Improved Detection of Dengue Virus Serotypes from Serum Samples – Evaluation of Single-Tube Multiplex RT-PCR with Cell Culture

Anita Chakravarti\textsuperscript{a}, Rajni Kumaria\textsuperscript{a}, P. Kar\textsuperscript{b}, Vineeta Vijay Batra\textsuperscript{c} and Vikas Verma\textsuperscript{a}

\textsuperscript{a}Department of Microbiology, Maulana Azad Medical College, Associated Lok Nayak Hospital and other Hospitals, Bahadur Shah Zafar Marg, New Delhi-110002, India
\textsuperscript{b}Department of Medicine, Maulana Azad Medical College, Associated Lok Nayak Hospital and other Hospitals, Bahadur Shah Zafar Marg, New Delhi-110002, India
\textsuperscript{c}Department of Pathology, Maulana Azad Medical College, Associated Lok Nayak Hospital and other Hospitals, Bahadur Shah Zafar Marg, New Delhi-110002, India

Abstract

Serological assays commonly used for the diagnosis of dengue are feasible only five or six days after the onset of illness; moreover, they involve antigenic cross-reactivity between four serotypes. However, viraemia present during the acute phase of the illness can be detected by cell culture. Being a complicated and time-consuming procedure, it cannot be used as routine diagnostic assay. In this study, the efficacy of multiplex RT-PCR (mRT-PCR) for serotypic characterization of dengue virus was evaluated using serum samples directly. The sensitivity of the assay was compared with the gold standard method of virus isolation by cell culture with serotypic characterization by indirect immunofluorescent antibody test (IFAT). Serum samples collected within 5–7 days of illness from 240 patients, clinically suspected of having dengue infection, were processed directly by mRT-PCR. An aliquote of each of these samples was simultaneously inoculated in C6/36 cell culture for the propagation of virus for serotypic characterization by IFAT. Serum samples collected within 5–7 days of illness from 240 patients, clinically suspected of having dengue infection, were processed directly by mRT-PCR. An aliquote of each of these samples was simultaneously inoculated in C6/36 cell culture for the propagation of virus for serotypic characterization by IFAT. ELISA/rapid immunochromatography test was performed to find out the presence of anti-dengue IgM antibodies. 44 patients were found to be positive for one of the four serotypes by mRT-PCR and 39 by IFAT on examination of culture fluid and infected cells respectively. However, 76 patients were found positive for dengue virus serotypes by mRT-PCR performed directly on serum samples, thus indicating significantly higher sensitivity of this method. The most predominant serotype detected during this study period was DENV-2, followed by DENV-3. This study proposed mRT-PCR as a method of choice for rapid, cost-effective and sensitive confirmatory diagnosis in the early phase of dengue infection.

Keywords: Dengue fever, dengue haemorrhagic fever, IFAT, multiplex RT-PCR, serum samples.

Introduction

Dengue fever and dengue haemorrhagic fever (DF/DHF) has emerged as the most important arboviral disease of mankind in terms of both morbidity and mortality. Dengue virus belongs to the genus, Flavivirus, and family, Flaviviridae, with four serologically related but antigenically distinctive serotypes (DENV-1, 2, 3, 4). An acute infection of dengue is generally asymptomatic, may sometimes be present with classical dengue fever, a mild illness, or a severe manifestation,
dengue haemorrhagic fever. The significant expansion in urban populations along with increased mobility has greatly contributed to the geographical expansion of the vector, *Aedes aegypti*. Consequently, a dramatic increase in the frequency of dengue has been recorded in the last decade. The severity of the problem is clearly reflected by the changed ratio of DHF to DF from 0.9 in the year 1980 to 20.4 in 2004. Worldwide, approximately 100 million cases of dengue are reported annually, resulting in approximately 500 000 cases of DHF with an estimated 50 000 deaths.

