One-Step RT-PCR protocols improve the rate of dengue diagnosis compared to Two-Step RT-PCR approaches

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Received in revised form 24 October 2003; accepted 12 November 2003

Abstract

Dengue is the most important arboviral disease transmitted to humans. In our laboratory, we have been working on the standardization of the polymerase chain reaction (PCR) diagnosis of this disease. In this work, we compared five commercial kits regularly used on reverse-transcription polymerase chain reaction (RT-PCR) protocols: two Two-Step kits (SuperScript II RT/Super Mix kit and reverse transcription system/Taq DNA polymerase) and three One-Step kits (ready-to-go RT-PCR Beads kit, QIAGEN One-Step RT-PCR kit, and AcessQuick RT-PCR system). Thirty-one serum samples of patients with clinical diagnosis of dengue fever (DF) were analyzed by RT-PCR and serology. RNA extraction was done with the QIAamp Viral RNA kit, and cDNA synthesis and PCR done according to the manufacturer’s protocol for the five kits. Out of the 31 serum samples collected from patients suspected of having dengue, 27 were IgM-positive, confirming the dengue diagnosis. Out of those, 24 were positive by the ready-to-go RT-PCR Beads kit, 25 were positive by AcessQuick RT-PCR system and 27 were positive by QIAGEN One-Step RT-PCR kit. On the other hand, only six samples were positive by the SuperScript II RT/Super Mix kits and 10 were positive by reverse transcription system/Taq DNA polymerase kit. The best performance observed with the One-Step kits was confirmed in spiked samples with known quantities of dengue-1 virus since they detected up $1 \times 10^2$ PFU/ml, while the most sensitive Two-Step kit detected up $1 \times 10^4$ PFU/ml. These data show that One-Step RT-PCR kits yielded a higher rate of dengue virus detection than the Two-Step kits and correlated well with the serological diagnosis.

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Keywords: Dengue; Diagnosis; RT-PCR; One-Step kits; Two-Step kits

1. Introduction

Dengue fever (DF) and dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) are caused by any of the four serotypes of dengue virus (serotypes 1–4), and are transmitted between humans primarily by the Aedes aegypti and Aedes albopictus mosquitoes (Henchal and Putnak, 1990). Their worldwide distribution and incidence rates in the Americas have substantially increased from the 1980s to this day (Anonymous, 2002). In Brazil, the incidence rate of dengue has been rising every year and currently dengue has been detected in the whole country. From the beginning of the epidemic to the year 2000, DEN-1 and DEN-2 viruses had been responsible for all cases of dengue in Brazil but since the introduction of DEN-3 in December 2000 (Miagostovich et al., 2002), this serotype has been detected in several states of the country.

According to World Health Organization, the public health measures aimed to control dengue should include the development and standardization of a precise and rapid diagnostic test (Anonymous, 1998; Gubler, 1998). PCR may assume an important role on the rapid diagnosis of dengue infections but, since all reverse-transcription polymerase chain reaction (RT-PCR) protocols are standardized locally, all of the steps involved in this test must be well...
standardized to be used on clinical specimens. Two important steps on this diagnostic procedure are the cDNA synthesis and the PCR protocol itself. There are commercial kits that may be used in both occasions and they may offer some advantages over "in-house" protocols.

Due to lack of studies that indicate which is the best kit for RT-PCR diagnosis of dengue, we compared five RT-PCR kits (two Two-Step kits and three One-Step kits) regularly used on these experiments. Our data show that the One-Step RT-PCR approaches were much more efficient than the Two-Step ones to detect dengue-1 virus genome.

2. Materials and methods

2.1. Clinical samples

Samples of patients with clinical diagnosis of acute dengue fever attended on the Health Care Centers of the Borborema City, São Paulo State, Brazil, were sent to the Laboratory of Molecular Virology, School of Medicine of Ribeirão Preto, University of São Paulo, within 3 days after the onset of illness for PCR evaluation.

