

ASSESSMENT OF DIAGNOSTIC CRITERIA FOR DENGUE VIRUS INFECTION
BY HEMAGGLUTINATION INHIBITION SEROLOGY ON SERA
COLLECTED AT SIX MONTH INTERVALS

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OBJECTIVE : To develop data that can be used to define diagnostic criteria for dengue virus infections using HAI serologic techniques on sera collected at six month intervals at the Phibunprachasan School.

BACKGROUND : From June 1977 until the present, blood specimens have been collected at 6 month intervals from a cohort of children attending a local public school (see AFRIMS Annual Progress Report, October 1978 to September 1979, pp. 118-122). The routine procedure used has been to perform HAI serology on all the sera within a few months after collection, tabulate the results, and compare the titers obtained to those obtained on the preceding serum specimen from the same child. Therefore, paired samples (from the beginning and end of each six month study period) are not assayed simultaneously.

For serologic diagnosis of acute infections it is widely accepted that paired sera (usually 2 to 4 weeks apart) should be assayed simultaneously. However, this approach required investing twice as much work in an already cumbersome project (approx. 20,000 HAI antibody titrations per year). Furthermore, we had no assurance that the widely accepted criteria of a four-fold rise (accepted for one antigen with sera spaced at 2 to 4 weeks) were applicable to our study in which we tested five antigens on sera separated by 6 months; true biological fluctuations in antibody levels and antibody lability with storage might lead to serious interpretation problems. We therefore sought to develop data to define reasonably sensitive and specific criteria for primary and secondary dengue infections.

Two basic types of criteria can be used to define a seroconversion to flavivirus antigens when five antigens are used : either the absolute magnitude of the titer rise or the number of antigens showing a certain minimum titer rise. Figure 1A shows that of children with seroconversion from no antibody to at least a 1:20 titer or greater between June 1977 and January 1978, some showed a rise to only 1, some to 2, and others 3, 4 or 5 antigens. Figure 1B similarly shows the proportion of paired specimens from children with pre-existing antibody in the June 1977 specimen in which there were 4 x or greater rises to 1, 2, 3, 4 or 5 antigens.

METHODS :

1. Assay of serum pairs by PRNT serology.

To develop satisfactory criteria for a primary seroconversion, all available serum pairs which showed HAI seroconversion from negative (<1:10 to

all 5 antigens, D1, D2, D3, D4, JEV) to positive ($>1:20$ to at least one antigen) were retested by PRNT₅₀. 67 serum pairs were found to have sufficient sera to perform all assays (out of a total of 114 possible serum pairs).

To develop criteria for a secondary seroconversion, serum pairs with positive antibody ($>1:20$) in the first specimen were examined. 13 serum pairs which had shown an HAI four fold or greater rise to only one antigen and 8 serum pairs which had shown an HAI four fold or greater rise to all 5 antigens were tested by PRNT₅₀.

2. Titration of duplicate samples.

Three types of laboratory variability in determining the HAI titer of a specimen were investigated using sera collected in June 1977 and found to be positive for dengue antibody at that time to at least one antigen ($>1:20$).

Inter-observer variation : Two experienced laboratory technicians (P and M) who have worked together for several years independently read and recorded the HAI titer end point on 98 specimens tested against all 5 antigens (490 titer determinations).

Intra-run variation : HAI titer determinations of 154 sera (770 titrations) were performed by P and M, then the same 154 specimens (770 titrations) were repeated the same week, using the same serum extraction preparation and antigen lot and dilution that had been used earlier the same week.

Inter-run variation : 157 sera which had been assayed for HAI antibodies in 1977 (785 titer determinations) were repeated two years later. The two determinations shared no steps in common; the serum extraction was repeated, and new buffers and different mouse brain antigen lots and dilutions were used. Results are expressed as the percent of specimens which showed differences between the two runs compared.

