Original Article

Evaluation of two ELISA Assay Kits against RT-PCR for diagnosis of Dengue Virus Infection in a Hospital Setting in Karachi, Pakistan

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Abstract

Objective: To evaluate two commercially available ELISA-based kits against RT-PCR for the diagnosis of dengue virus infection in a Tertiary Care center in Karachi.

Methods: During the 2006 Dengue outbreak, sera were collected from patients clinically classified as dengue fever and graded according to WHO grading. Out of these, 83 samples were selected randomly and analyzed using two different commercial kits (PanBio versus Calbiotech) and were compared with RT-PCR. Clinical charts of the inpatients were also reviewed. Statistical significance was considered at $P \leq 0.05$.

Results: Clinically, a total of 29 (69%) in-patients were diagnosed with dengue haemorrhagic fever, the remaining 13 (30.9%) were diagnosed as dengue fever. Diagnostic PCR was positive in 73 (87.9%) of the total 83 patients. PanBio capture ELISA had a sensitivity of 83.5%. Calbiotech on the other hand, had a sensitivity of 50.7%. The association of PanBio assay with PCR was found to be statistically significant ($p<0.001$).

Conclusion: Although RT-PCR is considered as gold standard for diagnosis of dengue virus infection, serological methods play important role in diagnosis as they are cost effective and easily available, especially in dengue endemic countries. Sensitivity and specificity of commercial kits can be variable; therefore evaluation of commercial ELISA kits is important in local setting (JPMA 59:390; 2009).

Introduction

Rapid and accurate methods for the diagnosis of dengue fever are important for optimum patient management and institution of early preventive strategies.[1] Viral isolation, nucleic acid detection and serological tests for detection of virus specific antibody are currently available for diagnosis of dengue virus (DEN) infection.[2,3] In the developing countries, due to limited resources, serological analysis by commercially available kits (ELISA based) is the main stay in diagnosis.[4]

ELISA has been used to detect acute phase (IgM) and convalescent phase (IgG) antibodies as well as detection of DEN antigens. IgM detection by ELISA is by far the most common test used for establishing serodiagnosis . In keeping with disease pathogenesis, detection of newly formed DEN antibodies (IgM) in primary infection is variable and is often not detectable until after viremia ends or fever subsides. Similarly IgM production is much lower in secondary DEN infections.[5,6]

Examination of paired serum samples is considered most reliable for establishing sero-diagnosis in primary infections.[7] In clinical practice however, only a single serum sample is available from a suspected patient for diagnosis. Thus it is important to know sensitivity and specificity of commercial serologic tests to diagnose dengue infection in single serum sample from a febrile/clinically suspected patient. This information is especially relevant for centers where other diagnostic options are not available.

In the current study we compared two commercial ELISA based Kits with RT-PCR for its sensitivity and positive predictive value to diagnose dengue in a single serum sample received from suspected dengue patients. In addition clinical charts of In-patients were also reviewed to correlate serological results with clinical findings.

Materials and Methods

The study was conducted at Aga Khan University Hospital (AKUH), a 550-bedded tertiary care center with fully equipped state of art clinical Microbiology Laboratory located in the metropolitan city of Karachi, Pakistan. During 2006 dengue fever out break, AKUH received sera of clinically suspected cases for diagnosis. 83 serum samples were randomly selected and were analyzed using two different Commercial kits (PanBio Vs Calbiotech). The results of these kits were compared with RT-PCR. In addition clinical charts of the in-patients were also reviewed to compare the results with the clinical findings.

1) PanBio Capture ELISA: The PanBio Den Duo (Cat No: E-DEN01D) assay is a combination kit in a microtiter plate format that is designed to detect both IgG and IgM antibodies to DEN in separate reactions by use of capture method. The assay was performed according to the

manufacturer's instructions. The plates were read using dual wavelength spectrophotometer with reference filter set between 600-650nm. The results were interpreted in the light of control sera (cut-off calibrators) and index value (PanBio Unit) was calculated as recommended by manufacturers. Acute dengue virus infection was defined if patient's sera were positive for high molar concentration of IgM alone or IgM with low molar concentration of IgG (<22 PanBio Units). The serum samples positive for IgG > 22 PanBio Units were considered as secondary cases as suggested by the manufacturers. Since Calbiotech assay detects only IgM antibodies against DEN, we compared the ability of two tests to provide diagnosis, based solely on IgM results on a single serum sample received from clinically suspected dengue infected patients.

