

Evaluation of a Commercial Real-Time PCR Kit for Detection of Dengue Virus in Samples Collected during an Outbreak in Goiânia, Central Brazil, in 2005[∇]

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In the past 2 decades, dengue has reemerged in Brazil as a significant public health problem. Clinicians demand a diagnostic test with high sensitivity that is applicable during the early symptomatic phase. We aimed to test two distinct molecular methods on samples from suspected dengue cases during an outbreak in Central Brazil. Acute-phase serum specimens from 254 patients suspected of having dengue were collected during 2005 in the city of Goiânia, Central Brazil. Samples were blindly evaluated by real-time and multiplex PCR in addition to routine immunoglobulin M serology and virus culture. Overall, acute dengue was confirmed by serology, multiplex PCR, or virus isolation for 80% of patients (203/254). Another four patients presented real-time PCR-positive results as the unique marker of dengue. Higher real-time PCR positivity levels and viral loads were observed in the early symptomatic phase of disease (≤ 5 days) than after this period. Multiplex and real-time PCR assays presented a high kappa agreement (0.85). According to multiplex PCR, 60 samples harbored dengue virus type 3 (DEN-3), 4 samples harbored DEN-2, and 1 sample displayed a pattern compatible with a double infection with DEN-2 and -3. The dengue virus real-time kit was found to be practical and adjustable for high throughput, to display the best performance in the early symptomatic phase of dengue cases, and to be valuable for confirming dengue diagnosis in a timely manner.

Dengue reemerged in Brazil in the 1990s as a significant public health problem, with thousands of cases, the introduction of new serotypes (dengue virus type 2 [DEN-2] and DEN-3), and hemorrhagic cases, including casualties (7, 8, 10).

The confirmatory diagnosis of dengue is routinely performed by an immunoglobulin M (IgM) test on samples collected 1 week after the onset of symptoms. This is an “a posteriori” analysis, of limited use for prompt diagnosis of the patient during the early symptomatic phase. In theory, molecular tests are able to fulfill this purpose during the window period, by directly assessing the presence of viral RNA in plasma/serum samples from subjects with suspected dengue. Actually, reverse transcriptase PCR (RT-PCR) was found extremely practical in the recent outbreak of severe acute respiratory syndrome, when medical staff could quickly avoid the adoption of quarantine measures for dengue RNA-positive patients (1).

Several RT-PCR methods for dengue RNA detection, including both conventional and real-time PCR, have been described in the literature (11). “In-house” methods usually show excellent performance but are hard to transfer to other laboratories successfully. This variability in results derives from the

differences in suppliers, thermocyclers, and technician skills among laboratories. Commercial kits represent an alternative that can guarantee a certain homogeneity in results, which is important in establishing testing procedures to be adopted by many centers. Also, it is impossible for in-house assays to achieve the level of quality control of commercial kits when good manufacturing practices are rigorously observed.

We aimed to compare a commercial dengue virus real-time kit with an “in-house” multiplex test for a series of suspected dengue cases, recruited in a large urban area in Central Brazil, with cocirculation of DEN-1, DEN-2, and DEN-3 (2), in order to evaluate the performance of molecular assays in dengue diagnosis.

MATERIALS AND METHODS

Patients and samples. Patients presenting to a reference hospital and public health clinics in Goiânia (population, ~1,200,000), Central Brazil, with symptoms compatible with dengue according to WHO guidelines (14) were enrolled from January to June 2005. Two hundred fifty-four patients, (mean age, 31 years [standard deviation, ± 15 years]; 57.4% females) were clinically evaluated. Patients had blood collected during the acute and convalescent phases for laboratory diagnosis. Virus isolation was performed on blood samples (<7 days from the onset of symptoms), and serological tests were performed on paired serum samples collected at the onset of symptoms (acute phase) and 2 weeks after the initial symptoms (convalescent phase) using an in-house dengue IgM antibody capture enzyme-linked immunosorbent assay (ELISA) (6) at the local public reference laboratory (LACEN-GO, Brazil). Plasma was separated immediately after blood drawing and was kept frozen at -70°C until analysis. Samples from suspected dengue patients, collected during the symptomatic phase, were coded and sent to the virology laboratory, Instituto de Medicina Tropical da Univer-

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sidade de São Paulo, São Paulo, Brazil, for molecular analysis. Both laboratories were blind to laboratory and epidemiological data for the patients enrolled.

