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Analysis of Human Rotavirus G Serotype in Bangladesh by Enzyme-Linked Immunosorbent Assay and Polymerase Chain Reaction

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ABSTRACT

Distribution of human rotavirus G serotype was investigated by enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) with faecal specimens obtained from children with diarrhoea in Bangladesh. By ELISA, subgroup and G serotype were determined for 59.5% and 28.6% of group A rotavirus-positive specimens respectively. However, of the 120 specimens, the G serotype of which was not determined by ELISA, serotype of the 112 specimens was typed by PCR. In total, G serotype was assigned for 95.2% of all the specimens, showing the highest rate of G4 (41.7%), followed by G1 (23.2%) and G2 (14.9%). Twenty-four specimens showed mixed types, such as G2 with G1, G8 or G9, or G1 with G4. These results indicate that PCR combined with ELISA is highly effective for G serotyping of rotavirus.

Key words: Rotavirus; Diarrhea, Infantile; Enzyme-linked immunosorbent assay; Polymerase chain reaction; Serotyping

INTRODUCTION

Human rotavirus (HRV) is the major aetiologic agent of severe diarrhoea in infants and young children worldwide (1,2). Although detection of rotavirus from clinical specimens has become a routine diagnostic procedure, characterization of these strains for serotype or specific gene products has remained difficult despite the need for such information before the introduction of a human rotavirus vaccine.

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The rotavirus particle consists of outer and inner capsid, containing 11 segments of double-stranded RNA. The outer capsid is composed of a major glycoprotein VP7 and minor trypsin-sensitive protein designated VP4. Both proteins appear to be involved in virus neutralization and define different antigenic specificities. VP7 defines a G serotype of rotavirus, and four G serotypes (GI, G2, G3, and G4) are most frequently detected in wild strains of human rotavirus. The antigenic specificity of VP4, which is independent of G serotype specificity, has been designated as P serotype (1,3-7). VP6, the sole component of the inner capsid, carries group-specific and subgroup-specific antigens. In group A rotaviruses, two subgroup specificities, I and II, are recognized (8).

Due to high morbidity and mortality of children due to rotavirus infection, global effort to develop and

evaluate rotavirus vaccines is in progress; the development of a new tetravalent reassortant vaccine is now close to licensure in the USA (9,10). To design a vaccine strategy and to evaluate success of candidate vaccines, it is essential to know the G serotypes of prevailing rotavirus strains before and after its introduction.

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 (10,11). In epidemiological studies, rotavirus G serotype in faecal specimens has been determined mostly by ELISA, using G serotype-specific monoclonal antibodies (Mabs) directed to VP7 (12,13). However, as reported previously and observed also in this study, G serotypes of a considerable proportion of specimens could not be determined by ELISA probably due to lack of a sufficient amount of double-shelled particles therein.

Comparative analysis of the amino-acid sequences of VP7 proteins of various rotavirus serotypes has revealed that VP7 protein has six variable regions (A to F) in which amino-acid sequences are highly divergent among strains of different serotypes, but are highly conserved among strains of the same serotypes (16,17).

To compensate the afore-mentioned defect in ELISA, PCR, using serotype-specific primers set appropriately in these variable regions, was applied for G serotyping, and high sensitivity and specificity of the method have been demonstrated (14,15). Although in Bangladesh, distribution and frequency of HRV G serotype have been examined until 1990 (13,19,20), it is necessary to understand the long-term trend of G serotype distribution, since it often changes from year to year (21-23).

In the present study, rotavirus specimens of Bangladesh were analyzed by PCR, in addition to ELISA, to obtain more complete information of G serotype distribution during 1992-1994.

MATERIALS AND METHODS

Stool specimens and screening of group A rotavirus

In total, 1,260 stool specimens were collected from children, aged less than five years, with watery diarrhoea and/or vomition. The patients came mostly from rural areas around Mymensingh town, and the distribution of age is typical for rotavirus. There has been no marked seasonal variation in the occurrence of diarrhoea. These patients were admitted into the Mymensingh Medical College hospital and infectious disease ward of S.K. hospital in Mymensingh during 1992-1994. Group A rotavirus was first screened by latex agglutination commercial test kit (Rotalex, Orion Diagnostics, Finland) and was further confirmed by ELISA with Mab YO-156 which is reactive with antigenic epitope on VP6 of group A rotavirus (24).

