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ORIGINAL ARTICLE

A comparative evaluation of dengue diagnostic tests based on single-acute serum samples for laboratory confirmation of acute dengue

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Abstract

A prospective study was carried out to evaluate the sensitivity of dengue NS1 antigen-capture ELISA in comparison with dengue virus isolation, conventional RT-PCR and real-time RT-PCR for laboratory confirmation of acute dengue based on single-acute serum samples. Four primary health-care centres were involved to recruit patients with clinical diagnosis of dengue illness. Patient's demographic, epidemiological and clinical information were collected on a standardized data entry form and 5 ml of venous blood was collected upon consent. In the laboratory, six types of laboratory tests were performed on each of the collected acute serum sample.

Of the 558 acute serum samples collected from 558 patients with clinical diagnosis of dengue from mid-August 2006 to March 2009, 174 serum samples were tested positive by the dengue NS1 antigen-capture ELISA, 77 by virus isolation, 92 by RT-PCR and 112 by real-time RT-PCR. A total of 190 serum samples were tested positive by either one or a combination of the four methods whereas, only 59 serum samples were tested positive by all four methods. Thus, based on single-acute serum samples, 190 of the 558 patients (34.1%) were laboratory-confirmed acute dengue. The overall test sensitivity was 91.6%, 40.5%, 48.4% and 58.9% for dengue NS1 antigen-capture ELISA, virus isolation, conventional RT-PCR and real-time RT-PCR respectively. Statistically, dengue NS1 antigen-capture ELISA was the most sensitive and virus isolation was the least sensitive test for the laboratory confirmation of acute dengue based on single-acute serum specimens. Real-time RT-PCR was significantly more sensitive than the conventional RT-PCR.

Keywords: Dengue, virus isolation, molecular diagnostics, NS1 antigen-capture ELISA.

INTRODUCTION

Dengue fever (DF) and its more serious forms, dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS), is a mosquito-borne disease affecting humans in more than 100 countries in the tropical and subtropical regions of the world. The disease, caused by the dengue virus, is increasingly a public health threat to urban and suburban areas.[1-3] Dengue virus is an enveloped positive-sense single- stranded RNA virus. There are four serotypes of dengue virus (dengue serotype-1, 2, 3, and 4), all of which are capable of infecting humans and have been documented to cause severe disease conditions. Infection with one serotype does not confer cross-protection against the other serotypes but instead may lead to a serious form of disease through immune-pathological enhancement of disease. Infection with one serotype also results in the production of cross-reactive antibodies (both IgM and IgG) with other serotypes, contributing to diagnostic dilemma in the laboratory diagnosis of dengue based on serological platforms.[4-6]

Until lately, acute dengue virus infection is laboratory confirmed by one or a combination of the three basic laboratory diagnostic methods: dengue virus isolation and identification, detection of viral genomic sequence by a nucleic acid amplification technology assay (RT-PCR), and serological demonstration of a four-fold or greater rise in antibodies (IgM and/or IgG) titre to dengue virus. Recent scientific reports

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show that in acute dengue virus infection, the non-structural protein one (NS1) of the virus is secreted out of infected cells and could be detected circulating in patient's blood.[7,8] Commercial dengue diagnostic test kits based on antigen-capture ELISA were subsequently developed for detection of circulating dengue NS1 antigen as an added laboratory diagnostic method to provide early confirmation of acute dengue.[8,9] The sensitivity of the available commercial dengue NS1 antigen-capture ELISA kits were previously evaluated using panels of serum samples collected from patients with acute dengue that were already tested positive by RT-PCR and/or virus isolation.[10-12] This form of evaluation is in effect indirectly biased against the dengue NS1 antigen-capture ELISA. It did not take into consideration those serum samples from patients with acute dengue that were positive by dengue NS1 antigen-capture ELISA but would not be tested positive by RT-PCR and/or virus isolation. Thus, a prospective study was undertaken to evaluate the sensitivity of dengue NS1 antigen-capture ELISA in comparison with other available laboratory dengue diagnostic test methods (virus isolation and molecular diagnostics) for laboratory confirmation of acute dengue based on single-acute serum samples collected from patients seen in outpatient clinics.

