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Comparison of E and NS1 antigens capture ELISA to detect dengue viral antigens from mosquitoes

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ABSTRACT

Background & objectives: In the absence of an effective vaccine or specific antiviral therapy against dengue infection, the only available control measure remains focusing on the incrimination and reduction of vector (mosquito) populations to suppress virus transmission. Diagnosis of dengue in laboratory can be carried out using several approaches, however, their sensitivity and specificity vary from test-to-test. This study was conducted to evaluate the sensitivity and stability of viral envelope (E) and NS1 antigens detected by ELISA in dengue virus infected mosquitoes.

Methods: An in-house developed E-ELISA to detect dengue E antigens was first characterized by using cross-reactive monoclonal antibody (mAb) 42-3 and rabbit polyclonal antibodies as the capture and detector antibodies, respectively. The sensitivity of E-ELISA was compared with the Platelia Dengue NS1 Ag kit using experimentally infected or field-caught mosquitoes.

Results: Our results demonstrated that the E-ELISA was capable of detecting viral antigens with the sensitivity of 69.57, 100, 52.38 and 66.67% for DENV-1 to DENV-4 infected mosquito pools, respectively. This was comparable to the Platelia Dengue NS1 Ag kit, detecting 100% of DENV-1 infected mosquito pools. Among 124 field-collected mosquito pools collected in the vicinity of localized outbreak areas; both E-ELISA and NS1 Ag kit confirmed nine RT-PCR positive samples with sensitivity and concordance rate up to 100%.

Interpretation & conclusion: With the future potential of antigen capture ELISA to be used in the resource deprived regions, the study showed that E-ELISA has similar sensitivity and antigen stability as NS1 Ag kit to complement the current established virological surveillance in human. The improvement of the sensitivity in detecting DENV-3/4 will be needed to incorporate this method into routine mosquito surveillance system.

Key words Antigen-capture ELISA; dengue; mosquito; NS-1; RT-PCR

INTRODUCTION

Dengue virus (DENV) causes over 100 million human infections each year, with 2.5 billion people living in areas where the disease is endemic[1]. Infection with DENV can result in a series of consequences from asymptomatic infection to clinical manifestations ranging from dengue fever (DF) to the life-threatening complications of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS)[2]. This mosquito-borne disease is caused by four serotypes of dengue virus (DENV-1 to 4), which belong to the *Flaviviridae* family, genus *Flavivirus*[3]. The virus is transmitted to humans mainly by two mosquito vectors, *Aedes aegypti* and *Ae. albopictus*. In the absence of effective vaccine or specific therapy, current disease control measures mainly focus on the suppression of mosquito populations to reduce virus transmission[4].

Vector surveillance allows timely implementation of emergency mosquito control measures such as insecticidal fogging to kill adults and reduction in breeding sites to limit an impending outbreak. Hence, accurate, sensitive, rapid and cost-effective laboratory assays to identify virus infected mosquito pools are a critical component for dengue surveillance and research programme. The standard methods for detecting dengue virus in mosquito pools are by virus isolation in cell culture or suckling mice, or the extraction of viral RNA and its detection by a reverse transcriptase-PCR (RT-PCR)[5-6]. These methods are costly, time consuming and require specialized equipment and highly trained personnel in biosafety level (BSL2/3/4) facility that are not always available in developing regions of the world, where dengue imposes major health and economic burden.

The advantages of antigen-capture enzyme-linked immunosorbent assay (ELISA) as an alternative method

of arbovirus detection in mosquitoes include speed, high-throughput result and on-site application. Specialized rapid test kits, such as VecTest utilizing antibody targeting envelop (E) protein to capture virion particles, is available to detect viral antigen in mosquitoes for St. Louis encephalitis virus (SLEV)[7] and West Nile virus (WNV)[8]. Recent studies have shown that commercially available ELISA kit designed to detect DENV non-structural protein 1 (NS1) in human serum specimens could be used to detect DENV NS1 in infected *Ae. aegypti*[9-11]. However, these various ELISA methods have not been evaluated in terms of the sensitivity and stability of the viral antigens within mosquito pools. In this study, we expanded upon these studies and compared the sensitivity of antigen-capture ELISA in detecting E and NS1 antigens in dengue virus infected mosquitoes. In order to compare the sensitivity of antigen-capture ELISA in detecting E and NS1 antigens from mosquito pools, an in-house ELISA for detection of E protein (E-ELISA) was developed and the detection sensitivity was compared with the Platelia Dengue NS1 Ag kit (Bio-Rad Laboratories; Catalogue No. 72830). Our result suggested that E-ELISA bears high potential to be further developed into field deployable lateral flow assay for the implementation of virological surveillance of vector mosquitoes due to the stability of E-antigens.