2) Calbiotech (Catalog No. DE051M): The Calbiotech Inc. (Catalog No. DE051M), Dengue virus IgM ELISA test system is an enzyme linked immunosorbent assay for the detection of IgM antibodies to dengue virus in human serum or plasma. Performed according to the manufacturer's instructions, reading the optical density at dual wavelength with reference filter of 600-650nm. The antibody Index was calculated using O.D. value and the cut-off value (Calibrator OD x Calibrator Factor). Excess IgM antibody, with an antibody index of >1.1 was considered positive for acute dengue infection; values from 0.9 to 1.1 were taken as Borderline positive. Since Calbiotech assay detects only IgM antibodies against DEN, we compared the ability of two tests to provide diagnosis, based solely on IgM results on a single serum sample received from clinically suspected dengue infected patients.

3) Genotype detection by RT-PCR: Viral RNA was extracted from 140µl of serum samples using QIAamp viral RNA mini kit (Qiagen Germany Cat No: 52904) in accordance with the manufacturer's instructions and finally RNA was eluted in 50µl of nuclease free water.

Reverse transcription and amplification was done using the Reverse Enzyme Blend from Abgene ™ Company. (Reverse-iT™ RTase blend). The first round of the RT-PCR reaction included the reverse transcription and amplification using Dengue virus consensus group specific primers previously described by Lanciotti et al.[8] The PCR reaction was performed in Eppendorf Master cycler gradient (Germany) with thermocycler programmed to incubate for 1h at 47ºC, 94ºC for 2 minutes and then proceed with 45 cycles of denaturation(94ºC, 30 sec), primer annealing (55ºC, 30sec) and primer extension (72ºC, 2min) and then final extension (72º,10 minutes).

A second round of amplification using the genotype-specific primers along with consensus primer was performed using aliquot from first round product. The samples were subjected to 20cycles of denaturation (94ºC, 2minutes), primer annealing (55ºC, 30sec) and primer extension (72ºC, 2min) and then final extension at (72ºC, 10minutes). A 10µl portion of PCR product was subjected to electrophoresis using 1.5% agarose gel containing 0.1µg/ml ethedium bromide in Tris EDTA buffer.

4) **Clinical History and other Laboratory Tests:** Medical records of all in-patients were reviewed for demographic, clinical and laboratory data. Patients were categorized into DF and DHF according to the WHO severity grading scale[2] and as published previously.[9] Briefly, DF was defined as an acute febrile illness along with two or more of the following manifestations: headache, retro-orbital pain, myalgia, arthralgia, rash and leucopenia. DHF was defined as fever or history of acute fever, lasting 2-7 days along with haemorrhagic tendencies, evidenced by at least one of the following: petechiae, ecchymoses or purpura, bleeding from mucosa, gastrointestinal tract, haematemesis or melaena. In addition, thrombocytopenia with platelet count equal to or less than 100,000 cells per mm^3 and evidence of plasma leakage due to increased vascular permeability were taken into account. These included rise in haematocrit (greater than 20% above average for age and sex), pleural effusion and/or ascites. Patients with profound shock along with other features of DHF were classified as Dengue Shock Syndrome (DSS).

Data Management and Statistical Analysis:

For each of sample included in this study, the information on hospital medical record number/laboratory identification number, study identification number, age, gender, dengue IgM and PCR status was recorded on a register and subsequently entered in MS-Excel.

The clinical findings for the sub group of in-patients were recorded from the medical records on a semi-structured data extraction sheet designed for the study. The sheets were checked and edited for logical errors and missing information by trained and experienced medical research officer. The data was coded and entered in Epidata 3.0 (Odense, Denmark) and transferred to SPSS 15.0 (SPSS Inc., Chicago, IL, USA) for statistical analysis. In descriptive analysis mean and standard deviation of the continuous variables and percentages of the categorical variables were computed. Chi square and Fisher's exact tests were used for univariate comparisons where appropriate. A p value of less than or equal to 0.05 was considered as statistically significant.

Results

The mean age of the total 83 patients was 30.7 ± 16.2 years, ranging from 2 to72 years with most frequent (28.9%) age group being 21-30 years. There were 50 males and 33 females with a male to female ratio of 1.5. About 50.6% (n=42) of patients were severe enough to be hospitalized and

41(49.4%) were outpatients. Information on clinical and laboratory characteristics was obtained from all the inpatients. A total of 29 (69%) inpatients were diagnosed with dengue haemorrhagic fever and the remaining 13 (30.9%) were diagnosed with dengue fever. (Table-1)

Among 42 inpatients fever was the most common symptom (100%) at time of presentation followed by nausea and vomiting (52.4%), rash (50.0%), diarrhea (19.0%), haemorrhage (19.0%), myalgia (16.7%) and others.

