CLINICAL VALIDATION OF BIOTEK-M™ DENGUE AQUA KIT IN THE DIAGNOSIS OF DENGUE INFECTIONS IN THE PHILIPPINES

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Abstract. Owing to the non-specific signs and symptoms of patients during the acute phase of dengue illness, accurate detection of dengue virus (DENV) infection by a reliable yet simple laboratory test during the early phase of illness is crucial for proper management and better clinical outcome of patients. In order to address this need, a miniature loop mediated amplification (LAMP) kit, BIOTEK-M™ Dengue Aqua kit, was developed and evaluated at four hospitals in the Philippines among 517 patients greater than six months of age who presented with prior fever of no longer than seven days. Diagnostic accuracy was determined by comparing with the gold standard of a combination of dengue heminested RT-PCR, IgM ELISA, and NS1 and paired acute-convalescent IgG ELISA, with 91.7% meeting the criteria for true DENV infection. Sensitivity, specificity, positive predictive value, and negative predictive value were 81.9% (95% confidence interval (CI): 89.3-94.1), 85.7% (95% CI: 46.7-99.3), 99.7% (95% CI: 98.3-100), and 7.5% (95% CI: 3.3-15.4), respectively. The positive likelihood ratio was 6.549 (95% CI: 1.046-40.981) and the negative likelihood ratio was 0.207 (95% CI: 0.165-0.260). These results validate the application of BIOTEK-M™ Dengue Aqua kit for detection of early DENV infection and should be a useful tool in dengue control and elimination programs in the Philippines and other dengue endemic countries.

Keywords: BIOTEK-M™ Dengue Aqua kit, dengue, early detection, RT-LAMP assay

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INTRODUCTION

Dengue fever is ranked by the World Health Organization (WHO) as the most critical and most rapidly spreading mosquito-borne viral disease in the world, with a 30-fold increase in global incidence over the past 50 years (WHO, 2009). Dengue is included in the top ten threats to global health in 2019 (WHO, 2019). Mapping of global distribution of dengue by evidence-based consensus shows a list of 128 affected countries for which there is good evidence of dengue occurrence (Brady *et al*, 2012). Bhatt *et al* (2013) estimated 390 million cases of dengue infections occur annually around the world and 500,000 cases develop the more severe forms of the disease, resulting in 25,000 deaths. In countries such as the Philippines, dengue infections impose a substantial health burden (Undurraga *et al*, 2017).

While there is no specific treatment for dengue infection, early and accurate diagnosis allows early implementation of supportive management, which greatly improves outcome (Ahmed and Broor, 2014; Pok *et al*, 2015). An affordable, accessible, reliable and user-friendly dengue test would allow dengue-endemic countries to achieve not only efficient control but also a more effective surveillance of dengue infection. In many countries, the most common laboratory tests for dengue are dengue non-structural 1 (NS1) antigen capture ELISA and dengue antibody serologic IgM tests. Dengue NS1 antigen can be detected in blood of patients in the early stages of dengue infection, particularly during the first four days of illness (Sundaram *et al*, 2016), with reports of sensitivity and specificity of up to 100% when used from the first until the third day of infection (Kalayanarooj, 2011; de Costa *et al*, 2014; Anand *et al*, 2016; Sundaram *et al*, 2016; Ambrose *et al*, 2017). Dengue serologic tests are likewise frequently used with reported sensitivity of IgM ELISA ranging from 40.9-66.7%, with IgM detectable only after the end of the viremia or fever (Anand *et al*, 2016) or when febrile episode has already subsided, usually at the fifth day of onset of illness (Kalayanarooj, 2011; Shukla *et al*, 2017). PCR-based tests are likewise available but only in limited settings as they are expensive to perform, require highly sophisticated equipment and, thus, difficult to use in field settings where outbreaks occur (Neeraja *et al*, 2015).

A relatively new laboratory procedure, the loop-mediated isothermal amplification (LAMP) assay developed by Notomi *et al* (2000) has been recognized to offer several advantages, such as rapid simple detection procedures under isothermal conditions, highly specific using 4 different primers and recognizes 6 distinct regions on the target gene, and having a high amplification efficiency wherein target DNA can be amplified 10^9-10^{10} times, which overcome the shortcomings of existing PCR-based protocols as summarized in the review of Wang and Gubler (2018). A miniature LAMP-based Biotek-M™ Dengue Aqua Kit (Biotek-M™) based was recently developed in the Philippines to provide a reliable locally available dengue test kit applicable not only in hospital settings but also in the field and other primary health care settings where only basic laboratory equipment is available. An initial pilot test of Biotek-M™ conducted in

