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Detection of Group B and C Rotaviruses by Polymerase Chain Reaction

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We adapted the polymerase chain reaction (PCR) to detect the noncultivatable group B and C rotaviruses and introduced a simple and convenient technique to purify viral RNA from stool specimens. Double-stranded RNA present in stool extracts was purified by adsorption to hydrodroxyapatite and was used as the template for reverse transcription and polymerase amplification. Primer pairs specific for group B (gene 8) and group C (gene 6) rotaviruses were selected to amplify group-characteristic sizes of cDNA copies readily identifiable in ethidium bromide-stained agarose gels. These primer pairs were used separately in individual PCR assays or were pooled with a primer pair specific for group A rotavirus (gene 9) in a combined PCR assay for the simultaneous detection of all three rotavirus groups. The method was very sensitive and was used to identify both human and porcine strains of group B and C rotaviruses in stool specimens. A second PCR amplification with internal group-specific primers served to increase further the sensitivity of the test and to confirm the diagnostic results obtained in the first amplification.

Rotaviruses are important agents of gastroenteritis in humans and animals. Three of the six identified groups of rotavirus infect humans. Group A rotavirus, the most common cause of diarrhea in infants and young children throughout the world, accounts for a large percentage of pediatric hospitalizations because of dehydrating diarrhea (16). Group B and C rotaviruses are morphologically identical to the group A strains, but their RNA genes present distinct migration patterns on polyacrylamide gels and they are not detected by common enzyme immunoassays (EIAs) for group A virus (23). Group B rotavirus, also called adult diarrhea rotavirus (ADRV), became well known following major outbreaks of watery diarrhea in adults in the People's Republic of China that have occurred since 1982 (11, 15, 23). To date, these outbreaks have been confined to China, and seroepidemiologic studies have demonstrated that group B-specific antibodies in human sera are rarely found outside of China (5, 15, 19, 22). In the veterinary field, infections with group B rotavirus have been identified in cattle, pigs, sheep, lambs, and rats. Group C rotavirus has been found in humans and piglets with severe diarrhea in many countries. Most cases in humans have been sporadic or in small clusters, but outbreaks have recently been reported in England (4, 8) and Japan (17). The finding of a low prevalence of antibody to group C rotavirus in humans (3, 23) and a high prevalence in pigs (23, 25) has led to the suggestion that group C rotavirus might be an emerging zoonotic infection in humans.

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The non-group A rotaviruses have been identified either by the characteristic electrophoretic patterns of their genomes or by the detection of rotavirus by electron microscopy that does not react in the common EIA for group A rotavirus. Group B-specific immunoreagents, including monoclonal antibodies, have been made and reported to be useful in EIAs (7, 15, 18).

We recently applied the polymerase chain reaction (PCR) technique to amplify and type group A rotavirus (12). The current study extends our previous study to provide a novel, sensitive, and specific method to detect group B and C rotaviruses individually and in a combined assay for all three groups of human rotaviruses. The availability of this test may be useful in studies on the transmission, epidemiology, and surveillance of these viruses.

MATERIALS AND METHODS

Viruses. For group B rotaviruses, 20 stool specimens were collected from patients involved in eight outbreaks that occurred from 1984 to 1987 in several provinces of the eastern part of the People's Republic of China. These included four specimens from Qingdao in 1984, two each from Changchum and Yanbian in 1985, two from Xinan in 1986, three from Qinghuangdao in 1986, four from Qinghuangdao in 1987, one from Chengde in 1987, and two from Huludao in 1987. Most outbreaks involved hundreds of cases of severe diarrhea, with two deaths reported in Qinghuangdao in 1987 (11). The source of infection could be traced to the local water supply in three outbreaks: Qinghuangdao in 1986, Chengde in 1987, and Huludao in 1987.

