Selective Detection and Differentiation of Subgenus B Adenoviruses (Types 3, 7 and 11) by Polymerase Chain Reaction

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Summary

Application of the polymerase chain reaction (PCR) method for detection of subgenus B adenoviruses (types 3, 7 and 11) was investigated. It is based on a simple (nonnested) PCR using primer pairs specific for the hexon-coding region. The PCR allowed amplification of DNA from subgenus B adenovirus prototype strains (types 3, 7 and 11) and adenovirus isolates (types 3 and 7), whereas it did not amplify DNA from subgenus A (type 31), C (types 1, 2, 5 and 6), D (types 8, 19 and 37), E (type 4) and adenovirus isolates (types 1, 2, 5 and 6). These results suggest that subgenus B adenoviruses (types 3, 7 and 11) are detectable selectively by means of PCR with primer pairs developed in this study. Amplified fragments from adenovirus types 3, 7 and 11 could be differentiated with restriction endonuclease analysis with Rsa I.

Introduction

Human adenoviruses constitute a large family with 49 serotypes identified so far. These serotypes are classified into six subgenera (A to F) on the basis of biochemical, immunological, and morphological criteria1-2). Subgenus B of adenoviruses has been divided into two DNA homology groups: B1 and B23). Both groups are associated with occasional severe infections and fatalities4-5). In 1996, adenovirus type 7 was isolated from fatal pneumonic cases in children with basal ailments in the heart or lung, showing a new aspect of emerging infectious diseases in Japan4).

In the serotyping of subgenus B adenovirus isolates by the neutralizing test, attention should be paid to a possible cross-reaction arising between type 7 and types 3 or 114). Recently, PCR has been applied successfully in adenovirus detection in clinical specimens6-8). In this paper, we describe a new PCR method for selective detection of subgenus B adenoviruses (types 3, 7 and 11). This rapid method facilitates identification of these adenoviruses and may be useful for epidemiological surveillance.

Materials and Methods

1. Prototype strains
Prototype strains of human adenovirus types 1-8, 11, 12, 19, 31 and 37 were obtained from American Type Culture Collection (Rockville, MD USA) or National Institute of Infectious Diseases (Tokyo, Japan).

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2. Isolation and serotyping of adenoviruses from clinical specimens

Adenovirus isolates were obtained by inoculation of clinical specimens (nasopharyngeal swabs and feces), which had been collected for surveying etiological agents in Hyogo Prefecture, onto 80% confluent monolayers of Hep-2 cells in duplicated wells of a 24-well plate (Nippon Becton Dickinson, Tokyo, Japan). When a cytopathic effect was evident, the isolates were serotyped by neutralization assay using antisera obtained from National Institute of Infectious Diseases (Tokyo, Japan) or Denkaseiken (Tokyo, Japan).

3. Viral DNA preparation

Viral DNA was prepared by a modification of the method described by Shinagawa et al. Briefly, Hep-2 cell cultures were prepared in 25 cm² tissue culture flasks (Nippon Becton Dickinson, Tokyo, Japan) and infected with adenoviruses. When most cells showed cytopathic effect, the culture was removed and washed twice with PBS (−). The cells were lysed with 1 ml of 0.6% SDS/10 mM EDTA and transferred into a 1.5 ml centrifuge tube. Most of cellular DNA was removed as precipitate by adding 0.3 ml of 5 M NaCl, mixing well, keeping on ice for 3 hr and centrifuging at 15,000 rpm for 15 min. The supernatant was transferred into 2 ml centrifuge tube and incubated with 200 µg of proteinase K (GIBCO BRL, Rockville, MD USA) at 37°C for 1 hr. The proteinase K treated sample was extracted twice with an equal volume of phenol/chloroform mixture. After precipitation with one volume of isopropanol, DNA was resolved in 50 µl of distilled water.

4. Primers and PCR amplification

Figure 1 shows the oligonucleotides used as primers for PCR amplification. Primer pairs, designated as Hyogo 1997 U and Hyogo 1997, were newly designed on the basis of hexon sequencing of adenovirus types 3 and 7. The other primer pairs (HexAA 1885 and HexAA 1913) were the general ones described by Allard et al. Both of these primer pairs generate 301-bp PCR products. All primers were synthesized by GIBCO BRL (Rockville, MD USA).

