Detection and Genotyping of Astroviruses by RT-PCR and Sequencing

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Abstract

Three primer pairs, AV244Mon and 82b, AV-15Gr and AV-12Gr, and Beg and End, were used for the detection of standard astroviruses (serotype 1 to serotype 7) in cell culture and astroviruses isolated from 89 diarrheal stool specimens negative for pathogenic bacteria, rotavirus and adenovirus by the reverse transcription-polymerase chain reaction (RT-PCR). All the standard strains and two isolates were detected by primer pairs AV244Mon and 82b, and AV-15Gr and AV-12Gr. All serotypes except serotype 4 were detected by the primer pair Beg and End. The molecular sizes of the RT-PCR products obtained by Beg and End were almost the same among the different serotypes except serotype 6. Sequence analyses of the nucleic acid in the regions between the primer pairs AV244Mon and 82b, and between Beg and End from PCR products of the standard strains and from the isolates of the two stool specimens revealed that each serotype had a unique sequence. These results indicate that the RT-PCR is useful for detecting and genotyping astroviruses.

Introduction

Human astroviruses are non-enveloped, single stranded RNA viruses that were first identified in 1975 by electron microscopy in stool specimens from children with diarrhea¹⁾. Astroviruses account for 2 to 8% of diarrheal episodes in children²⁾. Not only sporadic cases but also outbreaks have been reported to be associated with astroviruses^{3,4)}. So far, seven human astrovirus serotypes have been identified serologically⁵⁾. They were adapted to grow in tissue culture^{6,7)}. An enzyme immunoassay (EIA) for astroviruses has been developed⁸⁾; however, the antibodies for EIA are difficult to obtain commercially. Moreover, specific antibodies for each serotype are not available. For those reasons, it has been difficult to determine the serotype of astroviruses in stool samples. Recent nucleotide sequence analyses of the gene encoding the capsid protein of astrovirus serotypes 1⁹⁾, 2¹⁰⁾, 4¹¹⁾, and 6¹²⁾ enabled us to detect and genotype astroviruses in stool samples by the reverse transcription-polymerase chain reaction (RT-PCR). Universal primers for detecting serotypes 1 to 7 have been developed^{13,14)}. We have also made a common primer pair which detects all serotypes except serotype 4¹⁵⁾.

In this study we attempted to detect astroviruses in patients without bacterial or adenovirus-gastroenteritis in Japan by using the three published primer pairs. The results were compared with those obtained by EIA^{8,16)}. The PCR products were further examined by direct sequencing in the two parts of the capsid protein-coding regions.

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Materials and Methods

Astroviruses

Astroviruses OxAsT1 (serotype 1), OxAsT2 (serotype 2), 2684-90, (serotype 3), OxAsT4 (serotype 4), OxAsT5 (serotype 5), Symonds (serotype 6) and Rana (serotype 7) cultured on CaCo2 cells or LLCMK2 cells were used as standards⁷⁾.

Stool specimens previously determined to be free of bacteria, rotaviruses and adenoviruses were divided into two groups (Table 1). The first group was composed of 66 diarrheal samples obtained in the winter of 1991 and 1992 from outpatients in a pediatric clinic of Maizuru Kyosai Hospital located in central Japan. The other group was composed of 23 samples from various areas in Japan (5 samples from Ehime, 3 from Aichi, 10 from Kitakyushu, 3 from Niigata and 2 from Yokohama). All were collected in winter from 1991 to 1994. The ages and clinical symptoms of the patients were not known for all samples.

Extraction of RNA and RT-PCR

Viral RNA was extracted from stool specimens with guanidine thiocyanate and glass powder as previously described^{17,18)}. The RNA was reverse transcribed in a reaction buffer containing the desired first PCR primer mixture, a deoxynucleotide mixture, avian myeloblastosis virus reverse transcriptase, and a ribonuclease inhibitor at 37°C for 1 hr. The PCR was conducted with 1 U of Taq DNA polymerase and the reverse transcribed product by 35 cycles (94°C, 1 min for denaturing; 45°C, 2 min for annealing; 72°C, 3 min for extension; and 72°C, 7 min for final extension). The PCR products were electrophoresed in agarose gel containing ethidium bromide and photographed under UV light.

For the primers used for the PCR, Beg and End were chosen from the reported nucleotide sequence of the genome from serotype 1¹²⁾ and serotype 2¹⁰⁾: Beg 5'-ACC GTG TAA CCC TCC TCT C-3' (6495-6513 in Ast1 or 6477-6495 in Ast2); End 5'-TCC TAC TCG GCG TGG CCG C-3, (6735-6717 in Ast1 or 6717-6699 in Ast2). AV244Mon was derived from the paper¹⁴⁾ and 82b was chosen from the paper¹⁴⁾ near the primer AV245Mon: AV244 Mon 5'-GGT GTC ACA CCA AAA CC-3'; 82b 5'-GTG AGC CAC CAG CCA TCC CT-3'. AV-15Gr and AV-12Gr were 3' end of the RNA¹⁹⁾: AV-15Gr 5'-CTT TTC TGT CTC TGT TTA GAT TA-3'; AV-12Gr 5'-TTT TTT TTT TTT TTT GC-3'. In addition to Beg and End, another primer "Prel", 5'-GAA GCG GAC TTT GA-3', was used with End for determining and sequencing serotype 4 because the standard serotype 4 astrovirus was not detectable with Beg and End.

