated at 56°C for 30 minutes prior to use. Serial 2-fold dilutions of serum A and B were made for both tests. Three replicate bottles were used for each serum dilution and the virus control in the macro-PRNT. Six replicate wells were used for each serum dilution and the virus control in the micro-PRNT.

In both tests, the arithmetic mean of the 6 (micro) wells or 3 (macro) bottles of control virus was used to calculate the percentage reduction for each individual well or bottle in a test. The serum dilutions used were transformed to logarithms and the percent plaque reduction to corresponding probit values. The 50% effective dose (ED₅₀, serum dilution which caused a 50% reduction in the number of plaques); 95% confidence limits of the ED₅₀; relative potency (RP) of serum in each of the two PRNT tests; and the 95% confidence limits of the RP were calculated by the method for a parallel line, graded response bioassay.

Human sera were collected from 4 study villages and an urban Chiangmai school during November 1970. Sera from chickens, ducks, cattle, buffaloes, pigs, and dogs from the 4 study villages was collected in November or July 1970. All human and animal sera tested were diluted to 1:10 and heated at 56°C for 30 minutes. A 1:20 dilution of serum was used to screen for Ingwavuma antibody in the micro-PRNT.

PROGRESS: In addition to BKM 705-70, 10 additional pools of 4 mosquito species (Culex vishnui, fuscocephala, tritaeniorhynchus, and Aedes linneatopennis) yielded Simbu group agents (which have not yet been tested for identity to Ingwavuma virus). All 10 pools were obtained from biting collections on cattle and buffalo at San Sae obtained on 22 May 1970. Since both unengorged and engorged mosquitoes were contained in these pools, these isolations may have contained engorged mosquitoes which fed on the same viremic host. No evidence of a possible vector role for these 4 species was thus obtained. The virus was reisolated from positive pools of all 4 mosquito species.

In order to utilize a micro-PRNT as an antibody assay in seroepidemiologic studies subsequently described, it was important to determine whether this assay measured Ingwavuma antibody similarly to the standard macro-PRNT. Therefore a standard undiluted hyperimmune rabbit Ingwavuma antiserum (serum A) and an unknown dilution of this antiserum (serum B) were used as described in methods section to compare the similarity of both tests in measuring antibody. Results of these tests are shown in Table 1 (serum A) and Table 2 (serum B). In both tests using both sera, at least 4 responses were obtained between 15-85% plaque reduction and plaque reduction responses bracketed 50% plaque reduction. Control plaque counts averaged 32 in the macro and 15 in the micro-PRNT.

Statistical analysis of these tests by the method for a parallel line, graded response bioassay are shown in Table 3. With serum A, the mean probit response and regression lines (Y intercepts) are in close agreement, and the neutralizing slopes of both tests were parallel. The relative potency of serum A (89%) is close to the expected 100%. Since the error variance in this comparison is quite small resulting in narrow confidence limits, the 95% confidence limits of the relative potency do not overlap 100%. The ED₅₀ of serum A was 501 by macro-PRNT and 564 by micro-PRNT. From a biologic standpoint this difference is small, and only because of small error variance of the test and the closeness of the mean probit responses to 5, it is a significant difference.

With serum B, the mean probit responses and regression lines are in close agreement and the neutralization slopes of both tests nearly parallel. The 95% confidence limits of the relative potency clearly overlap 100% and the ED₅₀ of serum B by both tests is remarkably close.

Neutralization slopes of the 2 sera by both PRNT tests are illustrated in the figures on pages 73 and 74. In both figures the mean points at each dilution are quite close to the estimated regression line, evidence of the small error in these tests. The closeness of the mean plaque reduction points of each PRNT for each serum indicate a similar slope of neutralization for each test and are compatible with the hypothesis that both tests are measuring identical antibodies in the same sera.

These results indicated that the micro-PRNT indeed will reproduce virtually identical results to the standard macro-PRNT test, providing at least 14 plaques are obtained in the control wells, at least 3 responses are obtained between 15-85% plaque reduction, and the responses bracket 50% plaque reduction.

Sera were initially tested at a 1:20 dilution in the micro-PRNT. Sera showing equal or greater than 50% plaque reduction were considered to contain antibody to Ingwavuma virus. Representative positive sera were further examined for antibody titers.

The results of testing serum obtained from 490 residents of the Chiangmai Valley in November 1970 (6 months after the initial isolation of the virus) is shown in Table 4. None of the 490 inhabitants tested possessed Ingwavuma antibody at a 1:20 serum dilution.

The prevalence of Ingwavuma antibody in various domestic animal species indigenous to the 4 valley villages studied is shown in Table 5. The prevalence of antibody in cattle, water buffalo, chickens and ducks was low. Further, the titers in animals considered positive (equal or greater than 50% plaque reduction) did not equal 1:40 in the 2 cattle, 4 buffaloes, 3 chickens, or 4 ducks tested.