MATERIAL & METHODS

Virus, cells and antibodies

DENV-1 strain Myanmar, DENV-2 strain New Guinea C, DENV-3 strain 98TW503 and DENV-4 strain H241, used in this study were propagated in *Aedes albopictus* clone C6/36 cells (the American Type Culture Collection, ATCC, Manassas, VA), and grown at 28°C in Dulbecco's minimum essential medium (DMEM), Gibco BRL, California, USA; supplemented with 10% fetal bovine serum (FBS), nonessential amino acids and sodium pyruvate. Vero cell culture for determining virus infectivity was grown at 37°C with 5% CO_2 in DMEM supplemented with 10% heat-inactivated FBS, nonessential amino acids and sodium pyruvate. Virus stocks were used to infect 80% confluent cells monolayers in DMEM medium supplemented with 2% FBS and incubated at 37°C until the day designated, at which stage the supernatant and cell monolayers were harvested.

DB42-3 monoclonal antibody (mAb 42-3), which recognizes flavivirus group cross-reactive epitopes of envelope (E) protein was generated and purified as previously described[12]. This mAb has high binding efficiency in the antigen-capture ELISA to E of all four serotypes of DENV. Mouse hyper-immune ascetic fluids (MHIAF) against DENV-1 strain Hawaii, DENV-2 strain New Guinea C, DENV-3 strain H87, and DENV-4 strain H241 were provided by US-Centres for Disease Control and Prevention (US-CDC, Fort Collins, Colorado). Polyclonal rabbits antisera to E of DENV-1 to DENV-4 were kindly provided by Dr Gwong-Jen J. Chang from US-CDC and was previously generated by repeat immunizations of rabbits with 100 µg of plasmid DNAs expressing premembrane/E genes from DENV-1 to DENV-4, respectively[13].

Mosquito inoculation and processing

A seven-day-old female *Ae. aegypti* (Strain Kaohsiung-Sanming) (Diptera: Culicidae) were infected with DENV-1, -2, -3 or -4 by intrathoracic injection as modified from the method described by Rosen and Gubler[14]. Each mosquito in groups of 100 received 0.017 µl inoculum containing 1×10^6 plaque-forming units (PFU)/ml, 7.5×10^6 PFU/ml, 2.5×10^5 PFU/ml and 2.5×10^5 PFU/ml of DEN-1, -2, -3 and -4, respectively. The infected mosquitoes were maintained by feeding with 10% sucrose solution, and kept at 28°C and 60–70% relative humidity for 14 days before harvesting. The infected mosquitoes were pooled with uninfected mosquitoes at designated ratios, with a maximum of 100 mosquitoes per pool, and stored at –70°C until tested. The pools were homogenized in an MM300 mixer mill (QIAGEN) at 25 Hz for 5 min in the presence of 1 ml BA-1 (DMEM with 3% bovine serum albumin) diluent and one 3-mm sterilized tungsten-carbide bead. Uninfected *Ae. aegypti* mosquito controls and extraction controls consisting of 1 ml cold BA-1 diluent were processed in parallel with each batch of samples. The clarified suspensions from the homogenates after centrifugation at 12,000 rpm for 10 min at 4°C were prepared for virus titer determination by plaque assay and antigen-capture ELISAs. 10-fold serial dilutions of the homogenates were inoculated onto monolayers of Vero cells grown in 6-well plates. After absorption, the cells were overlaid with nutrient agarose and the plaques were enumerated 5 to 7 days later after the addition of crystal violet.

