Evaluation of a commercial dengue combo rapid test kit for the detection of NS1 and IgM

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Abstract. Accurate and timely diagnosis is critical for dengue patient management due to no specific treatment is available for the disease. The use of rapid diagnostic tests (RDTs) could assist in disease screening because of their simplicity and inexpensiveness but nonetheless, the performance of these tests needs to be carefully evaluated. Here, we report the performance of RVR Dengue Combo NS1-IgG/IgM Rapid Test for detection of dengue NS1 antigen and dengue-specific IgM using 98 samples that were screened initially using Panbio Dengue IgM Capture ELISA and Panbio Dengue Early Rapid Test. The positive percent agreement (PPA) between RVR Dengue Combo NS1-IgG/IgM Rapid Test and the reference comparator tests was 77.8% (κ=0.748, P<0.001) and negative percent agreement (NPA) was 95.7% for NS1 (κ=0.748, P<0.001). As for IgM, the PPA and NPA was 54.5% and 100.0% respectively (κ=0.561, P<0.001). Combining both NS1 and IgM results using a logical OR operator gave the best compromised PPA and NPA of 75.0% and 97.2% respectively (κ=0.743, P<0.001). In another aspect, all dengue negative samples with high titer of rheumatoid factor (RF) and Hepatitis B surface Antigen (HBsAg) were tested negative for NS1, IgG and IgM using this RDT thus indicating no cross-reactivity to these interference factors.

INTRODUCTION

Dengue is endemic in more than 100 countries, particularly in Southeast Asia with an estimated 50-100 million cases occurring each year globally (World Health Organization, 2016). As one of most seriously affected areas by dengue, this region has suffered significant economic and disease burden. According to a study by Shepard *et al.* (2013a) the estimated total economic burden from year 2001-2010 in Southeast Asia region was the highest for Indonesia, followed by Thailand and Malaysia which represent 34%, 31% and 14% respectively of the total economic burden of Southeast Asia. In Malaysia, the annual medical cost for dengue was estimated at US$103.4m per year (Shepard *et al.*, 2013b). In addition, 330,891 dengue cases with 788 deaths were reported from 2014-2016, showing a spike in the number of cases as compared to previous years (Ministry of Health Malaysia, 2016).

The dengue virus belongs to the Flavivirus genus of the *Flaviviridae* family with four closely related serotypes, namely DEN-1, DEN-2, DEN-3 and DEN-4 (Gurugama *et al.*, 2010). These four serotypes can cause illness in humans ranging from dengue with and without warning signs to severe dengue. To date, specific treatment and highly effective vaccines for dengue remain elusive (Horstick *et al.*, 2015); and efforts to reduce the cases and death counts are mainly focused on prompt case detection and early patient management (Murray *et al.*, 2013).

Serological tests are commonly used for dengue diagnosis as they are simpler than virus isolation and nucleic acid detection. Detection of IgM in a single specimen with consistent clinical manifestations is widely used to establish a presumptive diagnosis (Simmons *et al.*, 2012). Meanwhile, observing the pattern of antibody responses is widely used to differentiate between primary and secondary dengue infections. However, this test is limited by the cross-reactivity withother circulating flaviviruses, particularly when working in regions where multiple flaviviruses co-circulate (Guzmán and Kourí, 2004).

As for antigen detection, the non-structural protein 1 (NS1) of the dengue virus is detectable within day 1 to 9 of illness in primary or secondary dengue infected patients (Alcon *et al.*, 2002; McBride, 2009) and its detection is sufficient for confirmatory diagnosis (Peeling *et al.*, 2010). Nevertheless, caution should be exercised for the interpretation of dengue NS1 results, since false-positive results have been reported for this dengue biomarker in a patient with acute Zika virus infection (Gyurech *et al.*, 2016) and regions of cross-reactivity with Zika virus has not been tested. With regards to both approaches, the use of combined dengue-specific antigen (NS1) and antibodies (IgM and IgG) have been shown to enhance the diagnostic rates for dengue (Dussart *et al.*, 2006; Wang and Sekaran 2010; Hu *et al.*, 2011).