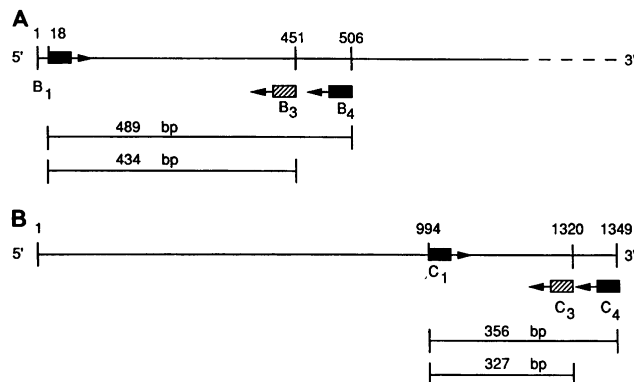

FIG. 1. Diagrams of gene 8 of a group B rotavirus (A) and gene 6 of a group C rotavirus (B), showing the size, direction, and position of the primers described in the text.

Group C rotaviruses were from patients with diarrhea in Thailand (four specimens) and Nepal (one specimen) that were previously analyzed by polyacrylamide gel electrophoresis (PAGE) and immunoelectron microscopy (21). Sixteen stool specimens were from patients with diarrhea that occurred in December 1989 and January 1990 in a nursery school in the northern district of Bristol, England, the same district in which an outbreak of group C rotavirus in a family in which there was a fatal case of disease has been described recently (8).

In addition to the human specimens, six non-group A porcine strains were analyzed that were collected from piglets experimentally infected with strain N338 (group B) or Cowden (group C) or naturally infected in Ohio and Thailand.

Strains of group A rotavirus were from our characterized collection (13).

Primers. The primers for group C and B rotaviruses were designed from the available sequence of gene 6 of a group C (Cowden) strain (2) and from unpublished sequence information for gene 8 of a group B strain. The sizes and positions of these primers are shown in Fig. 1, and their sequences (5′ to 3′) are as follows: C1, CTCGATGCTACTACAGAAT CAG; C3, GGGATCATCCACGTCATGCG; C4, AGCCAC ATAGTTCACATTTCATCC; B1, CTATTCAGTGTGTCG TGAGAGG; B3, CGAAGCGGGCTAGCTTGTCTGC; and B4, CGTGGCTTTGGAAAATTCTTG.

Primers Beg9 and End9, which were designed to amplify the complete gene 9 that encodes the VP7 protein of group A rotavirus, have been described previously (12).

PCR detection assay. The procedures for the preparation of viral RNA from stool specimens and amplification of rotavirus genes were those described previously for the gene 9 group A rotavirus (12), with the introduction of a simple RNA purification step. Briefly, 200 μl of a clarified 50% stool suspension made in 10 mM phosphate saline buffer was incubated with 1% sodium dodecyl sulfate and 100 μg of proteinase K per ml for 30 min at 56°C. The suspension was extracted with an equal volume of phenol-chloroform (1:1) and again with chloroform alone. The aqueous phase was then transferred to tubes containing 50 μl of hydroxyapatite (HA), vortexed three times, and spun for 3 min in an Eppendorf centrifuge. HA was prepared as described by Bernardi (1). The fluid was removed and the HA crystals were washed with 10 mM potassium phosphate pH 6.8. The RNA adsorbed was then eluted with 50 μl of 200 mM potassium phosphate by vortexing three times. After centrifugation, either 5 μl of the clear RNA solution was used directly for the reverse transcription (RT) and PCR amplification or the RNA was further concentrated by precipitation in ethanol. In the latter case, 0.1 volume of 3 M sodium acetate and 3 volumes of absolute ethanol were added to the eluate and kept at −70°C for 2 h. The precipitated RNA was collected by centrifugation, washed with 70% ethanol, dried, and suspended in the desired amount of water to be used in the RT PCR. These reactions were performed as described previously (12), using 30 cycles of 94°C for 1 min, 42°C for 2 min, and 72°C for 1 min and a final incubation at 72°C for 7 min. The primer pairs B1-B4, C1-C4, and Beg9-End9 were used separately or as a pool of primers in a first PCR detection assay.

PCR confirmation assay. A second amplification with a double-stranded DNA template formed in the first amplification was performed with one of the primers used for detection (first amplification) and a nested primer. Thus, primer pairs B1-B3 or C1-C3 were used to confirm the identifications of group B and C rotaviruses, respectively. The procedure was the same as that described previously for typing group A rotavirus as a second amplification (12).

