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Evaluation of laboratory tests for dengue diagnosis in clinical specimens from consecutive patients with suspected dengue in Belo Horizonte, Brazil

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ABSTRACT

Background: Dengue is a widely spread arboviral disease in tropical and subtropical regions of the world. Dengue fever presents clinical characteristics similar to other febrile illness. Thus laboratory diagnosis is important for adequate management of the disease.
Objectives: The present study was designed to evaluate the diagnostic performance of real-time PCR and serological methods for dengue in a real epidemic context.
Study design: Clinical data and blood samples were collected from consecutive patients with suspected dengue who attended a primary health care unit in Belo Horizonte, Brazil. Serologic methods and real-time PCR were performed in serum samples to confirm dengue diagnosis.
Results: Among the 181 consecutive patients enrolled in this study with suspected dengue, 146 were considered positive by serological criteria (positive NS1 ELISA and/or anti-dengue IgM ELISA) and 138 were positive by real-time PCR. Clinical criteria were not sufficient for distinguishing between dengue and non-dengue febrile illness. The PCR reaction was pre-optimized using samples from patients with known viral infection. It had similar sensitivity compared to NS1 ELISA (88% and 89%, respectively). We also evaluated three commercial lateral flow immunochromatographic tests for NS1 detection (BIOEASY, BIORAD and PANBIO). All three tests showed high sensitivity (94%, 91% and 81%, respectively) for dengue diagnosis.
Conclusion: According to our results it can be suggested that lateral flow tests for NS1 detection are the most feasible methods for early diagnosis of dengue.

1. Background

Dengue is a worldly common mosquito-borne disease. Next to 2.5 billion people are at risk of infection in the tropical and subtropical regions [1]. Close to 50 million infections occur globally every year [2]. Real prevalence of dengue is probably higher as in less developed countries notification is inefficient and diagnosis confirmation by laboratory assays is not always available. Viral transmission occurs most frequently through the bite of *Aedes aegypti* and *Aedes albopictus* mosquitoes. Four viral serotypes were identified, DENV-1, DENV-2, DENV-3 and DENV-4 [3]. Dengue clinical features vary from an undifferentiated febrile illness to the

Abbreviations: WHO, World Health Organization; DENV, dengue virus; DHF, dengue hemorrhagic fever; BVDV, bovine viral diarrhea virus; PCR, polymerase chain reaction; PPV, positive predictive value; NPV, negative predictive value; SLEV, Saint Louis encephalitis virus; YFV, yellow fever virus.

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severe hemorrhagic form (dengue hemorrhagic fever – DHF) that can lead to shock and death [4,5].

Dengue vector density varies according to rainfall [6]. Therefore, in Brazil, dengue incidence rises seasonally on summer after the raining period [7,8]. The incidence also varies significantly among years in the different regions of the country. From 2009 to 2011, the Department of Health Surveillance of the Ministry of Health of Brazil reported incidences of 205.5, 530.3 and 400.5 per 100,000 persons, respectively [9]. It is clear that dengue is an important health problem in Brazil. Thus, precise and early diagnosis is extremely relevant for adequate management of the disease. Dengue fever is characterized by unspecific symptoms and, in most cases, clinical presentation is similar to other febrile and viral diseases. Thus, clinical criteria are not ideal for the definitive diagnosis of dengue [4,10]. There are many diagnostic tools to detect an acute dengue infection, including virus isolation, RT-PCR and real-time PCR, viral genome sequencing, viral antigen detection and serologic methods [5,11,12]. In Brazil, serology is a common method used in public health services. RT-PCR and NS1 antigen detection by ELISA are sensitive methods for early detection of dengue virus infection [13] but they are not widely used in public health services in Brazil. Moreover, during epidemic periods in areas with high incidence rates, laboratory diagnosis is not always available. In the latter setting, physicians need to rely on clinical and epidemiological criteria to detect possible dengue cases which can lead to false diagnosis and failure to detect other viral pathogens with public health importance [14]. Recently, lateral flow immunochromatographic assays for NS1 antigen detection have been used on primary care services. Those tests are easy to perform, of low comparative cost when adopted for mass-surveys, and convenient for distribution to clinical facilities set far away for main healthcare centers in large countries, like Brazil. We aimed to determine their comparative usefulness in a real clinical epidemic situation.

