Identification of human and bovine rotavirus serotypes by polymerase chain reaction

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SUMMARY

The use of the polymerase chain reaction (PCR) for identifying serotypes of human and bovine rotaviruses was examined. In the identification of 115 human rotavirus samples in stools, results with PCR showed excellent agreement with results of serotyping by an enzyme-linked immunosorbent assay (ELISA) using serotype-specific monoclonal antibodies. Furthermore, the PCR showed a much higher sensitivity (93 %) than the ELISA test (82·6 %). The PCR method could also be applied for identifying the serotype of bovine rotaviruses.

INTRODUCTION

Rotaviruses are recognized as the major cause of severe gastroenteritis in infants and young children, and the young of a variety of mammalian and avian species [1, 2]. Fourteen serotypes have been defined by cross-neutralization tests [1–5]. In humans, four major serotypes (serotypes 1–4) and three minor serotypes (serotypes 8, 9, and 12) have been identified. The serotype specificity is associated largely with an outer capsid protein VP7, which is encoded by RNA segment 7, 8 or 9. An enzyme-linked immunosorbent assay (ELISA) using serotype-specific monoclonal antibodies directed against VP7 has recently been developed as a rapid and simple method for serotyping rotavirus strains in faecal samples (6–9), and the utility of the ELISA-serotyping has been widely recognized [10–15]. However, the serotype of rotaviruses cannot be determined by the ELISA-serotyping in a considerable portion (about 20–30 %) of faecal specimens. In order to identify the serotype specificity of the samples which cannot be typed in ELISA, a novel method is required.

Comparative analysis of the amino acid sequences of VP7 from different rotavirus serotypes shows that VP7 has six serotype-specific regions [16], which are designated A to F (amino acids 39–50, 87–101, 120–130, 143–152, 208–221, and 233–242). Amino acid sequences in these regions are well conserved among strains of the same serotype but differ considerably among strains belonging to different serotypes. Thus, each serotype has a characteristic, serotype-specific nucleotide

sequence in the six regions on the VP7 gene. Using the information on the serotype-specific nucleotide sequence, sequencing only the variable regions of VP7 gene and hybridization using serotype-specific oligonucleotides have been described as substitutes for serological methods [17, 18].

These substituted serotyping methods are indirect procedures predicting rotavirus serotype by examining the serotype-specific nucleotide sequences of VP7 gene. The PCR which has been successfully used for diagnosis of several viral diseases [19–21] was applied to assign rotavirus strains to serotype by Gouvea and colleagues [22]. In the present study, we have modified Gouvea's original method to increase the sensitivity mainly by using a second stage PCR routinely and using new specific primers for serotypes 2–4. We confirmed the specificity and sensitivity of PCR typing, and used this method to survey serotype distribution of human rotaviruses in the winter of 1990–91 in Sapporo, Japan. In addition, we applied PCR typing to bovine rotaviruses, in which the presence of three different serotypes (serotypes 6, 8 and 10) has recently been recognized.

MATERIALS AND METHODS

Virus strains

One hundred and fifteen rotavirus-positive stool specimens were obtained from infants with diarrhoea in the winter of 1990–91 in Sapporo, Japan. Twelve bovine faecal specimens positive for rotavirus were collected from young calves with diarrhoea in a dairy farm in Nakon Rachasima Province, Thailand. The following human and bovine rotavirus strains with different serotype specificities were used as reference strains: serotype 1 (strain KU), serotype 2 (strain S2), serotype 3 (strain YO), serotype 4 (strain ST-3), serotype 6 (strains NCDV and UK), serotype 8 (strains 69M and A5), serotype 9 (strain WI-61), and serotype 10 (strains 61A and KK3). The viruses were pretreated with 10 μg/ml of acetylated trypsin (type V-S from bovine pancreas; Sigma) and were propagated in MA-104 cells in the presence of trypsin (1 μg/ml). The viruses were harvested 1–3 days after infection.

ELISA

Detection of rotavirus in faeces was carried out by ELISA using YO-156 antibody, which reacts with the group A common antigenic epitope on VP6 of the inner capsid [23].

Serotyping of human rotaviruses by ELISA was performed as described previously [9, 15]. The ELISA employed anti-VP7 serotype-specific monoclonal antibodies (KU-4, S2-2G10, YO-1E2, and ST-2G7) as capture antibodies, rabbit antiserum (a pool of hyperimmune antisera to serotype 1 4 human rotavirus strains) as a detector antibody, and peroxidase-conjugated goat anti-rabbit IgG.

