COMPARISON OF ANTI-DENV/JEV IgG-mAb ENZYME-LINKED IMMUNOSORBENT ASSAY AND HEMAGGLUTINATION INHIBITION ASSAY

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Abstract. The hemagglutination inhibition assay (HAI) is commonly used for serological screening of dengue virus (DENV) and Japanese encephalitis virus (JEV) infections. However, HAI is time- and resource- intensive due to the multiple steps required for serum processing. This study evaluated the sensitivity and specificity of a monoclonal antibody (mAb)-based capture enzyme-linked immunosorbent assay (mAb-ELISA) in screening DENV- and JEV-specific IgG compared to the more traditional HAI. We selected 170 pairs of serum specimens obtained eight months apart during routine DENV surveillances in Kamphaeng Phet, Thailand, 2004-2005. These specimen pairs were tested by HAI to measure changes in neutralizing titers in individual subjects, followed by plaque reduction neutralization test for confirmation. Comparison of the HAI and anti-DENV/JEV IgG mAb-ELISA showed 100% specificity of IgG mAb-ELISA using 4G2 or the pair 2H2/J93 mAbs, and 92.9% and 97.6% sensitivity for 4G2 and 2H2/J93 mAb, respectively. Both anti-DENV/JEV IgG-4G2 and anti-DENV/JEV IgG-2H2/J93 mAb-ELISA correlated highly with DENV/JEV HAI. Hence, the anti-DENV/JEV IgG mAb-ELISA should be considered as an alternative screening tool to HAI for serological screening of DENV/JEV infection.

Keywords: dengue, ELISA, HAI, Japanese encephalitis

INTRODUCTION

Dengue virus (DENV) and Japanese encephalitis virus (JEV) are arthropod-borne and members of the family Flaviviridae. DENV infections can manifest as clinically non-apparent, undifferentiated febrile illness, classical dengue fever, or dengue hemorrhagic fever (Vaughn *et al*, 1997). JEV infections are the leading cause of mosquito-borne viral encephalitis in Asia (Halstead and Jacobson, 2003).

Laboratory diagnosis is necessary for confirmation and discrimination between DENV and JEV infections as clinical differential diagnosis is difficult. Hemagglutination inhibition assay (HAI), one of the most widely used laboratory methods,

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is employed to detect DENV- and JEV-specific antibodies based on their ability to inhibit red blood cell agglutination (Nisalak, 2015). However, HAI is time- and resource-intensive and, thus, there is a need for a faster and more affordable laboratory test.

Previous studies have demonstrated the feasibility of ELISA to rapidly diagnose infections. Innis *et al* (1989) developed an anti-IgM and -IgG enzyme-linked immunosorbent assay (MAC-ELISA) to detect DENV and JEV infections in paired serum specimens collected upon admission (acute) and discharge (convalescent) from hospitals. Monoclonal antibody (mAb)-based capture enzyme-linked immunosorbent assay (mAb-ELISA) is more rapid and implementable than HAI, making them ideal for laboratory testing or diagnosis of arboviral infections (Burke *et al*, 1987; Kuno *et al*, 1991; Johnson *et al*, 2000; Shu *et al*, 2003). Various laboratories have demonstrated the feasibility of using 4G2 and 2H2 mAbs for the detection of circulating DENV- and other flavivirus-specific IgG in serum of patients (Chan *et al*, 2012; Luo *et al*, 2013). JEV-specific J93 mAb has been used to confirm the presence of JEV in culture fluid by an antigen-capture ELISA system (Raengsakulrach *et al*, 1999).

The majority of DENV infections in children are thought to be non-apparent. A study in Kamphaeng Phet Province, Thailand of the epidemiology of non-apparent and symptomatic dengue infections in a cohort of children (aged 5-12 years) using pre- and post-surveillance serum specimens is being carried out to determine the rate of exposure to dengue and to provide diagnostic support (unpublished). In order to measure non-apparent dengue infections, the study relies on HAI for screening and plaque reduction neutralization test ($PRNT_{50}$) for confirmation (Endy *et al*, 2011). These two methods are the most commonly used in serological diagnosis.

HAI assay is a highly sensitive and specific test; however, in addition to high consumption of time and resources, HAI requires a high degree of technical skill. The 4G2 and combined 2H2/J93 mAbs have been used to develop a DEN/JEV IgG mAb-ELISA (Gentry *et al*, 1982; Henchal *et al*, 1982; Henchal *et al*, 1987). Here, we describe a more rapid and practical assay that may be used to replace HAI for screening purposes. We used 170 paired serum specimens (pre- and post-surveillance, approximately 7-8 months apart) collected during the Kamphaeng Phet study for our evaluation.