Agarose gels. PCR products (10 μl) were analyzed in 2% NuSieve–1% SeaPlaque agarose in Tris-borate buffer containing 0.5 μg of ethidium bromide per ml that was electrophoresed at 180 V for 30 min and photographed under UV light.

RESULTS

The use of HA to purify and concentrate viral double-stranded RNA from fecal extracts efficiently removed inhibitors of the RT PCR. It also proved to be a fast and convenient means of preparing rotavirus RNA for PAGE analysis, completely eliminating background in silver-stained gels (data not shown).

The PCR detection assay was performed for each individual rotavirus group in order to assess the specificity of each pair of primers, and then all six detection primers were combined in a single PCR assay for all three groups of rotavirus. Three specimens known to contain group A, B, or C rotavirus by PAGE and immunoelectron microscopy were selected for these tests, and the amplification results are shown in Fig. 2. Using the primer pair B1-B4 (PCR/B), only the group B sample (Chengde) produced the expected 489-bp DNA segment (Fig. 2, lanes B). Similarly, by using the primer pair C1-C4 (PCR/C), a 356-bp segment was produced with the group C rotavirus sample (Cowden) but not with the group A or B rotavirus samples (Fig. 2, lanes C). This same pair (C1-C4) gave a strong band of approximately 200 bp by amplification of the group B rotavirus and a weak band of 220 bp with the group A strain. These smaller segments, however, did not interfere with the test because they could easily be discerned from the diagnostic segment produced by amplification of group C rotavirus. An increase in the stringency of the PCR did not improve the results. As reported previously (12), the primer pair Beg9-End9 showed excellent specificity, amplifying only group A rotavirus (Fig. 2, lanes A).

When a combined PCR detection assay was performed with a pool containing all six detection primers, all three groups of rotavirus could be amplified, and each produced only the segment expected for its group (Fig. 2, lanes P). The cross amplification produced by the pair C1-C4 with group A and B viruses was less evident, because the presence of

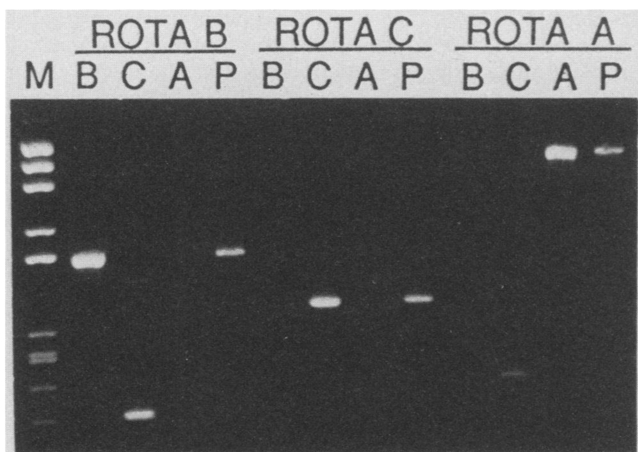

FIG. 2. PCR detection assay for group B, C, and A rotaviruses using the single pair of primers B1-B4 (lanes B), C1-C4 (lanes C), and Beg9-End9 (lanes A) or a pool of these three pairs of primers (lanes P). The marker (lane M) is a mixture of φX174*Hae*III-digested DNA and a 500-bp segment produced with control reagents from the GeneAmp kit (Cetus Corp.).