Thrombocytopenia was the most common laboratory finding present in 31 (73.8%) patients followed by raised aspartate aminotransferase (AST) in 26 (61.9%), raised alanine aminotransferase (ALT) in 24 (57.1%), monocytosis in 18 (42.9%), leucopenia in 11 (26.2%) and lymphocytosis in 10 (23.8%) patients. (Table-1)

Diagnostic PCR was positive in 73 (87.9%) of the total 83 patients. Of these 43 (58.9%) were DEN-2 and 30 (41.1%) were DEN-3. None of the patients was positive for DEN-1 and DEN-4 and co-infection with DEN-2 and DEN-3 was also not detected.

Sixty one (83.6%) of PCR positive samples showed IgM positivity with PanBio Capture ELISA assay, of these 13 (21.3%) patients had primary infection while 48 (78.7%) had secondary infection. Among IgM positives cases 51 patients had DEN-2 and while 10 were infected with DEN-3. PanBio performance was comparable with RT-PCR in all age groups (Figure) and in patients categorized as DF and DHF (Table 1).

PanBio capture ELISA had a sensitivity of 83.5%, specificity of 90.0%, positive predictive value (PPV) of 98.4% and negative predictive value (NPV) of 42.9%. Calbiotech on the other hand had a sensitivity of 50.7%, specificity of 60.0%, PPV of 90.2%, NPV of 14.3%. Overall percent accuracy was 84.3% for PanBio assay and 51.8% for Calbiotech assay (Table-2). The association of PanBio assay with PCR was also statistically significant ($p<0.001$) while that for Calbiotech assay was not significant ($p=0.738$) (Table-2).

Table-3 shows the detail of 36 Calbiotech false negative patients. Among these, 21 were infected by DEN-2, 15 by DEN-3. Similarly, based on clinical data from in-patients, 33.3% (n=5) of Calbiotech negative cases were diagnosed with DF while 10 (66.7%) had DHF. Majority of the false negative cases had low platelet counts (73.3%), raised ALT (66.7%) and raised AST (66.7%).

Table 1: General demographics, clinical features and Laboratory findings of patients with dengue virus infection diagnosed by different assays at Aga Khan University Hospital, Karachi, Pakistan.

Features	Overall n=83 n (%)	PCR (+) n=73 n (%)	PanBio (+) n=62 n (%)	Calbiotech (+) n=41 n (%)
Age Mean (±SD)	30.7 (16.2)	30.4 (15.4)	30.8 (15.7)	27.0 (13.9)
Gender				
Male	50 (60.2)	44 (60.3)	37 (59.7)	28 (68.3)
Female	33 (39.8)	29 (39.7)	25 (40.3)	13 (31.7)
Patient status				
Inpatient	42 (50.6)	36 (49.3)	32 (51.6)	24 (58.5)
Outpatient	41 (49.4)	37 (50.7)	30 (48.4)	17 (41.5)
Dengue severity *				
DF	13 (31.0)	10 (27.8)	9 (28.1)	6 (25.0)
DHF	29 (69.0)	26 (72.2)	23 (71.9)	18 (75.0)
Clinical features *				
Fever	42 (100.0)	36 (100.0)	32 (100.0)	24 (100.0)
Headache	4 (9.5)	3 (8.3)	2 (6.3)	1 (4.2)
Myalgia	7 (16.7)	7 (19.4)	5 (15.6)	5 (20.8)
Diarrhea	8 (19.0)	7 (19.4)	7 (21.9)	4 (16.7)
Nausea/Vomiting	22 (52.4)	19 (52.8)	17 (53.1)	10 (41.7)
Cough	6 (14.3)	6 (16.7)	6 (18.8)	4 (16.7)
Rash	21 (50.0)	18 (50.0)	16 (50.0)	14 (58.3)
Haemorrhage	8 (19.0)	8 (22.2)	0 (0.0)	6 (25.0)
Ascots	3 (7.1)	3 (8.3)	3 (9.4)	2 (8.3)
Laboratory findings *				
Thrombocytopenia	31 (73.8)	28 (66.7)	25 (59.5)	19 (45.2)
Leukopenia	11 (26.2)	9 (21.4)	9 (21.4)	7 (16.7)
Lymphocytosis	10 (23.8)	25 (59.5)	20 (47.6)	15 (35.7)
Monocytosis	18 (42.9)	15 (35.7)	15 (35.7)	14 (33.3)
Raised ALT	24 (57.1)	20 (47.6)	19 (45.2)	16 (38.1)
Raised AST	26 (61.9)	22 (52.4)	21 (50.0)	18 (42.9)

DF= Dengue fever, DHF=, Dengue hemorrhagic fever, DEN-2= Dengue genotype2, DEN-3= dengue genotype3,
ALT= alanine aminotransferase, AST= aspartate aminotransferase.
*Percentages are calculated among inpatients (n=42) only.