MATERIALS AND METHODS

Human serum samples

The study design and method have been previously described (Mammen *et al*, 2008). In brief, human serum samples were collected from a pediatric fever cohort, part of a prospective school-based study of approximately 2,000 children, 5-12 years old, in 12 schools, Kamphaeng Phet Province, Thailand, during 2004-2005. Serum specimens were collected from the entire cohort at the beginning of the surveillance period (June, S1) and at the end of the surveillance period (January or February of the following year, S2). Seroconversion between the two specimens (S1 and S2) is defined as a four-fold or more rise in HAI titers (Anderson *et al*, 2014). $PRNT_{50}$ was used to confirm the HAI results.

The study was approved by the Institutional Review Board of the Walter Reed Army Institute of Research (WRAIR), USA (Protocol number WRAIR#1042).

Selected samples

A total of 340 serum samples (170 acute and convalescent pairs) were randomly selected, consisting of 85 pairs with negative HAI and 85 HAI positive results. The HAI positive pairs included 20 paired serum specimens obtained from symptomatic dengue cases (DENV PCR-positive cases) and 65 pairs obtained from asymptomatic dengue cases (HAI and $PRNT_{50}$ seroconversion). Among the 85 HAI positive pairs there were 65 pairs positive for DENV and 20 for JEV.

HAI

HAIs were carried out using goose erythrocytes as previously described (Clarke and Casal, 1958). In short, DENV-1 (Hawaii), DENV-2 (NGC), DENV-3 (H87), DENV-4 (H241), and JEV (JaGAr01) antigens were extracted using a sucrose acetone-extracted method at concentration of 16 U/ml each. Serum titration was performed in 2-fold dilution steps starting at a dilution of 1:10. A non-inhibitory titer of 1:10 is annotated as <10 and calculated as 5. Seroconversion is defined as a 4-fold or greater rise in HAI titers for any of the four DENV serotypes and JEV between S1 and S2. A higher seroconversion titer for JEV that for DENV is marked as seroconverted for JEV.

$PRNT_{50}$

$PRNT_{50}$ was performed as described by Russell and Nisalak (1967) with some modifications. In brief, heat-inactivated S1 and S2 sera were diluted in four-fold serial steps (1:10 to 1:2,560) and mixed with an equal volume of each DENV-1 (16007), DENV-2 (16681), DENV-3 (16562), DENV-4 (C0036/06) and JEV (0423 vaccine strain) at $7x10^2$ PFU/ml before incubating at 35°C for 1 hour. The mixtures then were inoculated into 3-day old Rhesus monkey kidney epithelial LLC-MK2 cells (ATCC, Manassas, VA) ($4x10^4$ cell/ml) in a 12-well plate and incubated at ambient room temperature for 1 hour with shaking. Excess volume of the inoculum was removed prior to adding the first medium over a layer of low melting point agarose gel (LMP, Ultra PureTM LMP agarose; Invitrogen, Carlsbad, CA) and incubated for 4-6 days in a 5% CO_2 incubator at 35°C. Virus-infected cells were stained with 4% neutral red (Sigma, St Louis, MO) in a second medium over a layer containing LMP and incubated overnight. The number of plaques was manually counted and calculated using SPSS program for probit analysis at 50% reduction, which was identified as the highest dilution that reduces the number of plaques by 50% compared to the number of plaques in virus control well.

Anti-DENV/JEV IgG mAb-ELISA

The test was performed simultaneously using a 96-well flat-bottom microplates (one microplate to detect anti-DENV IgG and another to detect anti-JEV IgG). Two replicas of each test were performed using serum sample diluted either 1:400 or 1:1,600. Microplates were coated with 1:500 dilution of goat anti-mouse IgG (KPL, Gaithersburg, MD) in 0.018M carbonate buffer pH 9.0. After an overnight incubation at 4°C, plate wells were washed 5 times with phosphate-buffered saline pH 7.4 containing 0.5% Tween 20 (PBS-T). Then 5% skim milk in PBS containing 1% Tween 20 was added to each well and incubated for 1 hour at 37°C. Wells were washed 5 times with PBS-T before the addition of 2H2 mAb into the DENV assay plate, J93 mAb into the JEV assay plate or 4G2 mAb into both DENV and JEV assay plates. All mAbs were diluted 1:1,000. Plates were incubated as described above. Then DENV

Table 1
Anti-DENV/JEV IgG mAb-ELISA interpretation using ΔOD_{S2-S1} at 1:400 and 1:1,600 serum dilution with negative cut-off set at $A_{450\ nm} < 0.200$.