MATERIALS and METHODS

Population and serum samples

This study was carried out under the directive and funding support of the Ministry of Health Malaysia. The study protocol was designed in collaboration with the Institute for Medical Research. At the start of the study, a presentation on clinical, epidemiological and laboratory aspects of dengue and the study protocol was made to the health-care providers (doctors, nurses and laboratory technologists) of the collaborating primary health-care centres (Poliklinik Seksyen 7, Shah Alam; Poliklinik Kelana Jaya, Petaling Jaya; Poliklinik Sg. Buloh; Poliklinik Jinjang).

The recruitment of patients for this study was very much dependent on the clinical diagnosis of doctors treating the patients at the selected primary health-care polyclinics but the doctors were advised to use the WHO clinical criteria of acute dengue (fever with headache and/or myalgia and/or rash) as the guideline. Patients who presented with obvious respiratory illness (rhinitis, cough) or had a clinically known cause of fever were not included in the study. Upon consent, patients' demographic, epidemiological and clinical information were collected on a standardized data entry form. The date of onset of fever and the date of blood sample collection were also recorded in the data entry form for calculation of sample age. Sample age is defined as the interval in days between the date of onset of fever and the date of collection of blood sample. It was considered Day 0 if the blood samples were collected on the same day as the date of fever onset and Day 1 if the samples were collected the following day. After physical examination, five milliliters of venous blood was collected from each patient as part of the workout for laboratory diagnosis of acute dengue infection. The blood samples were allowed to clot at room temperature in the laboratories of primary care health centres. As soon as the blood had clotted, serum samples were separated and stored in a 4^0C refrigerator. Serum samples together with the completed standardized data entry forms were collected and transported to the National Public Health Laboratory the following morning. Six laboratory tests were performed on each acute serum sample and the excess sera were stored at -80 ^{0}C for future reference and further confirmatory or other tests, if needed. A second (convalescent) blood sample was requested and collected from the patient whose acute serum sample was tested positive by dengue NS1 antigen-capture ELISA but negative by virus isolation or any of the molecular diagnostics. Serological tests were subsequently carried out on the paired serum samples to check for sero-conversion or a four-fold or greater rise in antibody titre against dengue virus.

Laboratory Diagnostic Tests

Serological assays for the detection of anti-dengue IgM and anti-dengue IgG present in acute serum samples were carried out using a commercial IgM-capture ELISA kit and a dengue IgG-capture ELISA kit respectively (Panbio Diagnostics, Brisbane, Australia). Test procedures and interpretation of results were carried out in accordance with the manufacturer's instructions. For the anti-dengue IgG assay, the results were interpreted as primary dengue (negative) for Panbio units (P.U.) of 22 or less and secondary dengue (positive) for Panbio unit (P.U.) greater than 22.

Dengue virus RNA was extracted from acute serum samples using High Pure Viral Nucleic Acid Kit (Roche Diagnostic, Mannheim, Germany) according to the manufacturer's

protocol. Molecular detection of dengue virus RNA by conventional reverse-transcriptase polymerase chain reaction (RT-PCR) was performed using a set of pan-dengue generic oligonucleotide primers as described in Lanciotti *et al*.[13] Molecular detection of dengue virus RNA by real-time RT-PCR (rRT-PCR) was carried out in accordance with a published method by Chutinimitkul *et al*.[14]

Isolation of dengue virus from patients' serum samples was performed using C6/36 mosquito cell-line (ATCC CRL-1660). Detection for the presence of dengue virus infected C6/36 cells at the end of the 10th post-inoculation day was by an indirect immunofluorescence assay using a commercial anti-dengue monoclonal antibody complex covering all serotypes of dengue viruses (Chemicon Int. Inc. USA; Cat. No. MAB8705). The procedures of both types of tests were previously described in Chua *et al*.[15]