two hospitals in Metro Manila, Philippines from 2011 to 2012 using 117 blood samples during the first seven days of acute febrile illness of patients suspected to have dengue showed a sensitivity of 85% (95% CI: 75-92) specificity of 76% (95% CI: 63-86), positive predictive value (PPV) of 83% (95% CI: 72-90); negative (N)PV of 79% (95% CI: 66-88); positive likelihood ratio (PLR) of 3.545 (95% CI: 2.143-5.864) and negative (N)LR of 0.196 (95% CI: 0.109- 0.355) when compared to a dengue PCR-based assay (Berba *et al*, unpublished).

It is urgent to improve clinical management of dengue through early diagnosis. For a new dengue test to respond to the needs of health professionals and febrile patients in endemic countries who have fever and seeks to determine whether the fever is from dengue or other etiologies, the test must be: 1) reliable with acceptable diagnostic accuracy measures (high sensitivity and specificity measures as well as useful likelihood ratios); 2) accessible and feasible to set up in clinical settings with only basic laboratory capacities; and 3) affordable. The study evaluated clinical performance and determined accuracy measures of the new Biotek-M™ in diagnosis of DENV infection among patients presenting with acute febrile illness.

MATERIALS AND METHODS

Clinical validation protocol

In order to evaluate Biotek-M™ and determine diagnostic accuracy measures in an actual dengue infection setting, a clinical validation study using a prospective cross-sectional design was performed from July 2014 to November 2015. Four tertiary medical centers in Metro Manila, Philippines were selected as study sites, namely, The Medical City (TMC), a 700-bed private hospital; National Children's Hospital (NCH), a 250-bed government hospital; Philippine Children's Medical Center (PCMC), a 200-bed teaching government hospital; and Philippine General Hospital (PGH), a 1500-bed government teaching hospital. Patients suspected to have dengue and who met the inclusion criteria were invited to participate in the study. Inclusion criteria were age at least 6 months old, attended any of the four study sites within seven days of onset of acute fever, an axillary temperature ≥37.8°C at any time during the previous seven days, and dengue infection considered by attending physician.

In order to obtain sensitivity of 90% and specificity of 80% for validation of the Biotek-M™ test kit, and assuming prevalence of dengue infections among test subjects with acute febrile illness of 30%, with a 5% margin of error and a 95% CI, 462 participants were required (Edillo *et al*, 2015). In order to compensate for a possible drop-out of 10%, a recruitment goal of 500 participants was established.

Blood sample (3 ml from a child and 10 ml from a participant aged 18 and above) was collected from each participant and transported at 4°C within 24 hours to the University of the Philippines-National Institutes of Health (UP-NIH). Different dengue diagnostic tests (Biotek-M™, dengue hemi-nested RT-PCR (HRT-PCR), dengue IgM capture ELISA, and dengue NS1 antigen rapid tests) were independently performed by trained laboratory personnel assigned to

each test and blinded to results of the other tests. Results of the three dengue reference tests were relayed to the attending physician to guide patient management. If all the initial three dengue reference tests results were negative, patients were instructed to return 14 days later for additional blood collection (3 ml) for a dengue convalescent IgG test. Sera from acute patients were also subjected to dengue IgG assay. Patients' data, laboratory results, final clinical diagnosis, and patients' outcome were recorded.

Research protocol was approved by the Institutional Review Board, University of the Philippines. Manila and from the other participating hospitals (NIH 2010-047). Prior informed consent was obtained from each participant or from parents or legal guardian for a child (≤18 years of age).

Design of Biotek-M™ Dengue Aqua Kit

Biotek-M™ was developed by designing primers targeting dengue virus (DENV) NS5 gene using Primer Explorer v4 (Eiken Chemical Co Ltd, Tokyo, Japan) or through manual design (Table 1). The Biotek-M™ Dengue Aqua Kit (Fig 1) also included Biotek-M™ LAMP Heater, required for isothermal amplification of target gene using a dual temperature set-up normally used for LAMP reaction, namely, a first setting of 63-65°C and a stop reaction of 80°C, and Easyview LED Transilluminator to facilitate interpretation of results, positive samples producing a green

