Isolation of Flaviviruses from Blood Specimens from Palm Oil and Rubber Plantation Workers in Central Malaysia

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OBJECTIVES

1. To isolate and type flaviviruses from acute blood specimens of Malaysian palm oil and rubber plantation workers whose current illness has been shown to be due to a flavivirus by HAI serology.

2. To compare flavivirus isolation rates obtained by the tissue culture delayed plaque method, the mosquito inoculation method, and the suckling mouse intracerebral inoculation method.

BACKGROUND : During 1978-1979 the AFRIMS Department of Virology performed over 2,000 used HAI serologies in support of studies on pyrexia of unknown origin (PUO) conducted by the US Army Medical Research Unit, Kuala Lumpur. Although final tabulation of the coded HAI serology results is not yet available, approximately 15% of patients developed a four-fold rise to the representative flavivirus antigen (Tembusu) used. The actual virus type or types responsible for most of these infections remained unknown. Most of the patients in the KL-USAMRU PUO study had an acute blood specimen frozen in liquid nitrogen on admission to the study; these acute specimens were available for virus isolation attempts.

METHODS :

Blood specimens : All patients in the KL-USAMRU PUO study had an acute whole blood specimen frozen in liquid nitrogen at the bed-side. After the measurement of HAI antibody titers in acute and convalescent serum against Tembusu antigen was completed for all patients, a list of 84 patients was drawn up. These 84 fulfilled the following criteria: (1) 8x or greater rise in HAI titer against flavivirus antigen, (2) acute blood obtained with 7 days of onset of

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illness, and (3) at least 0.5 ml of acute blood frozen in liquid nitrogen remaining. The acute blood specimens for isolation were shipped frozen from Kuala Lumpur to Bangkok.

Isolation methods : The frozen whole blood specimens were thawed, diluted 1:4 in RPMI 1640, and disrupted by sonication. Aliquots of each specimen were then processed for viral isolation by (1) the delayed plaque method on LLC-Mk2 cells by standard methods, (2) by inoculation onto C6/36 *A. albopictus* cells and passage of the tissue culture supernatant fluid on day 7, (3) by intrathoracic inoculation into *Toxorhynchites splendens* mosquitoes and with detection of mosquito infection by (a) examination of head squash preparation for direct fluorescence with fluorescein conjugated broadly flavivirus reactive pooled convalescent serum from Dengue Hemorrhagic Fever patients, (b) plaquing of thorax-abdomen suspensions onto LLC-Mk2 cells, and (c) blind passage of the thorax-abdomen suspensions through C6/36 cells for one week with subsequent assay of the supernatant fluid by the direct plaque assay method on LLC-Mk2 cells, (4) by intracerebral inoculation of the litter of 1 to 2 day old suckling mice with observation for sickness or death. If no illness was observed, the mice were challenged on day 14 with 100 LD50 of virulent D-2 intracerebrally and observed for another 2 weeks. In litters showing even slight signs of sickness or death 1/2 of the litter was blind passed through at least another two mouse passages at two weeks intervals, with titration of pooled brains from the final mouse passage in LLC-Mk2 cells by the direct plaque method 14 days after inoculation.

The overall scheme of diagnostic maneuvers is presented in Figure 1. Separate personnel performed the mouse inoculation and challenge, the mosquito inoculation and head squash fluorescent assay, and the tissue culture passages plaquing; all results for a given method were completed without knowledge of results obtained by other personnel using other methods.

RESULTS : A total of 21 confirmed virus isolates were made from the 84 acute blood specimens assayed by all seven methods. All confirmed isolates were made by more than one of the diagnostic methods used and produced a passable plaque forming agent. An additional seven specimens were thought to be positive for virus based on preliminary assay results; in six of these the results of only one assay system was questionably positive and no plaque forming agent was isolated, and in the seventh results of two independent methods were questionably positive but no plaque forming agent was isolated. Results are presented in tabular form in Table 1.