Immunological assay for detection of dengue NS1 antigen was carried out using a commercial dengue NS1antigen-capture ELISA kit, PLATELIA™ DENGUE NS1 AG (Bio-Rad Corporate HQ, Hercules, USA). The test system is based on a one-step sandwich format microplate enzyme immunoassay for detection of dengue virus NS1 antigen in human serum or plasma. The test uses murine monoclonal antibody (MAb) for capture and revelation. If NS1 antigen is present in the sample, an immune-complex MAb-NS1-MAb/peroxidase will be formed. The test was performed and subsequent calculations were made strictly according to the assay procedure of the commercial Platelia Dengue NS1 antigen capture ELISA kit. Briefly, the acute serum specimens were allowed to warm up to laboratory ambient temperature (21-22 ^{0}C). Diluent, samples and controls (50 μl each) and 100 μl of conjugate were incubated for 90 mins at 37 ^{0}C within the respective microplate wells sensitized with MAb. After a six-times washing step, 160 μl of substrate was added into each well and incubated for 30 min at room temperature in the dark. The presence of immune-complex was demonstrated by a colour development and the enzymatic reaction was stopped by adding 100 μl of 1N H_2SO_4. The optical density (OD) reading was taken with a spectrophotometer at 450/620 nm and the amount of NS1 antigen present in an individual serum sample was determined by comparing the OD of the sample to the OD of the cut-off control serum.

Data management, analysis and interpretation
The demographic, epidemiological and clinical information together with laboratory data were tabulated in appropriate worksheets using the Microsoft Excel program and evaluated by Chi-square test using the Epi Info 6 (Center for Disease Control and Prevention, Atlanta) free computer program, for any statistically significant association. A probability (p) value of 0.05 or less was taken as the level of significant association for each ordinal variable with the relevant adjusting variables. A clinical case of dengue was laboratory confirmed if the patient's acute serum sample was tested positive for virus isolation and/or virus nucleic acids by molecular test (RT-PCR and/or real-time RT-PCR) and/or dengue virus NS1 protein.

RESULTS

This prospective comparative field evaluation involved 4 outpatient polyclinics of 4 respective health centres (Poliklinik Seksyen 7, Shah Alam; Poliklinik Kelana Jaya, Petaling Jaya; Poliklinik Sg. Buloh; Poliklinik Jinjang) situated within the Klang Valley of peninsular Malaysia. The analysis and results in this study are based on a data set collected from mid-August 2006 to March 2009. A total of 589 serum samples, consisting of 558 acute serum samples and 31 convalescent (2nd) serum samples, were collected from 558 patients with clinical diagnosis of acute dengue using WHO clinical criteria as guideline. Of the 558 patients, 344 were males and 214 were females (ratio of 1.6:1). Three hundred and forty seven patients were Malays, 80 patients were Chinese, 97 patients were Indians and 34 other patients were mainly foreign workers. The age of the patients ranged from 1.5 to 76 years old with a mean of 26 and standard deviation of 13.5 years.

Of the 558 acute serum samples, the number of samples tested positive by each type of laboratory test is shown in Table 1. Anti-dengue IgM-capture ELISA gave the highest positive rate (206/558, 36.9%) followed by dengue NS1 antigen-capture ELISA (174/558, 31.2%). Though anti-dengue IgM-capture ELISA gave the highest positive rate, the result was not considered as laboratory confirmation of acute dengue without a pair convalescent serum sample in this analysis but interpreted as recent dengue infection. At least 109 (19.5%) patients had recent secondary exposure to dengue virus by the positive detection of anti-dengue IgG above

TABLE 1: The number of acute serum samples from 558 patients with clinical diagnosis of acute dengue tested positive by each laboratory test method. The positive (reactive) result for anti-dengue IgG is equivalent to a value above the cut-off point for secondary dengue.

