JOURNAL OF CLINICAL MICROBIOLOGY, July 1993, p. 1735–1740
0095-1137/93/071735-06$02.00/0

Vol. 31, No. 7

Distribution of VP4 Gene Alleles in Human Rotaviruses by Using Probes to the Hyperdivergent Region of the VP4 Gene

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Received 25 January 1993/Accepted 8 April 1993

The rotavirus VP4 protein elicits the production of neutralizing antibodies and is known to play a role in inducing resistance to disease. At least five human rotavirus VP4 gene alleles have been described on the basis of antigenic polymorphism and/or nucleotide sequence differences. In the present study, we developed cDNA probes directed at the hyperdivergent region of the VP4 gene of the five described human rotavirus VP4 alleles (Wa, DS1, M37, AU228, and 69M) and used them in hybridization assays with human rotavirus strains from Latin America and Europe to determine the distribution of the VP4 gene alleles in nature. The Wa-like allele was detected most frequently, occurring in 57% of the 402 rotavirus strains tested, and the DS1-like allele was the next most common, occurring in 14% of the strains tested. The M37- and AU228-like alleles were detected in only 4 and 3% of the rotavirus strains tested, respectively, whereas the 69M-like VP4 gene allele was not detected. Several rotavirus strains from Europe did not react with any of the VP4 gene probes, although they did hybridize to a probe generated from a representative strain from the group. These data indicate the global distribution of various VP4 gene alleles and raise the possibility that other, unrecognized human VP4 alleles exist in nature because almost one-fourth of the strains could not be classified into any of the established VP4 groups.

Diarrheal illness has a striking impact worldwide, resulting in up to 5 million deaths in children under 5 years of age in the developing world (27). Over the last decade, rotavirus has been consistently recognized as a leading cause of the serious form of this disease in young children, and an active search for an efficacious vaccine is under way (4). The two outer capsid proteins of the virus, VP4 and VP7, which are both neutralization antigens, have been shown to play an important role in inducing resistance to the disease (18, 24). It has been suggested that the differences in the VP4 proteins between current rotavirus vaccine candidate strains and wild-type rotavirus strains may be related to the lack of consistent efficacy exhibited by such vaccine candidate strains (4, 20). In studies of the rhesus rotavirus vaccine and rhesus rotavirus × human rotavirus reassortants in young infants, the responses to VP4 have been predominantly homotypic (7, 16). Similarly, recent studies in infants undergoing natural primary rotavirus infection indicate that the responses to both VP4 and VP7 are mostly homotypic (7a).

VP4, which is encoded by the fourth viral gene, has been shown to exhibit antigenic polymorphism (15, 18, 28). Five VP4 gene alleles have been described in rotaviruses recovered from humans. The initial reports described two VP4 alleles (Wa- and DS1-like) which were present in virus recovered from children with symptomatic rotavirus infection and a third allele (M37-like) which was present in virus recovered from neonates who underwent asymptomatic infection in nurseries for newborn infants (5, 14) The Wa-like allele has been detected in strains with VP7 serotype 1, 3, or 4 specificity, while the DS1-like allele is associated with rotaviruses bearing VP7 serotype 2 specificity (5). The M37-like VP4 allele has been detected in selected rotaviruses bearing VP7 serotype 1, 2, 3, or 4 specificity (5, 13). Studies that have used RNA-RNA hybridization and gene sequence analysis of the VP4 gene have described two additional gene 4 alleles. Sequence data from strains K8 and 69M that were recovered from children with gastroenteritis in Japan and Indonesia, respectively, show that each of these strains has a unique gene 4 allele (25, 29). Strain AU228, also isolated in Japan, has been shown to have a gene 4 that is similar to that of strain K8 (22).

Studies of the antigenic diversity and distribution of VP4 have been hampered by the initial lack of serological reagents to these proteins. An alternative dot hybridization strategy has been developed to identify the different alleles of the VP4 gene (20). The VP4 gene has a region which is highly divergent among viruses with different alleles and which is highly conserved among viruses with the same allele (13). This hyperdivergent region has been used to distinguish the different gene 4 alleles of rotavirus strains by sequence analysis (25, 29) and probe analysis (20). Mapping of neutralizing monoclonal antibodies to VP4 suggests that strain-specific epitopes are also located in this region of the gene (21).

In the study described here, cDNA clones which encompass the hyperdivergent region of the different VP4 gene alleles were constructed and used as templates to synthesize probes by the polymerase chain reaction (PCR). The VP4

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allele-specific probes were used to determine the distribution of VP4 alleles present in rotavirus field strains.

MATERIALS AND METHODS

Rotavirus strains. The human rotavirus reference strains Wa (Wa-like VP4 allele), DS1 (DS1-like allele), and M37 (M37-like allele) have been well characterized (18, 19). Strain AU228 was isolated from an ill child in Japan (22) and has a VP4 gene that is similar to that of the K8 strain. Strain 69M was isolated from a child with gastroenteritis in Indonesia and has a VP4 gene which has been shown by sequence analysis to be distinct from the VP4 gene alleles described above (25).

These viral strains were prepared as described previously (6). Briefly, they were grown in MA104 cells in the presence of 1 μg of trypsin per ml, concentrated by ultracentrifugation, and then purified by trichloro-trifluoro-ethane extraction and CsCl gradient centrifugation.

Rotavirus field strains collected from infants with diarrhea from various previous studies conducted in Europe and Latin America were used to investigate the distribution and diversity of VP4 alleles (3, 8, 9, 11, 12). The specimens from Guatemala were provided by J. R. Cruz, those from Peru were provided by C. Lanata, and the Venezuelan specimens were provided by I. Perez-Schael and L. White.