Virus isolation. Virus was isolated by using a monolayer of *Aedes albopictus* C6/36 cells (5). Dengue virus isolates were identified by an indirect fluorescent antibody test using serotype-specific monoclonal antibodies (3).

RNA extraction. RNA was extracted in duplicate from 140 μ l of plasma by employing a viral RNA kit (QIAGEN, Germany). Elution was performed in 60 μ l according to the manufacturer's instructions.

cDNA synthesis and multiplex RT-PCR. cDNA was synthesized from 22 μ l of RNA, extracted as described above, plus 2.5 μ M random hexamers (N_6 ; Amer-sham, Brazil), 1 mM dithiothreitol, 1 U/ μ l RNase inhibitor (Invitrogen, Brazil), and 2.5 U of Moloney murine leukemia virus RT (Invitrogen, Brazil). This mixture was incubated for 5 min at 65°C and then for 30 min at 37°C, and RT was inactivated by a final incubation of 5 min at 95°C. PCR was performed as described elsewhere (4). Briefly, 5 μ l of cDNA was added to 20 μ l of a PCR mixture consisting of primers D1, TS1, and TS2 at 0.5 μ M, primers TS3 and TS4 at 0.125 μ M, 3 mM $MgCl_2$, 1 \times PCR buffer, 200 μ M deoxynucleoside triphosphates, and 1.25 U of Platinum *Taq* polymerase (Invitrogen, São Paulo, Brazil). The thermocycler (Mastercycler gradient; Eppendorf, Hamburg, Germany) profile was as follows: 94°C for 2 min; 40 cycles of 94°C for 45 s, 55°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 7 min. Fragments of different lengths are obtained from dengue virus serotypes, as follows: 482 bp for DEN-1, 119 bp for DEN-2, 290 bp for DEN-3, and 389 bp for DEN-4. Total RNAs extracted from C6/36 cell cultures infected with the respective dengue virus serotypes were used as controls. PCR products were run on 2% agarose gels, stained with ethidium bromide, and documented on a UV apparatus.

Real-time PCR. Duplicates (10 μ l each) of RNA eluate were directly applied to a commercial dengue virus real-time kit (RealArt; *artus*/QIAGEN, Germany) in a final volume of 25 μ l and were run on ABI 7300 real-time equipment (Applied Biosystems, Brazil). This method consists of a one-step PCR capable of detecting and quantifying all four serotypes in a single TaqMan-like assay using 6-carboxyfluorescein as the reporter fluorophore. An internal control is added to the samples before extraction. This molecule serves as a molecular target for amplification in a parallel assay with a distinct fluorophore (VIC). This allows for the identification of extraction failures and/or PCR inhibition. The dengue virus load is obtained by plotting the sample cycle threshold (C_T) on a curve generated by external quantified dengue virus RNA standards, also provided in the kit. This curve is generated in every run. The mean value for samples analyzed in duplicate was adopted.

Statistical analysis. Data were analyzed using SPSS, version 13.0 (SPSS Inc., Chicago, IL). Exploratory data analysis with box plot graphs was applied to evaluate viral loads stratified by the day of onset of symptoms. A Mann-Whitney U test was performed to compare the median viral loads by day of symptom onset, and a P value of ≤ 0.05 was considered statistically significant. The concordance of the positive and negative results between molecular tests was calculated using the kappa index. All patients or legal guardians gave informed consent, and the study received the approval of the Regional Ethical Committee.

RESULTS

A laboratory diagnosis of dengue virus infection was established by either a positive IgM capture ELISA result for the first and/or second sample, virus isolation, or multiplex PCR amplification. According to these criteria, 203 of 254 cases (80%) were found positive. Overall, real-time PCR gave positive results for 79 patients (31.1%); 57.8% (37/64) of those whose samples were obtained within 3 days of disease onset were found positive, and that proportion decreased to 39.5% (32/81) for samples obtained at 4 to 5 days and to 9.2% (10/109) for those obtained after 6 days. The sensitivity of real-time PCR was 37% (75/203) and its specificity was 92% (47/51) when the four samples presenting real-time PCR results as the only laboratory evidence of dengue virus were considered "false positives." Those four samples were collected from 1 to 4 days after disease onset. The performance of the four dengue diagnostic techniques on acute-phase samples, stratified by the day of onset of symptoms, is shown in Fig. 1. Multiplex PCR was positive for 65 patients (25.6%), with 60 samples harboring