2. Objectives

In the present work, we analyzed the performance of clinical data, real-time PCR and serologic tests for NS1 and anti-dengue IgM in serum samples from consecutive patients in a primary health care in an endemic area. Belo Horizonte is a large metropolitan area with approximately 5 million people in the Southeast region of Brazil. The aims of the present study were three-fold. Initial experiments were carried out to optimize a real-time PCR reaction against serum samples previously subjected to dengue virus isolation. Second, we evaluated the diagnostic performance of clinical data and real-time PCR for dengue diagnosis in an actual epidemic context. Third, in known dengue positive samples, we compared the diagnostic performance of three lateral flow tests for dengue NS1.

3. Study design

From January 2010 to March 2010, consecutive patients (at the age of 18 or older) with suspected dengue were enrolled in the study after giving written consent to participate. WHO guidelines were used for classifying dengue cases. Clinical data and venous blood samples were collected on the day of admission (acute sample). Six days after illness onset a second blood sample was collected (convalescent sample). According to our case definition, dengue positive cases presented NS1 ELISA and/or anti-dengue IgM ELISA positive tests. Cases with both NS1 ELISA and anti-dengue IgM ELISA negative tests were considered to have other febrile illness as cases with indeterminate result in one of the cited test plus negative result in the other test. Dengue NS1 Ag kit (Bio-Rad Laboratories) was used for NS1 detection in acute samples and anti-dengue IgM was detected in convalescent samples using MAC-ELISA (PanBio Diagnostics, Brisbane, Australia).

In order to evaluate the performance of the real-time PCR protocol we first compared some RNA extraction methods (data not shown). The most reproducible method was obtained by using the QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) as reported before [15]. First-strand cDNA synthesis was performed using MMLV Reverse Transcriptase in standard buffer (Promega, Madison, WI) and reverse primer 5′GGGTCTCCTCTAACCTCTAGTCCT3′. Individual real-time PCR reactions were carried out as previously described by Chien and colleagues [16] in the StepOne™ Real-Time PCR System (Applied Biosystems™). A sensitivity test was performed a priori using known positive samples subjected previously to dengue virus isolation by inoculation in C6/36 *A. albopictus* cells. Dengue virus serotypes were identified as previously described by Lanciotti et al. [17] with slight modifications [16]. The acute sample obtained from the consecutive patients was used for evaluating the performance of the real-time PCR.

Finally, we tested three lateral flow kits for NS1 antigen detection: Bioeasy – Dengue Eden Test Bioeasy (Standard Diagnosis, Pajan-dong, Korea), BIORAD – Dengue NS1 AG Strip (Bio-Rad, Marnes-la-Coquette, France) and PANBIO – Dengue Early Rapid (Inverness Medical, Sinnamon Park, Australia). We used acute samples from consecutive patients presenting positive results in real-time PCR, NS1 ELISA and anti-dengue IgM ELISA. For the specificity test we selected negative acute samples. Statistical analysis was performed in SPSS Statistics 17.0. Categorical and continuous variables where analyzed by Chi-square and Mann–Whitney respectively. A p value equal or smaller than 0.05 was considered statistical significant. Analysis of sensitivity, specificity, PPV, NPV and accuracy were carried out at http://www.openepi.com/.

4. Results

Initial experiments evaluated the sensitivity of the real-time PCR in 58 serum samples previously subjected to dengue virus isolation by culture in C6/36 cells. Serotypes were identified by multiplex PCR: DENV1 (30 samples), DENV2 (20 samples) and DENV3 (8 samples). Real-time PCR detected dengue virus in 52 samples (91% of total). This method also detected DENV4 virus (Fig. 1A).