2.2. Virus strain

Dengue-1 virus, Nauru Island strain, kindly provided by Dr. Robert E. Shope, (University of Texas, Galveston, TX) was used as a positive control in this study. The virus was grown in C6/36 cells and the infection detected by IFA (Tesh, 1979). The infected tissue culture fluids were then used to produce RT-PCR positive controls.

2.3. Serology

All of the samples were tested by MAC-ELISA according to the manufacturer’s instructions (MRL® Diagnostics, CA) and described elsewhere (De Paula et al., 2002a).

2.4. RNA extraction

RNA extraction was done with the QIAamp Viral RNA kit, according to the manufacturer’s instructions, PCR amplification and cDNA synthesis and PCR done according to the protocols, 1 μl of the 5× reaction buffer and 31 μl of distilled H2O. PCR was done in a 50 μl final volume containing either 220 ng or 1 μg of cDNA, 0.1 mM dNTPs, 1 U of Taq DNA polymerase, 10 μl of 10× Taq buffer, and 50 pmol of the primer pair ProtCDen-1as (5′ AA-GACGG(GC)TCGACCGTCTTTCA 3′) and ProtCDen-1as (5′ CTGGTGGGAGGGTATGGCTAGAAA 3′), which anneal to a conserved region located on the structural protein C and it is specific for DEN-1. This protocol was also done on all of the samples with an increased cDNA concentration (≈1 μg). We used only the DEN-1 specific pair of primers since we knew first hand that the circulating virus in Borborema city was the DEN-1 serotype. RT-PCR and amplicon detection were performed as previously described (De Paula et al., 2002b).

2.5. Two-Step kits

The first cDNA strand was synthesized in a mix containing 3 μl (~220 ng) of cDNA, 0.1 mM of dNTPs, 1 U of Taq DNA polymerase, 10 μl of 10× Taq buffer, and 50 pmol of the primer pair ProtCDen-1as. Detection of the amplicon was carried out as previously described (De Paula et al., 2002b).

2.5.2. Reverse transcription system/Taq DNA polymerase (Promega)

On this protocol, the first-strand cDNAs were synthesized at 37 °C for 1 h using 200 units of M-MLV reverse transcriptase, 1 μg of total RNA, 0.1 mM dNTP, 25 pmol of pHV primers (Amersham-Pharmacia), 10 μl of 5× of M-MLV reaction buffer and 31 μl of distilled H2O. PCR was done in a 50 μl final volume containing either 220 ng or 1 μg of cDNA, 0.1 mM of dNTPs, 1 U of Taq DNA polymerase, 5 μl of 10× buffer B, and 50 pmol of the primer pair ProtCDen-1as and ProtCDen-1as. Detection of the amplicon was carried out as previously described (De Paula et al., 2002b).

2.6. One-Step kits

Dengue RNA was reverse-transcribed into cDNA and a portion of the protein C region of DEN-1 genome was amplified by using the One-Step RT-PCR method in which reverse transcription and DNA amplification take place in an one-tube assay using a 50 μl assay we tested three different RT-PCR kits: the ready-to-go RT-PCR Beads kit (Amersham Biosciences, São Paulo, Brazil), the AccessQuick RT-PCR system (Promega, Madison, WI), and the QIAGEN One-Step RT-PCR kit (QIAGEN). In all three protocols, 1 μg of total RNA was used and the reaction included the random primers pHV, and ProtCDen-1as, ProtCDen-1as primers, at the concentrations described before. The AccessQuick RT-PCR system (Promega), which allows variable Mg2+ concentrations, was optimized in the concentration of 1 mM Mg2+. In all cases, after reverse transcription according to the manufacturers’ instructions, PCR amplification of dengue-1 genome was described before.

2.7. RT-PCR detection of dengue-1 virus on spiked samples

Human sera, negative for dengue antibodies, were spiked with dengue-1 virus, Nauru Island strain, at concentration ranging from 103 to 106 PFU/ml of dengue-1 per 140 μl. RNA extraction was done with the QIAamp Viral RNA kit, and cDNA synthesis and PCR done according to the protocols described before.
2.8. Data analysis

Analysis of variance (ANOVA), t-tests and the Bonferroni’s multiple comparison test were used to determine whether the differences among the five protocols were statistically significant. Statistical analysis were performed by using Graphpad Prism for Windows, version 3.0 (Graphpad Software Inc., San Diego, California).