DEN-3, 4 samples harboring DEN-2, and 1 sample showing a pattern compatible with a double infection with DEN-2 and DEN-3. Sixty-three samples were reactive and 173 were non-reactive by both PCR assays, with a kappa agreement of 0.85 (Table 1). Virus culture detected DEN-3 in 43 samples and DEN-1 in 1 sample. One hundred sixty samples were negative, 2 were not evaluated due to cell culture contamination, and 48 were not tested because specimens had been collected after 6 days of symptom onset, giving a positivity percentage of 21.5% (44/204). A clear trend for higher virus recovery rates in the early days of symptoms was observed, as shown in Fig. 1. The combination of IgM serology and real-time PCR accounted for at least 90% of the positive results among the 203 confirmed cases (all positive tests) regardless of the day of symptom onset (Fig. 2). Four samples, including a DEN-1 isolate, displayed virus isolation as the only laboratory marker. When real-time PCR and virus isolation were compared, a concordance of 0.80 was found (164/204), with 37 samples positive and 127 samples negative by both methods. Seven samples were culture positive but real-time PCR negative, and 33 exhibited the opposite pattern, i.e., real-time PCR positive but virus culture negative.

Viral loads ranged from 23 to 955,714,286 copies/ml, with a decreasing viremia trend for samples drawn after the fifth day of disease onset (Fig. 3). Median viral loads were 5,854,075 copies/ml on the first day of symptoms, 440,142 copies/ml on days 2 and 3, and 1,929 copies/ml from day 6 on. Median viral loads in samples from the first 5 days of onset of symptoms differed significantly from those in samples obtained after 5 days ($P < 0.05$ by the Mann-Whitney U test).

DISCUSSION

The dengue virus real-time kit was found to be practical and adjustable for high throughput. The "in-house" multiplex PCR presented a slightly lower analytic sensitivity but proved to be a valuable tool for epidemiological studies, allowing characterization of circulating dengue virus serotypes. The real-time kit displayed some analytical features rated as desirable in the routine laboratory diagnosis. One of these was its reproducibility, as measured by the standard deviation between replicates in the same run, which was always lower than 1 C_T . A second desirable feature was the absence, in this series, of "gray-zone" C_T s, allowing us to confidently identify a sample as "positive" even for a high C_T and a low viral load. The presence of an internal control also avoids false-negative results, and we did not observe a single case of inhibition of its signal, except in samples with very high viral loads, where this is expected to occur. The multiplex PCR has the advantage of serotyping, which may be relevant for patient management and for public health as well. It is considerably cheaper than the real-time kit, since it is performed with homemade reagents. An important drawback of the multiplex PCR was the need to repeat several tests due to inconclusive bands of low intensity. In this instance we decided to repeat the assay in a monoplex format, i.e., containing primers only for the suspected dengue virus serotype (band size). All repetitions showed that the first results were indeed false positives. The serotypes identified are consistent with the laboratory surveillance of dengue virus in the same city, where the majority of dengue cases in recent years were attributable to DEN-3 (2). This dominance of

