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# Diagnosis of Rotavirus Infection with Cloned cDNA Copies of Viral Genome Segments

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**The diagnostic potential of cloned cDNA copies of human rotavirus (strain WA) genome segments for the detection of rotavirus in clinical specimens has been determined. A hybridization assay in which a mixture of $^{32}$P-labeled cDNAs representing the 11 rotavirus segments was used as a probe compared favorably with three frequently used diagnostic tests for rotavirus in terms of both specificity and sensitivity. Significantly, clinical isolates could be readily distinguished when cloned cDNA copies of individual genome segments were used independently as a probe. In assays in which genome RNA from rotaviruses of known subgroups and serotypes were tested, cloned probes that encode nonstructural viral proteins hybridized efficiently to genome RNAs of all strains, whereas cloned probes corresponding to genome segments 6 and 9 exhibited the potential for differentiating strains of different subgroups and serotypes. Cloned cDNA copies of rotavirus genome segments therefore offer considerable potential for improved general diagnosis of rotavirus in clinical specimens, as well as for epidemiological studies in which virus isolates can be distinguished on the basis of nucleotide sequence homology of individual genome segments.**

As causative agents of acute gastroenteritis in humans and young domestic livestock, rotaviruses, members of the family *Reoviridae*, are responsible for many million infant deaths annually in developing countries and are of considerable economic importance to farmers and stock breeders (for reviews, see references 3 and 7). The rotavirus genome consists of 11 segments of double-stranded RNA which range in molecular weight from $0.46 \times 10^6$ to $2.6 \times 10^6$. cDNA copies of the genome segments of human rotavirus (strain Wa) have recently been cloned (8) and characterized (1, 8, 17, 19; see Fig. 2). In this communication, we examine the potential of a dot hybridization assay in which cloned cDNAs are used as a probe for the detection and differentiation of rotaviruses in clinical specimens.

Initially, the sensitivity and specificity of the hybridization assay were determined by hybridizing the cloned rotavirus cDNAs to immobilized genome RNAs purified from other members of the family *Reoviridae*. $^{32}$P-labeled cDNAs representing sequences of all 11 human rotavirus genome segments were hybridized to denatured genome RNA of a plant reovirus (wound tumor virus), human reovirus (Dearing strain, serotype 3), bovine rotavirus (NCDV Lincoln strain), and homologous human rotavirus (Wa strain). As little as 1 ng of homologous genome RNA could be detected with this method, whereas hybridization to the genome RNAs of the plant and human reoviruses was negative even at high (>50 ng) RNA concentrations (Fig. 1A). As expected, the human rotavirus cDNAs did hybridize to the bovine rotavirus genome RNA, but at one 10th the level obtained in the homologous hybridization reaction. No signal was observed when labeled plasmid pBR322 DNA was used as a probe in hybridization reactions with either purified genome RNAs or extracts of stool specimens.

A panel of 31 fecal specimens obtained from patients with gastroenteritis were tested for rotavirus by dot hybridization assay and by three other, more frequently used methods. Polyacrylamide gel electrophoresis followed by silver staining (PAGE-SS) (6, 14) was used as the reference test because of its high sensitivity, the unambiguous nature of positive results, and the quantity of information achievable with respect to the relatedness of viral isolates (6, 9, 10, 20). The results of the dot hybridization assay were in good agreement with those obtained by the PAGE-SS assay, with the exception of samples 12, 19, and 20 (Table 1). For two of these specimens (no. 12 and 19), the PAGE-SS assay suffered from a high level of background staining, whereas in the case of sample 20, the apparent concentration of rotavirus genome RNA, as indicated by the dot hybridization assay, was below that detectable by the PAGE-SS assay (<20 ng). The counterimmunoelectrophoresis assay (5) showed good agreement with the combined results of the PAGE-SS assay and the dot hybridization assay, except for a false-negative result in sample 7. On the other hand, the enzyme-linked immunosorbent assay (21) suffered from a high level of apparent false-positive results and low-positive values that were difficult to interpret.

A linear relationship existed between the hybridization signal and the dilution of the crude fecal nucleic acid fraction over a 100-fold dilution range (Fig. 1B). The concentrations of rotavirus genomic RNA in stool specimens were calculated by comparison with standard curves generated with known concentrations of purified genome RNA and were in good agreement with those obtained with the PAGE-SS assay (Table 1), ranging from 65 to >2,500 ng of RNA per ml of a 10% fecal solution.