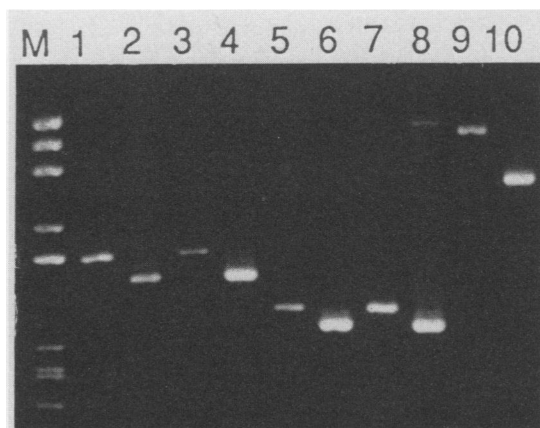

FIG. 3. PCR confirmation assay. A 1/100th amount of the DNA obtained in the first amplification and shown in lanes 1 and 3 for group B rotavirus, lanes 5 and 7 for group C rotavirus, and lane 9 for group A rotavirus was submitted to the PCR confirmation tests (second amplification) for group B (lanes 2 and 4), C (lanes 6 and 8), or A (lane 10) rotavirus. The PCR confirmation test for group A rotavirus constitutes the PCR typing method described by Gouvea et al. (12) and indicates a serotype 1 for the strain tested. The marker (lane M) is a mixture of φX174*Hae*III-digested DNA and a 500-bp segment produced with the control reagents from the GeneAmp kit.

primers with a perfect, or almost perfect, match in the primer pool preferably annealed to the template. However, amplification in the presence of the pool of primers seemed to result in a small decrease in the sensitivity of the assay, as can be seen by the reduced amount of specific product shown in Fig. 2, lanes P.

The PCR detection tests were then applied to the stool specimens, and the results are shown in Table 1. Application of both the PCR/B and the combined PCR tests to the 20 Chinese specimens identified group B rotavirus in 19 samples and a group A rotavirus in the remaining sample (F22) that could not be amplified by the PCR/B test. Screening of the other human specimens by both PCR/C and the combined PCR tests gave identical results, indicating the presence of group C rotavirus in all 5 Asian specimens and in 13 of the 16 English ones. Ethanol precipitation of the remaining 45 μl of the eluate before PCR/C amplification, instead of using only 5 μl of the eluate directly for amplification, did not alter the detection results, but produced slightly stronger DNA bands for those samples already known to contain group C rotavirus. All seven porcine specimens were correctly identified as containing group B (four samples) or group C (three samples) rotaviruses.

Further identification of the products generated in the PCR detection assays was accomplished by a second amplification with group-specific nested primers B3 and C3 (Fig. 3). Primer pair B1-B3 or C1-C3 was used to amplify portions of the first PCR product in the confirmation assays for group B or C rotavirus, respectively. Aliquots (1.0 to 0.01 μl) of the first PCR products were amplified with the nested primers, generating large amounts of internal segments. All rotavirus samples identified as group B or C in the first amplification were confirmed by this assay. In addition, the three English samples that could not be diagnosed in the first PCR amplification produced the characteristic 327-bp internal segment for group C rotavirus. For group A rotavirus, the second amplification or PCR typing method (12) identified a serotype 1 strain in the Chinese sample F22 (Fig. 3, lane 10).

To evaluate the sensitivity of the assay, 100 μl of a stool extract containing group A rotavirus was added to 10 μl (2 μg) of purified ADRV (10) prepared in 10-fold serial dilutions. The mixtures were treated with HA, and 1/10th (5 μl) of the eluates were submitted to the combined PCR assay. Electrophoresis of 10 μl of the amplified products is shown in Fig. 4A. Corresponding amounts of purified ADRV RNA were tested directly in the PCR assay (Fig. 4B). The end-point dilution for the detection of group B rotavirus was the same in both experiments, indicating complete recovery of RNA from the adsorption and elution steps involved in the specimen preparation. Up to a 10^5 dilution of rotavirus B RNA produced a clearly visible segment on ethidium bromide-stained gels. This represents 2 pg of RNA tested, or 120 fg of template RNA, considering that gene 8 contributes approximately 6% of the total RNA of the virus. The reproducibility of the assay can be appreciated by the uniform amount of the 1,062-bp segment representing the amplification of group A rotavirus RNA present in the stool specimen (Fig. 4A, lanes 4 to 8).