Diagnostic Test Method	Number of samples tested positive (%)
Virus Isolation	77 (13.8)
RT-PCR	92 (16.5)
Real-time RT-PCR	112 (20.1)
NS1-Ag ELISA	174 (31.2)
Anti-dengue IgM	206 (36.9)
Anti-dengue IgG (>22 P.U.)	109 (19.5)

22 Panbio Units (P.U.) in their sera. Of the 77 dengue viruses isolated, 36 were serotyped as dengue virus serotype 1, 28 were dengue virus serotype 2, 10 were dengue virus serotype 3 and 3 were dengue virus serotype 4.

The number of acute serum samples tested positive by each respective laboratory method that is also correspondingly tested positive by other test methods is shown in Table 2. One hundred and seventy four serum samples were tested positive using the dengue NS1 antigen-capture ELISA, 77 by virus isolation, 92 by RT-PCR and 112 by real-time RT-PCR. One hundred and ninety serum samples were tested positive by either one or a combination of the 4 four methods. Only 59 serum samples were tested positive by all four methods. Of the 174 samples tested positive by dengue NS1 antigen-capture ELISA, 76, 83 and 100 samples were also positive by virus isolation, RT-PCR and rRT-PCR respectively. Only one sample was confirmed dengue positive through virus isolation but negative by dengue NS1 antigen-capture ELISA (77 - 76). This same isolate was also tested positive by conventional RT-PCR. Nine samples (92 - 83) were tested positive by RT-PCR but negative by dengue NS1 antigen-capture ELISA. There were 12 positive samples (112 - 100) by real-time RT-PCR (rRT-PCR) but negative by dengue NS1 antigen-capture ELISA. Of the nine samples positive by RT-PCR and 12 samples positive by rRT-PCR but negative for dengue NS1 antigen, five were positive by both molecular methods. Sixteen acute serum samples positive by dengue NS1 antigen-capture ELISA but negative by virus isolation or any of

TABLE 2: A cross-tab table to show the number of acute serum samples tested positive by each respective laboratory method which are also tested positive by other test methods

Number of samples tested positive	NS1-ELISA	Virus Isolation	RT-PCR	rRT-PCR	IgM	IgG
NS1-ELISA	**174**	76	83	100	111	26
Virus Isolation	76	**77**	62	65	30	8
RT-PCR	83	62	**92**	86	38	13
rRT-PCR	100	65	86	**112**	54	15
IgM	111	30	38	54	**206**	87
IgG	26	8	13	15	87	**109**

the molecular tests were confirmed to be true positives by demonstration of sero-conversion in five samples and a four fold or greater rise in antibody titre in 11 of the 16 paired sera. Thus, based on single acute serum samples, 190 (174 + 1 + 9 + 12 - 1 - 5) of the 558 patients (190/558, 34.1%) were laboratory confirmed acute dengue cases. The overall sensitivity for laboratory confirmation of acute dengue by dengue NS1 antigen-capture ELISA, virus isolation, conventional RT-PCR and rRT-PCR was 91.6% (174/190), 40.5% (77/190), 48.4% (92./190) and 58.9% (112/190) respectively. Statistically, dengue NS1 antigen-capture ELISA was the most sensitive test and virus isolation was the least sensitive test for laboratory confirmation of acute dengue based on single-acute serum specimens ($\chi 2 = 119.54$, df = 3, p = 0.0000). Real-time RT-PCR was significantly more sensitive than the conventional RT-PCR [$\chi 2$ (Mantel-Haenszel) = 4.22, p = 0.03989].