E antigen-capture ELISA (E-ELISA)

Microtitration plates (Nunc Maxisorp, Nunclon Nunc, Roskilde, Denmark) were coated overnight at 4°C with 50 µl (per well) of mAb 42–3 at the concentration of 27 ng/ml in phosphate-buffered saline (PBS) for E-specific antigen capture ELISA. Wells were washed with PBS-Tween buffer (PBS, 0.05% Tween 20) and 200 µl (per

well) of blocking buffer (PBS, 0.05% Tween 20, 3% milk) was added and incubated at room temperature for 2 h. Mosquito homogenate samples were added to wells (50 µl/well) and incubated for 2 h at room temperature. The wells were washed again and incubated for 1 h at 37°C with 50 µl/well of anti-E polyclonal rabbit antiserum from individual serotype of dengue viruses (diluted in blocking buffer). The wells were washed again and incubated for 1 h at 37°C with 50 µl/well of peroxidase-conjugated goat anti-rabbit antiserum (Jackson Immuno Research Laboratories) (diluted 1/5000 in blocking buffer). After three further washes, each well received 50 µl of a freshly prepared 3,3',5,5'-tetramethylbenzidine solution (TMB microwell peroxidase substrate system; Kirkegaard and Perry Laboratories). The colour reaction was stopped after 10 min with 25 µl/well of 2 N sulfuric acid, and the plates were read at 450 nm optical density (OD_{450}). Negative controls were measured when the reaction was carried out in the absence of antigens, which were referred to the mock infection culture supernatant for *in vitro* infection assay and un-infected mosquito homogenates for viral spiked and infected mosquito samples.

One-step RT-PCR

The viral RNA was extracted from 140 µl of the supernatants from field-caught mosquito pool homogenates using a QIAamp viral RNA kit (QIAGEN Inc.) according to the manufacturer's suggested protocol. RNA was eluted in 50 µl of nuclease-free water. A total of 5 ml of RNA was subjected to one-step RT-PCR using the protocol as described[15]. Briefly, the RT-PCR amplification was assayed in a 50 µl reaction mixture containing 5 µl of RNA, 25 pmol of the mD1 and D2 amplimers, and reagent components of a one-step RT-PCR kit (Qiagen Inc.). The amplification involved the following steps: Reverse transcription at 50°C for 30 min; one cycle of initial denaturation of the reverse transcriptase and activation of the HotStart Taq polymerase at 95°C for 15 min; 55°C for 15 sec, and 72°C for 30 sec; 34 cycles at 95°C for 15 sec, 55°C for 15 sec, and 72°C for 30 sec; and one cycle of 10-min extension at 72°C. After amplification, a 5 µl portion of each product was analyzed by agarose gel electrophoresis.

Reproducibility and stability evaluation of the E-ELISA

To evaluate the reproducibility of E-ELISA, two separate ELISA plates coated with 42-3 mAb, and estimated the intra-plate and inter-plate assay performance by repeated testing of the three E-antigen positive specimens for five times in one plate and five times in the second plate, respectively. The assay results were used to calculate the coefficient of variation (CV) and statistical analysis.

To analyze the thermal stability of the E antigens within the mosquitoes that can be detected by E-ELISA, 14-day post-infected mosquitoes were divided into three groups. The control mosquito group was frozen immediately at –70ºC and the mosquitoes in the thermal stability groups were held at room temperature with sealed aluminum foil pouches containing desiccants for the designated duration before freezing at –70ºC. Uninfected mosquitoes (50 per group) were held under the same conditions. Control and experimental mosquito groups were processed and E-ELISA performed as indicated in previous sections to determine if the assay performance was similar in different storage conditions and durations.

Detection of field-collected mosquito samples

As part of an entomological surveillance programme in high risk districts with the repeat history of dengue outbreaks or high vector density in Kaohsing City, southern Taiwan, *Ae. aegypti* and *Ae. albopictus* mosquitoes were collected for field study at weekly intervals during September to December 2010 from both indoor (inside houses) and outdoor (in front and back yards). In general, over 50% of the wards of the city were visited at least once per month and whenever a dengue case was reported. The house and its surrounding neighbours were visited at least once within seven days of reported index case. At each visit, a cluster of 50 houses was surveyed and inspected; and mosquito collections were made in the first floor (including basement) and outside surroundings, for adult *Aedes* mosquitoes, using small sweeping nets. All adult mosquitoes collected from each visit were identified and pooled by species and gender according to the premise of each visit. Collected specimens were mailed to the laboratory with insulated ice packed boxes and immediately stored at –70°C until tested.