Rotavirus genome RNAs from clinical specimens collected in upstate New York during a 4-month period in the fall and winter of 1983 and 1984 exhibited electrophoretic

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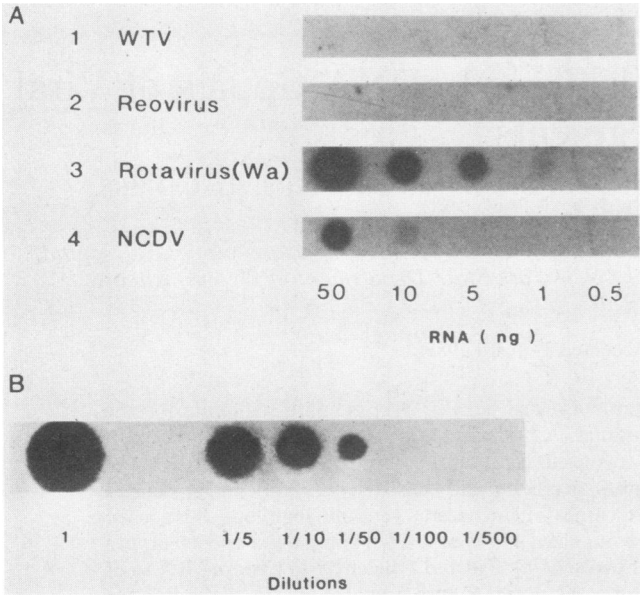


FIG. 1. (A) Hybridization of cloned rotavirus cDNA with *Reoviridae* virus genome RNAs. RNAs extracted from purified wound tumor virus (16), reovirus type 3, rotavirus strain Wa, or bovine rotavirus (NCDV, Lincoln strain) or from stool specimens were serially diluted with 20 mM Tris-hydorchloride (pH 8.0)–1 mM EDTA. Portions (20 μl, containing 0.5 to 50 ng of RNA) were heat denatured at 100°C for 5 min, diluted with 50 vol of cold 20 × SSC (1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate), and applied by filtration apparatus (Minifold; Schleicher & Schuell, Inc.) onto nitrocellulose filter sheets (Schleicher & Schuell). Under these conditions, more than 95% of 3′-[$^{32}$P]pCp-labeled human reovirus RNAs were reproducibly immobilized on nitrocellulose filters. The filters were then baked for 4 h at 80°C in vacuo. Immobilized RNAs were incubated in a plastic bag containing 5 ml of hybridization mixture composed of 5× SSC, 50% formamide, 20 mM Tris acetate (pH 7.4), 0.5% sodium dodecyl sulfate, 125 μg of yeast tRNA, and $10^6$ cpm of probe DNA which was labeled with $^{32}$P by nick-translation (specific activity, $2 \times 10^7$ cpm/μg). The probe DNA was denatured by heating at 100°C for 5 min before addition to the hybridization solution. After 16 h of hybridization at 37°C in the dark with constant rocking, the filters were washed three times in a small tray with 100 ml of 2× SSC for a total of 90 min at 37°C. The washed filters were dried briefly under a lamp and subjected to autoradiography at −70°C for 4 to 8 h with an intensifying screen. (B) Detection of rotavirus genome sequences in serially diluted fecal samples. RNA was extracted by phenol from the fecal material (10% suspension) obtained from sample 8 (Table 1) and subjected to dot hybridization under conditions as described in the legend to Fig. 1A. Autoradiography of the hybridized filter was performed for 24 h.

mobility profiles similar to those of strain Wa (subgroup 2 [11]) (Fig. 2A). Results of recent studies indicate that considerable sequence variation can exist for rotavirus isolates that exhibit similar genome RNA electrophoretic migration profiles (22, 23). Consequently, we tested the utility of the dot hybridization assay with individual genomic cDNAs used as a probe to differentiate between isolates that exhibit similar but distinguishable electrophoretic migration patterns.

