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Detection of Animal and Human Group B Rotaviruses in Fecal Specimens by Polymerase Chain Reaction

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A combined reverse transcriptase reaction-polymerase chain reaction (RT-PCR) was developed to achieve the sensitive detection of group B rotaviruses (GBR). Sequences derived from genomic segment 3 of the IDIR (intestinal disease of infant rats) strain of GBR permitted the detection of ≥0.08 pg of purified IDIR genomic RNA (4,000 genome copies). Primers complementary to the terminal sequences of gene 11 of GBR strain ADRV (adult diarrhea rotavirus) allowed for the detection of as little as 0.008 pg of purified ADRV genomic RNA. Detection of heterologous strains of GBR was also observed with these primer pairs. IDIR gene 3 primers recognized ≥8 pg of RNA from bovine GBR obtained from a variety of geographic locations. RNA from IDIR, but not bovine GBR, strains was detected by means of RT-PCR with ADRV gene 11 primers. Neither set of GBR primers was reactive in RT-PCR with fecal specimens containing group A rotaviruses or fecal specimens from uninfected controls. This RT-PCR assay permits the sensitive and specific detection of a variety of GBR in fecal specimens.

During the last decade, group B rotaviruses (GBR) have been identified as the etiologic agents of diarrhea in humans as well as in a variety of animal species (1). Epidemics of human diarrhea caused by the ADRV strain of GBR have been described in China (10). In addition, the IDIR strain of GBR has been reported to infect small numbers of humans outside of China (6). These GBR are antigenically and genetically distinct from group A rotaviruses (GAR), which are usually associated with wintertime outbreaks of infantile diarrhea in temperate climates (16). Thus, GBR cannot be detected by assays, such as the currently available commercial immunoassays, specific for GAR antigen. Immunoassays and immune electron microscopy for the detection of GBR have been developed (14, 15, 22). However, GBR are not cultivatable in vitro, so antiserum reagents directed against GBR are not widely available. Alternate techniques for the identification of GBR are available, but these methods also present difficulties in their application. Detection of rotavirus by means of direct electropherotype analysis usually requires the presence of ≥10 ng of viral RNA (3, 9). The electropherotype profiles of GBR as well as other non-group A rotaviruses can also be difficult to interpret, requiring the use of confirmatory assays (16). Nucleic acid hybridization has proved useful for GBR identification, but this method requires the use of specific cloned probes (4, 5). Furthermore, although hybridization is a relatively sensitive technique, requiring as little as 500 pg of GBR RNA for detection (5), more than 10^7 genome copies of GBR are needed to obtain this quantity of RNA.

Our understanding of the epidemiology and transmission of GBR would be aided by the development of a sensitive and specific GBR assay which could be performed without access to scarce GBR-specific reagents. The polymerase chain reaction (PCR) offers such a possibility (17). We have recently developed an RNA amplification assay which combines a reverse transcriptase (RT) reaction and a PCR (RT-PCR) for the identification of GAR in fecal specimens (23). The RT-PCR has permitted the detection of GAR at concentrations significantly lower than those required for detection by means of alternate diagnostic procedures. We have now developed an RT-PCR which specifically detects GBR in fecal specimens and offers substantially increased sensitivity relative to those of previously available assays.

MATERIALS AND METHODS

Viruses. The IDIR strain of GBR from Baltimore, Md., was propagated in infant CD-1 rats (Charles River Laboratories) as previously described (22). Calf fecal specimens containing GBR were kindly provided by Alfonso Torres (New York State College of Veterinary Medicine, Cornell University, Ithaca) (21). Two different electropherotypes were observed among the GBR isolates provided by A. Torres. Additional calf fecal specimens containing bovine GBR strains D522 and D531 were kindly supplied by D. R. Snodgrass (Animal Diseases Research Association, Edinburgh, Scotland) (19). A human fecal specimen containing the ADRV strain of GBR was obtained from an ill patient in China and was provided by Zhao-Yin Fang (Institute of Virology, Chinese Academy of Preventive Medicine, Beijing, China).

Fecal specimens containing the NCDV (Nebraska calf diarrhea virus) strain of GAR were also obtained from an infected gnotobiotic calf. Strain SA-11 (serotype 3) of GAR was obtained from H. Malherbe (Salt Lake City, Utah) and propagated in MA-104 tissue culture cells (24). Fecal specimens were also obtained from uninfected calves aged 1 day to 8 weeks and housed on a farm in Maryland. We have not previously detected GBR infection in animals from this farm, and examination of these calf fecal specimens by means of polyacrylamide gel electrophoresis (PAGE), electron microscopy, and immunoassay has not revealed GBR (5).