DENV plate		JEV plate		Interpretation
ΔOD_{S2-S1} 1:400	ΔOD_{S2-S1} 1:1,600	ΔOD_{S2-S1} 1:400	ΔOD_{S2-S1} 1:1,600	
≥0.200	≥0.200	≥0.200 or <0.200	≥0.200 or <0.200	Den
<0.200	≥0.200	≥0.200 or <0.200	≥0.200 or <0.200	Den
≥0.200	<0.200	<0.200	<0.200	Den
		<0.200	≥0.200	Den
		≥0.200	<0.200	Den
		≥0.200	≥0.200	JE
<0.200	<0.200	<0.200	≥0.200	JE
		≥0.200	<0.200	JE
		≥0.200	≥0.200	JE
<0.200	<0.200	<0.200	<0.200	Neg

Den, dengue; JE, Japanese encephalitis; Neg, negative.

plate was treated with a pool of 16 HA U of each of the four DENV serotype antigen and the JEV plate with 60 HA U of JEV antigen. Both plates were incubated as described above and wells washed 5 times with PBS-T. The test sera were diluted 1:400 and 1:1,600 in PBS containing 5% skim milk and 1% Tween 20 and added into each well and incubated as described above. Goat anti-Human IgG-HRP diluted 1:50,000 was added into each plate and incubated as described above. In between each incubation period the plates were washed 5 times with PBS-T. SureBlue™ TMB 1-Component Microwell Peroxidase Substrate (TMB), (KPL, Gaithersburg, MD) was added to each well and incubated for 10 minutes. The reaction was terminated by adding 0.2 M sulfuric acid to each well and $A_{450\ nm}$ was measured in a microplate spectrophotometer.

Final interpretation of the IgG mAb-ELISA requires the results of differences in $A_{450\ nm}$ values between S2 and S1 (ΔOD_{S2-S1}) of the 1:400 and 1:1,600 serum dilutions (Table 1). For the determination of the cut-off values for the assay, the mean ΔOD_{S2-S1} + 3 SD (0.200) of HAI negative pairs was used.

RESULTS

Comparison of HAI with anti-DENV/JEV 4G2 and 2H2/J93 ELISAs

Eighty-five pairs of each HAI-positive (for JEV or any DENV serotype) and 85 pairs of HAI-negative serum samples were selected from a cohort in a prospective school-based study to compare the sensitivity and specificity of the anti-DENV/JEV IgG mAb-ELISA with HAI results. The anti-DENV/JEV IgG mAb-ELISAs were plated with either 4G2 mAb (against flavivirus complex) or 2H2 and J93 mAbs (against DENV complex and JEV, respectively). HAI positivity was determined by a four-fold rise in titer between S1 and S2 samples. The 85 HAI-positive pairs included 65 pairs positive

Table 2
Comparison of anti-DENV/JEV 4G2 and 2H2/J93 ELISAs and HAI for detection of dengue virus (DENV) and Japanese encephalitis virus (JEV) infections.

Anti-DENV/JEV	IgG result	HAI result			Total
		DENV	JEV	Neg	
4G2 ELISA	DENV	61	0	0	61 (94%)
	JEV	0	18	0	18 (90%)
	Neg	4	2	85	91
Total		65	20	85	170
2H2/J93 ELISA	DENV	63	0	0	63 (97%)
	JEV	0	20	0	20 (100%)
	Neg	2	0	85	87
Total		65	20	85	170

DENV, dengue virus; JEV, Japanese encephalitis virus; Neg, negative.

Table 3
Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of anti-DENV/JEV 4G2 and 2H2/J93 ELISAs.

Assay	% sensitivity	% specificity	PPV	NPV
4G2 ELISA	93%	100%	100%	93%
2H2/J93 ELISA	98%	100%	100%	98%

for DENV (any serotype or combination of serotypes) and 20 pairs positive for JEV.

The overall sensitivity of the 4G2 ELISA was 93% (79/85 HAI positive pairs) (Table 2). The sensitivity for detection of anti-DENV and anti-JEV IgG was 94% (61/65 HAI positive) and 90% (18/20 positives), respectively. Six HAI-positive paired-samples were not detected by the 4G2 ELISA. The overall sensitivity of the 2H2/J93 ELISA was 98% (83/85 HAI-positive pairs). The sensitivity for detection of anti-DENV and anti-JEV IgG was 97% (63/65 HAI positives) and 100% (20/20 HAI positives), respectively. Of the 6 HAI positive and 4G2 ELISA negative paired samples, 2 (samples 29 and 37) also failed to be detected by the 2H2/J93 ELISA. The specificity for both ELISAs was 100% as all 85 HAI negative paired-samples were negative using the 4G2 and 2H2/J93 ELISAs.