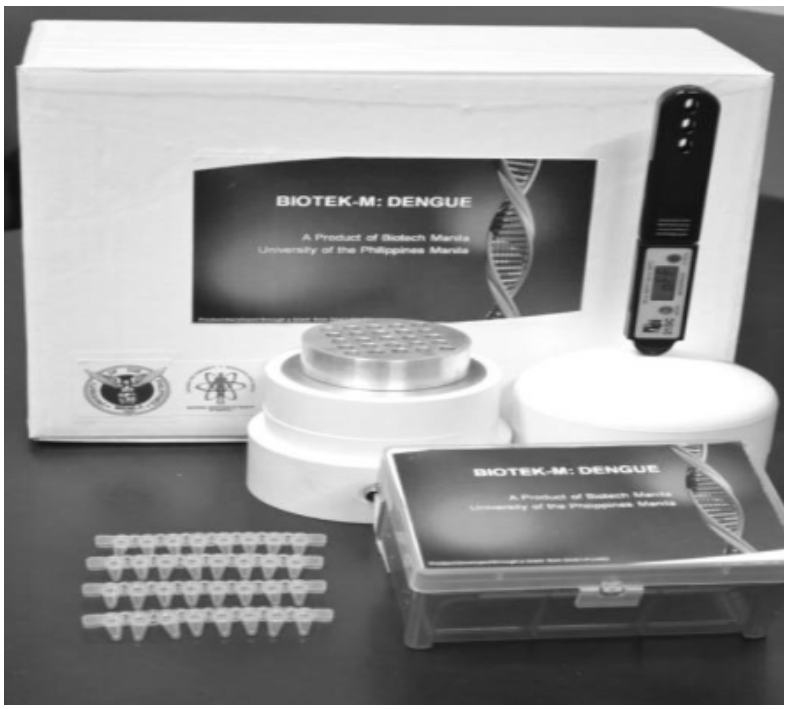

Fig 1 - Biotek-M™ Dengue Aqua Kit

Table 1
Primer sequences targeting NS5 gene of dengue virus used in development of Biotek-M™ test kit

Primer name	Sequence (5'→3')
NS5-F3	AGAACAAAAGACCCAGATGT
NS5-B3	ACTCTCCTAATTTTTTCTCCTTC
NS5-FIP	CCATTGGTTTCATCGTGAAAACGGGAAGAGTTCACAAGAAAGTC
NS5-BIP	AATCAGCAGGAGCTGTGAAGATGTCATTTTAACACACGCACATT
NS5-FLP	GGAAGAGTTCACAAGAAAGTC
NS5-BLP	TCATTTTAACACACGCACATT

light and negative an orange light. The instrument (10 cm wide × 12 cm high; weighs 700 g) can process 50 samples simultaneously.

Laboratory methods

In situations when blood samples were not sufficient to conduct all four tests, dengue HRT-PCR assay was first conducted, followed by the Biotek-M™ test, then, if sufficient sample was available, dengue IgM ELISA and finally dengue NS1 antigen rapid test.

Biotek-M™ test

The reaction mixture (25 µl) contained 0.384 pmol each of primers NS5-FIP and NS5-BIP, 0.16 pmol each of primers NS5-F3 and NS5-B3, 1.4 mM dNTPs, 0.8 M betaine, 0.1% Tween 20, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, 10 mM KCl, 20 mM Tris-HCl pH 8.8, 16 U *Bst* DNA polymerase (Lucigen, Middleton, WI), 0.125 U Moloney murine leukemia virus reverse transcriptase (Clontech Takara, Mountain View, CA), and specified amount of target RNA. The solution was incubated at 63°C for 60 minutes in a heating block. Analysis of each patient blood sample was performed in a set of six tubes, four of which has the primer mixture for each serotype, one for positive control (dengue cDNA) and one for negative control (PCR water only). A positive test is considered when there is a color change from orange to yellow green after addition of 2µl of 500 units SYBR Green I nucleic acid gel stain (Invitrogen, Eugene, OR).

Reference laboratory test 1: HRT-PCR

RNA was extracted from 140 µ of virus-infected tissue culture fluid or human serum using a Nucleospin Virus Kit (MACHEREY-NAGEL GmbH & Co KG, Dueren, Germany), according to manufacturer's protocol. RNA was eluted in 60 µl of the manufacturer's suggested elution buffer. RNA was subjected to HRT-PCR using primers located at the junction region of DENV capsid and pre-membrane genes as previously described (Chien *et al*, 2006). In brief, 3 µl aliquot of RNA, 0.5 µM each of mD1, rTS1, mTS2, TS3, and rTS4 primers, and one step RT-PCR Kit mixture (Invitrogen, Eugene, OR) to constitute a 25-µl reaction volume. Thermocycling, conducted in a conventional T100 PCR machine (Bio-Rad Laboratories, Hercules, CA), was performed as follows: 50°C for 30 minutes; 95°C for 15 minutes, 55°C for

15 seconds and 72°C for 30 seconds; 34 cycles of 95°C for 15 seconds, 55°C for 15 seconds and 72°C for 30 seconds; and a final heating at 72°C for 10 minutes. A 5 µl aliquot of reaction solution was analyzed by 15% agarose gel-electrophoresis and stained with Gel Red Nucleic Acid Gel Stain (Biotium, Fremont, CA). Serotype was determined by amplicon size: DENV serotypes 1, 2, 3, and 4 of 208, 119, 288 and 400 bp (redesigned) respectively (Chien *et al*, 2006).