All 21 of the confirmed virus isolates have been identified by the plaque reduction neutralizing antibody method in LLC-Mk2 cells. Nineteen isolates are dengue type 3 and two are dengue type 2. Although the range of diagnostic methods used was reasonably broad, no other flaviviruses were isolated, only dengue.

The relative sensitivity and specificity of the seven methods used to detect flaviviruses are presented in Table 2. There was 100% agreement between results obtained by fluorescent antibody staining of head squash preparations of inoculated mosquitoes with results obtained by plaquing thorax-abdomen

suspensions of inoculated mosquitoes on LLC-Mk2 cells; 21 specimens were positive by both these methods, and none was positive by only one of these two methods without the other being positive also. Passage of the mosquito thorax-abdomen suspensions through C6/36 cells did not improve the assay.

The suckling mouse intracerebral inoculation method with virulent virus challenge on day 14 detected 15 confirmed positives with only 3 unconfirmed ("false") positives. In most of these litters the mice developed sub-clinical infections, because in only 4 was illness or death detectable with serial mouse brain passage. Greater than 25% mouse survival was used as a positive result in the virulent virus challenge test. This cut-off value was chosen as giving the least overlap between confirmed positives and negatives (see Figure 2).

Cell culture isolation methods fared poorly in this comparative trial. The delayed plaque method on LLC-Mk2 cells detected only 43% of the confirmed positives, while passage through C6/36 cells before direct plaquing on LLC-Mk2 cells detected only 24%. As a check for the possibility that an unusually insensitive line of LLC-Mk2 cells was used, a new line which had been frozen and stored 5 years ago was brought out and tested. Aliquots of the twelve frozen blood specimens which were positive by mosquito inoculation but negative by LLC-Mk2 inoculation on the first run were reinoculated onto the new line of LLC-Mk2 cells and assayed for virus by the delayed plaque method. Only two of the specimens originally negative on LLC-Mk2 cells were positive for transmissible plaque forming virus on the new cells.

Quantitative relationships between results obtained with independent assays were noted. This can best be appreciated by comparing dengue virus detection rates found with the various assays against the number of mosquito head squash preparations showing fluorescence. When 0 of 4 head preparations were positive, other assays were rarely positive, and when positive they remained unconfirmed. If 1/4 to 3/4 were positive, a low but significant proportion of these specimens were positive by other assays, and with blood specimens in which 4/4 mosquito heads were positive the highest isolate rates for other techniques were found (see Figure 3). The proportion of mosquito head squash preparations showing fluorescence was also quantitatively related to the number of plaques produced by thorax-abdomen suspension pools of the same mosquitoes (see Table 3). Four of 4 head squashes showing fluorescence was typically associated with "too numerous to count plaques" on LLC-Mk2 cells while the thorax-abdomen suspension from pools with 1 to 3 positive heads gave fewer (<200) plaques. (X^2=8.416, p = .004).