The sensitivity, specificity, positive value (PPV) of the anti-DENV/JEV IgG mAb-ELISA compared with HAI was 100% for both the 4G2 mAb and 2H2/J93 mAbs (Table 3). The sensitivity, specificity, negative predictive value (NPV) for 4G2 mAb and 2H2/J93 mAbs was 93% and 98%, respectively.

Comparison of 4-fold rise of HAI assay with anti DENV/JEV 4G2 and 2H2/J93 ELISAs

Although there were few discrepan-

Table 4
HI-serology typical of HAI and anti-DENV/JEV 4G2 and 2H2/J93 ELISAs.

HAI typic	Interp	Number of paired sera		
		HAI	4G2 ELISA	2H2/J93 ELISA
HAI-negative	Neg	85	85 (100%)	85 (100%)
HAI-monotypic	Den	4	2 (78%)	2 (89%)
	JE	14	12	14
	Neg	0	4	2
HAI-ditypic	Den	4	2 (89%)	4 (100%)
	JE	5	5	5
	Neg	0	2	0
HAI-multitypic	Den	57	57 (100%)	57 (100%)
	JE	1	1	1

Den, dengue; Interp, interpretation; JE, Japanese encephalitis; Neg, negative.

cies between HAI and ELISA results, we attempted to establish an association between the strength of the HAIs and the ELISA results. The distribution of paired serum samples based on their HAI positivity, *ie*, based on whether the serum pairs were HAI positive for a single DENV serotype or JEV (monotypic) and for either JEV and a DENV serotype or two DENV serotypes (ditypic) or more than two (multitypic) demonstrated HAI assays were multitypic, with 100% correspondence to the ELISA assays, whether using 4G2 or 2H2/J93 Abs (Table 4). However, mono- and ditypic HAI tests have less correspondence to 4G2 ELISA (78% and 89%, respectively) and monotypic HAI test to 2H2/J93 ELISA (89%).

Analysis of ELISA false negative samples

In order to further analyze the 6 HAI positive paired samples found to be negative by 4G2 ELISA we combined data collected from the HAI tests, $PRNT_{50}$ and 4G2 ELISAs but failed to discover any discrepancies in the serology assays that could explain the phenomena (Table 5). The HAI results were confirmed by $PRNT_{50}$, and, with the exception of pair 37, both assays gave the same interpretation. Of note, however, these false negative 4G2 ELISA samples were all collected from subjects who were asymptomatic on both collection occasions.

Similarly, the two false negative 2H2/J93 ELISA sample pairs were collected from asymptomatic subjects and showed only slight discrepancies in their HAI and $PRNT_{50}$ results (however, pair 37 seroconverted to JEV using the $PRNT_{50}$ assay) (Table 6).

DISCUSSION

Among available serological assays, HAI and the $PRNT_{50}$ are the most commonly used techniques for the diagnosis of DENV and JEV infections, including current, recent and past; however, serum sample processing for HAI assay is time-consuming, involving multiple steps (Inoue *et al*, 2010). In this study, we evaluated the utility of anti-DENV/JEV IgG mAb-

Table 5
Results of 6 paired false negative samples using HAI, $PRNT_{50}$ and anti-DENV/JEV 4G2 ELISA.

Pair No.	Log No.	HAI					HAI Interp	$PRNT_{50}$					$PRNT_{50}$ Interp		ΔOD_{av} of 4G2 ELISA4G2				Interp
															DENV plate		JEV plate		
		D1	D2	D3	D4	JE		D1	D2	D3	D4	JE		Comment	1:400	1:1,600	1:400	1:1,600	
21	S1	320	80	40	80	160	Den	1,783	578	1,703	46	14	Den	Asymp	0.103	0.159	0.003	0.027	Neg
	S2	640	320	40	320	320		>2,560	2,018	2,329	607	14							
29	S1	40	40	80	40	80	Den	673	149	169	17	<10	Den	Asymp	0.151	0.055	0.087	0.012	Neg
	S2	80	80	320	80	160		677	343	507	116	<10							
37	S1	160	40	160	160	80	Den	1,509	537	493	24	<10	JE	Asymp	0.008	0.012	0.152	0.088	Neg
	S2	320	160	320	160	160		>2,560	1,864	1,059	14	365							
38	S1	<10	<10	<10	10	40	JE	<10	<10	<10	<10	121	JE	Asymp	0.091	0.000	0.196	-0.005	Neg
	S2	<10	<10	<10	10	160		20	<10	20	<10	828							
39	S1	20	20	160	40	80	Den	67	173	49	<10	<10	Den	Asymp	0.081	0.066	0.023	-0.005	Neg
	S2	20	80	640	80	80		256	1,000	152	34	<10							
43	S1	<10	<10	<10	<10	<10	JE	32	<10	<10	<10	<10	JE	Asymp	0.025	0.002	0.086	0.022	Neg
	S2	<10	<10	<10	<10	20		<10	<10	<10	<10	26							