Reference laboratory 2: IgM/IgG capture ELISA

IgM capture ELISA (MAC-ELISA) was performed using a Pan-Bio Dengue IgG/M Capture ELISA kit (Abbot, Frehold, NJ). In short, after warming all reagents to ambient temperature (20-25°C), 10 µl aliquot each of negative control, positive control, calibrator, and patient sample is added to 1 ml of sample diluent followed by 0.5 µl aliquot of diluted (1:250) antigen. The required volume of diluted antigen was then removed and an equal volume of Mab tracer added, and within 10 minutes 100 µl aliquot each of diluted patient's sample and controls were added into respective microwells and incubated for 60 minutes at 37±1°C. Wells were then washed before addition of 100 µl of antigen-Mab tracer and incubated for another 60 minutes. After washing, 100 µl aliquot of 3,3′,5,5′-Tetramethylbenzidine (TMB) was added to each well and incubated for 10 minutes before the reaction was terminated by a stop solution. A 450 nm was measured (iMark™ Microplate Absorbance Reader, Bio-Rad Laboratories, Hercules, CA).

Cut-off value was determined from average A_{450nm} of calibrator (in triplicate) and adjusted according to manufacturer's instructions. An index value was then calculated by dividing the sample absorbance by cut-off value. Values of calibrator, negative and positive controls within the manufacturer's specification are considered acceptable, and test was repeated if the values do not meet the specifications. Results are interpreted as shown in Table 2.

Reference laboratory 3: Dengue NS1 antigen rapid test

Dengue NS1 Ag STRIP (Bio-Rad Laboratories, Hercules, CA), an immunochromatographic test (ICT) for rapid detection of DENV NS1 antigen,

Table 2
Interpretation of index values in dengue IgM/IgG capture ELISA

Index value	Result	Interpretation
<1.8	Negative	No detectable antibodies
1.8-2.2	Equivocal	Test of sample should be repeated in duplicate. If at least one of the duplicates is equivocal, result should be reported as equivocal.
>2.2	Positive	Presence of elevated antibodies

IgM: Immunoglobulin M; IgG: Immunoglobulin G; ELISA: Enzyme-linked immunosorbent assay

was employed as previously described (Lima *et al*, 2010). In brief, a drop of migration buffer was added to 50 µl aliquot of serum in specimen tube and a strip was placed in tube. Following a migration time of 15 minutes, an appearance of "T" and "C" lines indicates a positive result while appearance of "C" line alone a negative result. If "C" line is not present, the test is considered invalid and has to be repeated. Strips giving ambiguous (faint color at "T" line) or negative results were replaced in the tube for a further 15 minutes and re-evaluated.

Statistical analysis

Accuracy measures were computed at $\alpha = 0.05$, 95% CI, power of 80% and a margin of error of ±2. Accuracy measures of Biotek-M™ test were calculated using the following equations:

Sensitivity = A/(A+C)
Specificity = D/(B+D)
PPV = A/(A+B)
NPV = D/(C+D)
PLR = sensitivity/(1-specificity)
NLR = 1- (sensitivity/specificity)

where A, B, C, and D are defined in Table 3.

RESULTS

Patients' demographic profiles and clinical presentations

Patients (n = 519) agreed to participate in the study, with mean ± standard deviation (SD) age of 17 ± 12 years, ranging from 6 months to 72 years of age, and 56.2% males (Table 4). Patients were recruited during the first to seventh day of onset of illness and the blood samples were taken on (mean) third day of illness. Clinical diagnosis

Table 3
Values used in determination of sensitivity, specificity, positive predictive and negative predictive value of Biotek-MTM test compared to reference standard laboratory dengue tests

BIOTEK-M™	Combination of standard laboratory dengue tests	
	Positive (any of Dengue RT-PCR and/or Dengue serology and/or Dengue NS1 tests)	Negative (Dengue RT-PCR, serology, NS1, and convalescent IgG)
Positive	A	B
Negative	C	D