Although rapid diagnostic tests (RDTs) are generally less sensitive than ELISA, it features some operational advantages that are highly desirable for resource-limited settings and as point-of-care tests. They are relatively inexpensive, simple and produce faster results. However, despite all these attributes, performance characteristics of RDTs such as sensitivity and specificity may be compromised for the sake of rapidity and simplicity of the test. Therefore, accurate evaluations need to be carried out to determine the validity of the RDT intended to be used by using other reference methods that have been validated such as ELISA, PCR, and virus isolation.

In this study, we assessed the accuracy of a commercial RVR Dengue Combo NS1-IgG/IgM Rapid Test by comparing its performance with two widely used dengue tests, namely Panbio Dengue IgM Capture ELISA and Panbio Dengue Early Rapid Test. We also combined the results from NS1 and IgM detection using a logical OR operator as a general strategy that allows dengue diagnosis throughout the normal temporal spectrum of patient presentation. Moreover, cross-reactivity tests for patient samples with high titer RF and HBsAg were also performed to observe any interference towards the dengue tests caused by autoimmune-related antibodies or other virus antigen.

MATERIALS AND METHODS

Clinical specimens

This study was approved by the Medical Research and Ethics Committee (MREC), Malaysia with reference number: NMRR-16-1635-32194 (IIR). All samples were originally collected from patients admitted to various health institutions in the state of Sabah, Malaysia including Queen Elizabeth Hospital (QEH), Queen Elizabeth II (QEH II), Beaufort Hospital, Ranau Hospital, Sipitang Hospital, Sabah Women's and Children's Hospital (HWKKS) and some health clinics between the period of September and November 2015.

A total of 98 specimens were retrospectively selected for the evaluation after meeting the inclusion criteria: suspected dengue with clinical symptoms including febrile illness, requested for dengue tests, presence of sample collection date and sufficient sample volume (>100 µl). Samples that were determined to be equivocal (n=7, index value >0.9 and <1.1) by Panbio Dengue IgM Capture ELISA were excluded.

All selected samples (n=98) were characterized for dengue (NS1 and IgM) using the comparator tests (Panbio Dengue Early Rapid Test and Panbio Dengue IgM

Capture ELISA), of which 40 samples were determined to be dengue positive (Table 1). These dengue positive samples included a total of 18 samples that were detected positive for dengue NS1 antigen by Panbio Dengue Early Rapid Test and 22 samples that were detected positive for dengue IgM by Panbio Dengue IgM Capture ELISA. 60% and 40% of the dengue positive samples were males and females respectively and the median age was 29.0 (1–73 years). Meanwhile, a total of 58 samples were negatively tested for both dengue NS1 and dengue IgM by Panbio Dengue Early Rapid Test and Panbio Dengue IgM Capture ELISA respectively. This included a panel of 10 dengue negative samples that containing high titer of an autoimmune biomarkers (RF, n=5) and a viral infection biomarker (HBsAg, n=5). All 98 selected samples were subjected to RVR Dengue Combo NS1-IgG/IgM Rapid Test for the evaluation which was conducted at a laboratory in QEH.

Panbio Dengue IgM capture ELISA
The Panbio Dengue ELISA IgM test (Alere, USA) is based on ELISA format and was conducted according to the manufacturer's instruction. An index value (N/C) was calculated by the ratio of sample absorbance (N) and cut-off value (C). For Panbio Dengue IgM capture ELISA, an index value greater than 1.1 is considered positive and less than 0.9 negative.

Panbio Dengue Early Rapid Test
The Panbio Dengue Early Rapid Test (Standard Diagnostic, Korea) is a one-step assay based on lateral flow immunoassay which is designed for qualitative determination of dengue virus NS1 antigen in human serum, plasma or whole blood. All samples subjected to Panbio Dengue Early Rapid Test were tested accordingly to the manufacturer's instruction. Results were interpreted 15–20 minutes after sample addition. Positive result is indicated by the appearance of two-colored bands at the both "T" and "C" lines whilst negative result is indicated by the presence of a single colored band at "C" line.