PCR-typing

Virus in 200 μl of 10% stool suspension or virus-infected culture fluid was disrupted by incubation at 55 °C for 30 min with 50 μl of disruption solution containing 50 mM Tris–HCl (pH 8·0), 5 mM EDTA, 5% Nonidet P-40 and

Table 1. *Location in the VP7 gene and sequences of oligonucleotide primers*

Primer name		Sequence (5'–3')	Product length (base pairs)	Location	
				Nucleotide nos.	Strain (serotype)
C1	(−)	GGTCACATCATACAATTCTAATCTAAG		1039–1062	SA11 (3)
C2	(+)	GGCTTTAAAAGAGAGAATTTCCGTCTGG	1062	1–28	Wa (1)
S1	(+)	CAAGTACTCAAATCAATGATGG	749	314–335	Wa (1)
S2	(+)	GACTACAATGATATTACTAC	657	406–425	S2 (2)
S3	(+)	GACGCGACGTTGCAATTG	582	481–498	SA11 (3)
S4	(+)	TCAAACGACAAATACAGCTA	394	669–688	ST-3 (4)
S6	(+)	GATTCTACACAAGAACTAGA	582	481–500	NCDV (6)
S8	(+)	GTCACACCATTTGTAAATTCG	885	178–198	69M (8)
S9	(+)	CTAGATGTAACTACAACTAC	306	757–776	WI-61 (9)
S10	(+)	CTAGAACAGAAATAAACGA	749	314–322	61A (10)

500 μg/ml proteinase K. Proteinase K was then inactivated by heating at 95 °C for 10 min. After phenol extraction, chloroform extraction and ethanol precipitation, rotaviral double-stranded RNA (dsRNA) was suspended in 100 μl of distilled water. Five stool specimens negative for rotavirus were also processed as described above for negative controls.

Procedure for PCR-typing was similar to the method developed by Gouvea and colleagues [22]. In order to increase the sensitivity and specificity, amplification was carried out in two stages. A first amplification of the full-length VP7 gene was followed by a second amplification of the DNA fragments with length specific for each serotype using nested primers and the copy of the full-length VP7 gene as the template.

The first amplification was carried out as follows. Extracted dsRNA (1 μl) was added to 100 μl of reaction buffer containing 10 mM Tris–HCl (pH 8·3), 40 mM KCl, 1·5 mM MgCl$_2$, 200 μM each of dATP, dCTP, dGTP and dTTP, 5 mM dithiothreitol, 3·5% dimethyl sulfoxide (DMSO), RNase inhibitor (5 units), and 100 pmol each of two primers (C1 and C2) listed in Table 1. The mixture was heated at 97 °C for 5 min and rapidly cooled in ice, and 10 units of avian reverse transcriptase (Seikagaku Kogyo, Tokyo) and 2·5 units of Tth DNA polymerase (Bokusui Brown Inc.) were added. The reaction mixture was covered by one drop of mineral oil, and the tube was placed in a thermal cycler (PC-700; Astec Co. Tokyo). Following the incubation for reverse transcription at 42 °C for 30 min, samples were subjected to one cycle of 5 min at 94 °C, 3 min at 40 °C, and 3 min at 72 °C, 25 cycles of 1 min at 94 °C, 2 min at 40 °C, and 3 min at 72 °C and a final cycle of 1 min at 94 °C, 2 min at 40 °C, and 10 min at 72 °C.

The second amplification was performed by using the same reaction buffer (100 μl) except that the different primer sets (25 pmol each) and the first amplification product (1 μl, undiluted or diluted 1:100) were included as the template. A mixture of primers (S1, S2, S3, S4, S8, S9 and C1 for human rotavirus, or S6, S8, S10 and C1 for bovine rotavirus) listed in Table 1 were used. The denaturation and reverse transcription steps were omitted. The second amplification consisted of 25 cycles at 94 °C for 1 min, at 42 °C for 2 min and at 72 °C for 1 min. The final extension was allowed to continue for 7 min. PCR products (10 μl)
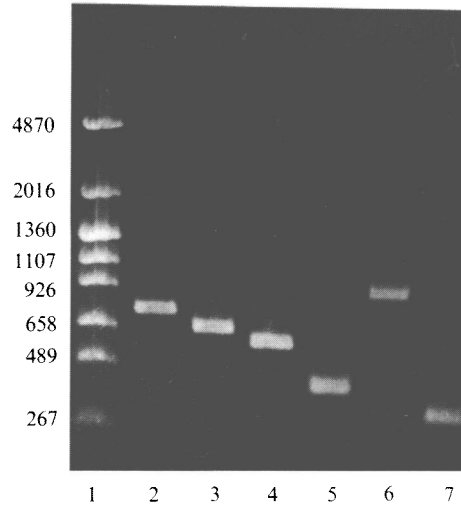
Fig. 1. PCR typing of representative human rotavirus serotypes. Viral RNA extracted from the virus-infected culture fluids was used as the template for PCR. Lanes: 1, molecular weight marker; 2, serotype 1 (strain KU); 3, serotype 3 (strain S2); 4, serotype 3 (strain YO); 5, serotype 4 (strain ST-3); 6, serotype 8 (strain 69M); and 7, serotype 9 (strain WI-61).