RT-PCR: reverse transcriptase-polymerase chain reaction; NS1: nonstructural protein 1; IgG: Immunoglobulin G

A: the number of true positive or positive Biotek-M tests among patients with positive dengue infection based on a positive result from any one of the reference laboratory dengue tests; B: the false positive or positive Biotek-M tests among those patients with no dengue infection based on all negative results of all standard reference dengue tests; C: the false negative or negative Biotek-M tests among patients positive to have dengue based on a positive result from any one of the reference laboratory dengue tests; D: true negative or negative Biotek-M test result among those with negative results of all standard reference dengue tests

Table 4
Demographic profiles, clinical diagnosis and treatment outcome of patients enrolled in the study

Parameter	Value
Age, years	
Mean ± standard deviation	17 ± 12
Median	14
Mode	5
Range	0.5-79
Male gender, percent (*n* = 519)	56.2
Day (mean) following illness onset when blood sample was drawn	3.5 (95% CI: 3.4-3.6)
Day of illness when blood sample was drawn, number of patients (%) (*n* =519)	
1	26 (5.0)
2	83 (16.0)
3	164 (31.6)
4	145 (27.9)
5	69 (13.3)
6	22(4.2)
7	10 (2.0)
Number of patients at study hospital, number	
TMC	323
NCH	160
PCMC	25
PGH	11
Clinical diagnosis (based on medical record), number	
Dengue infection	
without warning signs	254
with warning signs	211
severe	32
dengue shock syndrome	2
dengue encephalitis	1
dengue hepatitis	1
Other viral infection	
viral exanthem	2
systemic viral illness	10
upper respiratory tract infection	2
Chikungunya	1

Non-viral illness	
acute pharyngitis	2
typhoid fever	1
community acquired pneumonia	1
Leptospirosis	1
urinary tract infection	2
Successful treatment outcome, number (%) (n = 519)	
as outpatient	24 (4.6)
as inpatient	495 (95.4)

CI: confidence interval; NCH: National Children's Hospital; PCMC: Philippine Children's Medical Center; PGH: Philippine General Hospital; TMC: The Medical City

of all 519 patients suspected to have dengue infection included: 254/519 or 49% clinically diagnosed to have dengue without warning signs; 211/519 or 41% were categorized as dengue with warning signs; 32/519 or 6% had severe dengue; all the rest had other viral or bacterial infections. Ninety-five percent of patients were admitted for a (mean) stay of 5 days and no mortality was reported from any of the four participating hospitals.

Prevalence of laboratory-confirmed DENV infection among patients

The battery of dengue tests were performed on all 519 blood samples. Two specimens that did not undergo Biotek-M™ test were excluded and 21 samples that were not subjected to all four assays due to insufficient blood were categorized as inconclusive dengue status (Fig 2). Fourteen patients who were negative in all four dengue tests and failed to come back for a follow-up test on day 14 post-illness onset were unable to be categorized as true negative dengue status. Thus only 8/517 (1.5%) were categorized as true negatives while 474/517 (91.7%) were categorized as true positives. Prevalence (based on 517 samples) of laboratory confirmed DENV infection was 91.7% (95% CI: 89.3-94.1).

Accuracy measures of Biotek-MTM test compared to combined gold standard reference laboratory tests

For determination of accuracy measures of Biotek-M™ test, only 474 true dengue positive and 8 true dengue negative results were used; 35 samples designated as inconclusive dengue status and two that did not undergo Biotek-M™ test were excluded. In the clinical validation study of the 482 samples, 90 (18.7%) and 8 (1.6%) produced positive and negative results respectively in all reference DENV tests, 182 (37.7%) were positive in two reference tests, and 20 (4.1%), 24 (5.0%) and 154 (31.9%) positive in DENV RT-PCR, rapid DENV NS1 antigen, and DENV IgM test, respectively (Table5). However, there were 86 samples that were laboratory-confirmed DENV cases based on positive results in any one or a combination of reference DENV tests