ELISA

ELISA for subgrouping and serotyping of HRV was carried out according to the procedure described previously, employing subgroup I- and II-specific and G serotype 1-, 2-, 3- and 4-specific monoclonal antibodies (10,12,24). Briefly, the ELISA employed anti-VP7 serotype-specific monoclonal antibodies, namely KU-4, S2-2G10, YO-1E2 and ST-2G7, as capture antibodies, rabbit antiserum (a pool of hyperimmune antisera to serotype 1-4 HRV strains) as detector antibody, and peroxidase-conjugated goat anti-rabbit IgG.

Extraction of double-stranded RNA (dsRNA)

Rotavirus dsRNA was extracted by the method of Gentsch *et al.* (17) with some modifications. Three hundred µL of 10% stool suspension were mixed well with an equal volume of fluorocarbon and centrifuged at 8000 g for 5 minutes. The 250 µL of the aqueous phase, 320 µL of 6 M guanidine thiocyanate and 10 µL of RNA matrix in RNAID kit (BIO 101, Inc., LaJolla) were added and mixed on a mixer for 10 minutes. The mixture was centrifuged at 900 g for 1 minute, and the pellets were washed three times with 400 µL of wash buffer supplied in the RNAID kit by sequential centrifugation at 2000 g, 3500 g, and 8000 g. The final pellet was dried under vacuum, suspended in 50 µL of distilled water, and incubated at 65 °C for 10 minutes. The suspension was centrifuged at 8000 g for 1 minute, and the supernatant was stored at -20 °C until use.

PCR typing

Rotavirus dsRNA was extracted from stool suspension or virus-infected tissue culture fluid in the same way commonly used for RNA electropherotyping in polyacrylamide gels (25). Virus in 200 µL of 10% stool suspension or virus-infected culture fluid was disrupted by incubating at 55 °C for 30 minutes with 50 µL of disrupting solution, containing 50 mM Tris HCl (pH 8.0), 5 mM EDTA, 5 percent Nonidet P-50, and 500 mg/mL proteinase K. Proteinase K was then inactivated by heating at 95 °C for 10 minutes. After phenol and chloroform extraction and ethanol precipitation, rotavirus dsRNA was suspended in 100 µL of distilled water. Stool specimens, negative for rotavirus, were also processed as described above for negative control.

The procedure for PCR typing was similar to the method developed by Gouvea *et al.* (15). To increase the sensitivity and specificity, amplification was carried out in two stages. Amplification of the full-length VP7 gene in the first stage was followed by a second amplification of the DNA fragments using serotype-specific primers and the copy of the full-length VP7 gene as template. Serotype-specific primers were set in variable regions, so that the PCR products of different sizes are amplified depending on different G serotypes (16).

RESULTS

Subgrouping and serotyping by ELISA

Subgrouping and serotyping of group A rotaviruses in stools collected from children hospitalized with watery diarrhoea and/or vomition were carried out with an ELISA with subgroup and serotype-specific monoclonal antibodies. Of the 1,260 specimens, 168 (13.3%) were positive for group A rotavirus. Of the 168 rotavirus-positive specimens, subgroup was determined for 100 specimens (59.5%) with 48 specimens (28.6%) each being subgroup I and subgroup II specificity, and 4 specimens (2.4%) showed dual subgroup specificity. As to the G serotype specificity, 48 (28.6%) rotavirus-positive specimens were determined in ELISA. The results are shown in the table.

Of the 48 specimens, the serotype of which was determined, 6 were assigned to serotype G1, 12 to serotype G2, 2 to serotype G3, and 27 to serotype G4. One specimen reacted with G2+G4. The remaining 120 specimens remained undetermined.