Table 1. Kuala Lumpur Flavivirus Isolates

SEATO Number	KL Number	Tissue Culture Isolation: LLC-Mk2 Delayed	Tissue Culture Isolation: PassC6/36 LLC-Mk2 Direct	FA #(+)/ tested	Mosquito Inoculation: Direct Plaque Thorax-Abdomen on LLC-Mk2 Confirmed	Mosquito Inoculation: Thorax-abdomen C6/36 Direct Plaque on LLC-Mk2 cells	Suckling mouse inoculation: Virulent virus challenge: # protection/# challenged	Suckling mouse inoculation: Sickness or death	Total # assays postive
1724/79	11579	TNTC	0	4/4	50	$10.7x10^2$	3/6	0	5
1725/79	7863	36	0	2/4	74	$4.3x10^2$	5/9	+	6
1728/79	7810	0	0	2/4	138	$2.4x10^2$	8/11	0	4
1729/79	1123	0	0	4/4	TNTC	$9.9x10^2$	3/10	0	4
1730/79	11546	88	0	4/4	TNTC	$7.8x10^2$	6/11	0	5
1731/79	7820	0	0	2/4	122	$10x10^2$	6/9	0	4
1733/79	7850	TNTC	0	4/4	49	$5.5x10^2$	8/11	0	5
1734/79	7828	0	0	2/4	169	$5x10^2$	2/12	0	3
1739/79	7792	30	0	4/4	TNTC	$2x10^3$	10/11	+	6
1754/79	7835	0	0	3/4	79	$8x10^3$	3/12	0	4
1755/79	7881	0	0	4/4	TNTC	$1.2x10^3$	3/11	0	4
1756/79	7802	72	$3x10^2$	4/4	TNTC	0	12/12	0	5
1757/79	9145	33	0	2/3	106	$2.2x10^3$	10/12	+	6
1758/79	7826	0	0	4/4	TNTC	$1.4x10^3$	8/9	0	4
1762/79	7811	0	0	2/4	109	$3x10^3$	3/11	0	4
1765/79	7819	60	$7x10^2$	4/4	58	$4.1x10^3$	7/13	0	6
1775/79	7867	0	0	2/4	106	$9x10^2$	2/15	0	3
1784/79	7572	0	$7x10^{-1}$	2/4	TNTC	$11.4x10^2$	2/12	0	4
1785/79	7535	0	11x10	4/4	TNTC	$24.5x10^2$	1/12	0	4
1787/79	11502	$1.5x10^2$	$4.6x10^2$	4/4	TNTC	0	2/12	0	4
1800/79	11554	0	0	4/4	TNTC	$3x10^2$	4/6	+	5
				UNCONFIRMED**					
1748/79	7857	0	$6x10^2$*	0/4	0	0	0/12	0	1
1752/79	11557	0	0	0/4	0	0	2/5*	0	1
1771/79	11540	0	0	0/4	0	0	3/9*	0	1
1773/79	8069	0	0	0/4	0	0	ND	+*	1
1778/79	11555	0	$1.8x10^2$*	0/4	0	0	2/7*	0	2
1797/79	11519	0	0	0/4	0	$8x10^2$*	0/11	0	1
1805/79	9164	0	0	0/4	0	0	ND	+*	1

* Unconfirmed positive result

** An additional 56 specimens were negative in all seven assay systems

Table 2. Comparative Sensitivity and Specificity of Assays Used to Detect Flaviviruses.

	# Confirmed positive	# Unconfirmed positive	Relative sensitivity	Relative specificity
Mosquito inoculation; Head squash fluorescent stain	21	0	1.00	1.00
Mosquito inoculation; Thorax-abdomen direct plaque on LLC-Mk2 cells	21	0	1.00	1.00
Mosquito inoculation; Thorax-abdomen Pass C6/36 Direct Plaque on LLC-Mk2 cells	20	1	.95	.95
Suckling mouse inoculation; Protection against virulent D-2 challenge	15	3	0.71	0.83
Delayed plaque on LLC-Mk2 cells	9	0	.43	1.00
Pass C6/36 Direct Plaque on LLC-Mk2 cells	5	2	.24	0.71
Suckling mouse inoculation; Illness or death	4	2	0.09	0.66

Table 3. Relationship Between Proportion of Head Squash Preparations Positive by Fluorescent Antibody Staining and Thorax-abdomen Direct Plaque Counts on LLC-Mk2 Cells.