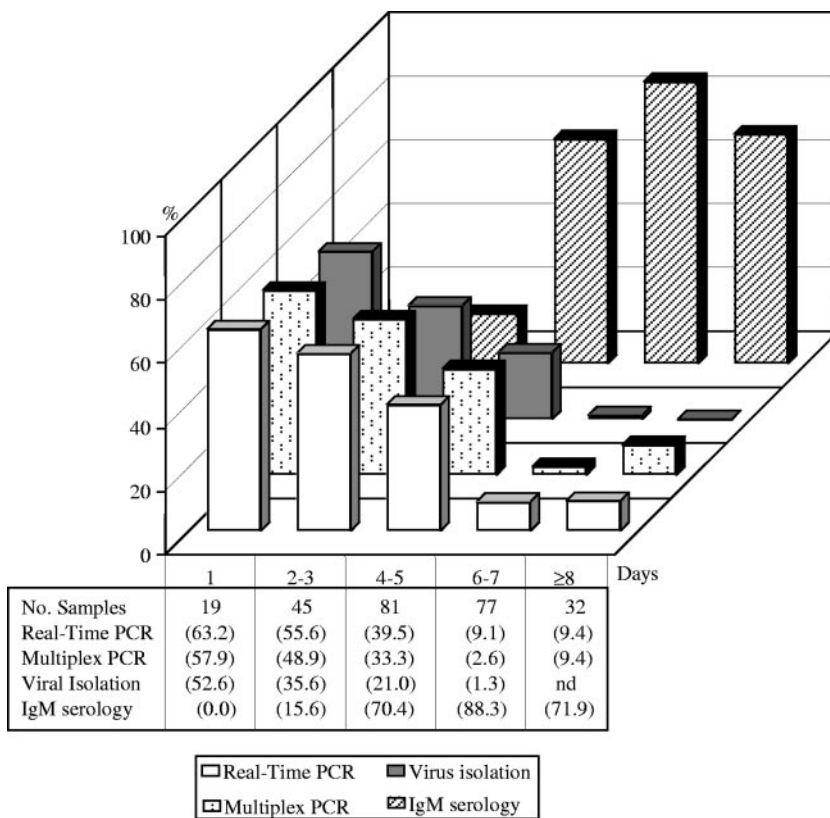


FIG. 1. Comparison of the performance of four diagnostic techniques (real-time PCR, multiplex PCR, viral isolation, and IgM serology) for suspected dengue cases according to day of onset of symptoms. Data are percentages of samples found positive by each method. nd, not done.

DEN-3 was preceded in time by DEN-1 outbreaks and occasional DEN-2 cases, so it is reasonable that a few cases of these two serotypes are still detected.

Because we performed both molecular assays in parallel, and beginning from the same RNA extract, the performance of the two could be accurately compared, leading to our conclusion of the slightly higher analytical sensitivity of the real-time kit, since samples containing fewer than 10,000 copies/ml of dengue virus RNA were not consistently reactive in the multiplex assay. Of note, four samples were reactive exclusively by real-time PCR. They presented viral loads of 228, 857, 994, and 2,318 copies/ml, respectively. All of them were collected during the first 4 days of symptoms, so it is reasonable to conclude that they represent cases of low-level viremia at the collection date,

below the sensitivity of our multiplex PCR assay, and that the patients had not developed IgM yet. We could not obtain follow-up samples, which would probably have revealed positive IgM results, in these cases.

Our results demonstrate the diagnostic value of molecular assays for dengue virus, provided that samples are drawn in the early days of symptoms, between days 1 and 3. In this period, the value of IgM detection in addition to PCR detection is low. After this critical phase, IgM is more useful than PCR, a situation attributable to the short course of viremia and the lag between viremia and the development of acute-phase antibodies (IgM).

A similar study was carried in Brazil by Poersch and coworkers (9), comparing nested PCR and qualitative real-time PCR for the diagnosis of dengue in the acute phase. In agreement with our results, they reported a superior sensitivity for real-time PCR, advocating its use by the health system in combination with IgM for late samples.

The dengue virus real-time kit showed high performance in the early symptomatic phase of dengue cases and is valuable for confirming dengue diagnosis in a timely manner. It needs to be established whether viral load could be a determinant of disease outcome (12, 13).

The goal of our work is to offer a fast (6-h), reliable, and affordable test for dengue in order to provide support for clinical decisions, improving the quality of the management of confirmed cases and avoiding investigative procedures for neg-

TABLE 1. Comparison of multiplex and real-time PCR results for 254 patients with suspected acute dengue

Result by Mx-PCR ^a	No. of samples with the following result by real-time PCR ^b :		Total
	Pos	Neg	
Pos	63	2	65
Neg	16	173	189
Total	79	175	254

^a Mx-PCR, multiplex PCR; Pos, positive; Neg, negative.

^b Kappa agreement with multiplex PCR results, 0.85.