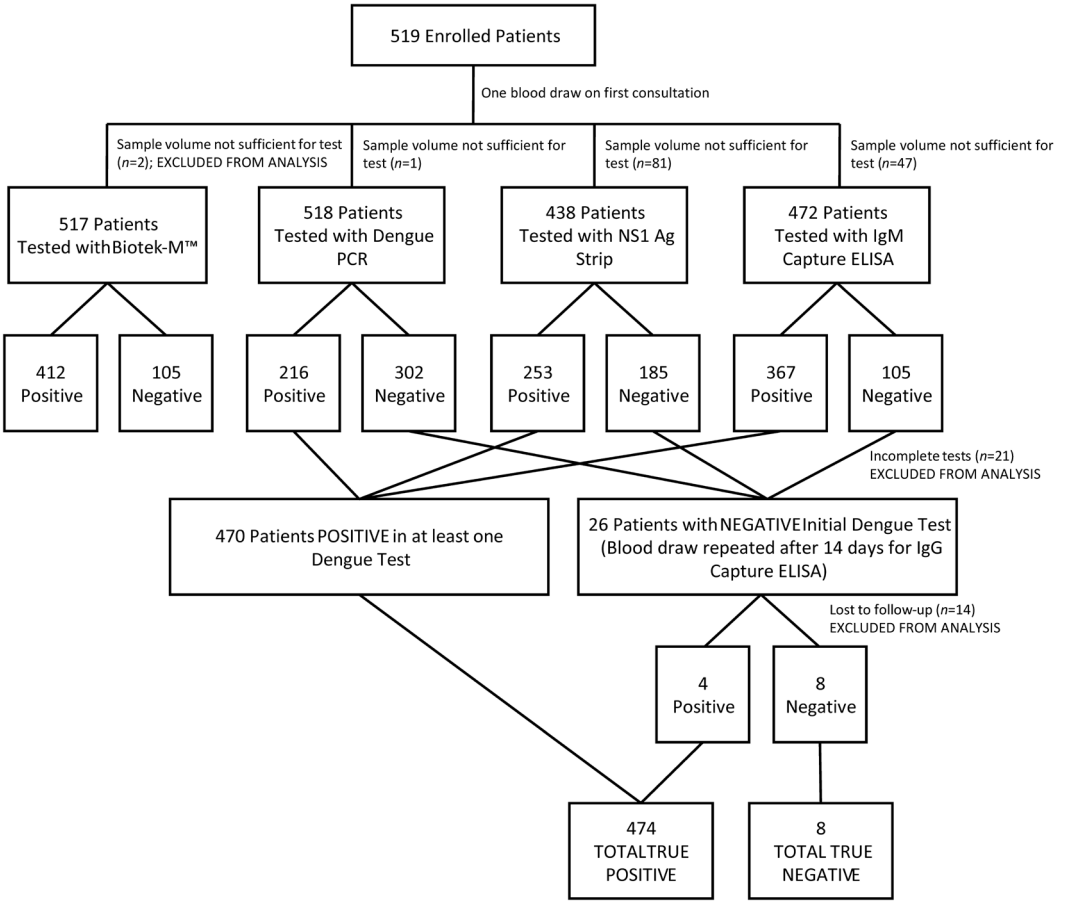

Fig 2 - Work flow and results of diagnosis of blood samples employing Biotek-MTM and reference standard laboratory tests

PCR: Polymerase chain reaction; NS1 Ag: Non-structural Protein 1 antigen; IgM: Immunoglobulin M; IgG: Immunoglobulin G; ELISA: Enzyme-linked immunosorbent assay

but were negative by the Biotek-M™ test.

Thus, sensitivity, specificity, PPV, NPV, PLR, and NLR of Biotek-M™ test were 81.7% (95% CI: 78.0-85.2), 87.5% (95% CI: 46.7-99.3), 99.7% (95% CI: 98.3-100), 7.5% (95% CI: 3.3-15.4), 6.549 (95% CI: 1.046-40.981), and 0.207 (95% CI: 0.165-0.260), respectively.

DISCUSSION

There is an urgent need for highly specific yet affordable dengue diagnostic tests that can be used for clinical management, surveillance and outbreak investigations and for facilitating early interventions to treat patients and control dengue epidemics. When a new diagnostic test is evaluated, choice of ref-