Direct sequencing

The PCR products obtained with the combination of primers AV244Mon and 82b, and Beg (or Prel) and End were sequenced directly by using a dideoxynucleotide sequencing kit (Perkin Elmer Applied Biosystems, Foster City CA).

Results

The PCR with 3 sets of primers

As is shown in Fig. 1, standard astroviruses from serotypes 1 to 7 were detected by RT-PCR with the primer combination AV244Mon and 82b (366pb), and the primers AV-15Gr and AV-12Gr (77bp). Astrovirus serotype 4 was not detected by primers Beg and End. The molecular size of the product of serotype 6 obtained by RT-PCR with primers Beg and End (218bp) was smaller than those of serotypes 1, 2, 3, 5 and 7 (241bp). The PCR product of serotype 4 obtained with primers Prel and End (299bp) was larger than those of serotypes amplified by Beg and End (details not shown).

Detection of astroviruses by EIA and RT-PCR

As shown in Table 1, astroviruses were detected in two of 89 clinical samples by RT-PCR with

Table 1 Detection of astroviruses by EIA and RT-PCR in patients with nonbacterial, nonrotavirus- and nonadenovirus gastroenteritis in Japan

Year	No. of samples	Place	EIA	RT-PCR		
				AV244Mon-82b	AV-15Gr-AV-12Gr	Beg-End
1991—1992	66	Maizuru	1	1	1	1
1991—1994	23#	5 areas	not done	1	1	1

Ehime, 5 samples ; Aichi, 3 samples ; Kitakyushu, 10 samples ; Niigata, 3 samples ; Yokohama, 2 samples.

Fig. 1 The first PCR analyses of cultured astrovirus serotypes 1 to 7, OxAsT1, OxAsT2, 1684-90, OxAsT4, OxAsT5, Simonds, Rana, respectively. (1) Primers were Mon244 and 82b. From the left, Hinf I- and Bgl-I digested PBR328 DNA size marker (M), serotype 1 to 7 standard astroviruses and negative control (N). All serotypes were detected. (2) Primers were Beg and End. From the left side, Hinf I-digested ϕ X 174 DNA size marker (M), serotype 1 to 7 standard viruses and negative control (N). Serotypes 1, 2, 3, 5, 6 and 7 were detected. The PCR product of serotype 6 was smaller than those of serotypes 1, 2, 3, 5 and 7. Serotype 4 was not detected. (3) Primers were 15Gr and 12Gr. From the left, Hinf I-digested ϕ X 174 DNA size marker (M), serotype 1 to 7 standard viruses and negative control (N). All serotypes were detected.

three sets of primers (AV244Mon-82b, 15Gr-12Gr and Beg-End).

DNA sequence analysis of the region between AV244Mon and 82b

As shown in Fig. 2, DNA sequences were different among the serotypes. Two Japanese strains (AS115 and AS153) were quite similar to standard serotype 1. Homologies among different serotypes were quite low, while homology was more than 90% within serotype 1 (S1, AS115 and AS153).

DNA sequence analysis of the region between Beg and End

As shown in Fig. 3, DNA sequences were different among the serotypes. Homologies among different serotypes in the region between AV244Mon and 82b were higher than those in the region between Beg and End (Fig. 2 and Fig. 3).

Discussion

Detection of astroviruses in clinical samples has been difficult for the following reasons: (1) It is sometimes difficult to differentiate astroviruses from other small round viruses such as parvovirus, caliciviruses and picornaviruses by electron microscopy²⁰⁾ (2) Polyclonal and monoclonal antibodies for EIA⁸⁾ are not easy to prepare. Serotyping of seven serotypes has required much more work than detection of astroviruses by immunofluorescence tests, immune electron microscopy and EIA^{5, 14, 21)}.

Recent developments in RT-PCR for detection and serotyping of rotaviruses in stool specimens have accelerated the study of astroviruses^{13, 14, 19)}. In our study three different sets of primers were used to detect astroviruses in cell cultures and stool specimens. Primers AV244Mon and 82b are

[illegible]

Three hundred and forty-eight nucleotides between primers AV244Mon and AV245Mon have been sequenced in serotypes 1 to 7⁽⁴⁾. The genotypes based on the sequence analysis are consistent

Fig. 3 DNA sequence analysis of the region between Beg and End.