Electropherotypes. Direct electropherotype analysis of rotavirus double-stranded genomic RNA was performed by means of the procedure described by Herring et al. (9). Extracted RNA segments were separated by electrophoresis through 7.5% polyacrylamide gels and stained with silver nitrate for visualization.

Fecal specimens. Liquid fecal samples were transferred to

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sterile glass vials with individual disposable pipettes. Solid samples were diluted 1:1 (vol/vol) with 0.01 M phosphate-buffered saline (pH 7.4)–0.01 M $CaCl_2$–0.01 M $MgCl_2$ prior to transfer. Samples were stored at −70°C until tested.

Purification of rotavirus RNA. CsCl purification of ADRV and the subsequent extraction of genomic RNA have been described by Fang and co-workers (7).

IDIR and bovine strains of GBR were not stable in CsCl gradients, so large-scale preparation of genomic RNA from fecal specimens was performed by means of CF-11 cellulose chromatography (20).

Preparation of 50-μl portions of fecal specimens and tissue culture lysates for subsequent testing with RT-PCR has been described in detail elsewhere (23). In brief, a 50-μl portion of thawed fecal specimen was combined with 50 μl of 0.01 M phosphate-buffered saline (pH 7.4)–0.01 M $CaCl_2$–0.01 M $MgCl_2$ in a 1.5-ml polypropylene microcentrifuge tube. After the addition of 200 μl of extraction buffer (0.2 M glycine, 0.1 M Na_2HPO_4, 0.6 M NaCl, 1% sodium dodecyl sulfate [pH 9.5]), the solution was extracted with phenol-chloroform and the RNA was purified with CF-11 cellulose. The resulting RNA pellet was washed and suspended in 25 μl of 1× TE buffer (10 mM Tris hydrochloride [pH 7.4], 0.1 mM EDTA).

Amplification of rotavirus RNA from fecal samples. The RT-PCR procedure was performed by methods developed in our laboratory (23). Adherence to protocols to diminish the possibility of false-positive results was strictly observed throughout all RT-PCR steps (11, 12). A 10-μl aliquot of the resuspended RNA was boiled for 5 min, cooled on ice, and combined with 40 μl of RT reaction mixture such that the final solution contained 200 μM each deoxyribonucleotide triphosphate, 1 μM each oligodeoxyribonucleotide primer, 50 mM Tris hydrochloride (pH 8.3), 5 mM $MgCl_2$, 75 mM KCl, 10 mM dithiothreitol, 0.1 μg of bovine serum albumin per μl (Bethesda Research Laboratories, Gaithersburg, Md.), and 100 U of Moloney murine leukemia virus RT (Bethesda Research Laboratories). After 60 min of incubation at 37°C, the reaction was stopped by boiling for 5 min, and the contents were cooled on ice. Ten microliters of each RT reaction product was added to a 90-μl reaction mixture containing 1.0 μM each oligodeoxyribonucleotide primer, 200 μM each deoxyribonucleotide triphosphate, 50 mM KCl, 10 mM Tris-Cl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, and 2.5 U of *Taq* polymerase (Perkin Elmer Cetus, Norwalk, Conn.). The PCR was performed on this mixture by use of 25 cycles on a DNA thermocycler (Perkin Elmer) with denaturation for 1 min at 94°C, reannealling for 1 min at 55°C, and elongation for 1 min at 72°C. Identical times and temperatures for PCR cycles were used for all primer pairs. Amplified DNA was identified following electrophoresis of a 10-μl aliquot of the PCR mixture through a 7.5% polyacrylamide gel and staining with silver nitrate. Positive reactions were identified by the visualization of a band of characteristic size on the gel. In some cases, the nature of the amplified DNA was confirmed by hybridization with a ^{32}P-labeled probe by established techniques (4, 5).

For determination of the sensitivity of the RT-PCR with purified RNA, the RNA was quantified by means of optical density measurements and confirmed by comparison of RNA bands with nucleic acid standards following PAGE and silver nitrate staining. Serial dilutions of the RNA were made in TE buffer and added to reaction mixtures as described above.