were separated by electrophoresis on 1 % agarose gel and visualized with UV light after staining with ethidium bromide. In each PCR run, the sample without template was included to confirm no contamination.

RESULTS

PCR typing of human rotaviruses

Fig. 1 shows the results obtained by PCR on culture fluids of cells infected with reference strains of human rotavirus representing six serotypes, 1–4, 8 and 9. The PCR products from strains with different serotype specificity exhibited different migrations which corresponded to the expected size of DNA fragments.

The serotype of the rotavirus in 115 human stool samples was assigned by both PCR typing and ELISA-serotyping. As shown in Fig. 2, the serotype of the rotavirus in stools was readily detected via PCR-typing. PCR with rotavirus-negative controls did not show any DNA bands (results not shown). Table 2 shows the comparison of the sensitivity and the specificity between ELISA-serotyping and PCR typing. By ELISA, 95 of 115 samples (82·6 %) could be serotyped: 83 were serotype 1, 5 were serotype 2, 7 were serotype 3; no serotype 4 strain was detected. In contrast, by PCR typing, the serotype of 107 of the 115 samples (93 %) was determined: 96 were serotype 1, 4 were serotype 2, and 7 were serotype 3. There was no disagreement in the determination of serotype of 91 strains whose serotype was identified by both methods.

Twenty samples could not be serotyped by ELISA (Table 2): 11 samples showed reactivity with more than two serotype-specific antibodies and 9 did not react with any serotype-specific antibodies (Table 3). Of these 20 samples, 14 were
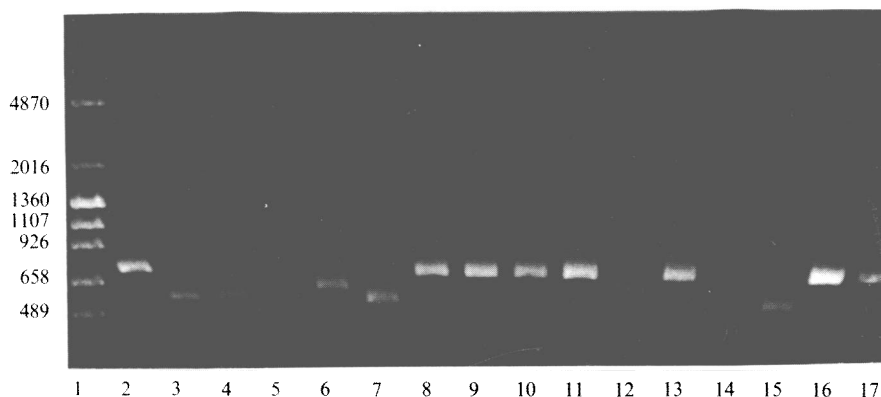
Fig. 2. PCR typing of human rotaviruses in stools. Lanes: 1, molecular weight markers; 2, stool sample GO-164 (assigned to serotype 1); 3, TA-823 (serotype 3); 4, TA-831 (serotype 3); 5, TA-842 (serotype 3); 6, TA-853 (serotype 2); 7, TA-855 (serotype 3); 8, GO-166 (serotype 1); 9, GO-172 (serotype 1); 10, GO-177 (serotype 1); 11, GO-180 (serotype 1); 12, MI-173 (serotype 2); and 13, TA-795 (serotype 1); 14, TA-803 (serotype 1); 15, TA-858 (serotype 3); 16, AK-108 (serotype 1); 17, AK-110 (serotype 1).