The specificity of real-time PCR was assessed evaluating culture samples containing other flavivirus (yellow fever virus – YFV-, bovine viral diarrhea virus – BVDV and Saint Louis encephalitis virus – SLEV). Real-time PCR melt curves showed an YFV-unrelated unspecific curve (possibly primer-dimers) and no amplification products with BVDV samples. The amplification products of SLEV virus samples showed two-peak melting curves, in which one peak was similar to that detected for dengue samples (Fig. 1B).

Clinical information and samples for RT-PCR and NS1 ELISA were collected from 181 consecutive patients with suspected dengue on admission. 124 samples were collected for IgM ELISA at the convalescent period from patients that were enrolled in the study on admission and returned at the convalescent period (6 days of symptoms) or patients that were admitted at the convalescent period.

The mean age of subjects was 43 years and 67% were women. According to the case definition used in this study, 81% were confirmed dengue cases (146 patients). The frequency of most clinical and demographic characteristics was equal in patients with acute dengue as compared to non-dengue acute illness. Platelets counts and hematocrit values were similar in both groups (Table 1).

Interestingly, subjects with dengue sought medical attention earlier when compared to non-dengue subjects (3.0 versus 4.2 days). Exanthema was more frequently reported in patients with dengue. Fever, headache, retro-orbitary pain and myalgia were the

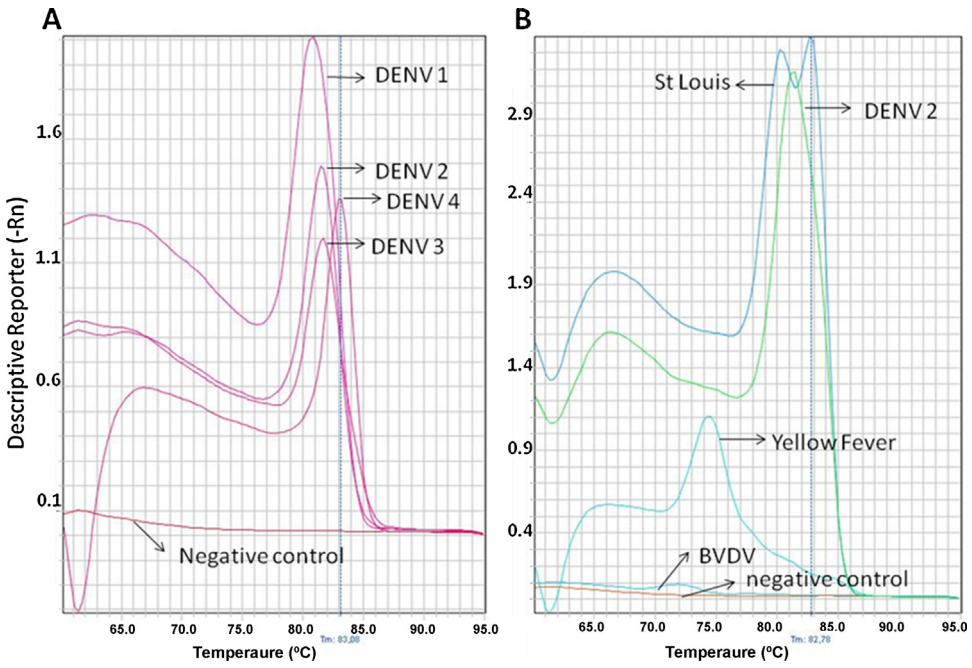

Fig. 1. Real-time PCR melting curves of positive control amplification products (A) and flavivirus samples (B).

Table 1
Epidemiological characteristics of patients enrolled in the study.

Clinical, demographic and laboratorial features	Dengue	Non-dengue	*p*
Number of patients	146 (80.7%)	35 (19.3%)	
Mean age in years	44 (±16)	42 (±17)	0.332
Gender (M/F)	46 (31%)/100 (69%)	14 (40%)/21 (60%)	
Days after illness onset	3.01	4.20	0.014
Platelets counts	136,896	127,818	0.522
Hematocrit	44.31	43.74	0.599
Secondary dengue (sic)	13 (8.9%)	3 (8.6%)	0.626
Comorbidity (diabetes, asthma, hypertension, gastritis, hypothyroidism) (%)	69 (47.3%)	18 (51.4%)	0.658
Systemic artherial hypertension (%)	60 (41.1%)	15 (42.9%)	0.849

most frequent symptoms among dengue cases but they occurred equally in both groups (Table 2). 1 patient developed DHF.