The cDNAs of strain Wa genome segments encoding polypeptides comprising the ribonucleoprotein viral core (segments 1, 2, 3, and 6) and several nonstructural polypeptides (segments 5, 10, and 11) hybridized efficiently with each of the isolates examined (Fig. 2B). In contrast, cloned cDNA copies of genome segments encoding outer shell polypeptides (segments 4 and 9) and the remaining nonstructural polypeptides (segments 7 and 8) varied in efficiency of hybridization to individual isolates. In particular, cDNAs of genome segments 4 and 9 hybridized poorly to each of the isolates. Of some interest was the correlation between the electrophoretic migration of genome segment 5 in isolate B (Fig. 2A) and the high level of hybridization of strain Wa genome 5 cDNA to the genome sequence in the same isolate. Of the isolates examined in this study, only isolate B contained a genome segment 5 that co-electrophoresed with genome segment 5 of strain Wa.

To investigate whether the potential exists for using the individual cloned probes to differentiate rotavirus strains belonging to different subgroups or serotypes, a hybridization assay was performed on genome RNA of human strains of known serotypes and subgroups as well as bovine and equine strains (Fig. 3). Under hybridization conditions of low stringency, cDNA probes encoding nonstructural polypeptides (containing cDNA copies of genome segments 7, 8, 10, and 11) efficiently hybridized to all rotavirus strains. However, cloned cDNA copies of genome segment 9, which encodes the serotype determinant VP7, and genome segment 6, which encodes the subgroup determinant VP6 (11), hybridized poorly to genome RNAs of other rotavirus strains even under conditions of low stringency. These results suggest that probes specific for genome segments 7, 8, 10, or 11 would provide effective reagents for general screening of clinical specimens for the presence of rotavirus, whereas probes specific for genome segments 6 and 9 have the potential for distinguishing rotavirus strains of different serotypes and subgroups.

TABLE 1. Comparison of different assays for the detection of rotavirus in clinical specimens

| Sample no. | Result obtained by[a] | | | | |
|---|---|---|---|---|---|
| | ELISA | CIE | PAGESS | Dot hybridization | |
| | | | | Score | Amt (ng/ml)[b] |
| 1 | ++++ | + | ++++ | ++++ | 1,700 |
| 2 | ++++ | + | +++ | +++ | 1,000 |
| 3 | ++++ | + | ++ | ++ | 665 |
| 4 | ++++ | + | ++++ | ++ | 520 |
| 5 | ++++ | + | ++++ | + | 200 |
| 6 | +++ | + | ++++ | + | 400 |
| 7 | +++ | − | + | + | 135 |
| 8 | ++ | + | ++++ | ++++ | 2,500 |
| 9 | ++ | + | ++++ | +++ | 1,000 |
| 10 | ++ | + | ++ | + | 153 |
| 11 | ++ | + | ± | + | 65 |
| 12 | + | QNS | − | + | 250 |
| 13 | + | − | − | − | 0 |
| 14 | + | − | − | − | 0 |
| 15 | ± | + | ++++ | ++++ | 1,500 |
| 16 | ± | NSP | − | − | 0 |
| 17 | ± | QNS | − | − | 0 |
| 18 | ± | − | − | − | 0 |
| 19 | − | − | − | +++ | 1,000 |
| 20 | − | − | − | + | 100 |
| 21–31 | − | − | − | − | 0 |

[a] Results are shown as estimated viral RNA concentrations, in nanograms per milliliter of 10% fecal solution, and are also denoted by the following symbols: −, <100 ng/ml; +, 100 to 500 ng/ml; ++, 500 to 1,000 ng/ml; +++, 1,000 to 1,500 ng/ml; ++++, >2,000 ng/ml. Abbreviations: QNS, quantity not sufficient; NSP, nonspecific; ELISA, enzyme-linked immunosorbent assay; CIE, counterimmunoelectrophoresis.

By the cDNA dot hybridization assay, rotavirus genome RNA was conveniently detected in small volumes (equivalent to 20 μl) of 10% fecal samples at concentrations as low as 20 to 50 ng of genome RNA per ml, appearing not to suffer from high levels of false-positive results sometimes obtained with the enzyme-linked immunosorbent assay (12; Table 1).