Table 5
Comparison of Biotek-M™ and reference standard laboratory dengue tests

Result of dengue reference tests	Number of samples	Day following illness onset when blood sample was drawn	Positive Biotek-M™ test		Negative Biotek-M™ test	
			n	Day of illness when Biotek-M™ was done	*n*	Day of illness when Biotek-M™ was done
		mean ± SD		mean ± SD		mean ± SD
Positive for dengue infection						
All the following were positive:						
RT-PCR +NS1 + acute IgM	90	3.4 ± 0.2	77	3.4 ± 0.2	13	3.6 ± 0.5
These two tests were positive:						
RT-PCR + NS1	60	2.4 ± 0.3	56	2.4 ± 0.3	4	2.0 ± (0.0)
PCR + acute IgM	41	3.5 ± 0.4	35	3.4 ± 0.4	6	3.8 ± 0.3
NS1 + acute IgM	81	3.8 ± 0.2	70	3.7 ± 0.3	11	4.2 ± 0.8
Only one test was positive:						
RT-PCR	24	2.0 ± 0.3	21	2.0 ± 0.3	3	1.7 ± 0.5
NS1	20	3.1 ± 0.6	17	3.0 ± 0.6	3	4.0 ± 1.6
Acute IgM	154	4.2 ± 0.2	112	4.2 ± 0.2	42	4.1 ± 0.4
Convalescent IgG	4	1.8 ± 0.8	0	0	4	1.8 ± 0.8
Negative for dengue infection						
RT-PCR +NS1 + acute IgM + paired IgG	8	3.4 ± 1.3	1	2.0*	7	3.6 ± 0.9
Total	482	3.5 ± 0.1	389	3.5 ± 0.1	93	3.6 ± 0.3

*Only one measurement was made

SD: Standard deviation; RT-PCR: Real-time polymerase chain reaction; PCR: Polymerase chain reaction; NS1: Non-structural protein 1; IgG: Immunoglobulin G; IgM: Immunoglobulin M

erence laboratory standard test(s), usually called the "gold standard(s)" is a crucial factor (Peeling *et al*, 2010). In complex illnesses there may be various reference tests and in the case of dengue infection the reference test depends on phase of dengue illness at which the test is conducted. Laboratory methods to confirm DENV infection during the early stages of the disease (from first to fifth day of onset of febrile illness) include DENV isolation, DENV RNA detected by RT-PCR and DENV non-structural antigen (*eg* NS1) detection when the virus is typically present in blood or blood-derived fluids, such as serum or plasma (WHO, 2009). Beyond seven days of symptom onset is referred to as the convalescent phase of dengue when serology (*eg* IgM ELISA) is the method of choice for diagnosis, and RT-PCR and NS1 tests are not recommended (WHO, 2009; CDC, 2019). In addition, patients whose initial dengue RT-PCR and/or NS1 and/or IgM antibody tests are negative during the first seven days of illness should have a convalescent sample tested for IgM or IgG antibody, with a 4-fold rise in titer compared to that at the acute phase a diagnosis of dengue infection.

In the present study, as there is no single test which can serve as single true gold standard, a combination of four reference laboratory tests were selected as gold standards, namely, DENV RT-PCR, DENV NS1 antigen, DNV IgM, and, when needed, paired acute-convalescent DENV IgG tests. A dengue case is diagnosed a true positive if patient's blood tests positive in any of DENV RT-PCR test and/or DENV NS1 antigen test and/or DENV IgM and/or rise in DENV IgG in convalescent titre based on the definition of a confirmed true positive case of dengue (CDC, 2015). A case is diagnosed DENV negative when all the above (including convalescent serology) tests are negative.

The likelihood ratios (LRs) are considered the best and most stable measures of diagnostic accuracy involving comparison of the likelihood of detecting a disease in a subject to that of not detecting the disease (McGee, 2002). A PLR >10 and NLR of ~ 0.1 are indicative of high reliability of a diagnostic test. The Biotek-M™ test, a new LAMP-based innovation kit from the Philippines, performed well in the clinical validation study with promising results in detecting DENV infection in patients six months or greater in age within the first seven days of fever onset. Biotek-M™ test demonstrated PLR and NLR of 6.54 and 0.21 respectively. When compared to other published studies of dengue diagnostic tests using LAMP technology, our study is the only one reporting LRs in addition to standard test measures as sensitivity and specificity, employed the largest number of cases over a longer duration from onset of fever (an important improvement as patients may come for diagnosis at different phases of dengue illness), and used a combination of four recommended reference dengue tests (Peeling *et al*, 2010) (Table 6).

As a screening test, it would be desirable if the new Biotek-M™ test is able to detect infection of all DENV infections shown to be positive in one or more of the four reference DENV tests. One sample was Biotek-M™ test

positive despite being negative in all four reference tests. RT-PCR has been reported to have an approximate limit of detection of 100 viral RNA genome copies (Lanciotti *et al*, 1992; Lau *et al*, 2015), while RT-LAMP has a limit of detection of about ten viral RNA genome copies (Teoh *et al*, 2013). In the present study, the limit of detection of Biotek-M™ was 50 DENV genome copies while that of RT-PCR was 1,000 DENV genome copies (data not shown). On the other hand, occurrence of negative Biotek-M™ test despite a positive finding from DENV reference tests was surprising, and was not due to the period of fever onset when blood sample was taken. Further studies are needed to determine the underlying reasons as these findings place a lower sensitivity than expected in the newly developed Biotek-M™ test.