PCR typing

G serotyping by PCR employing G1, G2, G3, G4, G8 or G9-specific primers was carried out for 120 specimens which were untypeable in ELISA.

The PCR products from strains with different serotype specificity exhibited different migration which corresponded to the expected size of DNA fragments.

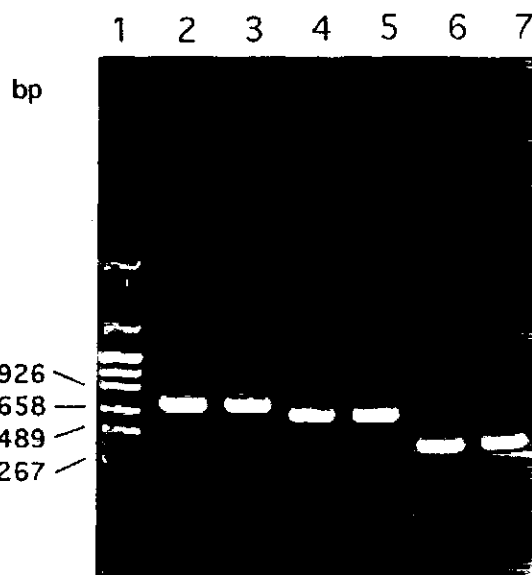

Fig. Agarose gel electrophoresis of PCR product derived from specimens with representative G serotype. Lane 1, molecular weight marker; lane 2 and 3, G serotype 1 specimens (749 bp); lane 4 and 5 G serotype 2 specimens (657 bp); lane 6 and 7, G serotype 4 specimens (394 bp)

The figure shows the PCR products amplified from HRV specimens with representative G serotypes. In total, 112 (93.3%) of the 120 rotavirus-positive specimens, whose G serotype was not detained by ELISA, were typed by PCR (Table). Of these, 33 were serotype G1, 13 serotype G2, 43 serotype G4, 2 serotype G1+G2, 17 serotype G1+G4, 3 serotype G2+G4, and 1 reacted with G2+G9. Eight specimens could not be serotyped by PCR (Table).

DISCUSSION

To understand the epidemiologic features of individual rotavirus serotypes prevailing worldwide, especially in developing countries, and to make use of the results for vaccination programme, worldwide surveys on the

Table. Frequency of HRV G serotype as determined by ELISA and PCR

Method for serotyping	No. of specimens examined	G serotype (%)								
		G1	G2	G3	G4	G1+G2	G1+G4	G2+G4	G2+G9	ND[†]
ELISA	168	6 (3.6)	12 (7.1)	2 (1.2)	27 (16.1)			1 (0.6)		120 (71.4)
PCR	120[*]	33 (27.5)	13 (10.8)		43 (35.8)	2 (1.7)	17 (14.2)	3 (2.5)	1 (8.3)	8 (6.7)
ELISA or PCR	168	39 (23.2)	25 (14.9)	2 (1.2)	70 (41.7)	2 (1.2)	17 (10.1)	4 (2.4)	1 (0.6)	8 (4.8)

[*] *G serotype of these specimens was not determined by ELISA;* [†] *Not detected*

distribution of rotavirus serotypes are essential. A large number of surveillance of rotavirus serotypes, performed globally by ELISA with serotype-specific monoclonal antibodies against four important rotavirus serotypes, have provided useful information on the rotavirus-associated epidemiology (12,13,16,27). At present in human rotaviruses, four major G serotypes have been identified among the 14 human and animal rotaviruses (1,28). Serotype is, by definition, a classification based on neutralization of viral infectivity. Rotavirus G serotyping has been performed recently by ELISA (4,10,11), using G serotype-specific monoclonal antibodies which are not yet available for all serotypes.