The sensitivity of detection of GBR was also determined with serial dilutions of infected feces. The quantity of viral double-stranded RNA present in fecal specimens was determined by comparison with known quantities of nucleic acid following PAGE and silver nitrate staining (8). Portions of fecal specimens were serially diluted in another fecal specimen which was known to be nonreactive by means of the RT-PCR. Fifty-microliter portions of these dilutions were extracted, and the RNA was CF-11 purified and added to RT-PCR mixtures as described above.

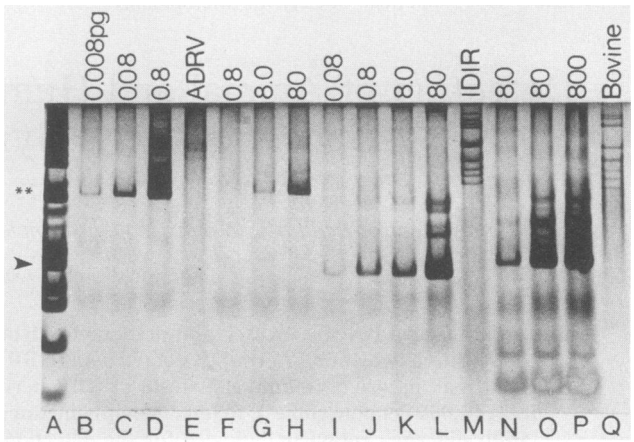

FIG. 1. Polyacrylamide gel of purified GBR RNA and RT-PCR samples. Lane A contains *Hae* III restriction digest fragments of DNA from bacteriophage φX174 as markers. Lanes E, M, and Q contain GBR genomic RNAs from ADRV, IDIR, and bovine strains, respectively. RT-PCR was performed as described in Materials and Methods with various concentrations of viral RNA, as indicated above the lanes. Samples (10 μl) of each reaction were subjected to PAGE, and nucleic acid bands were visualized by means of silver staining. Lanes B to D represent reactions with primers 2 and 2R and purified ADRV RNA. Lanes F to H represent reactions with primers 2 and 2R and purified IDIR RNA. Lanes I to L represent reactions with primers 1 and 1R and purified IDIR RNA. Lanes N to P represent reactions with primers 1 and 1R and purified bovine GBR RNA. The arrowhead indicates the position of the 276-bp product following PCR with primers 1 and 1R. Asterisks indicate the position of the 631-bp product following PCR with primers 2 and 2R.

Oligonucleotide primers. Primer 1 consisted of bases 898 to 920 in the positive strand of IDIR gene 3: (5′)-ATCATGG AGGCCGGCCACAGACT-(3′). Primer 1R was derived from the complementary segment between bases 1175 and 1150 of IDIR gene 3: (5′)-CTAGAAGTATCTATCTGTGCAAA GCC-(3′). Primer 2 consisted of bases 1 to 19 in the positive strand of ADRV gene 11 (2): (5′)-GGTATATAAAAGTC AGTAG-(3′). Primer 2R was derived from the complementary segment between bases 631 and 614 of ADRV gene 11 (2): (5′)-GGGTTTTTTAAATATAAC-(3′).

RESULTS

Oligonucleotide primers for RT-PCR were chosen from a region of IDIR genomic segment 3 which was previously shown to exhibit nucleic acid hybridization with bovine strains and ADRV strains of GBR (5, 18). A characteristic band of 276 bp was observed on PAGE after reaction of primers 1 and 1R with IDIR RNA (Fig. 1). As little as 0.08 pg of purified genomic RNA was detectable by this reaction. IDIR RNA in fecal specimens from infected rat pups could also be detected if present in concentrations of ≥0.8 pg. By comparison, 10 ng of genomic RNA was required to identify clearly the rotavirus electropherotype after direct extraction of RNA and silver staining by the technique of Herring et al. (9).