An aliquot (1 µl) of the appropriately diluted DNA preparation was used as the template. Amplification reactions were conducted in 50 µl of reaction mixture containing 0.5 µM each of the primer pairs, 200 µM each dideoxynucleotide, 1.25 U Taq polymerase (TaKaRa Shuzo, Shiga, Japan), and 1X PCR buffer (GIBCO BRL).

Fig. 1 Oligonucleotide sequences used in this study. Hyogo 1997 U and Hyogo 1997 are newly synthesized and used as primer pairs with PCR A. HexAA 1885 and HexAA 1913 are used as primer pairs with PCR B. The sequences are shown to illustrate their homology. Nucleotides that are identical to the preceding nucleotides sequence are marked by asterisks (*).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Numbering</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sence primer</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyogo 1997 U</td>
<td>5'-GCCCAATGGCATAATGCACATCG-3'</td>
<td>18855-18858</td>
</tr>
<tr>
<td>HexAA 1885</td>
<td>5'-<em><strong>G</strong>G</em><strong>T<em>T</em></strong>*******-3'</td>
<td>18882</td>
</tr>
<tr>
<td><strong>Antisense primer</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyogo 1997</td>
<td>5'-AAGCACCCCGGCAGATCATAAAGA-3'</td>
<td>19136-19156</td>
</tr>
<tr>
<td>HexAA 1913</td>
<td>5'-C<em><strong><em>G<strong>G</strong></em>G</strong></em>*****T-3'</td>
<td></td>
</tr>
</tbody>
</table>

* The numbering refers to the nucleotides' positions based on those of Adenovirus type 2.
10 mM Tris-HCl, pH 8.3, 50 mM KCl and 1.5 mM MgCl$_2$. The PCR was carried out using a cycle of 94°C for 30 seconds, 60°C for 30 seconds and 75°C for 30 seconds, and was continued for 30 cycles; in the first cycle, the denaturation step continued for 7 minutes at 94°C, and in the last cycle, the extension step continued for 5 minutes at 75°C. PCR using the newly synthesized primer pairs (PCR A) and PCR using the general primers$^7$ (PCR B) were performed in parallel. Five µl of the final reaction product was analyzed in a regular 1.5% agarose gel and stained with etidium bromide.

5. Restriction enzyme analysis of PCR products

Aliquots of 10 µl of PCR A reaction amplimer were digested for 6 hr with 10 U of Rsa I under conditions specified by its manufacturer (New England Biolabs, Beverly, MA USA). Enzyme digests were loaded onto 12% polyacrylamide gel and stained with etidium bromide.

Fig. 2 Effect of Mg$^{2+}$ on PCR. DNA from prototype strain of adenovirus type 3 was amplified, using Mg$^{2+}$ concentrations ranging from 0.5 to 4.5 mM (left to right, with 0.5 mM increments; lane 1-9). PCR A and PCR B were performed parallelly using primers (Hyogo 1997 U and Hyogo 1997) and primers (HexAA 1815 and HexAA 1913) respectively. L; 100 bp DNA Ladder (New England Biolabs, Beverly, MA USA).

1. Optimizing the PCR

The newly developed PCR for subgenus B adenoviruses was tested with DNA from prototype adenovirus type 3 using different Mg$^{2+}$ concentrations (0.5-4.5 mM). The bands of 301-bp were clearly visible with 1.5 mM concentration of Mg$^{2+}$ (Fig. 2 PCR A). However, the general primers tested under the same conditions did not amplify DNA fragments when Mg$^{2+}$ concentration was less than 2 mM (Fig. 2 PCR B). From these preliminary experiments, 1.5 mM MgCl$_2$ was used thereafter.
2. Selective detection of subgenus B adenoviruses by the newly developed PCR

The two PCR methods were tested for adenoviruses prototype strains (types 1–7, 11, 19, 31 and 37). PCR A amplified 301-bp fragment with adenovirus types 3, 7 and 11. However, DNA from adenovirus types 1, 2, 4, 5, 6, 19, 31 and 37 (non-subgenus B adenoviruses) did not amplify by the PCR A. In contrast, the PCR B amplified DNA from non-subgenus B adenoviruses. Therefore, selective detection of subgenus B adenoviruses (types 3, 7 and 11) was possible by means of the PCR A with primer pairs developed in this study (Fig. 3).