There are differences in the inherent sensitivity and specificity of the various platforms employed for detecting dengue infection particularly regarding the time during the febrile phase of the illness when the diagnosis is made (Ambrose *et al*, 2017). This highlights the concern that currently no single assay can accurately detect DENV across the different stages of the illness, and in clinics and hospitals, physicians have resorted to using a combination of tests, *eg* DENV NS1 antigen together with DENV IgM tests, to optimize dengue diagnosis, resulting in increased health cost. There is a need for a "one-stop" rapid DENV infection test that has acceptable sensitivity and specificity regardless of the phase of the illness and is affordable in resource-limited areas with high prevalence of DENV infection (Rathakrishnan and Sekaran, 2013). Biotek-M™ test is applicable for blood samples taken from day 1 to day 7 of onset of fever, and has the advantages of rapid turn-around time, lower limit of detection, and simple operating procedure.

In addition to the above-mentioned limitations of the Biotek-M™ test, there was a loss of 14 participants to the 14-day follow-up tests and 37 samples that could not undergo the complete battery of DENV diagnostic tests. Exclusion of patients who do not meet criteria for either true positive or negative DENV infection creates a spectrum bias in diagnostic testing (Kohn *et al*, 2013). Loss of follow-up cases can be corrected by facilitating logistics of bring back patients to the hospitals after discharge. Insufficient amounts of blood samples will require improvement in quality control of testing stations.

In summary, the new miniature locally developed Biotek-M™ test designed for early diagnosis of dengue with acute febrile illness was subjected to clinical validation and achieved sensitivity of 81.9%, specificity of 85.7%. positive predictive value of 99.7%, negative predictive value of 7.5%, positive likelihood ratio of 6.549, and negative likelihood ratio of 0.207, values indicative of the reliability of new LAMP-based test for clinical application. The weakness of the Biotek-M™ test was the false negative results (18%), which needs to be further investigated. Overall, Biotek-M™ meets the characteristics of a practical, rapid and portable instrument for blood detection of dengue during the crucial early stage of the illness and may contribute to the improvement in delivery of patient care

Table 6
Diagnostic accuracy measures of LAMP-based dengue tests reported in the literature

Reference	Number of samples	Mean age (years)	Day following illness onset when test was conducted	Reference test	Sensitivity*	Specificity	Likelihood ratio
Zhou *et al* (2019)	153	36.5	Within five days	RT-PCR (NS1 target) + NS1 antigen	95%	No data	No data
Lopez-Jimena *et al* (2018)	31	No data	No data	RT-PCR (no specified target) + nested-PCR (no specified target)	96%	100%	No data
Lau *et al* (2015)	189	No data	No data	IgG or IgM	100%	100%	No data
Dauner *et al* (2015)	44	No data	No data	RT-PCR (no specified target)	86%	93%	No data
Neeraja *et al* (2014)	300	No data	Within seven days (acute phase)	RT-PCR (prM target)	93% (vs NS1 antigen test)	95% (vs NS1 antigen test)	No data
			After seven days (convalescent phase)	NS1 antigen	100% (vs RT-PCR test)	100% (vs RT-PCR test)	

Teoh *et al* (2013)	305	No data	No data	qRT-PCR (no specified target) + IgM	92%	100%	No data
Sahni *et al* (2012)	279	No data	No data	RT-PCR (prM target)	100% (vs NS1 antigen test)	87% (vs NS1 antigen test)	No data
				NS1 antigen	49% (vs IgM test)	92% (vs IgM test)	
				IgG or IgM testing	100% (vs RT-PCR test)	100% (vs RT-PCR test)	
Lu *et al* (2012)	42	No data	No data	RT-PCR (no specified target) + IgG or IgM	95.25%	100%	No data
Li *et al* (2011)	93	No data	Within seven days	RT-PCR (universal primers for DENV 1-4)	No data	No data	No data
Parida *et al* (2005)	83	No data	Within seven days	RT-PCR (no specified target) + IgM	100%	93%	No data

*Within 95% confidence interval

RT-PCR: Real-time polymerase chain reaction; NS1: Non-structural protein 1; IgG: Immunoglobulin G; IgM: Immunoglobulin M; DENV: Dengue virus

and in dengue control programs in areas in the world heavily burdened by dengue infection.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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