# of mosquito heads out of four positive by fluorescent antibody staining	Direct plaque count of thorax-abdomen suspension on LLC-Mk2 cells		
	TNTC	<200	
4/4	9	3	/12
<4/4	1	8	/9
	10	11	/21

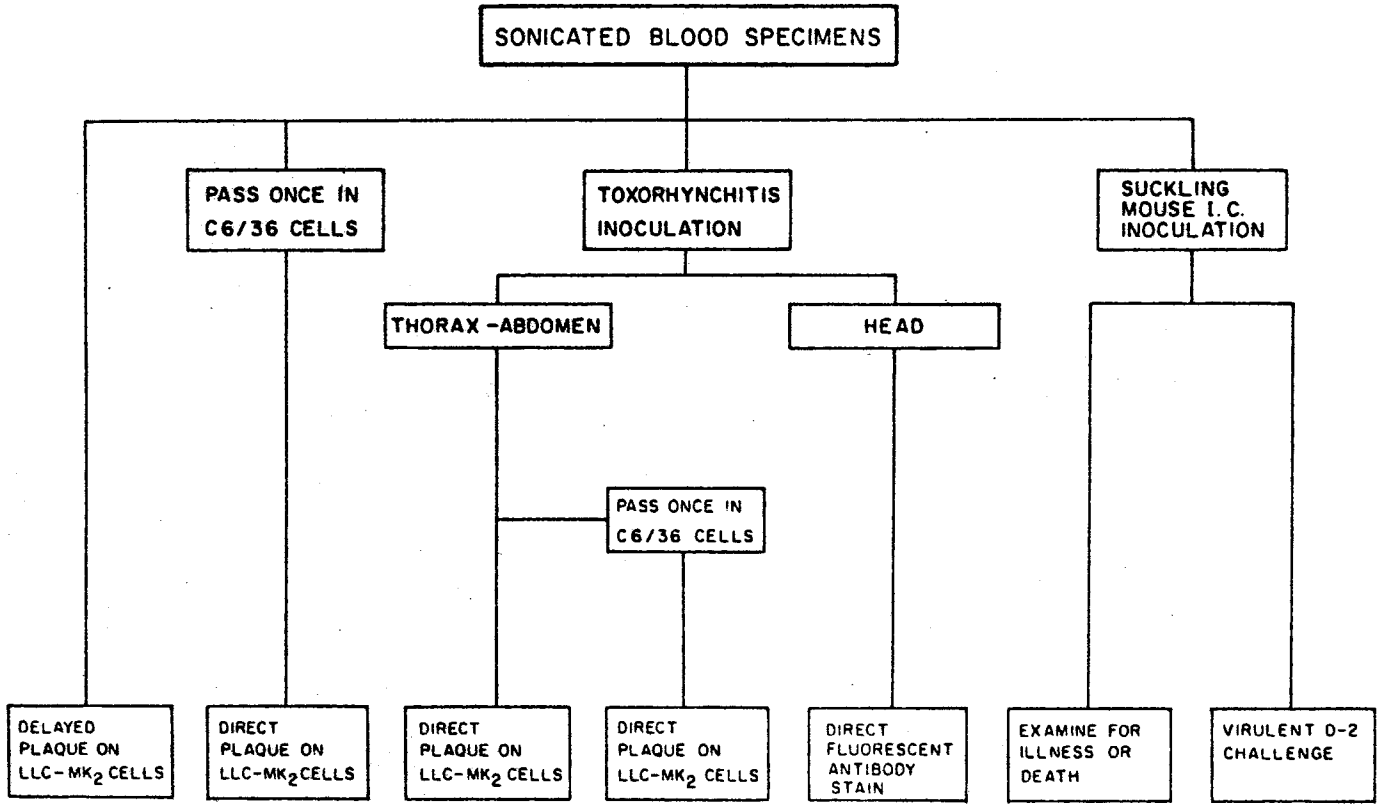

Figure 1. Summary of Methods Used to Isolate Dengue Viruses.