Table 2: Comparative analysis of ELISA based assays with PCR, for patients presenting with signs and symptoms suggestive of dengue fever at Aga Khan University Hospital Karachi, Pakistan.

		PCR		Total	% Sens	% Spec	% NPV	% PPV	% Acc
		Positive n = 73	Negative n = 10						
Calbiotech	Positive	37	4	41	50.7	60.0	14.3	42.8	51.8
	Negative	36	6	42					
PanBio	Positive	61	1	62	83.5	90	90.2	98.4	84.3
	Negative	12	9	21					

Sens= Sensitivity, Spec= Specificity, NPV= Negative Predictive Value, PPV= Positive Predictive Value, Acc = Accuracy.

Discussion

Dengue virus infection has emerged as a great challenge for public health, worldwide.A rapid and accurate method for the diagnosis is important for both the clinician and the patient. In Pakistan dengue virus infection has been reported since 1993. Due to non-availability of diagnostic tests locally, the laboratory diagnosis in the past reports was performed in the collaborating centers abroad.[10,11] The year 2005-2006 however, witnessed an unprecedented increase in epidemic DHF activity in the country with a large number of cases being reported from Karachi. In view of immense pressure to ensure rapid diagnosis, many simple ELISA based commercial kits were imported at national and local hospital levels. Although most of these kits have shown to have good sensitivity and specificity when standardized using paired sera,[12] in clinical practice usually, only single sample is available to give confirmed diagnosis. Thus it is imperative to evaluate the performance of commercial kits in less then ideal situations such as non-availability of paired sera and limited access to advance molecular techniques such as PCR to analyze the final results.

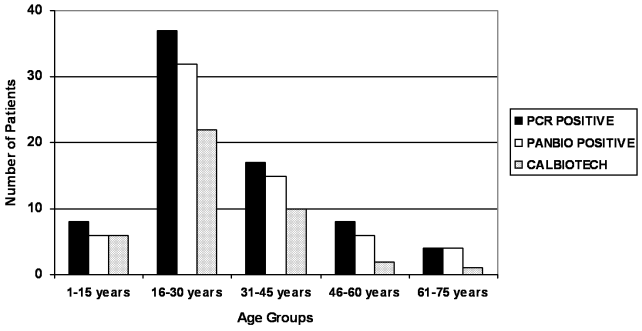

Figure: Distribution according to age and type of diagnostic test used at Aga Khan University Hospital.

We evaluated two commercially available ELISA based kits against RT-PCR in a diagnostic laboratory setup. Dengue virus identification by molecular methods such as RT-PCR, has higher sensitivity and specificity, and is increasingly being used for the diagnosis. Various RT-PCR protocols for dengue virus detection are in practice, however, we utilized the well known two-step nested RT-PCR protocol developed by Lanciotti et al,[8] having the advantage of detecting and differentiating four dengue serotypes.

Table 3: Characteristics of patients labeled as false negative on Calbiotech ELISA assay, at Aga Khan University Hospital Karachi, Pakistan.

Features	False (-) by Ch. ELISA n=36	%
Gender		
Male	20	55.6
Female	16	44.4
Den-type		
DEN-2	21	58.3
DEN-3	15	41.7
Patient status		
Inpatient	15	41.7
Outpatient	21	58.3
Dengue severity +		
DF	5	33.3
DHF	10	66.7
Platelets count +		
Normal	4	26.7
Low	11	73.3
Leukocyte count +		
Normal	12	80
Low	3	20
ALT level +		
Normal	3	33.3
Raised	6	66.7
AST Level +		
Normal	3	33.3
Raised	6	66.7

+ Information not available for all the patients.

Serological diagnostic methods provide a rapid cost effective diagnosis and anti-dengue IgM and IgG antibodies are often found from sera of the patients with acute infection. However, IgM levels may not be detected in secondary infections. At the same time, cross reaction between dengue antibodies and other flaviviral antigens may also occur, and this complicates the diagnosis, if other flavivirus infections cannot be ruled out. Despite these limitations, serology is still a main stay in diagnosis of dengue infections in many dengue

endemic countries. Thus it is imperative to evaluate the performance of commercial kits in local setting.