Minimum infection rate (MIR) was expressed as a number per 1000 mosquitoes. MIR assumes that when a pool tests positive, just one mosquito in that pool was actually DENV-infected. The number of positive pools relative to the mosquitoes tested was examined, and this relationship was extrapolated onto the general mosquito population in the environment. The net result was the estimate of the minimal degree of virus infection in the mosquito population. This study does not require ethical approval.

Statistical analysis

The Student's *t*-test was used for comparisons between normally distributed continuous variables while the Mann-Whitney *U*-test for comparisons between continuous variables, not normally distributed. Chi-square analy-

sis was used for comparisons among proportional data. The receiver operating characteristic (ROC) curve analysis, a plot of the sensitivity versus false positive rate (100% specificity), was applied to determine the cut-off value of the antigen capture ELISA, and the accuracy of discrimination was measured by the area under the ROC curve (AUC). The statistical significant of the CV results was determined based on the method developed by Reed[16] with 1.5 for the factor of *k* which stands for the ratio of the difference between two assays was used. The obtained *p*-value of CV helps to determine what magnitudes of difference can be expected by chance alone when the particular CV is in effect. For all statistical analyses, SPSS version 10.0 software package was used.

RESULTS

Characterization and cut-off determination of E-ELISA

To establish a specific and sensitive E-ELISA for detecting dengue E antigens, mAb 42-3 and rabbit polyclonal antibodies against E from four serotypes of dengue viruses were used as the capture and detector antibodies, respectively. The checkerboard analysis of dilution series of capturer and detector antibodies was performed to optimize the reaction conditions of the assay. The optimal concentrations for mAb 42-3 were determined to be 27 ng/ml and 1 : 3200, 1 : 800, 1 : 200 and 1: 400 for rabbit polyclonal antibodies against DENV-1 to 4, respectively.

Then, the above optimal concentrations for both mAb and rabbit sera were used to determine the positive cut-off value for detecting E-antigen from the mosquito pools. One DENV infected and 49 uninfected mosquitoes per pool were processed and 25, 35, 23 and 23 pools of DENV-1 to DENV-4 infected mosquitoes were evaluated, respectively. The mosquito homogenates were evaluated concurrently by E-ELISA and the virus plaque assay. The homogenates from the equal number of non-infected mosquito per pool were used as negative control. The assays were repeated in three replicates. As shown in Fig. 1, the infective titers of each serotype of DENV, highly correlated with the OD_{450} readings. Area under ROC curve (AUC) was used to determine the best fit cut-off value. As shown in Table 1, specimens were considered to be positive if their OD values were greater than the mean OD_{450} value plus three standard deviations (SD) of the negative control. This criterion with the highest AUC was used as the cut-off values for the following study.

The reproducibility of E-ELISA was evaluated on two different plates with five replicates per plate. As shown in Table 2, the intra-CVs of three samples tested for DENV-1 to DENV-4 in this assay were 1.89, 1.69, 8.03, and 1.19% with statistical significance ($p< 0.01$), respec-