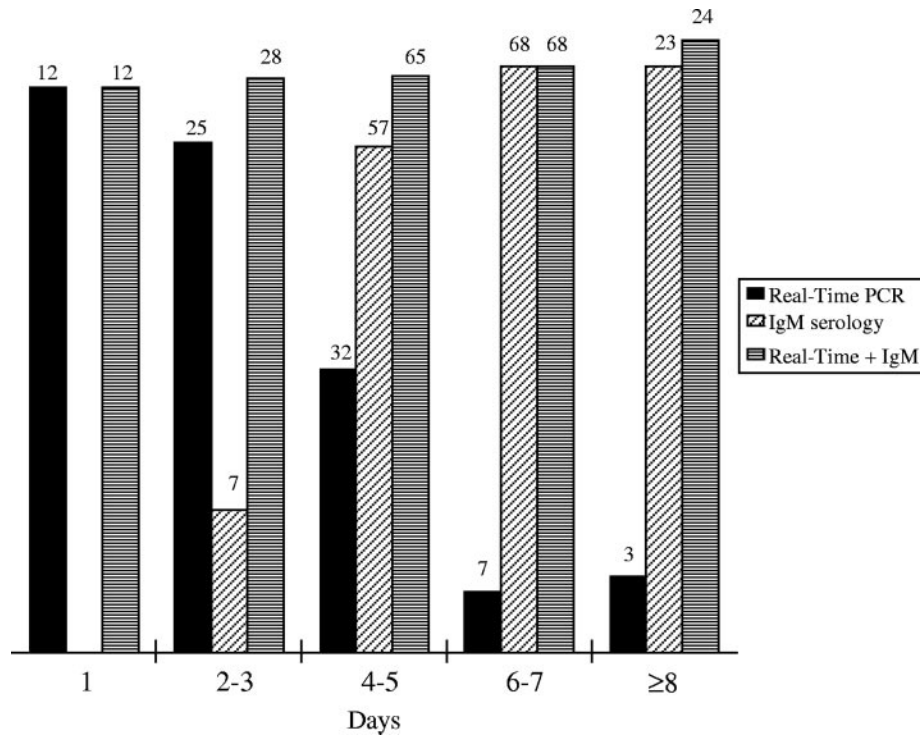


FIG. 2. Comparison of the performance characteristics of IgM serology, real-time PCR, and IgM serology combined with real-time PCR for a selected subset of 207 patients with confirmed dengue diagnoses (note that for four samples, positive results were obtained only by virus isolation [see Results]). Absolute numbers of positive results are given on top of each bar.

ative cases. Overall, this may represent a significant economy of resources for the health system. In this work, samples were processed a posteriori, i.e., they were collected between January and June 2005 and actually analyzed 6 months later, except for the IgM test, which, according to the protocol currently in

use in our country, is performed 1 week after fever onset. Although the costs of molecular diagnostic tests are usually higher than those of serological tests, the processing of batches of samples allows the achievement of an economy of scale. We foresee the use of real-time PCR in a few specialized centers, strategically located and receiving samples from surrounding cities. If the system is properly planned, the number of samples will not be the limiting factor, as is the case today for IgM serology, for which the samples in queue exceed the installed laboratory capacity, leading to delayed diagnoses.

The level of laboratory sophistication required is the same as that which we already have in Brazil for viral load and resistance testing for human immunodeficiency virus, so the system may benefit from the equipment and human resources now in place.

Due to the cost, the commercial kit presented here may not be affordable for the Brazilian health system. Developing or adapting the “in-house” real-time PCR tests described in the literature is a possibility we are pursuing now. The key step that demands further development is RNA extraction. Manual extraction, as adopted in this work, is time-consuming, expensive, and prone to contamination. We are seeking automated or semiautomated methods in a microplate format, allowing simultaneous extraction from as many as 96 samples, so that the process, from serum/plasma specimens to RNA, is completed in less than 1 h. If extraction is coupled with a fast one-step real-time PCR protocol of about 2 h, we can foresee a daily routine of 96 tests with a turnaround time of 4 h, which can cope with the demand of populated regions experiencing dengue outbreaks from time to time.

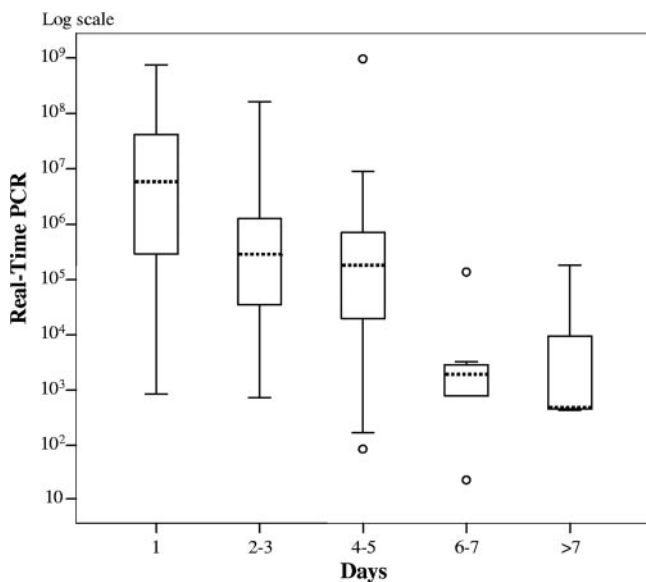


FIG. 3. Box plot showing viral loads of samples positive by real-time PCR according to day of onset of dengue symptoms. Dotted lines, medians; open circles, outliers. Note that values are given on a log scale.

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