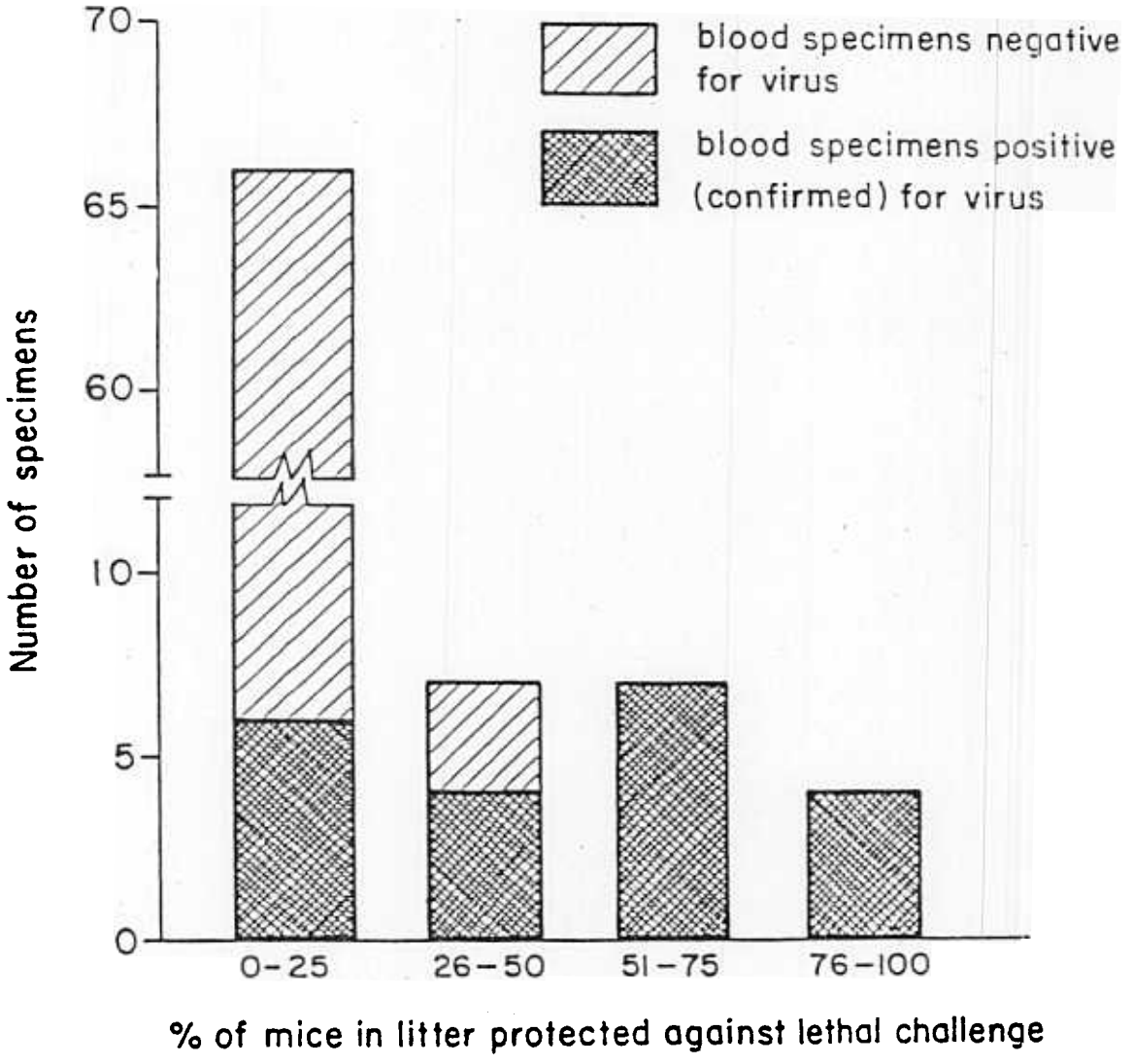

Figure 2. Graph of Number of Virus Positive and Virus Negative Blood Specimens Protecting Suckling Mice Against Subsequent Lethal Challenge with a Mouse Virulent Dengue Type-2 Challenge.