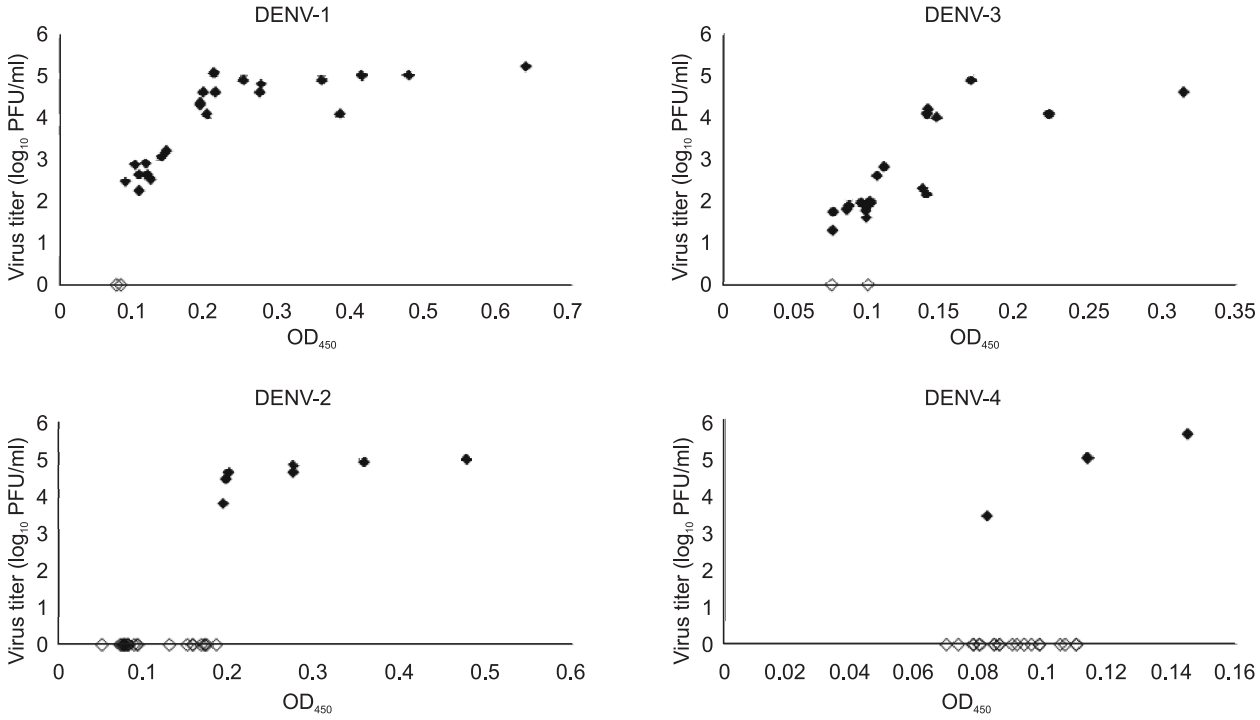

Fig. 1. Correlation between virus infectivity and E-ELISA reactivity in virus-infected mosquito pools. One DENV intrathoracically infected mosquito and 49 uninfected mosquitoes per pool were processed and the pool numbers were 25, 35, 23 and 23 for DENV-1 to DENV-4, respectively. The infective positive and negative pools were labeled as solid and open diamonds, respectively.

Table 1. Determination of the cut-off values of the antigen capture ELISA

Serotype[a]	Cut-off value	% Sensitivity	% Specificity	AUC[b] (95% CI)[c]
DENV-1	Mean ± 2 SD	69.57	0	34.8 (2.8–66.8)
	Mean ± 3 SD	69.57	100	100 (81.2–100)
	Mean ± 4 SD	65.52	100	82.6 (60.4–100)
DENV-2	Mean ± 2 SD	100	84.38	70.8 (32.5–100)
	Mean ± 3 SD	100	93.75	90.2 (0–100)
	Mean ± 4 SD	66.33	93.75	63.5 (24.2–100)
DENV-3	Mean ± 2 SD	66.67	50	58.3 (13.6–100)
	Mean ± 3 SD	52.38	100	76.2 (50.7–100)
	Mean ± 4 SD	38.1	100	69 (39.4–98.7)
DENV-4	Mean ± 2 SD	66.67	85	75.8 (0–100)
	Mean ± 3 SD	66.67	100	83.3 (0–100)
	Mean ± 4 SD	66.67	100	83.3 (0–100)

[a]Serotype of dengue viruses used to inject intrathoracically into the mosquito. The results were obtained from 25, 35, 23 and 23 mosquito pools for DENV-1 to DENV-4, respectively; [b]AUC: Area under (ROC) curve; [c]CI: Confidence interval.

tively. In addition, the inter-CVs for DENV-1 to DENV-4 were 2.13, 2.3, 2.67 and 4.52% with statistical significance ($p< 0.01$), respectively.

Sensitivity of the E-ELISA versus the Platelia Dengue NS1 Ag kit

Among the 106 mosquito pools inoculated by DENV-1 to DENV-4, 23 out of 25 (92%), 7 out of 35 (20%), 21 out of 23 (91%) and 3 out of 23 (13%) pools were positive by viral plaque assays, respectively. Using the positive pools from viral plaque assay as the gold standard, the sensitivity of the E-ELISA in detecting viral antigens was 69.57, 100, 52.38 and 66.67% for pools of DENV-1 to DENV-4, respectively. Furthermore, 100% of the sensitivity was observed for all four serotypes of DENV when the infective titers were greater than 10^3 PFU/ml. The positive mosquito pools were further subjected to the Platelia Dengue NS1 Ag kit and the sensitivity was 67% in detecting DENV-4. The low sensitivity of NS1 Ag kit in detecting DENV-4 was similar to result from E-ELISA. Additionally, 100% detection rates wee found for NS1 Ag kit in detecting DENV-1 to 3 infected mosquito pools.