In contrast, the prevalence of Ingwavuma antibody in dogs and pigs in the 4 study villages was higher. Most of the dog and pig sera found positive for antibody gave 100% plaque reduction at the 1:20 screening dilution. Representative sera from 11 dogs and 16 pigs all showed antibody titers of $\geq 1:160$ upon further testing.

Sequential bleedings of domestic animals in the Chiangmai Valley was not reproducibly accomplished during the 1970 study; consequently serum from serologically positive domestic animals obtained prior to November 1970 was not available to test. Thus it is not known whether some of these animals developed Ingwavuma antibody during 1970 when the virus was isolated in the Valley.

To measure the force of JEV infection in the Valley during the 1970 season, sentinel pigs (which lacked antibody to JEV) were purchased from farms in Nakorn Panom and Saraburi and placed in the four study villages. Four of these sentinel pigs bled after residence in the Valley were found to have Ingwavuma antibody in sera obtained in November 1970. Serum obtained in April 1970 (one pig) and July 1970 (3 pigs) was tested for Ingwavuma antibody to determine if Ingwavuma infection occurred while these animals were quartered in Chiangmai Valley. All 4 sentinel pigs had Ingwavuma antibody in sera obtained before shipment to the Valley.

These findings for Ingwavuma virus in Thailand are similar to those found for other arboviruses of the Simbu group. Many of these viruses have been isolated from Culex species, and antibody prevalence studies have shown that large domestic animals (cattle, goats, or pigs) are important hosts. With most Simbu group viruses, man appears to be a rare and probably incidental host. We found no evidence of human Ingwavuma infection in Chiangmai Valley.

Table 1.
Comparison of macro with micro-PRNT (serum A)

Dilution ^a	Macro-PRNT			Micro-PRNT		
	No. of plaque ^b	Average	% plaque reduction	No. of plaque ^c	Average	% plaque reduction
20	0,0,0	0	100	0,0,0,0,0,0	0	100
40	0,0,0	0	100	0,0,0,0,0,0	0	100
80	1,0,2	1	97.2	2,0,0,0,2,0	0.67	95.6
160	6,4,9	6	81.25	3,2,2,1,3,3	2.33	84.2
320	9,13,14	12	62.5	5,6,6,5,5,5	5.33	65.4
640	20,17,16	18	43.7	8,9,9,9,8,8	8.17	49.4
1280	25,26,23	25	21.9	13,10,11,12,10,11	11.11	27.9
2560	28,29,31	29	9.4	15,16,16,16,15,15	15.5	0

^aReciprocal of serum dilution

^bControl plaque count in macro-PRNT 34,34,28 — average 32

^cControl plaque count in micro-PRNT 15,16,15,16,15,15 — average 15.4

Table 2.
Comparison of macro with micro-PRNT (serum B)

Dilution ^a	Macro-PRNT			Micro-PRNT		
	No. of plaque ^b	Average	% plaque reduction	No. of plaque ^c	Average	% plaque reduction
10	0,0,0	0	100	0,0,0,0,0,0	0	100
20	1,0,0	0	100	0,0,1,1,0,1	0.5	96.8
40	2,2,4	3	90.6	6,2,1,4,2,2	2.83	81.7
80	5,6,7	6	81.25	6,4,6,6,4,4	5	67.5
160	15,18,15	16	50.0	3,6,8,8,7,8	6.67	56.7
320	20,22,20	21	34.4	12,9,9,10,9,8,	9.5	38.3
640	25,26,25	25	21.9	13,11,10,12,13,10	11.5	25.3
1280	29,30,29	29	9.4	16,15,14,14,14,16	15	0

^aReciprocal of serum dilution

^bControl plaque count in macro-PRNT 30,34,32 — average 32

^cControl plaque count in micro-PRNT 15,15,15,16,15,16 — average 15.4

Table 3.
Summary of Ingwavuma virus PRNT analysis, comparison of macro-with micro-PRNT

	Serum A		Serum B	
	<u>Macro</u>	<u>Micro</u>	<u>Macro</u>	<u>Micro</u>
Dilutions Used	160-1280	160-1280	80-640	80-640
Mean Probit Response	5.08	5.18	4.93	4.90
Parallelism Validity	Valid		Borderline	
Curvature Validity	Valid		Valid	
Error Variance	.039		.060	
Combined Slope	-1.8248		-1.7965	
Y intercept	9.927	10.021	9.067	9.042
Relative Potency (%)	89		103	
95% CL Rel. Potency (%)	86-92		98-109	
ED ₅₀	501	564	183	178
95% CL ED ₅₀	486-516	552-567	176-192	173-183

Table 4.
Antibody to Ingwavuma virus in the villagers and school children in Chiangmai Valley