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100
S1      :      :      :      :      :      :      :      :
AS115   ACCGTGTAACCCCTCCTCAACTCTCGTAAATCAAGGAATGACAATGACGCGTCCACAAGGATAGCAGCGCGCATTCGCCACGCTTCCGATAGGAT
AS153   primer: Beg *****
S2      T**A***A***C*****G**GA***C**A***A***A***TAA*****T*****A**CTGCGT**G**AAC
S3      T**A***A*****G**GA***C**G*****A**CAA*****G*****TAGCGT**A***
S4      *CTCT*GT***CAGG*C*TCAG**GG**GCGTGCTACGT*TAT*G*T*T*AGCATA***A**T*T*GAGT*GGGAAG*
S5      T*****A*T**C*****G**GA***C*****AA**CA*CA*TAA***G*****A**GCG***C**AT*
S6      *****A*C*****G*****G**GAT**C**A**C*T***TA**AAT*****T*****CAAATT*C**AACC
S7      T**A***A***C*****G**GA***C**A***T***TA**CAA*****G*****TTGCGT**C**AACA

200
S1      :      :      :      :      :      :      :      :
AS115   CAAGCGTGGAGTATACATGGACCTGCTTCTCGGGGTAAGCCAGGCAATGCGTCTCATGCGTGTGAAGAGGCACGCAAGACGATGGGGAAATC
AS153   *****
S2      G*****CA**C**G*****C***C**T*TCG**GA***G*****AA**C*****T**A*****T*G*GCA*CA*TCAG
S3      A*****T*****C*****CA**T**TC**TCA*****A*****C*****CATGGGCACTA*TCAA
S4      GGT*AACAATCAAC*AGCCAG*ACATCCAGGAATTTCCTGAGATGTTGTTG**GCA*GAG**CA**AT*CGT*T*TATGTG*GC***AACCAACGTGCG*
S5      G*G***CA*T**T***AT*****T*G**T**CC*TG*****ATGCA*****C*****GC*A*****T**GC*ATA*TCAT
S6      T*G*****A*CCT*CA*AT*****G*C*C*CA*C*ATT*T*TG*CG*CA***C*****G*****A**CA*T**AA**T**C**CTTTC***AAGC---
S7      A*****CA*T**C*****CT**T**CC*CTCG*****T*****A*****A*****CCATG*GCACTA*TCAT

S1      :      :      :      :
AS115   AATCCCTGCACATCTGGAAGCCGCGGCCACGCCGAGTAGGA
AS153   *****
S2      CTGG**AAACTT*****GA**
S3      *TG**TAATGT*****GA**
S4      TCAAT*AAACTT*****GA**
S5      G*C*TACAA**T**A*****
S6      -----
S7      *TG**TAATGT**GGA*****

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S1-S7 are standard viruses which were cultured.
AS115 and AS153 were isolated from stools in Japan.

with the serotypes. Moreover, a dendrogram demonstrating the genetic relatedness of 44 astroviruses among serotypes 1 to 7 was shown in that paper. In contrast we were able to analyze the samples not only by primers AV244Mon and 82b but also by primers Beg and End. Moreover, analyses of two strains of astrovirus type 1 (AS115 and AS153) revealed a genotype quite similar to that of standard serotype 1 obtained by sequencing PCR products with both primer sets.

As in our previous studies of rotaviruses^{17,18)}, we will be able to detect astroviruses from the second PCR products in more samples even in the small volume of the first RT-PCR products. Full DNA sequence determinations of astrovirus capsid regions are in progress in our laboratory and serotype-specific primers are being designed as well for serotypes 3, 4, 5, 6 and 7 as for serotypes 1 and 2 which were reported previously¹⁵⁾. Genotyping should be perfected in the near future not only by sequencing but also by combination of the first and second PCR.

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逆転写－遺伝子増幅法および遺伝子解析によるアストロウイルスの 検出と遺伝子型の決定について

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要 旨

AV244Mon と 82b, AV-15Gr と AV-12Gr, Beg と End の 3 種のプライマーセットを用いた逆転写－遺伝子増幅法により, 細胞培養由来の標準アイトロウイルス (1 型から 7 型) および病理菌陰性・ロタウイルス陰性・腸管アデノウイルス陰性の 89 の下痢便からアストロウイルスの検出を試みた。AVMon と 82b および AV-15Gr と AV-12Gr ではすべての標準株と 2 下痢便でアストロウイル

スが検出できた。Beg と End では 4 型を除き検出可能であった。また Beg と End では 1, 2, 3, 5, 7 型は同じ分子サイズであったが 6 型はそれより小さかった。AV244Mon と 82b 領域, Beg と End の領域の遺伝子配列はそれぞれ型特有であった。これらのことから逆転写－遺伝子増幅法および遺伝子解析はアストロウイルスの検出と遺伝子型の決定に有用であると思われた。