When two rotavirus groups were amplified together, a competition existed in which, with increased amounts of rotavirus B RNA in the mixture, the level of detection of rotavirus A declined (Fig. 4A, lanes 3 to 1). This competition, which favored the amplification of a smaller segment (in

TABLE 1. PCR detection and identification of rotavirus serogroups

Serogroup	No. of samples (source)	No. of samples identified by:		
		PCR/B (origin)	PCR/C	PCR/combined (serogroup)
B	20 (China)	19	0	19 (B) +1 (A)
	4 (pigs)	4	0	4 (B)
C	5 (Asia)	0	5	5 (C)
	16 (England)	0	13	13 (C) + 3 (C)[a]
	3 (pigs)	0	3	3 (C)

[a] Three samples were identified as group C after second amplification.

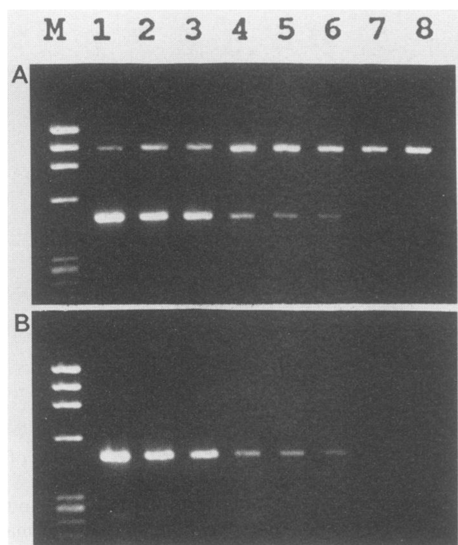

FIG. 4. Sensitivity of the combined PCR assay. A 10-fold serial dilution of purified ADRV RNA was added to an extracted stool specimen containing group A rotavirus and treated with HA as described in the text. (A) Amplified products of 5-μl aliquots of eluates subjected to the combined PCR detection assay. (B) The same assay shown in panel A with corresponding amounts of pure ADRV RNA. Amounts of total ADRV RNA in the PCR test ranged from 200 ng (lanes 1) to 2×10^{-7} ng (lanes 8). Lanes M, φX174*Hae*III-digested DNA marker.

this case, the group B-specific product) at the expense of a larger one (group A product), only affects the detection of group A rotavirus when the latter is present in a relatively small quantity, such as in this experiment.

DISCUSSION

The PCR technique is particularly well suited for the detection of viruses that cannot be cultivated in vitro. Like tissue culture, it is an amplification technique, measuring not the nucleic acid actually present in the clinical specimen but copying it 10^6-fold for detection. In our previous work (12), we applied the PCR technique to amplify the VP7 gene of the common group A rotavirus in order to generate nucleic acid material for molecular studies and to identify the virus type in clinical specimens that failed to be serotyped by EIA with monoclonal antibodies (13). The procedure used to prepare viral RNA from stool specimens involved deproteinization with phenol-chloroform followed by ethanol precipitation, which is the method commonly used to prepare rotavirus genomic RNA for PAGE analysis. This treatment, however, was not sufficient to eliminate substances that inhibit the subsequent enzymatic reactions with reverse transcriptase and *Taq* polymerase. These inhibitors were present in only a small number of specimens, which had to be diluted prior to RT PCR, and the sensitivity of the method was proportionally reduced. Removal of these inhibitors was important in rendering the PCR technique more sensitive, and several methods have been described. Xu and colleagues (27) used glass in the presence of sodium iodide and Wilde et al. (26) used chromatographic cellulose fiber (CF-11) to selectively adsorb rotavirus RNA from the stool extracts, dramatically increasing the sensitivity of the RT PCR with stool specimens. We chose HA for the same purpose and obtained similar results. With the present method, however, we used the eluted RNA directly in the RT PCR, bypassing ethanol precipitation. In practical terms, this represents a marked reduction in sample manipulation and the time required for the test. Adsorption, washing, and elution were performed in the same tube containing the extracted RNA. Further concentration of the eluted RNA by ethanol precipitation did not improve virus detection.