At present, laboratory diagnosis of dengue is mainly dependent on serology using IgM capture enzyme-linked immunosorbant assay (MAC-ELISA). Antigenic cross-reactivity between members of the *Flaviviridae* family reduces the specificity of the assay. Moreover, dengue-specific IgM antibodies (a marker of present infection) are detectable only 6–10 days following onset of the illness. Owing to the availability of freely circulating viable virus particles in the blood for initial five days after onset of the disease, detection of virus is thus most appropriate alternative for early diagnosis.

Virus isolation by cell culture has been merited as “gold standard for diagnosing dengue”. However, serotype characterization by the conventional method of indirect immunofluorescent (IF) staining of infected C6/36 cells (Ae. albopictus origin) requires an incubation period of minimum seven days. In view of the fact that mortality rate as high as 20% is reported among complicated dengue cases, it is immensely important to have a rapid and sensitive laboratory assay for conclusive diagnosis in the acute phase of infection. Complicated and time-consuming cell culture procedure, thus, has a restricted scope as routine diagnostic assay. A number of studies have been conducted to evaluate the potential of multiplex RT-PCR (mRT-PCR) for the detection of DENV-RNA using different regions of genome. The present study conducted at the Maulana Azad Medical College, New Delhi, attempted to determine the efficacy of multiplex RT-PCR to detect DENV serotypes directly from patients’ sera.

**Materials and methods**

**Clinical samples**

A total of 240 patients, having febrile illness consistent with dengue, were enrolled in this study conducted from January 2002 to January 2005 following WHO inclusion criteria. Blood samples were collected within eight days of the onset of the fever, aseptically processed for the separation of serum and immediately transferred to –70 °C deepfreezer till processed further for mRT-PCR and cell culture. All the samples were screened by ELISA (Pan Bio, Australia, or Cal Biotech Inc., CA, USA) / dengue duo rapid strip test (Pan Bio, Windsor, Australia) for the presence of dengue virus-specific IgM antibodies.

**Prototype dengue virus strain**

DENV-4 (NIV-642069) serotype-specific strain, obtained from the National Institute of Virology, Pune, India, was used as positive control in the present study. The virus was propagated into Ae. albopictus C6/36 cells (obtained from the National Centre for Cell Sciences, Pune, India) maintained in Leibovitz’s Medium (L-15) at 28 °C, containing 100 units of penicillin, 75 units of streptomycin, and 2% fetal bovine serum (Sigma Aldrich Co., USA). The cell cultures were processed every day till seven days post-inoculation, infected cells were screened by indirect immunofluorescent antibody test (IFAT) and culture supernatants were screened simultaneously by mRT-PCR for the presence of the virus serotypes.
Improved Detection of Dengue Virus Serotypes from Serum Samples

Virus isolation from clinical samples

200 µl of serum sample from each of the clinically suspected patients was inoculated into confluent monolayer of the C6/36 cell culture and gently rotated every 10 minutes for one hour at 28 °C for even absorption of the virus by cells. After seven days, cells were examined for DENV serotypes by both IFAT and mRT-PCR.[6,12] The negative controls were mock-infected C6/36 cells incubated with phosphate buffered saline (PBS), instead of clinical samples.

Dengue virus serotyping by IFAT

Infected cells were harvested by centrifugation at 1500 × g for 10 minutes at 4 °C and spotted in quadruplet on the Teflon-coated slides. Cells in each well were incubated with monoclonal antibodies (dilution 1:10) against one of the four dengue serotypes in a moist chamber at 37 °C (monoclonal antibodies were kindly provided by the Division of Vector-Borne Viral Diseases, CDC, USA). After 30 minutes, slides were washed thrice with PBS pH 7.5 for 10 minutes each and incubated at 37 °C for 30 minutes with FITC-labelled goat antimouse IgG antibodies (Santa Cruse, USA; dilution of 1:100) in the humid chamber again. Slides were washed with PBS as mentioned earlier, mounted with PBS-buffered glycerol and examined for the presence of fluorescence[6] by Leitz immunofluorescent microscope (400× magnification).