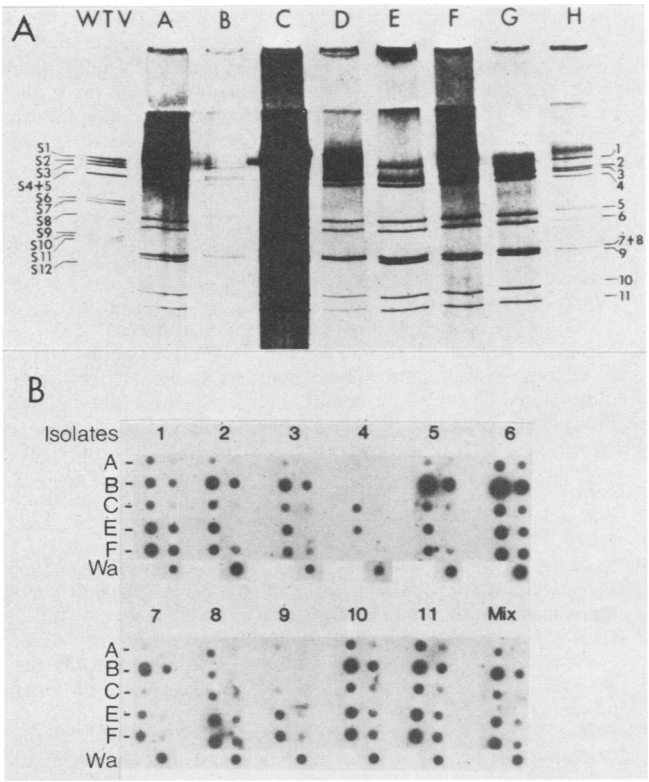


FIG. 2. (A) Polyacrylamide gel electrophoresis and silver staining of human rotavirus double-stranded RNA extracted from fecal samples collected in Albany, Buffalo, and Rochester, N.Y., from October 1982 to January 1983. Lane WTV, 200 ng of wound tumor virus double-stranded RNA; lanes A and E, isolates from Albany; lanes B and D, isolates from Buffalo; lanes F and H, isolates from Rochester. Electrophoresis was performed at room temperature at 250 V for approximately 2 h. Double-stranded RNA was visualized by silver staining (6, 14). (B) Dot hybridization with cloned cDNAs of individual genome segments. The 11 individual genome cDNAs (1 μg each) were labeled with $^{32}P$ by nick-translation and purified by a small Sephadex G-100 column. The estimated sizes (in base pairs) of viral genome cDNA inserts are: segment 1, ~3,000; segment 2, 900; segment 3, 3,100; segment 4, 500; segment 5, 1,620; segment 6 (1), 1,350; segment 7, 1,074; segment 8, 1,059; segment 9 (18), 1,060; segment 10 (17), 750; and segment 11 (8), 666. Heat-denatured RNA samples from the stools of five rotavirus-positive patients (lanes A through E) were applied to 11 separate nitrocellulose filter sheets. For each sample, RNAs of two concentrations (one- and fivefold dilutions) were examined. Each nitrocellulose filter was hybridized to one of 11 individual genome cDNAs to examine the hybridization specificity. Approximately $2 \times 10^6$ cpm of $^{32}P$-labeled cDNA probe was used for each dot hybridization. The mixed probe was prepared by combining equal radioactivities of 11 genome cDNAs to give a total of $2 \times 10^6$ cpm. Samples A, B, C, E, and F were the same as those shown in Fig. 2A. As controls, the individual genome segments of human rotavirus (Wa strain), which had been separated by 5% polyacrylamide gel electrophoresis, were hybridized under the same conditions (designated Wa in the figure). Autoradiography was carried out for 18 h.

Although we observed no hybridization of plasmid sequences in any of the fecal specimens examined during the course of this study, this problem may arise when a larger sampling of specimens is tested. To circumvent this possibility during routine use of this method, it is recommended that cloned cDNA inserts rather than the recombinant plasmids be used as a probe. The dot hybridization assay allows the processing of large numbers of samples requiring only phenol extraction, heat denaturation by boiling, and spotting onto nitrocellulose membranes. One person can easily process 50 to 100 specimens in 36 h. An addition advantage of the method may be the ability to apply samples to nitrocellulose membranes in field situations for later processing at a centralized, fully equipped facility. Recently, Pedley and McCrae have reported a similar dot hybridization technique for detection of field isolates of bovine rotaviruses (18).