RVR Dengue Combo NS1-IgG/IgM Rapid Test
This combo RDT is manufactured by RVR Diagnostics Sdn. Bhd. (Malaysia) and is based on lateral flow immunoassay format. This test cassette is divided into two sides; the left side is the Dengue IgG/IgM rapid test and on the right side is the Dengue NS1 Rapid Test. The presence of dengue IgG, IgM and NS1 can be detected by the appearance of a burgundy colored line at the corresponding test line (i.e. dengue IgG, IgM and NS1 at the "G", "M" and "T" test line respectively). All tests using this RDT were carried out in accordance to the manufacturer's instruction.

Table 1. Serum samples used for RVR Dengue Combo NS1 & IgG/IgM test kit evaluation

Variables	N (%) or Median (range)
*Dengue Positive**	40 (100.0)
Male sex	24 (60.0)
Female sex	16 (40)
Age (years)	29.5 (0.167–73)
Dengue NS1 positive	18 (45.0)
Dengue IgM positive	22 (55.0)
*Dengue Negative**	58 (100)
Dengue NS1 negative	23 (39.7)
Dengue IgM negative	25 (43.1)
Dengue NS1/IgM negative but positive for both rheumatoid factor (RF)	5 (8.6)
Dengue NS1/IgM negative but positive for hepatitis B antigen (HBsAg) test	5 (8.6)

* Dengue positive sera and known dengue negative sera as determined to be positive or negative respectively by the comparator dengue tests, Panbio Dengue Early Rapid Test and Panbio Dengue IgM Capture ELISA.

Dengue Negative Sera with RF and HBsAg

Avitex RF test is a rapid latex agglutination kit for the detection of rheumatoid factor (RF) in human serum. For RF test, samples were processed as described by the manufacturer. Method comparison with EIA RF test resulted in 100% specificity and 100% sensitivity.

The Architect HBsAg Qualitative II assay is a one-step immunoassay for the qualitative detection of HBsAg in human serum or plasma. All samples subjected to this assay were tested accordingly to the manufacturer's instruction. Samples with readings of more than 1.0 S/CO were considered reactive while samples with readings below 1.0 S/CO were considered non-reactive.

Data Analysis

Performance characteristics evaluation for RVR Dengue Combo NS1-IgG/IgM Rapid Test was done according to the guidelines reported by Banoo *et al.* (2010). Tabulation, management, and analysis of raw data were carried out using Microsoft Excel (Microsoft Inc., WA, USA). Statistical analysis was performed with SPSS Statistics version 24 (IBM, NY, USA). The measure of agreement between RVR Dengue Combo Rapid Test and comparator tests was performed using Cohen's Kappa (κ). Additionally, positive percent agreement (PPA) and negative percent agreement (NPA) were also determined using the following formula:

$$PPA\ (\%) = a\ /\ (a + c) \times 100\ \%$$
$$NPA\ (\%) = b\ /\ (b + d) \times 100\ \%$$

Where:
a = number of true positives
b = number of true negatives
c = number of false negatives
d = number of false positives

RESULTS

Performance evaluation

The combo RDT was evaluated through a two-step analysis; first, by the individual NS1 and IgM test component of RVR Dengue Combo NS1-IgG/IgM Rapid Test separately and second, by the combination of both test components (NS1 or IgM). Samples tested were classified as dengue NS1 positive (n=18) and dengue IgM positive (n=22) based on the test results by Panbio Dengue IgM Capture ELISA and Panbio Dengue Early Rapid Test. We also combined both types of sample (dengue NS1 and dengue IgM positive, n=40), which represent the total dengue positive samples, in our analysis.

The percentage of positive detection for each of the dengue biomarker in the dengue positive samples by RVR Dengue Combo NS1-IgG/IgM Rapid Test is summarized in Table 2. For individual component analysis, dengue NS1 positive sera were identified NS1 positive in 77.8% samples and IgM positive in 16.7% samples using this combo RDT. As for dengue IgM positive sera, only 54.5% of samples were positive for IgM using this combo RDT whilst 45.5% were NS1 positive. Combination of both NS1 and IgM test components of RVR Dengue Combo NS1-IgG/IgM Rapid Test (using logical OR operator) increased

Table 2. Crosstab of number of sample tested positive for known dengue positive with RVR Dengue Combo NS1-IgG/IgM Rapid Test