Additionally, the number of uninfected mosquitoes in a pool (49, 74 and 99) did not affect the sensitivity of E-ELISA in detecting the presence of a single infected mosquito. For convenience, all further work with experimentally infected mosquitoes was done with pools of 50 (one infected and 49 uninfected mosquitoes).

Stability of viral antigens

In order to analyze the stability of E- and NS1-antigens in the infected mosquitoes, intrathoracically infected mosquito pools were stored in –70°C immediately or kept at room temperature for 7 and 14 days before storing in –70°C for further processing. The results were shown in Table 3. The detection sensitivities were similar for both E-ELISA and NS1 kit without statistical significance when pool specimens were kept at room temperature for 7 or 14 days as compared to the pool stored in –70°C immediately ($p> 0.05$). Interestingly, the detection sensitivity of E-ELISA on DENV-3 increased slightly for the pools stored in room temperature for 7 and 14 days with statistical significance ($p< 0.01$). This result confirmed that both E- and NS1-antigen remained stable for at least 14 days at room temperature and could still be detected by E-ELISA and NS1 kit, respectively. Our observation suggested that strictly maintaining cold-chain for field application is not essential for transporting the field-collected mosquitoes when ELISA method is used for antigen detection.

Antigen detection on field-collected mosquito pools

Due to the variable handling and storage conditions after mosquito trapping, which might result in virus inac-

Table 2. Reproducibility of E-ELISA

Repeatability	Serotypes[a]			
	DENV-1	DENV-2	DENV-3	DENV-4
Inter-assay CV[b]	1.89% ($p<0.0001$)	1.69% (p<0.0001)	8.03% ($p=0.0018$)	1.19% ($p<0.0001$)
Intra-assay CV[b]	2.13% ($p<0.0001$)	2.3% ($p<0.0001$)	2.67% ($p<0.0001$)	4.52% ($p<0.0001$)

[a]50-uninfected mosquito pool was spiked with 10^6 PFU/ml of virus-infected tissue culture media. Five pools per dengue serotype were processed and clarified homogenates were assayed by E-ELISA to estimate the inter- and intra-plate confidence variable of assay; [b]The value of CV was calculated based on the standard deviation and average of O.D. from five different pools and the *p*-value was determined based on the method proposed by Reed with $k = 1.5$.

Table 3. Effects of storage temperature on the stability of E-antigens in infected mosquitoes as determined by E-ELISA

Serotype	Treatment group[a]	No. of mosquito pools	E-ELISA Mean OD_{450}±SD[b]	NS1 Ag kit Mean OD_{450} ± SD[b]
DENV-1	Control	6	0.20±0.0089	1.72±0.372
	Day 7	10	0.18±0.0466	1.84±0.163
	Day 14	10	0.19±0.0423	1.86±0.242
DENV-2	Control	10	0.13±0.0170	2.16±0.127
	Day 7	10	0.12±0.0153	2.31±0.153
	Day 14	10	0.14±0.0326	2.41±0.326
DENV-3	Control	9	0.11±0.0247	1.81±0.246
	Day 7	10	0.17±0.0328*	1.65±0.382
	Day 14	10	0.16±0.0383*	1.57±0.383

[a]DENV-infected mosquitoes were harvested 14 days after intrathoracic injection. Control mosquito pools were frozen immediately at –70°C; Experimental pools were kept at room temperature with desiccants for 7 or 14 days and frozen at –70°C until use; [b]SD: Standard deviation; *Statistically significant (p<0.05) compared to control group by student's t-test.

tivation, virus isolation or plaque assay might not be feasible. A conventional one-step RT-PCR was used as the gold standard in our preliminary evaluation of antigen-capture ELISA on field-collected mosquitoes focused on the sensitivity and concordance of E-ELISA and NS1 Ag kit. The RT-PCR identified nine positive out of 124 field collected mosquito pools (7.25%) with the calculated minimum infection rate (MIR) of 21.69/1000. The RT-PCR positive pools were subjected to both E-ELISA and NS1 Ag kit and all nine pools were also found positive in both assays, yielding 100% sensitivity to both assays. The concordance of E-ELISA and NS1 Ag kit reached 100%.