DENV1 was the most prevalent serotype corresponding to 57% of total and 73% of real-time PCR positive samples (Table 3). DENV-4 was found in 4 samples, those are probably the first DENV4 reported cases in Belo Horizonte. DENV4 was first detected in Brazil in 1982 [18] and recently suggested to be circulating at low levels in the southeast region [19]. Three patients had a mixed DENV-1/DENV-3 infection which evolved as classic dengue fever.

Real-time PCR was positive in 138 samples (Table 4), whereas 94% (129 samples) were positive and 6% (9 samples) were negative on serological criteria. The primers used in the real-time PCR were designed from the conserved NS5 coding region of the dengue virus RNA genome. To assure that the real-time PCR was detecting dengue virus, we partially sequenced the genome of one of the dengue positive samples (GenBank: http://www.ncbi.nlm.nih.gov; accession: JQ24798; JF917098; JF917097).

Among 124 samples analyzed by IgM capture ELISA, 100 were positive. 57 patients enrolled on admission (32%) did not show up

Table 2
Correlation of symptoms presented by patients enrolled in the study.

Symptoms	Dengue	Non-dengue	*p*
Headache (%)	135 (92.5%)	34 (97.1%)	0.285
Retro-orbitrary pain (%)	114 (78.1%)	27 (77.1%)	0.904
Myalgia (%)	128 (87.7%)	31 (88.6%)	0.574
Arthragia (%)	50 (34.2%)	10 (28.6%)	0.702
Bleeding (%)	19 (13.0%)	2 (5.7%)	0.182
Exanthema (%)	56 (38.4%)	7 (20.0%)	0.041
Abdominal pain (%)	32 (21.9%)	9 (25.7%)	0.630
Loss of appetite (%)	68 (46.6%)	11 (31.4%)	0.105
Diarrhea (%)	41 (28.1%)	12 (34.3%)	0.469
Vomiting (%)	41 (28.1%)	10 (28.6%)	0.954
Dizzness (%)	76 (52.1%)	16 (45.7%)	0.500
Drowsiness (%)	72 (49.3%)	13 (37.1%)	0.195
Confusion (%)	11 (7.5%)	2 (5.7%)	0.523
Difficulty breathing (%)	14 (9.6%)	3 (8.6%)	0.576
Cough (%)	30 (20.5%)	11 (31.4%)	0.167
Fatigue (%)	68 (46.6%)	13 (37.1%)	0.313

Table 3
Correlation of real-time PCR results and dengue serotypes identified by multiplex PCR in serum samples from consecutive patients.

Multiplex PCR	Realtime PCR		Total
Serotype	Negative	Positive	
Negative	40 (93.0%)	4 (2.9%)	44 (24.3%)
DENV1	2 (4.7%)	101 (73.2%)	103 (56.9%
DENV2	0 (0.0%)	8 (5.8%)	8 (4.4%)
DENV3	0 (0.0%)	14 (10.1%)	14 (7.7%)
DENV4	0 (0.0%)	4 (2.9%)	4 (2.2%)
DENV1 and DENV 3	1 (2.3%)	2 (1.4%)	3 (1.7%)
Undefined	0 (0.0%)	5 (3.6%)	5 (2.8%)
Total	43 (100.0%)	138 (100.0%)	181 (100.0%)

Table 4
Correlation of real-time PCR results with serological diagnosis.

	Diagnosis by serological criteria NS1/IgM		Total
	Non-dengue	Dengue	
Negative real-time PCR	26 (74.3%)	17 (11.6%)	43 (23.8%)
Positive real-time PCR	9 (25.7%)	129 (88.4%)	138 (76.2%)
Total	35 (100%)	146 (100%)	181 (100%)

Table 5
Correlation of real-time PCR (A) and NS1 ELISA (B) results with diagnosis by all criteria. Here we considered as positive samples (dengue) those cases presenting positive real-time PCR and/or NS1 ELISA and/or anti-dengue IgM. Negative samples (non-dengue) were considered those cases presenting negative real-time PCR and negative or indeterminate serological tests.