Test sample	No. of sample tested positive (%) by RVR Dengue Combo NS1-IgG/IgM Rapid Test		NS1 or IgM
	NS1	IgM	
Dengue positive NS1	**77.8 (14/18)**	16.7 (3/18)	77.8 (14/18)
Dengue positive IgM	45.5 (10/22)	**54.5 (12/22)**	72.7 (16/22)
Combined Dengue positive NS1 and IgM	60.0 (24/40)	37.5 (15/40)	**75.0 (30/40)**

the detection percentage for dengue IgM positive sera, and the combination of dengue NS1 and IgM positive samples (72.7% and 75.0% respectively) but not for the dengue NS1 positive sera.

The PPA value for each dengue biomarker were determined using the results obtained from the corresponding test, e.g. results from the NS1 test component of RVR Dengue Combo NS1-IgG/IgM Rapid Test was compared with dengue NS1 positive sera determined by Panbio Dengue Early Rapid Test for NS1 detection. Overall, the RVR Dengue Combo NS1-IgG/IgM Rapid Test has lower PPA but comparable NPA with the comparator dengue tests for all dengue biomarkers investigated in this study (Table 3). The PPA for the detection of dengue NS1 (77.8%, 95% confidence interval [CI]: 58.6–97.0) was higher than IgM (54.5%, 95% confidence interval [CI]: 33.7–75.3) while combination of NS1 and IgM resulted in PPA of 75.0% (95% confidence interval [CI]: 61.6–88.4). The NPA for dengue IgM was 100% indicating that RVR Dengue Combo NS1-IgG/IgM Rapid Test showed similar specificity as the corresponding comparator test, Panbio Dengue IgM Capture ELISA. The NPA for dengue NS1 was marginally lower than IgM, which was 95.7% (95% confidence interval [CI]: 87.3–100) and combination of NS1 and IgM resulted in an NPA of 97.9% (95% confidence interval [CI]: 93.8–100).

Overall, the NPA for RVR Dengue Combo NS1-IgG/IgM Rapid Test was comparable with the comparator tests, with the IgM test component of RVR Dengue Combo NS1-IgG/IgM Rapid Test having similar specificity (100%) as Panbio Dengue IgM Capture ELISA. The NPA for NS1 detection

was marginally lower than IgM detection byRVR Dengue Combo NS1-IgG/IgM Rapid Test (95.7%), with only one positive result detected from the 23 dengue negative sera.

Cross-reactivity
All dengue negative samples with high titer values for RF and HBsAg (n=5 each) gave negative results for NS1, IgM and IgG when tested using RVR Dengue Combo NS1-IgG/IgM Rapid Test, indicating no cross-reactivity with these potential interfering factors in serological test.

DISCUSSION

The operational characteristics of the dengue tests used in the present study are summarized in Table 4. Both RDTs used in this study (RVR Dengue Combo NS1-IgG/IgM Rapid Test and Panbio Dengue Early Rapid Test) are based on the lateral flow immunoassay (LFIA). Both tests are more temperature-stable, simple and produce faster results (up to 16 times) than Panbio Dengue Capture ELISAs. However, the cost for the detection of dengue NS1 antigen and dengue-specific IgM/IgG antibodies in blood samples is significantly lower than the commercial dengue tests listed in Table 4, which saves RM 46.7 per patient. The utilization of this RDT for dengue diagnosis thus could reduce the economic burden of dengue, especially in hyperendemic areas such as Malaysia.

In this study, we were able to detect multiple dengue biomarkers when tested with RVR Dengue Combo NS1-IgG/IgM Rapid Test in dengue positive sera for example 45.5% known dengue IgM positive

Table 3. Level of agreement between RVR Dengue Combo Rapid Tests and the comparator tests for detection of dengue NS1, IgM and combined NS1/IgM

| Dengue Biomarker | RVR Dengue Combo | | Cohen's Kappa (κ) value |
	PPA, % (95%, CI)	NPA, % (95%, CI)	
NS1	77.8 (14/18)(58.6–97.0)	95.7 (22/23)(87.3–100)	0.748
IgM	54.5 (12/22)(33.7–75.3)	100.00 (25/25)	0.561
Combined NS1 and IgM	75.0 (30/40)(61.6–88.4)	97.9 (47/48)(93.8–100)	0.743

