Method

Identification of P[8]b Subtype in OP354-Like Human Rotavirus Strains by a Modified RT-PCR Method

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SUMMARY: In our previous study, a novel P[8] subtype, i.e., P[8]b was identified for human rotavirus strains MMC38 and MMC71 detected in Bangladesh, of which the P types could not be determined by conventional RT-PCR genotyping methods. In the present study, a modified multiplex RT-PCR method was developed to detect P[8]b as well as common human rotavirus P types. With this method, P[8]b was detected in three strains among the 26 rotavirus specimens which had been judged as mixed P types in the previous study in Bangladesh. The VP4 nucleotide sequences of these strains showed more than 98.9% identities to those of strains MMC38 and MMC71. The newly designed RT-PCR method was considered as useful for identifying P[8]b and avoiding misclassification by the conventional RT-PCR genotyping methods.

INTRODUCTION

Rotavirus, a member of the family Reoviridae, has 11 segments of double-stranded RNA encoding six structural proteins and six nonstructural proteins (1). The rotavirus outer capsid consists of two structural proteins VP7 and VP4, which have neutralization antigens and define G and P serotypes, respectively. Based on VP7 and VP4 gene sequences, rotaviruses are classified into G and P genotypes (types), respectively. VP8*, an N-terminal portion of cleavage products of VP4 with trypsin, is highly divergent among rotaviruses and associated with P types (2). The 24 G types and 33 P types of group A rotaviruses have been classified so far (3). In human rotaviruses, the major genotypes are G1, G2, G3, G4, and G9, which are combined with P[4], P[6], and P[8] (4). A large number of epidemiologic studies on human rotavirus conducted to date revealed that predominant G/P types are different depending on countries or regions, and change by year or season (4).

Recently, we have conducted a hospital-based survey of rotaviruses in sporadic diarrheal cases in children and adults in Bangladesh (5). In this study, P genotypes were not identified in the two strains, MMC38 and MMC71, with the RT-PCR developed for P genotyping, which has been commonly used for epidemiologic studies of human rotaviruses (6-8). Therefore, full-length VP4 gene sequences of these strains were determined and analyzed (9). VP4 sequences of MMC38 and MMC71 were genetically distinct from the previously known P genotypes, while relatively close to both P[4] and P[8], the two major P genotypes in human rotaviruses. On the other hand, the VP8* portions of MMC38 and MMC71 showed more than 93.9% nucleotide sequence identity to P[8] variants, i.e., OP354-like P[8] strains (10), being clustered into the same lineage with these strains. Thus, we proposed that the VP4 of these strains should be classified into a subtype of the P[8] genotype (P[8]b) that is distinct from that of common P[8] rotaviruses (P[8]a) (9).

Strain OP354 was reported for the first time in Malawi during 1998–1999 (10). Thereafter, rotavirus strains with OP354-like (P[8]b) VP4 were detected in various countries, e.g., India (11,12), Thailand (13), Vietnam (14), Bangladesh (9), and Finland, suggesting widespread distribution of P[8]b rotaviruses throughout the world. However, to understand the epidemiological nature of P[8]b subtypes in rotaviruses, it was necessary to establish a genotyping method to identify major P types of the human rotavirus, discriminating between P[8]a and P[8]b.

In the present study, we developed a genotyping method to identify P[8]b as well as four other major human P types (P[4], P[6], P[8]a, and P[9]) by a modified multiplex RT-PCR. With this method, we identified additional P[8]b rotaviruses among the Bangladeshi strains which had been classified as mixed P types in an earlier study (5).

MATERIALS AND METHODS

RNA extraction and modified RT-PCR genotyping: Rotavirus RNA was extracted from 10% stool suspension using RNAID kit (BIO101, Inc., La Jolla, Calif., USA) according to the manufacturer’s instructions. The 877 bp-cDNA fragment from VP4 gene was amplified RT-PCR as described previously (15) using common primers corresponding to nucleotide sequences of the VP4 gene that are well conserved in human rotavirus
strains (Table 1). This amplicon was used as a template in a second PCR with a pool of P-type-specific primers to generate fragments with type-specific length (Table 1). The primer mixture selected for the second amplification consisted of primers 1T-1, 3T-1, and 4T-1, described by Gentsch et al. (6), to detect P[8]a, P[6], and P[9] genotypes, respectively, and two newly designed primers P4-DS–1 and P8b-MMC38 specific to the P[4] and P[8]b genotypes, respectively. Sequences of these primers, their position, sizes of each PCR product, and reference strains are indicated in Table 1.