Extraction of dengue virus RNA and serotyping by multiplex RT-PCR

RNA was extracted by the modified method of Chomczynski et al.[13] using trizol reagent (Invitrogen Corp., Carlsbad, California, USA). Briefly, 250 µl of clarified cell culture supernatant of DENV-4 reference strain was mixed with 750 µl of trizol reagent and processed for RNA extraction. The resulting RNA precipitate was washed with 75% ethanol and dissolved in 25 µl diethylpyrocarbonate-treated water. Following the same procedure, DENV RNA was extracted from the serum samples as well as cell culture infected with these samples.

mRT-PCR was performed by method of Harris et al.[12] using 5 µl of extracted RNA as template. Briefly, the master mix contained 5 mmol/L dithiotritol, 1.5 mmol/L MgCl₂, 50 mmol/L KCl, 10 mmol/L Tris (pH 8.5), four dNTPs at a concentration of 200 mmol/L each, 30 pmol each of DENV-1 (Consensus primer) and TS-1, TS-2, TS-3 and DENV-4 primer (serotype specific primers for DENV-1, 2, 3 and 4 respectively), 200 units of MMLV reverse transcriptase (Invitrogen Corp.) and 1 unit Taq DNA polymerase (Invitrogen Corp.) per reaction. All sequences and locations of used primers were described in Harris et al. publication.[12] cDNA was synthesized by reverse transcription at 42 °C for 50 minutes followed by 40 cycles of PCR; each cycle consisting of denaturation at 94 °C for 30 seconds, primer annealing at 55 °C for one minute and primer extension at 72 °C for two minutes with a final extension at 72 °C for 10 minutes. The resulting serotype-specific DNA products were visualized on 1.5% agarose gel as serotype specific DNA band of 482 bp (DENV-1), 119 bp (DENV-2), 290 bp (DENV-3) and 389 bp (DENV-4) using ø X 174/Hae III digest DNA.

Results

Turn-around time for dengue virus serotyping by IFAT and mRT-PCR

To compare the time required for the detection of DENV serotypes by IFAT and mRT-PCR, both the procedures were conducted every day up
Improved Detection of Dengue Virus Serotypes from Serum Samples

to seven days post-inoculation of prototype serotype strain, DENV-4 at TCID $10^{5.5}$. A 389 b.p. DENV-4-specific DNA product could be detected on the second day by mRT-PCR conducted in cell culture supernatant, while fluorescence could be detected in the infected cells by IFAT only after four days, using DENV-4 serotype-specific monoclonal antibodies.

C6/36 cell culture and detection of DENV serotypes

The cell culture was consistently maintained for the cultivation of dengue virus from the serum samples. On processing of culture after seven days, IFAT could identify dengue serotypes in 16.3% (39/240) patients using serotype-specific monoclonal anti-dengue antibodies (Table 1), whereas mRT-PCR could detect DENV serotypes in 18.3% (44/240) cases (Table 1, Figure) (which included 39 samples found positive by IFAT). Out of the five samples found positive only by mRT-PCR, four cases belonged to serotype DENV-3 and one case was positive for DENV-4. The sensitivity of IFAT was found to be 88.6% in comparison to mRT-PCR in cell culture (Table 2). Standard serotype DENV-4, used as positive control, could be clearly detected while negative control showed no fluorescence, which ruled out the presence of any cross-contamination and also proved the validity and specificity of the procedure.

Detection of dengue virus serotypes in patients’ sera by mRT-PCR

Multiplex RT-PCR was able to detect the serotypes in a significantly higher number (31.6%; 76/240) of suspected cases when dengue virus RNA was isolated directly from the patients’ serum samples, and 32 patients were found to be positive for one of the four DENV serotypes