Increased sensitivity was achieved by using the second PCR amplification. DNA copies produced in the first amplification of viral double-stranded RNA obtained from three English specimens were not visible following the first amplification but produced the diagnostic group C segment upon the second amplification. The increased sensitivity with a second amplification has been demonstrated previously for PCR typing of group A rotavirus by using, as templates, the DNA copies of the entire gene 9 (produced in a first PCR amplification). Most PCR detection protocols involve hybridization of the PCR products with ^{32}P-labeled internal probes. This procedure increases both the sensitivity and the specificity over those obtained with ethidium bromide staining. Nevertheless, the same high specificity, and perhaps better sensitivity, can be achieved with a second amplification with internal primers as described here, with the additional advantages that only one kind of test and no radioactive material are used.

The construction of primers specific for group B rotavirus was limited by the availability of only a partial sequence for the gene 8 segment. Three primers designed for this gene, B1, B3, and B4, were able to anneal to both the human and animal strains tested, suggesting that these chosen regions might be conserved among the group B strains. Studies of additional rotavirus B strains will be necessary to confirm the universality of these primer sequences. The recent cloning of other genes of ADRV will provide alternative choices for primers in the future (9).

The primers for group C rotavirus were synthesized from the only sequence of gene 6 that was available (2). The gene-coding assignments for the group C rotavirus remain to be established, but if they are analogous to those of group A, gene 6 would code for the major inner capsid protein VP6 that contains a group-specific antigen(s). The three primers C1, C3, and C4 recognized all the human and porcine strains of group C rotavirus tested. Unexpectedly, primers C1 and C4 seemed to recognize similar sequences in the genome of group B strains, but the segment generated was distinctively smaller than the one predicted for group C rotavirus and, therefore, did not interfere with the diagnostic test. Furthermore, this cross-annealing, or cross-reaction, was greatly reduced in the combined PCR assay.

The combined PCR for the simultaneous detection of all three known human groups of rotavirus in a single assay presents advantages in terms of economy of time, reagents, and sample size required for the assay. Although its sensitivity seems to be slightly lower than those of individual PCR assays, in the present study, all rotaviruses detected in the individual assays were also detected in the combined assay. Furthermore, the group A rotavirus present in one of the samples obtained during the Changchum outbreak (F22) could be readily detected and later identified as a serotype 1 strain in the PCR typing assay. This sample, known to contain rotavirus by electron microscopy but negative by PAGE, gave inconclusive results when tested in EIAs for group A and B rotaviruses because of high nonspecific reactions in both assays (data not shown).

The ability of the combined PCR assay to detect different rotavirus groups in a sample might help uncover mixed

group infections. Mixed human rotavirus infections have been observed in many laboratories by using PAGE as a screening test, and in two surveys they accounted for 10% of the rotavirus-positive specimens (20, 24). The appearance of more than 11 double-stranded RNA segments within the characteristic size range of the rotavirus genome indicates the presence of more than one rotavirus strain or genotype. The rapid evolution of RNA viruses producing heterogeneous viral populations (14) and the coexistence of several rotavirus strains resulting in occasional multiple infections are thought to account for the presence of the extra segments; however, with rare exceptions (13), mixed infections have not been characterized. The sensitive PCR detection assay described here might prove to be useful in unmasking non-group A rotaviruses in possible cases of mixed infections with group A rotavirus. Because PCR preferentially amplifies smaller segments, it is expected that amplification of group C (356 bp)- and B (489 bp)-specific segments will preferably be generated over the one for group A (1,062 bp).

It is common practice to dilute the stool specimens received for viral analysis and to freeze the remaining suspensions for further tests. Probably because group A rotavirus is often excreted in large amounts and the conventional diagnostic methods (electron microscopy, PAGE, and EIA for group antigens) do not require complete or intact virions, detection was still successful after several cycles of freezing and thawing of the diluted samples. Degradation of the viral outer capsid (containing the type-specific antigens), however, becomes an important problem for serotyping rotavirus by EIA (6). We have noted degradation not only of the viral capsid but also of the double-stranded RNA of group A rotavirus in diluted fecal suspensions by PAGE analysis (unpublished data) and recently by PCR. Storage of samples in their natural state has been suggested for good viral preservation (6) and might be particularly important for the detection of group B and C rotaviruses.

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