However, in most studies, serotype of about 20-30 percent (in some cases over 50%) of rotavirus strains in stool specimen was left untyped. There may be several possible reasons for the reduced rate of serotyping, using serotype-specific monoclonal antibodies by ELISA. One major reason is that the serotype-specific monoclonal antibodies may not detect strains within a given serotype which have antigenic variation on VP7 protein (11,29,30), because monoclonal antibody is directed against a single epitope. The other reason is that the virion outer capsides, consisting of VP7 and VP4, are not stable in faeces, and the VP7 molecule released from the virion is considered to be a poor antigen, because the serotype-specific epitopes on VP7 were found to be conformational (29).

To compensate this defect in ELISA, PCR was applied for G serotyping, and high sensitivity and specificity of the method have been demonstrated (14,15). Although in Bangladesh, distribution and frequency of HRV G serotypes have been examined until 1993 (13,19,20,31), it is necessary to understand the long-term trend of G serotype distribution since it often changes from year to year (21-23), Gouvea *et al.* (15) first applied the PCR method for rotavirus serotyping by adding DMSO to the reaction buffer to prevent reannealing of viral dsRNA. In our study, we applied the Gouvea's method modified by Taniguchi *et al.* (16) where sensitivity of the reaction was increased. The concentration of DMSO was decreased to 3.5 percent, RNase inhibitor was added to the reaction buffer, second PCR was routinely employed, and serotype G2, G3 and G4-specific new primers were prepared (16). In the present study, rotavirus specimens in Bangladesh were analyzed by PCR, in addition to ELISA, to obtain more complete information on distribution of G serotype.

G serotyping by PCR employing G1-, G2-, G3-, G4-, G8- or G9-specific primers was carried out for 120 specimens which were untypeable in ELISA according to the procedure described previously (15). As a result, G serotype was assigned for 112 of the 120 specimens (Table). In total, 160 of the 168 rotavirus-positive specimens were typed by ELISA and PCR (Table), indicating the highest detection rate of G4 (41.7%), followed by G1 (23.2%), and G2 (14.9%). In the previous studies on HRV serotypes prevailing in Bangladesh, G1 or G2 was generally the predominant serotype, and an occasional increase of G4 was observed (13,19,20,31). G3 was always the rarest serotype as seen in our present study, suggesting that HRV with G3 is not endemic in Bangladesh. However, unlike previous findings, G4 was the commonest strain in the present study. Further investigation is needed to assess whether it was an occasional event. Although ELISA has been widely employed for G serotyping since the development of G serotype-specific Mabs (24), the rate of typeable specimens varies considerably depending on the study, e.g. approximately 70% in a Japanese study (12), in contrast to about 40% in the previous study in Bangladesh (20). These inconsistent results seem to be caused by difference in the amount of double-shelled particles in the stool samples, which may be influenced by condition of sample preservation, since a sufficient amount of virion is required for ELISA.

However, this deficiency of ELISA may be compensated by PCR G serotyping which requires only a little amount of viral genome, not virion itself. In this study, G serotype of 93.3% of the specimens which were untypeable in ELISA was indeed determined by PCR, and a similar increase in the typing rate by PCR, compared to ELISA, was reported (15). Hence, it should be emphasized that PCR is effective for G serotyping of rotavirus in combination with ELISA.

It was noted that mixed serotypes were found in 19.2% of the samples examined in PCR and that a minor serotype, G9, was found in a single specimen as a mixed type (G2+G9). The G9 serotype was also found in the stool specimens of Bangladesh in our previous study (unpublished data). Some genetic variation of G9-VP7 gene has been reported, and G9 virus is suggested to be prevalent much more than our previous estimation. Thus, it is probable that most undetermined G serotype of rotavirus may belong to G9. However, it is preferable to use some different set of primers to detect G9 by PCR because of its genetic variation. We have no data on electropherotype of the G9 rotavirus. In our previous report on HRV infections in Bangladesh, mixed patterns of RNA profiles were frequently observed, especially after flood (20). Mixed infecfion with different rotavirus strains may reflect frequent contamination of water with viruses and may facilitate generation of novel rotavirus strains through reassortment (32). Therefore, the frequency of mixed infection of rotaviruses and its influence against efficacy of rotavirus vaccine should be further investigated.

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