<table>
<thead>
<tr>
<th>Method of dengue virus detection</th>
<th>Type of sample</th>
<th>Time required for detection</th>
<th>Positivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFAT</td>
<td>C6/36 cells infected with serum</td>
<td>7–10 days</td>
<td>39/240 (16.3%)</td>
</tr>
<tr>
<td>mRT-PCR</td>
<td>Supernatant of C6/36 cell culture infected with serum</td>
<td>7–10 days</td>
<td>44/240 (18.3%)</td>
</tr>
<tr>
<td>mRT-PCR</td>
<td>Serum from clinically suspected patients</td>
<td>~6 hours</td>
<td>76/240 (31.6%)</td>
</tr>
</tbody>
</table>

Table 1: Comparison of mRT-PCR and IFAT for detection of dengue viruses

This table depicts the comparison of three methods of dengue virus serotype detection within 240 samples by direct mRT-PCR, mRT-PCR conducted on cell culture supernatant and IFAT conducted on C6/36 infected cells. Same samples were evaluated in a head-to-head manner.

Figure: Characterization of DENV serotypes on 1.5% agarose gel by the one tube multiplex RT-PCR

Lanes 1-4 showing: DENV-2, DENV-3, DENV-1 and DENV-4 respectively, from patients serum samples.
Lane 5: DENV-4 (the dengue standard serotypes) isolated from cell culture using C6/36 cell line.
Lane 6: ø X 174/Hae III digest DNA marker.
Lane 7: Negative control having PCR grade water in place of RNA template.
Improved Detection of Dengue Virus Serotypes from Serum Samples

**Table 2: Comparison of three dengue virus serotype detection methods:**
(i) direct mRT-PCR,
(ii) RT-PCR conducted on cell culture supernatant and
(iii) IFAT conducted on C6/36 infected cells

<table>
<thead>
<tr>
<th>Dengue virus serotypes</th>
<th>Serotypic distribution of patients infected with dengue virus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mRT-PCR in sera (n=76)</td>
</tr>
<tr>
<td>DENV-1</td>
<td>08</td>
</tr>
<tr>
<td>DENV-2</td>
<td>33</td>
</tr>
<tr>
<td>DENV-3</td>
<td>24</td>
</tr>
<tr>
<td>DENV-4</td>
<td>11</td>
</tr>
<tr>
<td>Total</td>
<td>76</td>
</tr>
</tbody>
</table>

n=Number of patients found infected with any one of the four dengue virus serotypes

exclusively by direct mRT-PCR only (Table 1). The sensitivity of both IFAT as well as mRT-PCR in cell culture was thus found to be comparatively much lower (sensitivity IFAT=51.3%; mRT-PCR in cell culture=57.9%) than of direct mRT-PCR. There was 100% agreement in the results obtained by all the three protocols. As the whole process of extraction of RNA followed by serotype identification could be completed in six hours only, it could be defined as a superior diagnostic approach, with a combination of rapidity and sensitivity (Table 2).

**Detection of dengue virus-specific antibodies**

Acute-phase sera of only two cases (samples collected after 5th day of illness) were found to be positive for dengue serotype-specific IgM antibodies. Both these cases were found positive by direct mRT-PCR only and none by cell culture. The serotypic characterization revealed one case to be positive for DENV-3, while the second case belonged to DENV-4 serotype.

**Duration of fever and detection of dengue virus serotypes**

Out of the 76 mRT-PCR-positive acute-phase sera, 10 cases (4.2%) were detected to be positive from the samples collected within 6–8 days of the onset of illness. However, none of these samples yielded any positive results by either IFAT or mRT-PCR conducted on cell culture (Table 3).

**Discussion**

Air travel has broadened the geographical areas infected with dengue virus and dengue cases are now reported not only from tropical countries but also from areas previously considered as non-endemic.\cite{14} India has
become endemic for all the four known DENV serotypes, resulting in increased susceptibility of the population to DHF.\cite{10,11} Serology, the common practice in laboratory diagnosis, has a limited role in early detection or serotype characterization. As the initial symptoms of dengue mimic those of malaria, typhoid and leptospirosis, which are endemic in the country, availability of a rapid and differential diagnosis at an early stage of infection is of utmost importance for better patient management.