Table 3 shows the number of acute serum samples of known sample age collected from 558 patients with clinical diagnosis of acute dengue and those that were tested positive by each of the test methods. Of the 558 acute serum samples, only 517 samples had known dates of onset of illness with corresponding dates of blood sample collection for the calculation of sample age (Column 2). Of the 190 patients who were laboratory confirmed to have acute dengue using either one or a combination of the four laboratory test methods, only 171 samples had known sample age (Column 3). The overall positive detection rate by dengue NS1 antigen-capture ELISA, virus isolation, conventional RT-PCR and rRT-PCR was 92% (158/171), 39% (67/171), 46% (79/171) and 57% (97/171) respectively. Thus, the Dengue NS1 antigen-capture ELISA not only gave a significantly higher overall positive rate for confirming acute dengue ($\chi 2 = 118.2$, df = 3, p = 0.0000), but also gave a higher positive detection rate (86-100%) across all categories of sample age. Virus isolation rate was poor for samples collected after the fifth day of illness. The detection rate for dengue virus nucleic acids by molecular methods (both RT-PCR and rRT-PCR) was low for blood samples collected on the day of onset of fever (sample age of Day 0) and after the sixth day of illness. Of the 40 serum samples collected from patients on the day of onset of illness, anti-dengue IgM was detected in 18 and anti-dengue IgG of more than 22 P.U. was detected in eight (Columns 8 and 9). Seven of the 18 positive anti-dengue IgM serum samples and three of the eight positive anti-dengue IgG serum samples were from patients confirmed to have acute dengue by other diagnostic tests (within parenthesis of Column 8 and 9). Collectively, 35 (18+6+11) patients had pre-existing anti-dengue IgM and 17 (8+3+6) patients had pre-existing anti-dengue IgG of >22 P.U. in their blood collected within three days of illness (Sample age Day 0 to Day 2). Of the 35 patients with pre-existing anti-dengue IgM and 17 patients with pre-existing anti-dengue IgG, 16 (7+2+7) and five (3+2) were respectively confirmed to have acute dengue.

DISCUSSION

In order to provide timely information for the management of patients with acute dengue and early public health control of dengue outbreaks, it is important to establish an accurate confirmation of acute dengue virus infection during the first few days of clinical symptoms. Recently, a newly available diagnostic platform based on detection of secreted dengue non-structural (NS1) protein present in patient's blood represents a new and promising approach for early laboratory confirmation of acute dengue infection. Commercial dengue diagnostic kits based on dengue NS1 antigen-capture ELISA have been developed which were evaluated and found to have an overall sensitivity of 88.7% to 93.4% with a high specificity of 100%[10-12] In previous studies, the sensitivity of the dengue NS1 antigen-capture ELISA was evaluated using panels of serum samples which were already tested positive by virus isolation and/or molecular diagnostic methods (RT-PCR and real-time RT-PCR). This approach of evaluation using a panel of known positive samples essentially biased against the sensitivity of the dengue NS1 antigen-capture ELISA method.[10-12] Despite the biased approach, dengue NS1 antigen-capture ELISA was found to be more sensitive than virus isolation and RT-PCR in laboratory diagnosis of acute dengue. In this comparative evaluation, dengue NS1 antigen-capture ELISA was found to be far more sensitive than virus isolation, conventional RT-PCR and real-time RT-PCR in laboratory confirmation of acute dengue as a whole and across all sample age groups (Table 2 and Table 3). This evaluation also confirmed that real-time RT-PCR was significantly more sensitive than the conventional RT-PCR.

Until recently, it is an acceptable practice to interpret a positive detection of anti-dengue IgM

TABLE 3: The number of samples with known sample age that were tested positive by each test method

Sample Age (Day)	Number of samples	Confirmed Acute Dengue	Number of samples tested positive by each method					
			NS1-ELISA (%)	Virus Isolation (%)	RT-PCR (%)	rRT-PCR (%)	IgM (*)	IgG (*)
0	40	9	9 (100)	4 (44)	1 (11)	3 (33)	18 (7)	8 (3)
1	39	10	9 (90)	7 (70)	6 (60)	8 (80)	6 (2)	3 (0)
2	58	16	16 (100)	8 (50)	11 (69)	12 (75)	11 (7)	6 (2)
3	93	35	32 (91)	24 (69)	23 (66)	26 (74)	23 (14)	12 (3)
4	97	35	30 (86)	16 (46)	19 (54)	23 (66)	41 (25)	21 (10)
5	74	32	31 (97)	6 (19)	11 (34)	17 (53)	36 (26)	19 (7)
6	50	25	22 (88)	2 (8)	5 (20)	5 (20)	35 (22)	14 (3)
7	37	7	7 (100)	0	3 (43)	3 (43)	16 (7)	9 (1)
8	11	2	2 (100)	0	0	0	5 (2)	4 (1)
9	6	0	0	0	0	0	1 (0)	1 (0)
10	7	0	0	0	0	0	0	1 (0)
11	4	0	0	0	0	0	0	0
14	1	0	0	0	0	0	0	0
Total	517	171	158 (92)	67 (39)	79 (46)	97 (57)	192	98