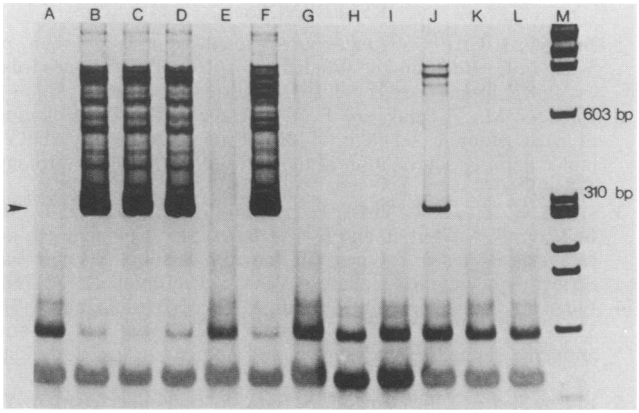

FIG. 2. Polyacrylamide gel of PCR products following reaction of bovine fecal specimens with IDIR gene 3-encoded primers. Double-stranded RNA was extracted from fecal specimens from infected and uninfected calves. Fecal inhibitory substances were removed by means of CF-11 cellulose chromatography. Extracted RNA was added to the RT reaction mixture containing primers 1 and 1R. Following the RT reaction, 10-μl samples were transferred to the PCR mixture as described in Materials and Methods. After completion of the amplification cycles, 10-μl samples of the RT-PCR mixture were visualized by means of PAGE and silver staining. The arrowhead indicates the position of the 276-bp PCR product. Additional RT-PCR assays of calf specimens were performed as described in the text, but the samples were visualized on other polyacrylamide gels. Lanes: A, GBR-infected calf specimen 75-63; B, GBR-infected calf specimen 76-17; C, GBR-infected calf specimen 79-60; D, GBR-infected calf specimen 80-3; E, GAR strain NCDV-infected calf specimen 81-4; F, GBR-infected calf specimen 81-11; G, uninfected calf specimen (calf age, 6 h); H, uninfected calf specimen (calf age, 212 h); I, uninfected calf specimen (calf age, 48 h); J, purified bovine GBR RNA (80 pg); K, GAR strain SA-11; L, RT-PCR without added RNA; M, size markers.

The ADRV strain of GBR hybridized with cDNA probes synthesized from IDIR genomic segment 3 but did not react in RT-PCR with IDIR primers 1 and 1R or with 13 additional primer pairs derived from IDIR gene 3 sequences. However, oligonucleotide primers complementary to the 5′ and 3′ termini of ADRV genomic segment 11 (primers 2 and 2R) provided sensitive detection of ADRV, with a PCR product of 631 bp (Fig. 1). RT-PCR with this primer pair was capable of detecting as little as 0.008 pg of purified ADRV genomic RNA. When fecal specimens containing ADRV were extracted and CF-11 purified, RT-PCR detected ≥0.08 pg of whole genomic RNA. ADRV primers 2 and 2R also permitted the detection of ≥0.8 pg of CF-11-purified IDIR RNA.

Bovine strains of GBR were evaluated for reaction with primer pairs complementary to ADRV or IDIR nucleic acid sequences. Bovine GBR RNA was not detectable by RT-PCR with ADRV primers 2 and 2R. However, bovine GBR RNA could be detected by RT-PCR with IDIR primers 1 and 1R when genomic RNA was present at ≥8 pg (Fig. 2). We also used RT-PCR to detect virus in fecal specimens from eight GBR-infected calves from the United States and Scotland. Fecal specimens from seven of these calves contained GBR, as determined by genome electropherotype analysis. All seven fecal specimens were reactive in RT-PCR with IDIR primers 1 and 1R. The eighth specimen showed no PCR product (Fig. 2A) and was also found negative for the presence of GBR by nucleic acid hybridization, genome electropherotype analysis, immune electron microscopy, and enzyme immunoassay. Fecal specimens from 10 uninfected calves as well as a calf infected with the NCDV strain of GAR did not react in RT-PCR. GBR strains from other sources, such as pigs and lambs, were not available for testing by RT-PCR with these primer pairs.

DISCUSSION

The sensitivities of these GBR RT-PCR assays are comparable to those reported for RT-PCR assays for GAR (23). As little as 0.08 pg of purified IDIR RNA (or approximately 4,000 genome copies) could be detected by RT-PCR with primer pairs 1 and 1R. Detection of IDIR RNA in extracted fecal specimens was almost as sensitive, requiring 0.8 pg of genomic RNA. Detection of ADRV was also quite sensitive, with primers 2 and 2R capable of detecting 0.008 pg of purified RNA or 0.08 pg of RNA extracted from fecal samples. We previously found that it was necessary to perform a chromatographic step in the stool extraction process to remove the inhibitory activity that greatly decreased the sensitivity of RT-PCR (23). The 10-fold-decreased detection of GBR in fecal specimens may have been due to residual inhibitory activity in the fecal extract. Alternatively, the decreased detection of GBR in fecal specimens may have resulted from losses of viral RNA during the extraction process.

