Rapid Detection of Dengue Viral RNA by Nucleic Acid Sequence-Based Amplification (NASBA)

by


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Abstract

The suitability of RNA amplification by nucleic acid sequence-based amplification (NASBA) for the detection of dengue viral RNA was investigated. A set of primers and probe were synthesized, based on a selected RNA sequence from the non-coding region at the 3' end of dengue viral RNA, and was used in the NASBA assay. The NASBA reaction product was then determined by agarose gel electrophoresis and electrochemiluminescence (ECL) signal count. The sensitivity of the NASBA assay was equal to 1 PFU/ml for all of four dengue virus serotypes. There was no false positive result with Japanese encephalitis (JE) virus. This method was used successfully to detect dengue virus in the infected tissue culture cells. This test will be useful for the detection of dengue viruses in the clinical specimens.

Keywords: Dengue virus, Nucleic Acid Sequence-Based Amplification (NASBA), RNA, detection, electrochemiluminescence (ECL).

Introduction

Dengue virus infection, a mosquito-borne disease, is a major cause of morbidity and mortality worldwide, especially in tropical and subtropical regions[4]. Dengue virus infection is caused by dengue virus (Family Flaviviridae, Genus Flavivirus), which has four antigenetically distinct serotypes (DEN-1 to DEN-4). Infection with any of the dengue viruses generally leads to different severity in the patients from mild febrile, dengue fever (DF) to dengue haemorrhagic fever (DHF) or complicated with shock, dengue shock syndrome (DSS)[2]. The most challenging
problem associated with patient management is rapid diagnosis in dengue cases.

Currently, the laboratory diagnosis of dengue virus infection is based on virus isolation and anti-dengue virus antibodies detection\(^{3}\). Isolation of dengue virus from patients’ sera collected in the acute phase of illness, or from mosquito vectors, can be accomplished with cell culture or mosquito inoculation, when the virus can be detected by using specific monoclonal antibodies\(^{4}\). These methods are sensitive but time-consuming with incubation periods ranging from 5 to 14 days. Serological diagnosis, such as haemagglutination inhibition test (HI), complement fixation (CF), neutralization test, and enzyme-linked immunosorbent assay (ELISA), are commonly used in most laboratories for the detection of antibodies. These methods are simple to perform but generally require paired serum samples for the measurement of four-fold or greater rising of antibody titers and the cross-reaction of the antibodies to another flavivirus may occur\(^{3}\).

Polymerase chain reaction (PCR) is a molecular technique that has facilitated the detection of several kinds of microorganisms including dengue virus\(^{6}\). PCR is performed rapidly, and is sufficiently sensitive for the detection of all four dengue virus serotypes in any kind of specimen; however, it generally requires specialized training and specific equipment as thermalcycler.

Recently, an alternative method to PCR, the nucleic acid sequence-based amplification (NASBA) has been developed. NASBA is an isothermal RNA amplification technique that is achieved by the action of avian myeloblastosis-reverse transcriptase (AMV-RT), T7-RNA polymerase and RNase-H\(^{7}\). In contrast to PCR, NASBA is performed without the use of specific equipment such as a thermalcycler. The amplification products can be detected by electrochemiluminescence (ECL) or agarose gel electrophoresis (AG) or enzyme-linked gel assay (ELGA). NASBA has already been successfully applied for the detection of viral RNA\(^{8,9,10,11}\) and other micro-organisms\(^{12,13}\).

In this study, attempts were made to develop NASBA as a new detection method for dengue virus, and to compare the procedures for detecting NASBA products by using ECL and AG.

**Materials and methods**

**Virus strains**

Virus seeds in C6/36 cell lines were obtained from the Department of Virology, Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok. DEN-1 (Hawaii), DEN-2 (New Guinea C), DEN-3 (H-87) and DEN-4 (H-241) and Japanese encephalitis (JE) virus were titrated in VERO cells by a standard plaque assay.

**Selection of primers and probes of NASBA**

The primers and probes used in this study are listed in Table 1. A pair of oligonucleotide primers is carrying T7-RNA polymerase as the specific tail that recognized the sequence at 5’ end of the target RNA. NASBA products derived from this pair of primers were calculated to be 203 nucleotides, including the primer sites.
Nucleic acid isolation

Nucleic acid release and isolation were performed as described by Boom et al., 1990(14). Briefly, 100 µl of serum sample was added to lysis buffer solution (consisting of 4.7 M GUSCN/ 46 mM Tris-HCL, pH 6.4/ 20 mM EDTA/ 1.2% (W/V) TritonX-100). Activated silica suspension (50 µl; 1mg/ml in 0.1 M HCL) was added. The silica pellet was washed twice with washing buffer (5.25 M GUSCN/ 50 mM Tris-HCL, pH 6.4), twice with 70% ethanol and once with acetone. The pellet was dried at 56°C for 10 min. Finally, nucleic acids were eluted with elution buffer (1.0 mM Tris-HCl, pH 8.5) and stored at -20°C.

Nucleic acid amplification

The reaction was performed as per the manufacure’s instructions (Organon Teknika, BV, Boxtel, the Netherlands). The reaction was performed with 20 µl of reaction mixture (consisting of 40 mM Tris-HCl, pH 8.5/12 M MgCl2/70 mM KCl/1.5% (v/v) of dimethyl sulfoxide/5 mM dithiotreitol/1mM of each dNTPs/2 mM of each ATP, CTP, UTP/1.5 Mm GTP/0.5 mM ITP/0.1 µg/µl of BSA/0.08 U RNase-H/32 U T7-RNA polymerase and 6.4 U AMV-RT/ 0.2 µM of each primer and 5 µl of isolation nucleic acid). Amplification products were stored at -20°C for further analysis.