**Amplification and sequence analysis of VP4 and VP7 genes:** Full-length VP4 and VP7 gene sequences of the strains MMC153, MMC183, and DH389 were determined by RT-PCR and direct sequencing, as described previously (9,15). Sequence analysis and comparison were carried out using the GENETYX-MAC (version 11.2), and multiple sequence alignment and phylogenetic analysis were performed using the MEGA software (version 4.1) with Kimura 2-parameter distances.

**Nucleotide sequence accession numbers:** The nucleotide sequence data reported in this study were deposited in the GenBank database under the accession numbers GQ869839 (VP4) and GQ869842 (VP7) for strain MMC153, GQ869840 (VP4) and GQ869843 (VP7) for strain MMC183, and GQ869838 (VP4) and GQ869841 (VP7) for strain DH389.

**RESULTS**

First, the cell culture-adapted reference strains were used to examine the specificity of the modified P-typing method. The results indicated that DNA fragments with the expected different sizes of 268 bp for M37 P[6], 346 bp for KU P[8]a, 392 bp for K8 P[9], 499 bp for DS-1 P[4], and 636 bp for MMC38 and MMC71 P[8]b were amplified (data not shown). When nucleotide sequences of these amplified products were directly determined, these DNA sequences were identical to those of strains representing individual P types (data not shown).

In the previous study, P genotypes of strains MMC38 and MMC71 were not determined as P[4], P[6], P[8], or P[9] by commonly used multiplex RT-PCR P-typing protocols reported (5). However, we observed multiple faint amplicons, i.e., non-specific products by the RT-PCR generated from the strains MMC38 and MMC71 which might be judged as mixed infection of P[4] and P[6], or P[4] and P[8], by mistake. Therefore, P typing with the newly established RT-PCR method was carried out for 26 stool samples which had been recorded as mixed P types in our previous study in Bangladesh (5), to know whether or not P[8]b is included among them. As a result, P[8]b was identified in three rotavirus samples (strains MMC153, MMC183, and DH389) in which 640 bp-fragment specific to P[8]b was generated (data not shown).

To analyze VP4 genes from the strains MMC153, MMC183, and DH389, full-length VP4 gene sequences of these strains were determined. The VP4 sequences of the three strains were almost identical to each other (99.1–99.2% identity at nucleotide level; 98.8–99.2% identity at amino acid level), and more closely related to those of P[8]b strains MMC38 and MMC71 (98.9–99.6% nucleotide and 98.2–99.2% amino acid sequence identities) than P[8]a strains, e.g., strain Wa (89.2–89.4% nucleotide and 92.1–92.6% amino acid sequence identities). These rotaviruses were assigned to G9 by G typing and their VP7 genes showed an extremely high level of nucleotide and deduced amino acid sequence identity (99.2–100% and 99.1–100%, respectively) to that of the G9P[8]b strain MMC38.

A phylogenetic tree was constructed on the basis of the full-length VP4 nucleotide sequences of the P[8]b strains with those of the P[4] and P[8]a genotypes (Fig. 1A). These newly identified P[8]b strains were clustered with strains MMC38 and MMC71. The VP8* portions of these strains showed more than 99.2% identities to each other, and more closely related to those of the P[8]b strains (94.8–99.1% nucleotide and 95.8–99.5% amino acid sequence identities). In the phylogenetic tree of VP8* sequence, the P[8]b viruses, including three newly identified strains, were located in a single cluster, including OP354 and OP354-like rotaviruses (Fig. 1B).

All the P[8]b rotaviruses which were identified in Bangladesh were detected in stool samples from children. The frequencies of P[8]b among children and the mixed P-types based on conventional P typing were 4.4% (5/113) and 11.5% (3/26), respectively.

**DISCUSSION**

In the previous study, some nonspecific amplicons were detected from a rotavirus with P[8]b subtype by P genotyping with RT-PCR (5). This finding indicated that the P type of these rotaviruses could not be determined correctly by the RT-PCR, which may cause diagnostic problems in the epidemiologic study of rotavirus strains.
viruses. Therefore, we developed a genotyping method to identify P[8]b as well as four other major human P types (P[4], P[6], P[8]a, and P[9]) by a modified multiplex RT-PCR.

P[8] has been found to be the most frequent P type among rotaviruses worldwide (4). As a component of immunization antigen, two currently available rotavirus vaccines have the P[8]a-VP4 (16), while P[8]b-VP4 is not included. Although it is not evident at present whether or not the rotavirus vaccines are efficient for
both P[8]a and P[8]b rotaviruses equally, in order to obtain any clues relevant to this issue, distribution of P[8]b rotaviruses should also be carefully checked while undertaking epidemiologic surveillance of rotaviruses. For this purpose, the newly established P-typing method in the present study will be useful, and will provide more accurate characteristics of P type distribution in epidemiologic studies of rotaviruses.

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REFERENCES