The sensitivity of GBR RT-PCR is substantially greater than that demonstrated for nucleic acid hybridization, which previously was the most sensitive means for the detection of GBR (5). While hybridization permits the identification of as little as 500 pg of GBR genomic RNA in fecal specimens, this amount is at least 100-fold more than that required for detection by GBR RT-PCR. The increased sensitivity offered by GBR RT-PCR is even greater when this assay is compared with other assays. Direct electropherotype analysis has frequently been used for the detection of GBR in fecal specimens, but electropherotyping generally requires ≥10 ng of genomic RNA to discern clearly the electropherotype pattern (3, 9). Electropherotype analysis is thus 100,000-fold less sensitive than is our RT-PCR for the detection of GBR.

To detect heterologous GBR strains, we selected PCR primers 1 and 1R on the basis of the results of hybridization experiments with IDIR cDNA clones (5). The region of IDIR gene 3 flanked by these primers appeared to hybridize most intensely with ADRV and bovine strains of GBR (unpublished data), indicating the conservation of nucleic acid sequences in this region. Indeed, primers 1 and 1R permitted RT-PCR with IDIR and bovine strains of GBR isolated from two different geographic regions of the United States (21) and two different bovine GBR strains from Scotland (19). In contrast, ADRV RNA was not detectable with primers 1 and 1R or any of 13 additional primer pairs complementary to sequences from other regions of IDIR gene 3. However, ADRV and IDIR were both detected by RT-PCR with primers complementary to the 5′- and 3′-terminal sequences of ADRV gene 11. Bovine GBR did not react with ADRV primers 2 and 2R. The specificity demonstrated by primer pairs 1-1R and 2-2R should permit one to distinguish among ADRV, IDIR, and bovine strains of GBR. Reaction with both primer pairs 1-1R and 2-2R would indicate the presence of IDIR, while ADRV would react only with 2-2R and bovine GBR would react only with 1-1R. The performance of RT-PCR with both primer pairs permits the detection of

these GBR with substantially greater sensitivity than that previously available by alternate techniques. Additional studies should be performed to determine whether this improved sensitivity will lead to increased detection of animals and humans who are infected with GBR.

The RT-PCR results observed with GBR strains heterologous to the sources of the primers are consistent with recent reports concerning the effects of primer-template mismatches on PCR (13). Single mismatches in the internal portion of a primer appear to have little effect upon PCR yield, but single mismatches at the 3′ terminus of a primer can diminish PCR sensitivity by 100-fold. Although the sequences of the bovine GBR strains are not known, the decrease in sensitivity observed in PCR with IDIR primers 1 and 1R and these heterologous GBR strains might be explained by these mismatches. Double mismatches in the 3′ terminus can decrease PCR yields even more dramatically than can single mismatches. Double mismatches might account for the total inability of IDIR primers 1 and 1R to recognize ADRV RNA and the failure of ADRV primers 2 and 2R to react with bovine GBR RNA. These results indicate that it may be difficult to identify a single primer pair which will react with all GBR strains. For selection of such primers, additional nucleic acid sequence analyses of diverse GBR strains will be needed.

While the use of RT-PCR can strikingly increase the sensitivity of detection of GBR, the procedure is also valuable because it does not require access to scarce GBR reagents. The sensitive detection of GBR in fecal specimens has previously been hindered by the limited supply of these reagents. GBR have not yet been adapted to growth in tissue cultures, and since the production of reagents requires the purification of virus from fecal specimens, the availability of GBR antigens as well as anti-GBR antisera has been limited. In contrast, RT-PCR can be performed with readily available reagents and custom synthesis of specific primer pairs. Thus, the detection of GBR in fecal specimens can be accomplished by RT-PCR without the need for anti-GBR antisera.

The principal disadvantages of the PCR are the cost of the enzyme and the equipment and the risk of generating false-positive results. While the relatively expensive thermocycler is not absolutely required for the performance of the PCR, manual performance of the PCR is very labor-intensive. Also adding to the labor-intensive nature of the PCR is the need for rigorous adherence to protocols to prevent the appearance of false-positive results (11, 12). Because of the tremendous amplification of the PCR, even the slightest amount of contamination can result in false-positive results. Careful attention to proper procedures and the inclusion of appropriate control specimens are mandatory for any PCR assay. However, if quality control procedures are faithfully maintained, multiple specimens can be analyzed by the PCR within just a few hours.

For laboratories familiar with PCR technology, the RT-PCR protocol allows the sensitive and specific detection of GBR in fecal specimens, in turn allowing for more widespread investigations for GBR in additional geographic regions, animal reservoirs, and human populations. These studies are likely to aid in defining the epidemiology of GBR and the clinical syndromes associated with GBR infections.

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