Nucleic acid detection

The reaction was carried out according to the manufacture’s instructions (Organon Teknika, BV, Boxtel, the Netherlands). The amplification products were diluted to 1:20 in detection diluent (1.0 mM Tris-HCl, pH 8.5/0.2g/l methylisothiazolone), incubated with biotinylated dengue virus-specific probe bound to 5 µg of streptavidin-coated paramagnetic beads and 3 x 1011 molecules of ruthenium-labelled oligonucleotides detection probe. Then 300 µl of assay buffer (100 mM tripropylamine, pH 7.5) was added before reading by an ECL reader (NASBA QR System, Model 2000; Organon Teknika, B.V., Boxtel, the Netherlands).

Agarose gel electrophoresis

NASBA amplification products were analysed by agarose gel electrophoresis and visualized with ethidium bromide staining.

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Table 1: Primers and probe for detection of dengue virus

<table>
<thead>
<tr>
<th></th>
<th>Sequence (5’to3’)</th>
<th>Position</th>
<th>Size*</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>aat tct aat acg act cac tat agg gga gac (T7 promotor) AGC AGG ATC TCT GGT CT</td>
<td>10638-10654</td>
<td>203 bp</td>
</tr>
<tr>
<td>P2</td>
<td>gat gca agg tcg cat atg agg gtt aga gga (ECL tail) GAC CCC TCC C</td>
<td>10511-10520</td>
<td>203 bp</td>
</tr>
<tr>
<td>Probe</td>
<td>AAA CAG CAT ATT GAC GCT GGG</td>
<td>10615-10638</td>
<td>154 bp</td>
</tr>
</tbody>
</table>

* Use DEN-2 (New guinea C) as the reference sequence; including primer sites
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Results

Sensitivity of NASBA

All dengue virus serotypes were amplified with P1 and P2 and gave a clear band of 203 bp by agarose gel electrophoresis (Figure 1).

Figure 1: Agarose gel electrophoresis of NASBA amplification products for detection of dengue viruses

[Lane 1: DEN-1, lane 2: DEN-2, lane 3: DEN-3, lane 4: DEN-4, lane 5: negative control, lane 6: Japanese encephalitis virus. Molecular weight markers are shown on the right; DNA sizes are given in base pairs]

The lower limit of detection of each dengue virus serotype by NASBA is shown in Figure 2. The detection limit for all dengue virus serotypes after NASBA reaction was confirmed as less than or equal to 1 PFU/ml.

Figure 2: Assessment of the detection limit for dengue viral RNA by agarose gel electrophoresis

[A) DEN-1 B) DEN-2 C) DEN-3 D) DEN-4]

[The 10-fold dilution series of each dengue virus serotype was prepared, prior to extraction. Lane 1: 10,000 PFU/ml, lane 2: 1,000 PFU/ml, lane 3: 100 PFU/ml, lane 4: 10 PFU/ml, lane 5: 1 PFU/ml, lane 6: 0.1 PFU/ml, lane 7: 0.01 PFU/ml, and lane 8: negative control. Molecular weight markers are shown on the right; DNA sizes are given in base pairs]

Amplified products of dengue viruses were tested by ECL signal count and all gave positive results. No false positive result was seen when water was used as negative control. The detection limit for all dengue virus serotypes after NASBA reaction was confirmed as less than or equal to 1 PFU/ml when amplified products were determined by ECL signal count (Figure 3).

[The 10-fold dilution series of each dengue virus serotype was prepared, prior to extraction. Lane 1: 10,000 PFU/ml, lane 2: 1,000 PFU/ml, lane 3: 100 PFU/ml, lane 4: 10 PFU/ml, lane 5: 1 PFU/ml, lane 6: 0.1 PFU/ml, lane 7: 0.01 PFU/ml, and lane 8: negative control. Molecular weight markers are shown on the right; DNA sizes are given in base pairs]

Specificity of NASBA

JE virus was extracted, amplified and detected in the same way as performed in the dengue-NASBA method described above. There was no NASBA reaction product as determined by agarose gel electrophoresis or ECL signal count. These results showed that the dengue NASBA method did not cross-react with the JE virus.

Discussion

In this study, we developed NASBA for the detection of dengue viral RNA. The results showed that dengue viral RNA can be extracted, amplified and detected directly from virus culture. One advantage of NASBA
is a continuous, isothermal process that does not require a thermalcycler. The constant temperature allows each step of the reaction to proceed as soon as an amplification intermediate becomes available. Thus, the exponential kinetics of the NASBA process, which are caused by multiple transcription of RNA copies from given DNA products, are intrinsically more efficient than DNA amplification methods which are limited to binary increases per cycle. It is an important research tool, particularly in the area of RNA, simplifying both RNA detection and direct sequencing. Products of NASBA are single stranded RNA, and thus can be applied to detection formats using probe hybridization without the denaturation step and contamination with genomic DNA is not amplified. The complete procedure can be undertaken in a single working day (allowing 2 hours for extraction and amplification, 1 hour for detection). In the present study, we were developing the NASBA reaction for the detection of dengue virus in clinical specimens.

Acknowledgement

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References