Table 2. *Comparison of the serotype designation of 115 rotavirus strains in stools as determined by ELISA and PCR*

| | | | ELISA results | | | | | |
| | | | Assigned to serotype | | | | | |
			1	2	3	4	Untyped	Total
PCR results	Assigned to serotype	1	82	0	0	0	14	96
		2	0	4	0	0	0	4
		3	0	0	5	0	2	7
		4	0	0	0	0	0	0
		Untyped	1	1	2	0	4	8
		Total	83	5	7	0	20	115

assigned to serotype 1 and 2 to serotype 3, by PCR; the remaining four samples could not be assigned to serotype by PCR (Tables 2 and 3). In contrast, only 8 samples could not be assigned to serotype by PCR, although 4 of the 8 samples could be serotyped by ELISA (Table 2). Thus, indirect serotype identification via PCR showed a higher sensitivity than serotyping by ELISA.

PCR typing of bovine rotaviruses

PCR was also applied to identify three different serotypes (serotypes 6, 8 and 10) of bovine rotaviruses by preparing serotype 6- and 10-specific primers in addition to the serotype 8-specific primer used for human rotavirus typing. Fig. 3 shows the PCR results obtained by using culture fluids of cells infected with serotype 6 (bovine UK and NCDV) strains, serotype 8 (bovine A5 and human 69M) strains and serotype 10 (bovine 61A and KK3) strains. Amplified DNA fragments from

Table 3. *PCR typing of human rotaviruses not typable by ELISA*

Reaction in ELISAs

Sample no.	Anti-VP6			Anti-VP4	Anti-VP7				Assigned serotype by PCR
	YO-156 (Common)	S2-37 (I)	YO-5 (II)	YO-2C2 (Common)	KU-4 (1)	S2-2G10 (2)	YO-1E2 (3)	ST-2G7 (4)	
TA824	+	+	+	+	+	+	+	+	1
TA860	+	+	+	+	+	+	+	+	1
GO144	+	+	+	+	+	+	−	+	?
TA833	+	−	+	+	+	+	−	+	1
GO154	+	−	+	+	+	+	−	+	1
AK119	+	−	+	+	+	+	−	+	1
GO146	+	−	+	+	+	+	−	+	1
TA785	+	−	+	+	+	+	−	+	1
GO171	+	−	−	+	−	+	−	+	?
AK107	+	+	−	+	−	+	+	+	?
TA833	+	−	+	+	−	+	−	−	3
GO126	+	−	+	+	−	−	−	−	1
GO152	+	−	+	+	−	−	−	−	1
TA814	+	−	+	+	−	−	−	−	?
MI202	+	−	+	+	−	−	−	−	1
TA817	+	+	−	+	−	−	−	−	1
GO180	+	−	+	−	−	−	−	−	1
MI183	+	−	+	−	−	−	−	−	1
TA795	+	−	−	−	−	−	−	−	1
TA864	+	−	−	−	−	−	−	−	3

* The reactivity of the monoclonal antibodies with the stool samples was based on the criteria described previously [15, 23]. Parentheses show the specificity of the monoclonal antibodies: YO-156, commonly reactive to group A rotavirus; S2-37, subgroup I-specific; YO-5, subgroup II-specific; YO-2C2, commonly reactive to group A rotavirus; KU-4, serotype 1-specific; S2-2G10, serotype 2-specific; YO-1E2, serotype 3-specific; ST-2G7, serotype 4-specific.
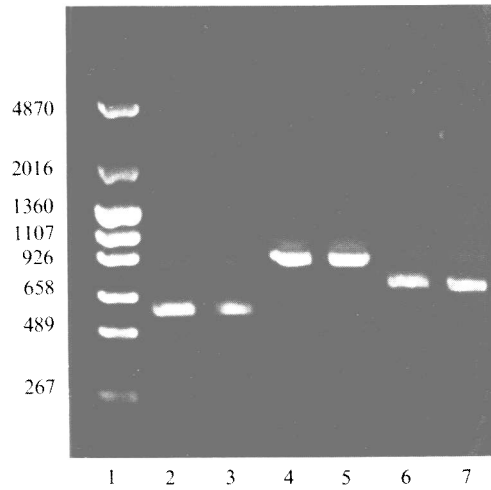
Fig. 3. PCR typing of bovine rotavirus serotypes. Viral RNA extracted from virus-infected culture fluids was used as the template for PCR. Lanes: 1, molecular weight marker; 2, serotype 6 (strain NCDV); 3, serotype 6 (strain UK); 4, serotype 8 (human strain A5); 5, serotype 8 (human strain 69M); 6, serotype 10 (strain KK3); and 7, serotype 10 (strain 61A).
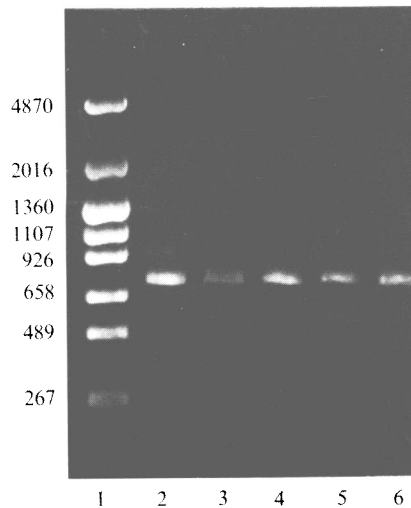