(*) = The figure within the parenthesis indicates number of serum samples tested positive from patients with confirmed acute dengue.

as indicative of acute dengue in the presence of corroborating clinical and epidemiological findings because MAC-ELISA is the only laboratory method widely and readily available in most hospital laboratories. This practice is probably still applicable for countries where there is no endemic transmission of dengue virus. In countries where dengue transmission is hyper-endemic with co-circulation of 4 serotypes of dengue viruses and up to 90% of infections presenting as subclinical or with mild symptoms but resulting in the production of anti-dengue IgM in the blood,[16-18] accepting a positive detection of anti-dengue IgM based on a single serum sample as indicative of acute dengue will lead to a false diagnosis. Thus, a single IgM assay should not be used in dengue endemic countries as a confirmatory test for current acute dengue especially if it is collected in early illness because IgM can persist for ≥60 days after an exposure to the virus. This principle is supported in this study by the finding of 18 patients with anti-dengue IgM in their blood collected on the day of onset of illness (Column 8, Table 3). Similarly, the presence of anti-dengue IgG of Panbio Units >22 units (equivalent to haemagglutination inhibition titre of >1:2560 dilution) in blood samples collected in the early phase of acute illness should not be interpreted as acute secondary dengue in dengue endemic areas. In this study, eight patients were found to have anti-dengue IgG of >22 P.U. in their blood samples collected on the day of onset of illness. Of these eight patients, three of them had dengue NS1 protein detected in their blood samples as well, and dengue virus was isolated from two of these three samples (Column 9, Table 3). This finding suggests that these three patients most probably had acute tertiary dengue taking into consideration the usual interpretation of the presence of anti-dengue IgG of >22 P.U. as recent secondary dengue. This finding is also supports the fact that infection with one dengue serotype does not confer cross-protection but can lead to serological diagnostic confusion. A previous study conducted by this laboratory showed that no dengue specific IgM was detected within the first two days of illness and only about 50% of patients had detectable dengue IgM in their sera on the 5th post fever day.[10] In this study, 35 patients had pre-existing anti-dengue IgM and 17 patients had pre-existing anti-dengue IgG of >22 P.U. in their blood collected within three days of illness. Sixteen of the 35 patients and five of the 17 patients were subsequently confirmed to have acute dengue (Table 3). The findings suggest these patients had recent primary and secondary dengue and a proportion of them were having acute secondary and tertiary dengue at the time of study.

In this study, only 190 of the 558 patients with clinical diagnosis of acute dengue were laboratory confirmed. This shows that about a third (34.1%) of the patients seen in outpatient polyclinics were correctly diagnosed as having acute dengue which is supported by laboratory results, a finding that could have important implications on dengue statistics, response and control if the diagnosis of acute dengue is purely based on clinical diagnosis without laboratory confirmation. This finding is in concordance with findings from other studies given the fact that clinical presentation of dengue fever often overlaps other infectious diseases which are endemic in the tropical region.[19-20]

Although positive detection of anti-dengue IgM was considered as recent dengue in this study, a proportion of patients with serologically positive anti-dengue IgM but negative by other test methods (virus isolation, dengue antigen and nucleic acids detection) could still be suffering from acute dengue. This could especially be true if the patients sought late treatment and their blood samples were collected after five days of illness where positive detection rate of other test methods would be very low by then.

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