Using $^{125}I$-labeled viral transcripts as hybridization probes, Flores et al. (4) reported the ability to detect as little as 8 pg of rotavirus RNA. While the transcript dot hybridization assay appears to be more sensitive than the cDNA dot hybridization assay, it should be emphasized that the latter allows cloned cDNA copies of individual genome segments to be independently used as a probe. Furthermore, it should be possible to increase the sensitivity of the dot hybridization assay by the use of single-stranded cDNA probes obtained by subcloning into M13 phage (15). Additional potential improvements include the use of $^{125}I$-labeled (2) or biotin-labeled (13) cDNA rather than $^{32}P$-labeled cDNA to increase the shelf life of the labeled probe as well as to provide greater compatibility with facilities currently available in most diagnostic laboratories.

The potential of cloned cDNA copies of the human rotavirus genome segments for use in epidemological studies is illustrated by the different efficiencies with which individual genome cDNAs hybridized to the same clinical isolates (Fig. 2B) and to rotavirus strains of known serotypes and subgroups (Fig. 3). The findings shown in Fig. 3, i.e., that the cDNAs of segments 6 and 9 can distinguish subgroups and serotypes, have subsequently been confirmed by experiments in which three representative human rotavirus RNAs (strains Wa, SA11, and S2) were cross-hybridized with four cDNA probes of segments 6 and 9 from strains Wa (serotype 1 and subgroup 2) and SA11 (serotype 3 and subgroup 1) (unpublished observations). Probe DNA from Wa segment 6, which encodes the group-specific antigen, hybridized only to Wa RNA, whereas probe DNA from SA11 segment 6 hybridized to RNAs of its parent and of S2 rotavirus (serotype 2 and subgroup 1). Probe cDNAs of segment 9, which encodes the type-specific antigen from Wa and SA11 rotavirus, hybridized only to their parental RNAs and did not hybridize to S2 virus RNA.

By employing the appropriate combination of genome cDNAs as a probe, it should be possible to use the dot hybridization assay not only as a standard diagnostic test but also for examining the degree of variation and distribution of rotavirus isolates within the general population. Furthermore, the high level of sensitivity achievable with the dot hybridization assay should permit the detection of rotavirus genome RNA in situations of limited infections. Clearly, the dot hybridization assay in which cloned cDNA copies of genome segments are used as a probe provides an additional accurate and sensitive method for detecting and differentiating rotaviruses in clinical specimens and, therefore, should contribute significantly to the improved diagnosis of rotavirus infections.

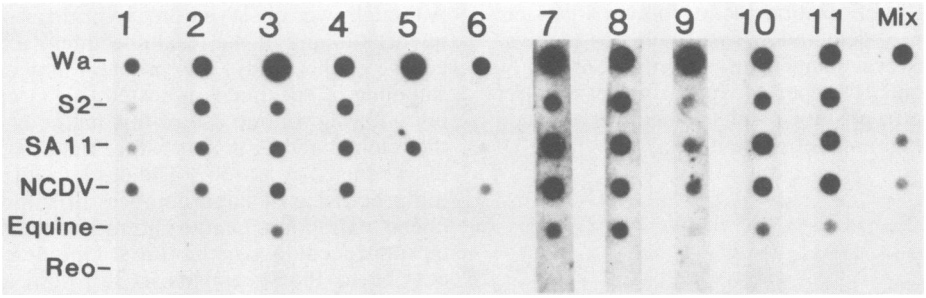


FIG. 3. Dot hybridization of $^{32}$P-labeled cDNA copies of individual rotavirus (Wa) genome segments to denatured genome RNAs of other rotaviruses under conditions of low stringency (35% formamide). The numbers at the top indicate the genome segment of which the probe is a copy. Wa, Human rotavirus strain Wa (serotype 1, subgroup 2); S2, human rotavirus strain S2 (serotype 2, subgroup 1); SA11, human rotavirus strain SA11 (serotype 1, subgroup 3); NCDV, bovine rotavirus NCDV Lincoln strain; Equine, equine rotavirus (serotype 3) field isolate passaged 18 times in MA104 cells (courtesy of Margaret Conner, Cornell College of Veterinary Medicine, Ithaca, N.Y.).

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