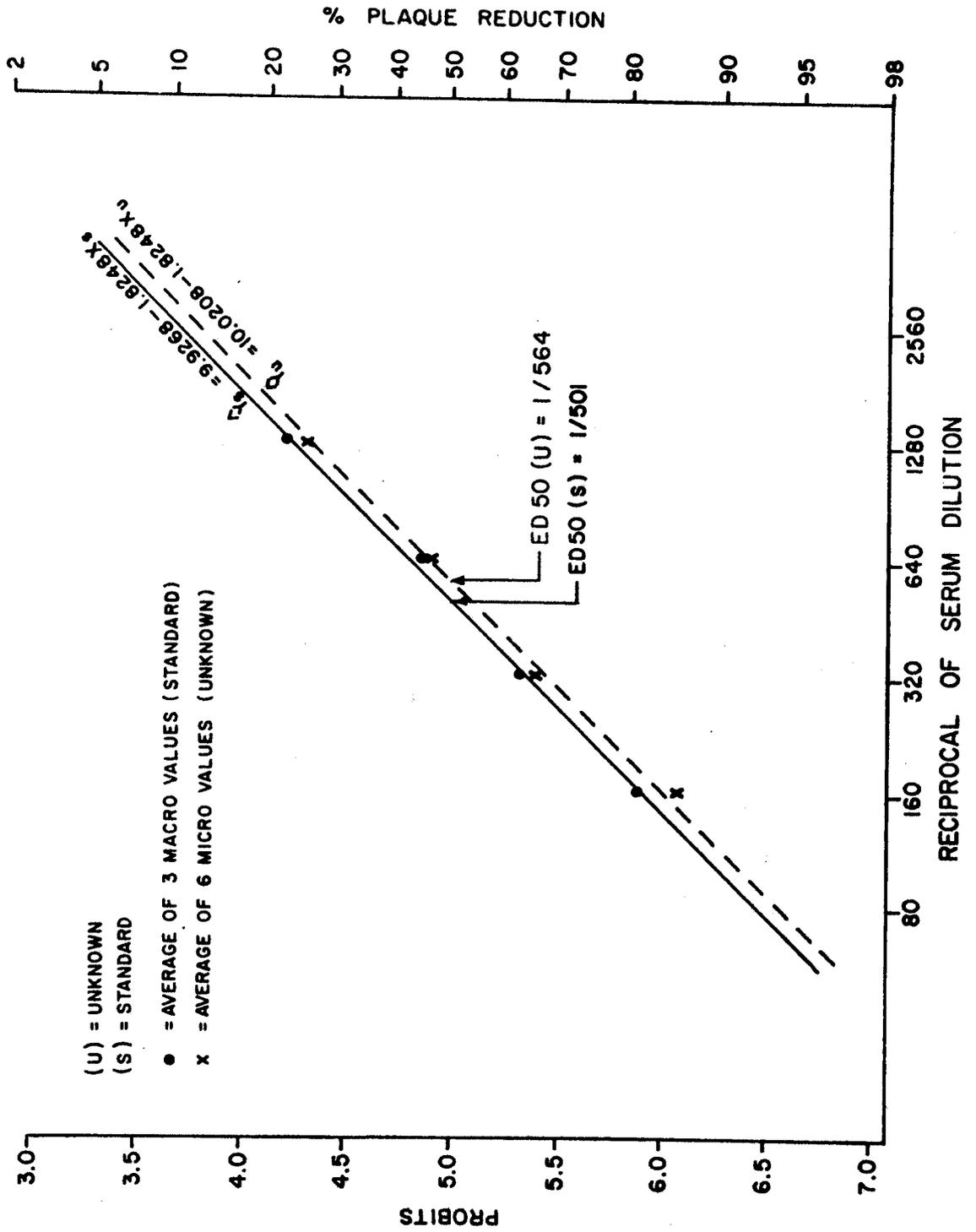
<u>Study area</u>	<u>No. studied</u>	<u>No. with Ingwavuma Antibody</u>	<u>Prevalence rate (%)</u>
(A) — Maerim	81	0	0
(B) — Sanpatong	79	0	0
(C) — Sankampang	81	0	0
(D) — Sarapee	91	0	0
(E) — Paug Chang School	158	0	0
Total	490	0	0

Table 5.
Antibody to Ingwavuma virus in the animal sera in Chiangmai Valley

<u>Species^a</u>	<u>No. studied</u>	<u>No. with Ingwavuma antibody</u>	<u>Prevalence rate (%)</u>
Bovine	50	2	4
Buffalo	50	4	8
Chicken	50	3	6
Duck	55	4	7.3
Dog	50	11	22
Pig (Local)	26	7	26.9
(Sentinel)	24	11	45.8

^aCollected from area (A) through (D)

INGWAVUMA VIRUS ANTIBODY; COMPARISON OF MACRO (s) AND MICRO (u) PLAQUE REDUCTION
 NEUTRALIZATION TESTS SERUM A.



INGWAVAMA VIRUS ANTIBODY; COMPARISON OF MACRO (S) AND MICRO (U) PLAQUE REDUCTION
 NEUTRALIZATION TESTS SERUM B.