Fig. 4. PCR typing of bovine rotaviruses in faeces. Lanes: 1, molecular weight markers; 2, faecal sample A10; 3, A20; 4, A26; 5, A31; and 6, A36. The PCR products from the five bovine faecal specimens migrated to the positions corresponding to the expected size (749 base pairs) of serotype 10.

bovine serotypes 6, 8 and 10 showed the migration in agarose gel corresponding to the expected characteristic sizes of 582, 885 and 749 base pairs, respectively. PCR typing was also carried out by using RNA from 12 bovine faecal specimens collected in Thailand. All were assigned to serotype 10 by the characteristic size of their PCR products (Fig. 4).

DISCUSSION

Global surveys of rotavirus serotypes are essential to obtain epidemiological data on rotavirus infection and to provide the fundamental knowledge needed to develop an effective vaccine. Surveillance of rotavirus has been performed worldwide by ELISA with monoclonal antibodies to the four major human serotypes and has yielded useful information on rotavirus epidemiology [10–15]. However, in most of these studies, the serotype of 20–30% (in some studies > 50%) of rotavirus in stool specimens could not be determined. There may be several reasons for this reduced efficiency in serotype determination. First, each of the serotype-specific monoclonal antibodies may not detect the strains within a given serotype which have antigenic variation within VP7 [24–26] because monoclonal antibody is directed against a single epitope. Secondly, the virion outer capsids consisting of VP7 and VP4 are not stable in faeces, and the VP7 molecule released from the virion is considered a poor antigen because the serotype-specific epitopes on VP7 are found to be conformational [26]. To overcome these problems, a substitute method of serotyping is required.

In the comparison with ELISA-serotyping using monoclonal antibodies, PCR-tying of rotaviruses was more sensitive and much quicker. Furthermore, PCR-typing has other advantages over the ELISA-serotyping. Because genomic RNA is more stable than the outer capsid of rotavirus particles, the PCR can be applied to stool suspension which have not been stored in a good condition. In developing countries, stool specimens for examination are not always preserved in a frozen or cooled state. In our experience, the serotype of the rotaviruses in stool suspensions which were kept for 14 months at room temperature was clearly assigned by the PCR method (data not shown). When a new serotype is suspected, the preparation of a monoclonal antibody specific for that new serotype is required. In contrast, PCR typing can soon be applied to the new serotype by preparing specific primers for the new serotype. Furthermore, PCR products can be used for further characterization by direct sequencing, cloning of the VP7 gene, etc. Thus, the PCR can be used as a powerful additional technique for characterization of rotaviruses. However, PCR-typing has a disadvantage compared to the ELISA-serotyping in the cost per sample and in the number which can be handled at one time.

Rotavirus VP4 also has an independent neutralization antigen [27, 28]. The presence of four kinds of VP4 types has been reported [29, 30]. Typing of human rotavirus VP4 by PCR would be quite useful for elucidating the combination of VP4 and VP7 neutralization antigens in each rotavirus strain.

Most bovine rotaviruses including reference strains NCDV and UK belong to serotype 6. However, several bovine rotavirus strains were recently assigned to serotype 8 or 10 [31–33]. In our previous genomic and serological analyses of Thai bovine isolates, we detected one serotype 8 and several serotype 10 bovine strains [32, 33]. The development of PCR typing in this study permitted the differentiation of these bovine rotavirus serotypes. Surveys using the PCR on serotype distribution of bovine rotavirus on a large scale will elucidate the epidemiological features of bovine rotavirus infections. Multiple serotypes have also been reported in equine and porcine rotaviruses: serotypes 3, 5, 13 and 14 in equine rotavirus and serotypes 1–5 and 11 in porcine rotavirus [1, 3, 4, 34]. PCR

could be also applied for serotyping equine and porcine rotaviruses in faeces (Pongsuwanna et al., unpublished).

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