With the aim of establishing a rapid, sensitive and cost-effective method, we evaluated the in-house standardized and modified mRT-PCR (adopted from the method of Lanciotti et al.\cite{8} and Harris et al.\cite{12}) with the traditional method of serotype detection by IFAT. Investigation of acute-phase serum samples in cell culture proved mRT-PCR as a comparatively more sensitive technique as 18.33% positivity was achieved by mRT-PCR as compared to 16.25% by IFAT, when aliquots of the same cell culture were tested simultaneously by these two methods. The underlying reason for this difference might be that surface protein of DENV are recognized and bound in direct proportion with monoclonal antibodies in the case of IFAT. In contrast, by mRT-PCR, viral RNA is converted to a much higher concentration of a comparatively more stable serotype-specific end product, and DNA, consequently, increases the sensitivity of the procedure. Our results were in contrast to those obtained by Oleveira et al.,\cite{10} who found 17 samples positive by mRT-PCR as compared to only two by IFAT following cell culture. Concordant results have also been reported by previous studies.\cite{15,16} The RT-PCR method of Lanciotti\cite{8} was observed to exceed the sensitivity of virus identification by IFAT when the two tests were simultaneously applied on aliquots of same samples.\cite{17}

Further passaging or longer incubation may help increase the viral load, which may increase the detection limit of IFAT. However, reducing the time required for final results is of high priority for laboratory diagnosis of DENV. To know whether mRT-PCR is able to detect the DENV serotypes earlier than IFAT characterization, a time-course experiment was conducted using a fixed dose of standard strain of DENV-4 serotype. mRT-PCR was able to detect the virus two days ahead of IFAT, further proving it as a more sensitive as well as faster detection procedure. Oliveria et al.\cite{10} in their study were able to detect viral RNA one day post-infection by RT-PCR as compared to four days required by IFAT. The difference in the detection period may be due to the difference in the strain used by them, or due to varied experimental conditions, although the conclusions of both the studies were in congruence.

In an attempt towards optimization of an accurate and rapid test, we further evaluated the sensitivity of this mRT-PCR protocol for direct detection of DENV serotypes in serum samples, with virus isolation by cell culture using aliquots of same sera. A significantly higher number of patients (33%) were detected positive for one of the four dengue serotypes by mRT-PCR of serum samples directly, including 22 patients serotyped only by this protocol. The two major factors underlying this improved sensitivity could be (i) the ability of RT-PCR to detect intact RNA inside the non-viable virus particles, which otherwise could not propagate in cell culture, resulting in false negatives by cell culture; and (ii) dissociation of antibody-virus complex while treating the serum samples with RNA extraction reagent containing phenol which could have resulted in the detection of causative serotypes in these eight otherwise antibody-positive samples. It is proposed that the presence of immune complex could have interfered with either entry and/or propagation of these virus particles in the C6/36 cells (Table 3). In comparison to cell culture, other advantages such as feasibility
of direct processing of serum by mRT-PCR for serotypic characterization makes it a rapid assay that can be very useful to clinicians for providing better and timely treatment to the patients. As there is no need to maintain the cell line, it further saves on the cost of expensive chemicals and skilled staff. This protocol of mRT-PCR obviates the limitation imposed by cell culture and serology and can rapidly detect all the four serotypes circulating in a region. Moreover, mRT-PCR also allows the detection of concurrent infections by multiple serotypes using a single-serum sample, as observed by us and others.[10,18-21] Therefore, this method should be used as routine diagnostic assay for simultaneous detection of all the serotypes in a single acute-phase sera as well as study of their geographical pattern.

The sensitivity and specificity of laboratory diagnosis would increase by adopting a combination of virus detection by mRT-PCR in the early acute phase of illness, along with serology on acute and convalescent blood samples.

Acknowledgement

We thankfully acknowledge the financial assistance provided to us by the Indian Council of Medical Research for conducting this research work.

References


Improved Detection of Dengue Virus Serotypes from Serum Samples