DISCUSSION

Many studies have been proposed to use antigen-capture ELISAs for identifying dengue viral E or NS1 antigens from infected positive mosquito pools[9, 17-19]. However, these assays have not been evaluated simultaneously to compare the sensitivity of the assays and stability of both E- and NS1-antigens. In this study, we first characterize E-ELISA to detect E-antigens because no commercial kit is available. The detection limit of the ELISA used in this study was similar to those reported for SLEV and WNV in mosquitoes[7-8, 20], although the determinations of the cut-off were different. In the current assay, the cut-off used was based on three independent in-plate negative control mosquito pools with at least 40 uninfected mosquitoes per pool. This cut-off determination had the highest AUC.

Then we compared the sensitivity and stability of antigens utilizing antigen-capture ELISAs to detect viral E- and NS1-antigens from infected mosquitoes. Our results suggested that the use of the E-ELISA described in this report was as sensitive as the Platelia Dengue NS1 Ag kit, which was further confirmed by using field-caught mosquito pools. The concordance rate of detecting viral antigens by E-ELISA and NS1 Ag kit was 100% from all nine RT-PCR positive mosquito pools. The antigen stability assay described in this study suggested that both E- and NS1-antigens were stable and could be detected by both E-ELISA and NS1 Ag kit for up to 14 days in ambient temperature, however, the lower detection of DENV-1 E-antigens by E-ELISA, compared to the Platelia Dengue NS1 Ag kit, was most likely due to the lower sensitivity of anti-E antibodies in detecting DENV-1 E- antigens. In this study, we demonstrated that this E-antigen capture ELISA has a good potential to develop into a reliable field deployable testing kit similar to the VecTest WN and SLE antigen assays[7, 21-22].

The variable sensitivity to detect viral antigens from four different serotypes of dengue viruses from mosquito pools suggested the variable sensitivity of the cross-reactive anti-E or -NS1 antibodies in capturing antigens. As shown in the study, lower sensitivity was observed in detecting E- and NS1-antigens from DENV-3 (52.38%) and DENV-4 (66.67%) infected mosquitoes by examining the virus titers from those plaque assay positive mosquito pools (which failed to be detected by ELISA). We found that those pools contained virus titers below 5×10^3 and 10^4 PFU/ml for DENV-3 and DENV-4, respectively. Further, improvement of the antigen capturing antibodies for E and NS1 by increasing the sensitivity for DENV-3 and DENV-4 will be needed for the future implementation of ELISA method into routine mosquito surveillance system.

A major concern in conducting the surveillance programme is the need for maintenance of a cold chain to avoid RNA or virus degradation. Our results suggested that E-antigens, similar to NS1 as reported by previous publications[20, 23–24], can be detected by E-ELISA even though the mosquitoes were kept under room temperature up to 14 days. The higher OD in detecting DENV-3 from mosquitoes kept in room temperature can be explained by the cellular disruption or antigen disaggregation from the infected mosquitoes kept at higher RTs, leading to increased higher reactivity in ELISA as previously observed by Tsai *et al*[20]. Our study suggested that storage of desiccated mosquito specimens up to 14 days did not affect the stability of the DENV antigens; thus, strict maintenance of the cold-chain environment between field sites and testing laboratories may not be essential. Comprehensively evaluating this protocol in the future could

demonstrate the way for expanding the geographic range accessible to dengue viral surveillance where dry ice or mechanical freezers are unavailable.

CONCLUSION

In summary, our study suggested that the E-ELISA has 100% detection rate for all four serotypes of DENV when the infective titers of the mosquito pools reached 10^3 PFU/ml. Due to the comparable sensitivity and antigen stability as NS1 Ag kit, the E-ELISA has the potential to be developed into a field deployable kit as VecTest WNV and SLEV antigen assay. The future efforts to improve the sensitivity of the antigen-capturing antibodies, particularly DENV-4 for both E-ELISA and NS1 Ag kit is crucial in order to incorporate this method into routine mosquitoes urveillance system and to complement the currently established active or passive virological surveillance in humans for rapid evaluation of dengue outbreak.

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