Table 4. Operational characteristics of the commercial dengue tests used in this study

Manufacturer	Product name	Analyte	Time taken to perform the test	Test format	Sample volume	Sample type	Kit stability (temperature)	Cost (per test, ringgit Malaysia)
Alere	Panbio Dengue IgM Capture ELISA**	IgM	4 h	ELISA*	10 µL	Serum	2–8°C	18.23
	Panbio Dengue ELISA IgG**	IgG	4 h	ELISA*	10 µL	Serum	2–8°C	20.83
Standard Diagnostic	Panbio Dengue Early Rapid Test**	NS1	15–20 min	LFIA*	100 µL	Whole blood, plasma or serum	1–30°C	21.60
RVR	Combo Dengue NS1-IgG/IgM Rapid Test	IgG, IgM & NS1	15 min	LFIA*	80–100 µL	Whole blood, plasma, or serum	2–30°C	14.01

* ELISA and LFIA denote enzyme-linked immunoassay and lateral flow immunoassay respectively.
** Commercial dengue tests that are routinely used in our lab in QEH, state of Sabah, Malaysia.

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sera also tested positive for NS1. Interestingly, all known dengue IgM positive sera were tested negative for NS1 using its comparator test, Panbio Dengue Early Rapid Test. However, in the absence of a reference dengue test, we were unable to determine if this was due to false positive detection (inferior specificity) by the NS1 test component of RVR Dengue Combo NS1-IgG/IgM Rapid Test or false negative (inferior sensitivity) of Panbio Dengue Early Rapid Test hence a limitation of our study. Furthermore, the sensitivity and specificity of a dengue assay is strongly influenced by the quality of the antigen and antibody used and can vary greatly between commercially available products.

In RVR Dengue Combo NS1-IgG/IgM Rapid Test, NS1 and IgM detection were combined due to the importance of both biomarkers in the diagnosis of acute dengue infection (Peeling *et al.*, 2010). The combinational strategy of both dengue NS1 and IgM results significantly increased the detection percentage of RVR Dengue Combo NS1-IgG/IgM Rapid Test for dengue IgM positive sera, but not for dengue NS1 positive sera, hence a limitation of this strategy and also indicates the usefulness of NS1 alone. However, when all known dengue positive sera (NS1 and IgM, n=40) were combined, the detection percentage of RVR Dengue Combo NS1-IgG/IgM Rapid Test increased to 75%, compared to individual detection of NS1 (60%) and IgM (37.5%).

We compared the performance of RVR Dengue Combo NS1-IgG/IgM Rapid Test with the comparator dengue tests by reporting on the positive percent agreement (PPA) and negative percent agreement (NPA), the two separate indices that are analogous to sensitivity and specificity in a diagnostic test (Cicchetti & Feinstein 1990), due to the absence of reference dengue test (Banoo *et al.*, 2010). Individually, the PPA for NS1 detection was substantially higher than IgM detection by RVR Dengue Combo NS1-IgG/IgM Rapid Test (77.8% vs. 54.5%). One of the possible factors that may contribute to

the difference in the PPA value was the test format of the corresponding comparator test, as ELISAs are known to be more sensitive than RDTs. In this evaluation, the NS1 test component of RVR Dengue Combo NS1-IgG/IgM Rapid Test performance was compared to one other RDT, Panbio Dengue Early Rapid Test while the IgM test component of RVR Dengue Combo NS1-IgG/IgM Rapid Test performance was compared to Panbio Dengue IgM Capture ELISA. In an evaluation performed by Fry *et al.* (2011), Panbio Dengue Early Rapid Test showed moderate sensitivity of 69.2% and 62.0% for dengue NS1 in study locations in Vietnam and Malaysia respectively. In another study, the evaluated sensitivity for Panbio Dengue IgM Capture ELISA by Blacksell *et al.* (2012) was higher (83.2%). Therefore, when comparing the performance of an RDT with ELISA, a much lower value of PPA is expected for the RDT, which was the case for the IgM detection. Similar result have been reported for other commercial RDT, SD Bioline Dengue Duo Rapid Test (sensitivity of 49.3% for IgM alone)(Vickers *et al.*, 2015).