3. Results

3.1. Clinical samples

Samples from 31 patients presenting with dengue symptoms that included thrombocytopenia and positive torniquete test were sent to our laboratory to investigate the etiology of these infections. Out of these samples, 27 were positive for dengue IgM antibodies on a second sample collected after 6 days of disease onset.

3.2. One-Step kits × Two-Steps kits

Out of the 27 IgM-positive serum samples, 24 were positive by the ready-to-go RT-PCR Beads kit, 25 were positive by AcessQuick RT-PCR system, and 27 were positive by QIAGEN One-Step RT-PCR kit. On the other hand, only six samples were positive by the SuperScript II RT/Super Mix kit, and 10 were positive by reverse transcription system/Taq DNA polymerase kit. Four samples were not positive by any kit but these samples were also IgM-negative (Table 1) for dengue viruses. No etiologic diagnosis was reached for these samples. There was a statistically significant difference among the One-Step and Two-Step kits (P < 0.05) while no statistically significant difference was detected among the three One-Step kits (P > 0.05). The same was true for the two Two-Step kits (Table 1).

RT-PCR was repeated using both Two-Step kits with a five-fold higher concentration of total cDNA than that initially used, since this was the only difference in terms of reagent concentrations among the One-Step and Two-Step kits used in this work. However, the results obtained in both experiments were exactly the same as the ones obtained with a smaller cDNA concentration ruling out that the better performance of One-Step protocols was related to the cDNA concentration. We can also rule out problems with the specificity of the primers since we have tested these primers in other occasions, and in our hands, they have performed as well as others described on the literature (data not shown).

3.3. RT-PCR detection of dengue-1 virus in spiked samples

As observed in Fig. 1, the One-Step kits detected smaller concentrations of dengue-1 virus than Two-Step kits. The QIAGEN One-Step RT-PCR kit detected up 1 × 10^4 PFU/ml, while the most sensitive Two-Step kit (reverse transcription system/Taq DNA polymerase) detected up 1 × 10^5 PFU/ml. The ability to detect smaller concentrations of dengue-1 virus might explain why the QIAGEN One-Step RT-PCR kit had the best performance among the kits tested on our experiments.

4. Discussion

Dengue virus infections often result in an acute, self-limited, viral disease but they may also induce a severe form of the disease characterized by an increase on the vascular permeability that may lead to a fatal outcome (Halstead, 1998). Adding to these findings, the worldwide distribution makes dengue the most important human arboviral disease in the world (Gubler, 1989).

RT-PCR has been developed for the diagnosis of several diseases, including dengue. The method is rapid, sensitive, and if well standardized, it can be used for dengue genome detection in human clinical samples, biopsies, autopsy tissues or mosquitoes (Deubel, 1997; Guzman and Kouri, 1996).

Due to the fact the WHO still considers RT-PCR for dengue diagnosis an experimental procedure that requires better standardization and field studies to validate it (Anonymous, 1998), we conducted a validation study of PCR-based dengue diagnosis in clinical samples collected in Brazil. RT-PCR was more sensitive than virus isolation allowing for a rapid detection and identification of dengue infections, proving that it can be used on the routine diagnosis of dengue (De Paula et al., 2002a).

Since we have been working on the standardization of RT-PCR for dengue diagnosis, from sample collection to amplicon detection, we decided to investigate which commercial kit was associated to the best performance for PCR amplification of dengue-1 genome. We compared five commercial kits that could be used on PCR protocols for dengue