Sera from 83 patients with clinical suspicion of Dengue fever and dengue hemorrhagic fever were analyzed using RT-PCR and the 2 available ELISA kits. PanBio ELISA was found to be more sensitive and specific than Calbiotech ELISA, similar results have also been reported in other studies as well.[13,14] We report statistically significant correlation of PanBio assay with RT-PCR ($p<0.001$). Serological results obtained from Calbiotech assay, did not show statistically significant association with RT-PCR ($p=0.738$).

When tested by Calbiotech ELISA; 33% of patients with Dengue fever and 66.7% patients with clinical features consistent with dengue haemorrhagic fever (DHF) were tested negative. Similarly, 58.3% patients infected with Den-2 and 41.7% of those infected with Den-3 were tested negative by this kit. These findings suggest that this assay overall had a poor negative predictive value (14.3%) and all negative tests must be reconfirmed by another kit and / or method.

Although our study has limitation of small sample size, however, our results have identified need for the re-evaluation of commercial kits for its performance in less then ideal situations such as non-availability of paired sera and limited access to advance molecular techniques such as RT- PCR before reporting the final results. In addition, clinical presentation such as fever, rash and nausea/vomiting and other laboratory tests like thrombocytopenia and raised liver enzymes should be considered before making the final diagnosis.

References

1. Sathish N, Vijayakumar TS, Abraham P, Sridharan G. Dengue Fever: its Laboratory Diagnosis, with Special Emphasis on IgM Detection. Dengue Bulletin 2003; 27: 116-25.
2. De Paula SO, Fonseca BA. Dengue: a review of the laboratory tests a clinician must know to achieve a correct diagnosis. Braz J Infect Dis 2004; 8: 390-8.
3. Kao CL, King CC, Chao DY, Wu HL, Chang GJ. Laboratory diagnosis of dengue virus infection: current and future perspectives in clinical diagnosis and public health. J Microbiol Immunol Infect 2005; 38: 5-16.
4. Cuzzubbo AJ, Endy TP, Nisalak A, Kalayanarooj S, Vaughn DW, Ogata SA, et al. Use of recombinant envelope proteins for serological diagnosis of Dengue virus infection in an immunochromatographic assay. Clin Diagn Lab Immunol 2001; 8: 1150-5.
5. Innis BL, Nisalak A, Nimmannitya S, Kusalerdchariya S, Chongswasdi V, Suntayakorn S, et al. An enzyme linked immunosorbent assay to characterize dengue infections where dengue and Japanese encephalitis co-circulate. Am J Trop Med Hyg 1989; 40: 418-27.
6. Branch SL, Levett PN. Evaluation of four methods for detection of immunoglobulin M antibodies to dengue virus. Clin Diagn Lab Immunol 1999; 6: 555-7.
7. Wichmann O, Hongsiriwon S, Bowonwatanuwong C, Chotivanich K, Sukthana Y, Pukrittayakamee S. Risk factors and clinical features associated with severe dengue infection in adults and children during the 2001 epidemic in Chonburi, Thailand. Trop Med Int Health 2004; 9: 1022-9.
8. Lanciotti RS, Calisher CH, Gubler DJ, Cheng GJ, Vorndam AV. Rapid detection and typing of dengue viruses from clinical samples by using reverse-transcriptase-polymerase chain reaction. J Clin Microbiol 1992; 30: 545-51.
9. Khan E, Siddiqui J, Shakoor S, Mehraj V, Jamil B, Hasan R. Dengue outbreak in Karachi, Pakistan, 2006: experience at a tertiary care center. Trans R Soc Trop Med Hyg 2007; 101: 1114-9.
10. Chan SY, Kautner IM, Lam SK. The influence of antibody levels in dengue diagnosis by polymerase chain reaction. J Virol Methods 1994; 49: 315-22.
11. Akram DS, Igarashi A, Takasu T. Dengue virus infection among children with undifferentiated fever in Karachi. Indian J Pediatr 1998; 65: 735-40.
12. Wichmann O, Stark K, Shu PY, Niedrig M, Frank C, Huang JH, et al. Clinical features and pitfalls in the laboratory diagnosis of dengue in travellers. BMC Infect Dis 2006; 6: 120.
13. Palmer CJ, King SD, Cuadrado RR, Perez E, Baum M, Ager AL. Evaluation of the MRL diagnostics dengue fever virus IgM capture ELISA and the PanBio Rapid Immunochromatographic Test for diagnosis of dengue fever in Jamaica. J Clin Microbiol 1999; 37: 1600-1.
14. Vaughn DW, Nisalak A, Solomon T, Kalayanarooj S, Nguyen MD, Kneen R, et al. Rapid serologic diagnosis of dengue virus infection using a commercial capture ELISA that distinguishes primary and secondary infections. Am J Trop Med Hyg 1999; 60: 693-8.