RESULTS : Table 1 shows the results of PRNT serology on specimen pairs which were HAI negative in the first specimen but positive for 0, 1, 2 or 3 antigens in the second specimen six months later. It can be seen that randomly chosen HAI negative sera (from pairs that do not show a seroconversion by HAI) are uniformly PRNT negative. However, HAI Negative sera selected on the basis of an HAI rise to only one antigen often have PRNT antibodies in the 1st specimen (8/23) or are false seroconversions (7/23). Only a minority of "one antigen primary seroconversions" are true. The proportion of pairs with true titer rises increases with the number of antigens seroconverted by HAI. Overall it is reasonable to estimate 16% of the HAI defined 1^o seroconversions are false primaries; 58% are true seroconversions, 19% are already antibody positive but are false seroconversions and 7% are secondary seroconversions masquerading as primary seroconversions.

Table 2 shows that serum pairs with PRNT verified seroconversions on the average showed four-fold rises to more antigens than did false seroconversions. D1 and D3 produced broadly reactive HAI sero-responses in every instance, compared to D4 which did so only sporadically (8/26, 31%) ($\chi^2 = 5.4$, $p = .03$) or D2 (2/5, 40%) ($p = N.S.$).

A similar analysis of secondary infections (as defined by HAI) is presented in Table 3. Of "one antigen secondary seroconversions", 77% are false positive and 23% true positive, while "5-antigen secondary seroconversions are all (100%) true secondary infections by PRNT also. From this data and the data in Figure 1B it is reasonable to estimate that 60 to 70% of the HAI defined secondary infections are in fact true secondary infections.

Table 4 summarizes variability in results of HAI titrations when inter-observer, intra-run, and inter-run results are compared. As the current procedures used in our serologic study are most comparable to "inter-run" comparisons, it can be seen that in specimens with pre-existing antibody, using the criteria of four-fold or greater rise to only 1 antigen would result in a false seroconversion rates of approximately 13% (26.7 ± 2); therefore a more rigorous criteria must be chosen to define secondary infections in this study.

Table 1. PRNT₅₀ antibody verification of "primary" seroconversions in specimens showing four fold or greater rises to 1, 2, or > 3 antigens by HAI

4 x HAI titer rise to N Ag's	Results of PRNT serology*				Total
	First specimen Ab Neg		First specimen Ab pos		
	No Δ	4 x ↑**	No Δ	4 x ↑**	
0	18	0	0	0	18
1	7	8	8	0	23
2	3	6	2	0	11
3	0	25	3	5	<u>33</u>
					<u>67</u>

* Criteria for positive : PRNT₅₀ ≥ 20

** Criteria for seroconversion : PRNT₅₀ or 4 x ↑

or

PRNT₅₀ < 20 → PRNT₅₀ ≥ 20

Table 2. Number of Flavivirus antigens with four-fold or greater increase by HAI in patients with virus infection types defined by PRNT₅₀ serology

<u>Type of PRNT₅₀ seroconversion</u>	<u>N</u>	<u># Flavivirus antigens with 4 x or greater increase by HAI</u>
Pos → no change	13	2.1 ± 1.7
Pos → 2° infection	5	5.0 ± 0
Neg → Neg	10	1.3 ± 0.5
Neg → D1	3	5.0 ± 0
Neg → D2	5	3.4 ± 1.8
Neg → D3	3	5.0 ± 0
Neg → D4	26	2.9 ± 1.6
Neg → JEV	0	-

Table 3. PRNT₅₀ antibody verification of "secondary" seroconversions in specimen pairs showing a four-fold or greater rise to 1 or 5 antigens by HAI

<u>4 x HAI titer rise to N Ag's</u>	<u>First specimen antibody negative</u>		<u>First specimen antibody positive</u>		<u>Total</u>
	<u>0 Δ</u>	<u>4 x ↑</u>	<u>4 Δ</u>	<u>4 x ↑</u>	
1	0	0	10	3	13
5	0	0	0	8	8

Figure 1.