Asym, asymptomatic; Den, dengue; DENV, dengue virus; Interp, interprétation; JE, Japanese encephalitis; JEV, Japanese encephalitis virus.

Table 6
Results of 2 paired false negative samples using HAI, $PRNT_{50}$ and anti-DENV/JEV 2H2/J93 ELISA.

Pair No.	Log No.	HAI					HAI Interp	$PRNT_{50}$					$PRNT_{50}$ Interp		ΔOD_{av} of 2H2/J93 ELISA				2H2/J93 Interp
															DENV plate		JEV plate		
		D1	D2	D3	D4	JE		D1	D2	D3	D4	JE		Comment	1:400	1:1,600	1:400	1:1,600	
29	S1	40	40	80	40	80	Den	673	149	169	17	<10	Den	Asymp	0.025	-0.03	0.147	0.072	Neg
	S2	80	80	320	80	160		677	343	507	116	<10							
37	S1	160	40	160	160	80	Den	1,509	537	493	24	<10	JE	Asymp	0.094	-0.067	0.076	0.064	Neg
	S2	320	160	320	160	160		>2,560	1,864	1,059	14	365							

Asym, asymptomatic; Den, dengue; DENV, dengue virus; Interp, interprétation; JE, Japanese encephalitis; JEV, Japanese encephalitis virus.

ELISA that can use 4G2 (flavivirus) or 2H2/J93 combination (dengue complex/JE) mAb for serodiagnosis and differentiation of DENV and JEV virus infections. The correlation between HAI test and anti-DENV/JEV IgG mAb-ELISA was high, as were the sensitivity and specificity of the anti-DENV/JEV IgG mAb -ELISA using 4G2 and 2H2/J93 mAbs. The advantages of the anti-DENV/JEV IgG mAb-ELISA over the HAI test and $PRNT_{50}$ are its expediency, ease of execution and requirement of a small specimen volume (5-10 µl) versus 80 and 120 µl required for HAI and $PRNT_{50}$, respectively. In addition, HAI is limited by the high cross-reactivity of flavivirus-specific antibodies in the serum, such as Zika virus-specific IgG making definitive diagnosis difficult (Shu *et al*, 2003; Lanciotti *et al*, 2008). However, it is not unlikely that the same difficulty occurs with the 4G2 and 2H2/J93 mAb-ELISAs.

Regarding discrepancies between HAI- and $PRNT_{50}$-positive and 4G2 and 2H2/J93 ELISA-negative samples (although small) collected from asymptomatic subjects, we can only postulate that the DENV and JEV neutralizing antibodies in these samples were not sufficiently heterogeneous or cross-reactive (all were mono- or ditypic HAI positive) as to be able to bind with sufficient affinity to the ELISA antigens. A previous study reported DEN IgG and JE IgG ELISAs results correlate highly with those of DEN HAI when using acute and convalescent serum samples (Inoue *et al*, 2010).

In summary, this study demonstrates that anti-DENV/JEV IgG mAb-ELISA correlated highly with HAI. Moreover, this mAb-ELISA is a simple, rapid and sensitive test that can be used for serological screening of DENV/JEV infections.

ACKNOWLEDGEMENTS

The authors thank Ms Napaporn Latthiwongsakorn, Ms Taweewun Hunsawong, Mr Yongyuth Poolpanichaupatam, Mr Somkiat Changnak and Ms Suwannee Chantharawiphak, Department of Virology, AFRIMS, Thailand for their laboratory technical support on $PRNT_{50}$ and HAI assays, and Ms Somporn Krasaesub for biostatistical assistance.

Material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the authors, and are not to be construed as official, or as reflecting true views of the Department of the Army or the Department of Defense. The investigators have adhered to the policies for protection of human subjects as prescribed in AR 70-25.

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