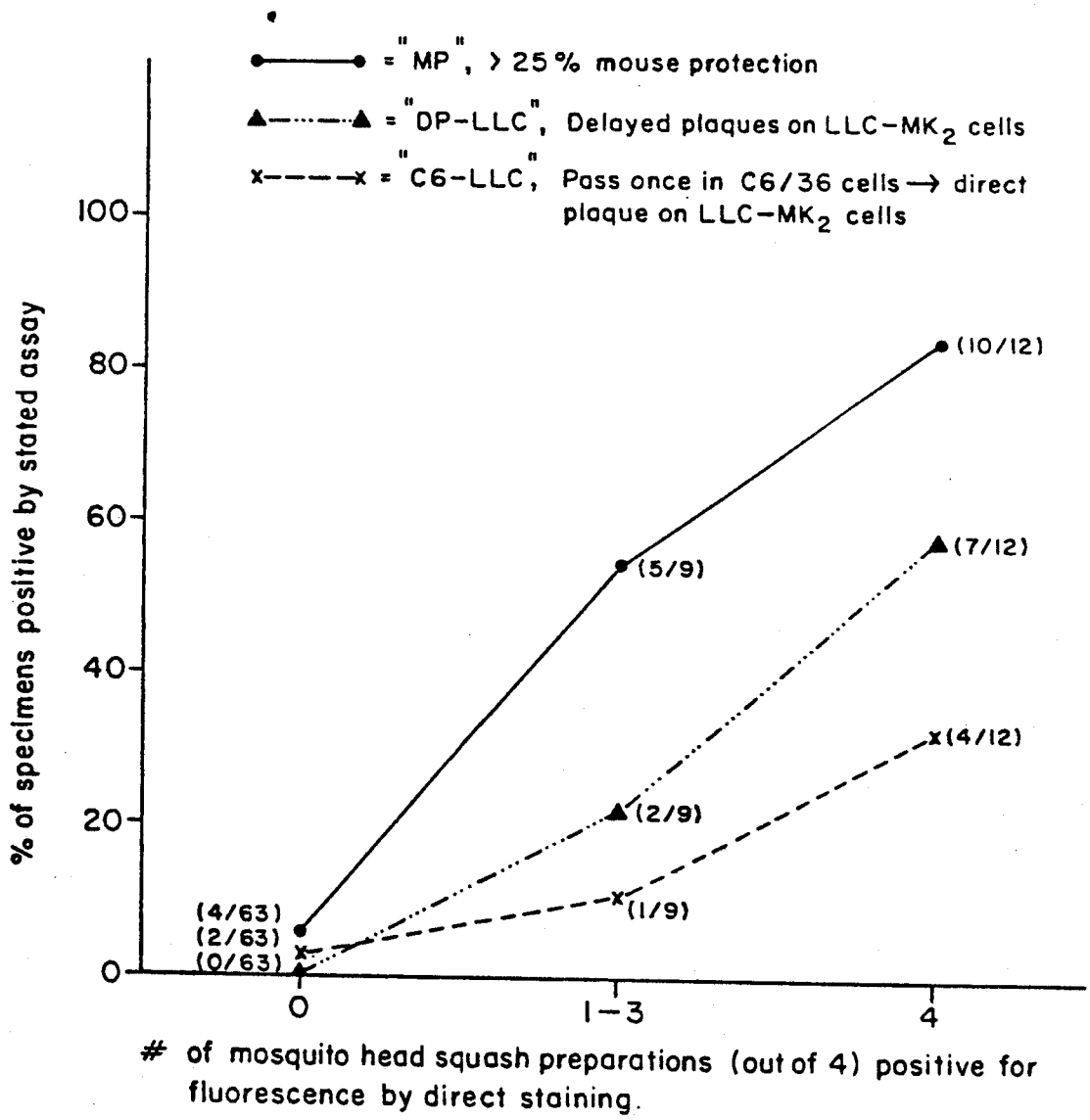

Figure 3. Graph of Quantitative Relationship between Mosquito Head Squash Fluorescent Antibody Assay and the Mouse Protection Assay, the Delayed Plaque on LLC-Mk2 Assay, and the C6/36 Cell Passage Followed by Direct Plaquing on LLC-Mk2 Cell Assay.