	Diagnosis by all criteria (PCR/NS1/IgM)		Total
	Non-dengue	Dengue	
Negative NS1	22 (88.0%)	14 (9.0%)	36 (19.9%)
Positive NS1	0 (0%)	137 (87.8%)	137 (75.7%)
Indeterminate	3 (12.0%)	5 (3.2%)	8 (4.4%)
Total	25 (100.0%)	156 (100.0%)	181(100.0%)
Negative PCR	25 (100.0%)	18 (11.5%)	43 (23.8%)
Positive PCR	0 (0%)	138 (88.5%)	138 (76.2%)
Total	25 (100.0%)	156 (100.0%)	181(100.0%)

on the convalescent period for second sample collection – thus IgM ELISA was not performed in serum of these patients.

As we found a strong correlation between real-time PCR and serological diagnosis ($p < 0.001$) we performed a complementary analysis to compare real-time PCR to NS1 ELISA. When we considered as dengue positive cases any test (real-time PCR, NS1 ELISA or anti-dengue IgM ELISA), NS1 ELISA and real-time PCR sensitivities were similar (88% for both; Table 5A and B). NS1 ELISA showed similar performance compared to real-time PCR particularly regarding sensitivity and accuracy parameters when we combine methods as reference tests (Table 6).

We compared three of available commercial NS1 rapid tests. From the acute samples of consecutive patients, we selected 67 specimens which simultaneously presented positive results for NS1 and anti-dengue ELISAs and real-time PCR and 10 samples with negative results for all tests. Table 7 shows that all tests present high sensitivity (94%, 91% and 81%). One method (BIOEASY) was simpler to perform as it did not require additional material besides a sample dispenser and the test strip. All tests showed 100% specificity when we tested negative samples. This is similar to the data given by the manufacturers.

5. Discussion

Few studies have demonstrated the performance of the real-time PCR in a real epidemic context. The epidemic situation faced in the study region from February to May in 2010 was one of the greatest epidemic peaks ever experienced in Belo Horizonte [9]. We addressed early diagnosis tools for dengue analyzing the performance of real-time PCR, NS1 ELISA and lateral flow tests for NS1 detection. Compared to NS1 ELISA, the real-time PCR showed similar sensitivity. Lateral flow tests for NS1 detection were the most feasible methods for dengue early diagnosis as they were simpler and less expensive than other methods.

Table 7
Sensitivity and specificity of commercial lateral flow tests for dengue NS1 detection in serum. A total of 77 samples were selected, 67 specimens were acute samples taken from patients with positive results in real-time PCR, NS1 ELISA and anti-dengue IgM ELISA methods and 10 acute samples were taken from patients with negative results in all tests.

TEST	Sensitivity	95% CI	Specificity	95% CI
BIOEASY	63 (94.03%)	85.63–97.65%	10 (100%)	72.25–100%
BIORAD	61 (91.04%)	81.81–95.83%	10 (100%)	72.25–100%
PANBIO	59 (88.06%)	78.17–93.82%	10 (100%)	72.25–100%

As it has been published elsewhere [10,20], it was evident in our study that symptoms and clinical features were not efficient for distinguishing dengue and other febrile illness. The only symptom reported more frequently by patients with confirmed dengue was exanthema but no more than 38% of patients presented that symptom. Interestingly, patients with dengue sought medical attention earlier than subjects with other febrile illness. This may be related to the intensity of pain and discomfort experienced by patients with dengue, as it is also known as “break bone fever” in Chinese medicine.