DNA from clinically isolated adenoviruses were examined by the PCRs A and B. All of the DNA from adenovirus strains serotyped 3 or 7 was selectively detected by the PCR A. The PCR B amplified 301-bp fragment DNA from adenoviruses typed 1, 2, 5 and 6 (Table 1).

3. Restriction enzyme analysis of PCR products generated by PCR A

Restriction enzyme analysis was performed using amplimers obtained with PCR A. Differentiation was possible based on specific patterns (types 3, 7 and 11) in electrophoresis gel after treatment with Rsa I (Fig. 4 and Fig. 5).

**Discussion**

In this paper, we described a new PCR method, PCR A, to detect selectively adenoviruses types 3, 7 and 11. The method amplifies the same region of the hexon gene identified as group-specific by Allard *et al.*. They reported primer pairs (HexAA 1885 and HexAA 1913) that anneal to the group-specific region of adenoviruses. They detected 18 of 18 adenovirus types representing all six subgenera by PCR. Their method is very simple and useful but the 3′ end of their downstream primer (HexAA 1913) does not exhibit precise base pairing with hexon-coding regions of adenovirus types 3.
Table 1  Comparison of PCR A (with Hyogo 1997 U and Hyogo 1997) and PCR B (with HexAA 1885 and HexAA 1913) for clinical isolates in Hyogo Prefecture.

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Number</th>
<th>PCR A Positive</th>
<th>PCR A Negative</th>
<th>PCR B Positive</th>
<th>PCR B Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adeno 1</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Adeno 2</td>
<td>5</td>
<td>0</td>
<td>5</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Adeno 3</td>
<td>48</td>
<td>48</td>
<td>0</td>
<td>0</td>
<td>48</td>
</tr>
<tr>
<td>Adeno 5</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Adeno 6</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Adeno 7</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>

Fig. 4  Comparison of Rsa I restriction patterns of amplified DNA of adenovirus prototypes 3, 7 and 11. L: 100 bp DNA Ladder.

Fig. 5  Schematic presentation of Rsa I restriction patterns of PCR products (adenovirus types 3 and 7).

bp 3p *3i *7p 7i
254 — —
139 — —
115 — —
71 — —
47 — — —
44 — —

*p; prototype strain
*i; clinically isolated strain

and 7 at their 3' ends. Although, the terminal match at the 3' end is critical for PCR amplification, a mismatched T at the 3' is known to allow amplification if the PCR is proformed in non-stringent conditions

In this study, their primers did not allow amplification of DNA from adenovirus type 3 with Mg2+ concentrations less than 2 mM. Morris et al. evaluated the same primers (Hex 1885 and HexAA 1913) on ocular specimens and reported that DNA prepared from adenovirus type 7 gave no amplified product with an annealing temperature of above 55°C.

We sequenced 2 and 8 strains of adenovirus type 3 and 7, respectively, which had been sampled in Japan, Nepal and Indonesia, and confirmed that newly synthesized primer pairs (Hyogo 1997 U and Hyogo 1997) exhibit precise base paring with target DNA of these isolates (unpublished data). Thus, PCR A was very sensitive to the detection of these two serotypes.

Amplimers of PCR A could be differentiated by endonuclease (Rsa I) analysis. Kase et al. described that Rsa I was capable of discriminating PCR (they used primers by Allard et al.) products of adenovirus types 3 and 7. In this study, we also found that adenovirus type 11 was distinguishable from types 3 and 7.

Further investigation must be done with adenovirus types 14, 16, 21, 34 and 35 (other subgenus B adenoviruses) to clarify whether these viruses can be detected and differentiated by our newly developed PCR method.
Acknowledgments

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References