Table 1

<table>
<thead>
<tr>
<th>Laboratories samples</th>
<th>MAC-ELISA</th>
<th>Two-Step P &gt; 0.05 (P = 0.0506)</th>
<th>One-Step P &gt; 0.05 (P = 0.7077)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SuperScript II RT/Super Mix kit</td>
<td>Reverse transcription system/Taq DNA polymerase</td>
<td>QIAGEN One-Step RT-PCR kit</td>
</tr>
<tr>
<td></td>
<td>Ready-to-go RT-PCR beads kit</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAC-ELISA</td>
<td>31</td>
<td>6/27 (22%)</td>
<td>24/27 (88%)</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>10/27 (37%)</td>
<td>27/27 (100%)</td>
</tr>
</tbody>
</table>

P < 0.05 (P = 0.0027).
Fig. 1. A 2% agarose gel electrophoresis showing the amplicons obtained after RT-PCR amplification of dengue-1 spiked samples following RNA extraction by One-Step and Two-Step kits. M: 100-bp DNA ladder; lane C: negative control (uninfected C6/36 cells); lanes 10^6–10^1 serially diluted samples containing several dilutions of dengue-1 virus. (A) Qiagen One-Step RT-PCR kit; (B) AcessQuick RT-PCR system; (C) ready-to-go RT-PCR beads kit; (D) reverse transcription system/Taq DNA polymerase; (E) SuperScript II RT/Superscript kit.

diagnosis. Out of 31 samples, only four samples were not positive by any kit but they were also shown to belong to patients with other febrile illnesses, since their samples were IgM-negative for dengue virus when tested on samples collected on the convalescent period. The One-Step kits, where reverse transcription and PCR are done on the same tube, were much more efficient to detect dengue-1 virus than the Two-Step kits, where both steps are done in separate. One logical explanation for these results could be the higher cDNA concentration contained on One-Step kits. However, we tested the Two-Step kits with a higher concentration of dengue-1 cDNA and obtained exactly the same results. Thus, due to the fact that there was no difference between the protocols with different concentrations of cDNA, the hypothesis that the better performance of the One-Step kits was related to DNA concentrations can be ruled out. Also, when normal human sera were spiked with dengue-1 dilutions ranging from 1 × 10^0 PFU/ml to 1 × 10^6 PFU/ml, the One-Step kits also showed a much better performance than the Two-Step kits.

The best performance observed with the One-Step kits may be due to the use of more effective thermostable enzymes than those used in the Two-Step kits. The QIAGEN One-Step RT-PCR kit had a better performance among all kits tested probably because it has two enzymes that provide highly and specific reverse transcription. Ominiscript reverse transcriptase is specially designed for reverse transcription of RNA concentrations greater than 50 ng, and Sensiscript reverse transcriptase is optimized for use with very small amounts of RNA (<50 ng), and as a DNA polymerase, this kit uses the HotStarTag DNA Polymerase, a thermostable enzyme that is designed for highly efficient
and specific RT-PCR amplifications (Grabensteiner et al., 2001). Even though this kit had a slightly better performance than the others, there was not a statistical difference among the three One-Step kits \((P > 0.05)\). Combining the results from spiking normal human sera with different concentrations of dengue-1 virus and the results of the RT-PCR on patient samples, it is likely that the better performance of the One-Step kits is related to their ability to detect the small viral load presented by some dengue patients. The reason why increasing the cDNA concentration did not improve the detection rate associated with Two-Step kits might be related to the fact that the increase in the total RNA in these samples may not represent an increase in the concentration of dengue-1 RNA. Another consideration to make is that even though a limited number of One-Step kits were used on this study, other One-Step kits might work as well as the ones tested here. Thus, the most important result of this study is that One-Step kits should be preferentially used for PCR amplification of dengue genomes if one wishes to improve the diagnostic rate of this test.

To our knowledge, this is the first study comparing the use of One-Step and Two-Step kits for dengue diagnosis. Our data show that the One-Step kits were more efficient on dengue virus detection than the Two-Step kits, and it was less laborious, faster, and eliminated the step of cDNA synthesis in a different tube, what could lead to a substantial decrease on sample contamination.

Acknowledgements

This work was supported in part by Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP), São Paulo, Brazil (Grants no. 99/09466-3). S.O.P. and C.M.L. were supported by FAPESP scholarships (Grants nos. 00/09287-0, and 02/00394-4, respectively).

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