Early diagnosis is important for the clinical management of dengue and may prevent unsatisfactory outcomes [11,21–23]. The performance of methods available for dengue diagnosis varies according to the disease period. Dengue virus and dengue viral products are detected in serum at the early illness period, consequently the sensitivity of dengue diagnostics methods as RT-PCR and real-time PCR, virus isolation or NS1 detection are higher at first days of illness [23]. On the other hand, serologic methods as anti-dengue IgM have better performance for dengue diagnosis at the convalescent phase (around 5–6 days post illness onset) [23–25], when higher levels of anti-dengue immunoglobulins are detected in blood. Hence this method presents a disadvantage: many patients do not return to the point of care for blood collection because they feel better. Dengue hemorrhagic syndrome manifests when fever decreases, which occur around that same period, thus an effective clinical approach would benefit from early diagnosis [21,23].

The real-time PCR had high sensitivity for dengue diagnosis. It correlated with serological diagnosis results used in this study for case definition (positive NS1 ELISA and/or positive anti-dengue IgM ELISA) and the overall performance of the method was satisfactory when compared to NS1 ELISA alone and serological methods

Table 6
Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and diagnostic accuracy of real-time PCR and NS1 ELISA compared to other methods alone or in combination.

Reference test/test	NS1/realtime PCR (%)	Realtime PCR/NS1 (%)	NS1 or IgM/realtime PCR (%)	Realtime PCR or IgM/NS1 (%)
Sensitivity	88.32	87.68	88.36	87.58
Specificity	61.36	62.79	74.29	89.29
PPV	87.68	88.32	93.48	97.81
NPV	62.79	61.36	60.47	56.82
Diagnostic Accuracy	81.77	81.77	85.64	87.85

in combination. Those data are in accordance to previous studies that described other real-time PCR protocols [16,26–28].

The real-time PCR used here displayed unspecific amplification of SLEV sequences, which clearly limits its overall applicability. It may be useful in setting of epidemic situations as the one occurring during the study period. It is interesting to note that other studies have used a similar design (i.e. the same or similar pair of primers) but have not tested the specificity against other flavivirus [16,29]. SLEV infection in humans is usually characterized by a subclinical or mild febrile illness, although the virus may also cause encephalitis. This is epidemiologically relevant in North America, where unpredictable epidemics occurred in the last decades [30]. In 2007, an outbreak of SLEV occurred concomitantly with a large DENV-3 outbreak in the state of São Paulo [14,31,32]. Thus, not only plain nucleic acid amplification data, but also analysis of the melting curves of the amplified products, which displayed different profiles for Dengue and SLEV, should be considered for diagnostic purposes based on real-time PCR, as SLEV mild cases display undistinguishable acute febrile illness mimicking dengue fever specially in the acute phase of infection [14,33]. We also suggest that in an uncommon clinical context (for example a suspected case of viral encephalitis) a specific qRT-PCR for Saint Louis should be performed. Therefore the use of our method should be limited in patients presenting non usual clinical symptoms. Taking in consideration the complexity of real-time PCR and the particular issue of specificity of our method, detection of NS1by lateral flow tests was more practicable and very accurate for early diagnosis of dengue.

Commercial kits designs of lateral flow tests vary regarding sample required (blood, plasma or serum), additional reagents and material used. Our data showed that all kits we have tested presented good sensitivity and specificity for dengue diagnosis. However, the simplest (from the technical point of view) and least expensive method (BIOEASY) had higher sensitivity. The latter test can be performed with blood, plasma and serum samples, it only requires a sample pipette and no buffer is used. Additionally, it may be run at bedside using total blood skipping the centrifugation step. BIORAD is designed for NS1 detection in human plasma and serum and PANBIO requires serum samples. Therefore, the latter two tests need a centrifugation step, additional material and reagents, which is time and money consuming.

In this study our aim was to perform a comprehensive evaluation of methods for dengue diagnosis in consecutive patients during an epidemic period. In conclusion, a real-time PCR was optimized for clinical use, showing good diagnostic performance. No advantage of real-time PCR over rapid NS1 detection was evident. Tests for NS1 have diagnostic performance over 80% in real life detecting disease (performances of 2 tests were higher than 90%). NS1 should be used in a real life epidemic setting.

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Competing interests

None declared.

Ethical approval

The work was approved by the Research Ethics Committees of Federal University of Minas Gerais (UFMG) and Municipal Health Department of Belo Horizonte, Minas Gerais, Brazil. Subjects gave informed consent to participate.

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