To assess the overall agreement between RVR Dengue Combo NS1-IgG/IgM Rapid Test and the comparator dengue tests, Cohen's kappa values were determined. Using common interpretation (Viera & Garrett 2005) of the values, our data showed that there was substantial agreement (κ=0.748, P<0.001) between RVR Dengue Combo NS1-IgG/IgM Rapid Test and Panbio Dengue Early Rapid Test for NS1 detection but only moderate agreement (κ=0.561, P<0.001) between RVR Dengue Combo NS1-IgG/IgM Rapid Test and Panbio IgM ELISA for IgM detection.

In serological tests, the positive results of dengue-specific IgM in a single sample is useful to identify probable dengue cases (Peeling *et al.*, 2010) but not for infection confirmation. Nevertheless, combining the test results for antigen and antibody in both RDT and the reference assay is a well-established strategy to provide acceptable accuracy of dengue diagnosis (Blacksell *et al.*, 2011). Based on our combination

results for NS1 and IgM detection for this RDT, both PPA and NPA values were averaged at 75.0% and 97.9% respectively with a kappa value (κ) of 0.743 (P<0.001), indicating substantial agreement with the comparator tests.

Another concern related to the accuracy of serological tests is potential interferences from other immune responses such as autoimmunity or systemic conditions that could generate false-positive results (Hunsperger *et al.*, 2009). It has been shown that the generation of auto-antibodies against platelets, endothelial cells and coagulatory molecules are dengue-associated and molecular mimicry between these molecules with NS1, prM, and E proteins could be a possible cause for cross-reactivity of anti-NS1, antiprM and anti-E Abs, respectively, with host proteins (Wan *et al.*, 2013). We performed cross-reactivity test of a common auto-immune biomarker, RF for rheumatoid arthritis. Previously, Jelinek and co-workers reported 13 false positive cases for dengue IgM detection in samples with a mean value of 404.2 IU/ml for RF (Jelinek *et al.*, 2000). In our study, all RF samples with mean detection value of 320.0 IU/ml were negatively tested by RVR Dengue Combo NS1-IgG/IgM Rapid Test indicating no cross-reactivity between the detecting components of this RDT with the tested autoantibody markers. Similar results were obtained when the sera from patients with Hepatitis B infection (mean detection value = 4522.2 S/CO) were tested with this RDT that concluded no cross-reactivity for this antigen. Previously, Berlioz-Arthaud *et al.* (2008) reported interferences of HBsAg and RF when tested with Panbio Dengue duo IgM and IgG rapid strip test, leading to false-positive results.

Our study had several limitations. First, the performance of RVR was compared with one other RDT (for NS1) and ELISA (for IgM), not with other robust diagnostic methods such as PCR and virus isolation as the reference dengue test. The two comparator tests used in this evaluation were also reported to have mixed performance, especially in patients tested after 6 days of symptoms, as well as in NS1 detection and secondary infection (Hunsperger *et al.*, 2014). Hence, in the absence of reference dengue test, we reported the values of PPA and NPA for the performance comparison, instead of the traditional sensitivity and specificity. Second, there are no information about dengue serotypes and infection type (primary or secondary) for the infected sera used in this study. Since these two variables affect dengue biomarkers, such information can help better interpretation of our data. Third, the level of cross-reactivity for other circulating flaviviruses such as West Nile virus, Japanese encephalitis virus and yellow fever virus is unknown for this combo RDT since such cross-reactivity test was not conducted and future work will include this.

Based on the limited data obtained from the present investigation, we conclude that this RDT is a specific assay relative to Panbio Dengue IgM capture ELISA and Panbio Dengue Early Capture with no cross-reactivity observed for autoimmune markers ANA and RF, as well as HBsAg. Due to lower PPA values, confirmation with other reliable methods such as ELISA is necessary for the negatively tested samples with suggestive clinical indication for dengue infection. For this RDT, we propose the use of a combination of NS1 and IgM test components for dengue confirmation due to best compromised value for both PPA and NPA, as compared to individual detection of the test components.

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