Preparation of RNA. Single-stranded RNA transcripts were produced by in vitro transcription of the rotavirus-purified cores as described previously (6). The partially purified virus was added to a transcription mixture, which contained 2.5 mM CTP, 2.5 mM GTP, 2.5 mM UTP, 10 mM ATP, 0.5 mM *S*-adenosylmethionine, 12 mM $MgCl_2$, and 100 mM Tris-acetate buffer (pH 8.0). Bentonite (0.1%) was added to inhibit any RNases that were present, and 0.5 mM EDTA was added to activate the viral transcriptase. Transcription was performed at 42°C for 6 to 8 h, after which time the bentonite was pelleted by centrifugation. The supernatant containing the viral single-stranded RNA was then extracted with phenol-chloroform and precipitated with ethanol.

Double-stranded RNA from either stool or tissue culture-adapted material of the field strains was prepared by phenol-chloroform extraction and ethanol precipitation.

Preparation of VP4 gene clones. The single-stranded RNA transcripts served as templates for cDNA synthesis by reverse transcription by using the primers deduced from the conserved sequences of the VP4 gene. The method of Gubler and Hoffman (17) was used to generate DNA clones.

PCR-generated probes. PCR was used to generate ^{32}P-labelled DNA probes from each of the clones with VP4 gene allele-specific primers, as described elsewhere in detail (20). A common primer (primer 1 in Fig. 1), 5′-GATGGTCCT TATCAACC-3′, which is homologous to nucleotides 205 to 221, was used for all of the VP4 gene alleles. "Reverse" primer 2, 3′-CCTGCAAAGTACCACTTTGTGG-3′ (complementary to nucleotides 533 to 554), was used for the VP4 alleles Wa, DS1, and M37. Specific reverse primers 3 and 4 were developed for the amplification of the fourth gene of AU228 and 69M, respectively. Primer 3, 3′-TGACCAT AGTTCCTCGCTGCGG-5′ (complementary to nucleotides 533 to 554), was used for strain AU228, and primer 4, 3′-TGAATATTACCTCTCTGTGGTT-5′ (complementary to nucleotides 535 to 556 except for base 552), was used for strain 69M.

The PCR-generated probes were retrieved by electrophoresis through 1.4% low-melting-point agarose gels and recovered by the method of Feinberg and Vogelstein (2). Alternatively, unlabelled DNA amplified in the same way was later labelled with [^{32}P]dATP by using a random primer labelling kit (Prime-a-gene; Promega Corp., Madison, Wis.), as specified by the manufacturers.

Hybridization analysis. The double-stranded RNA was denatured by boiling for 3 to 5 min and was then immediately chilled on ice for 2 to 5 min. Several identical nylon membranes (Nytran; Schleicher & Schuell, Keene, N.H.) were prepared by dotting 1 μl of the RNAs onto the membranes; this was followed by exposure to short-wave UV light for 5 min. Control RNAs from the five laboratory strains, each possessing a previously characterized VP4 gene allele, were similarly dotted onto the membranes. Hybridization was performed as described previously (3, 20). Briefly, the membranes were prehybridized for 1 to 2 h at 54°C in a mixture containing 50% formamide, 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 40 mM sodium buffer (pH 6,5), 0.2% sodium dodecyl sulfate (SDS), and 2× Denhardt's solution. A second prehybridization step was conducted in fresh solution containing 25 μg of boiled salmon sperm DNA per ml. Hybridization was carried out in a similar solution containing 10% dextran sulfate and 3×10^6 to 5×10^6 cpm of the appropriate VP4 probe. Hybridization was carried out at 54°C for 12 to 24 h and was terminated by washing the membranes four to five times in 2.5× SSC with 0.2% SDS at room temperature and washing them twice in 1× SSC–0.1% SDS at 54°C. The membranes were dried at 37°C and exposed to X-ray film for 12 to 24 h.

RESULTS

Specificities of the probes. The nucleotide sequence of the original cloned DNA corresponding to each of the PCR-amplified hyperdivergent regions of the VP4-specific probes was determined. The nucleotide sequences of the cloned DNA from each strain were consistent with the sequences published for viral strains Wa, DS1, M37 (15), AU228 (22), and 69M (26) (Fig. 1). A difference of greater than 30% was found when the nucleotide sequences of the hyperdivergent regions of all of the VP4 alleles were compared; for the Wa-like and DS1-like pair, however, the difference was only 17% (Table 1). However, as shown in Fig. 2, each of the five strains could be differentiated by hybridization.

Sensitivities of the probes. The sensitivity of the assay was evaluated by hybridizing the probes to serial 10-fold dilutions of the well-characterized laboratory strains of rotavirus. The probes were found to be capable of detecting as little as 10 ng of RNA applied to the nylon membranes (data not shown).

Analysis of field specimens of human rotaviruses. A total of 402 rotavirus-positive diarrheal stool specimens from Europe and Latin America were evaluated with the PCR VP4 probes to assess the distribution of the different VP4 alleles in nature; 313 specimens (78%) could be successfully classified by the probes (Table 2). The Wa-like VP4 allele was observed most frequently in all geographic areas examined, occurring in 229 of the 313 specimens (73%), while the DS1 allele was identified in 57 specimens (18%). The M37 VP4 allele was observed in viruses from 18 patients (6%), all of whom were infants (including four newborns) with rotavirus illness.

Strains bearing the AU228-like VP4 allele were identified in six specimens from Latin America and three specimens

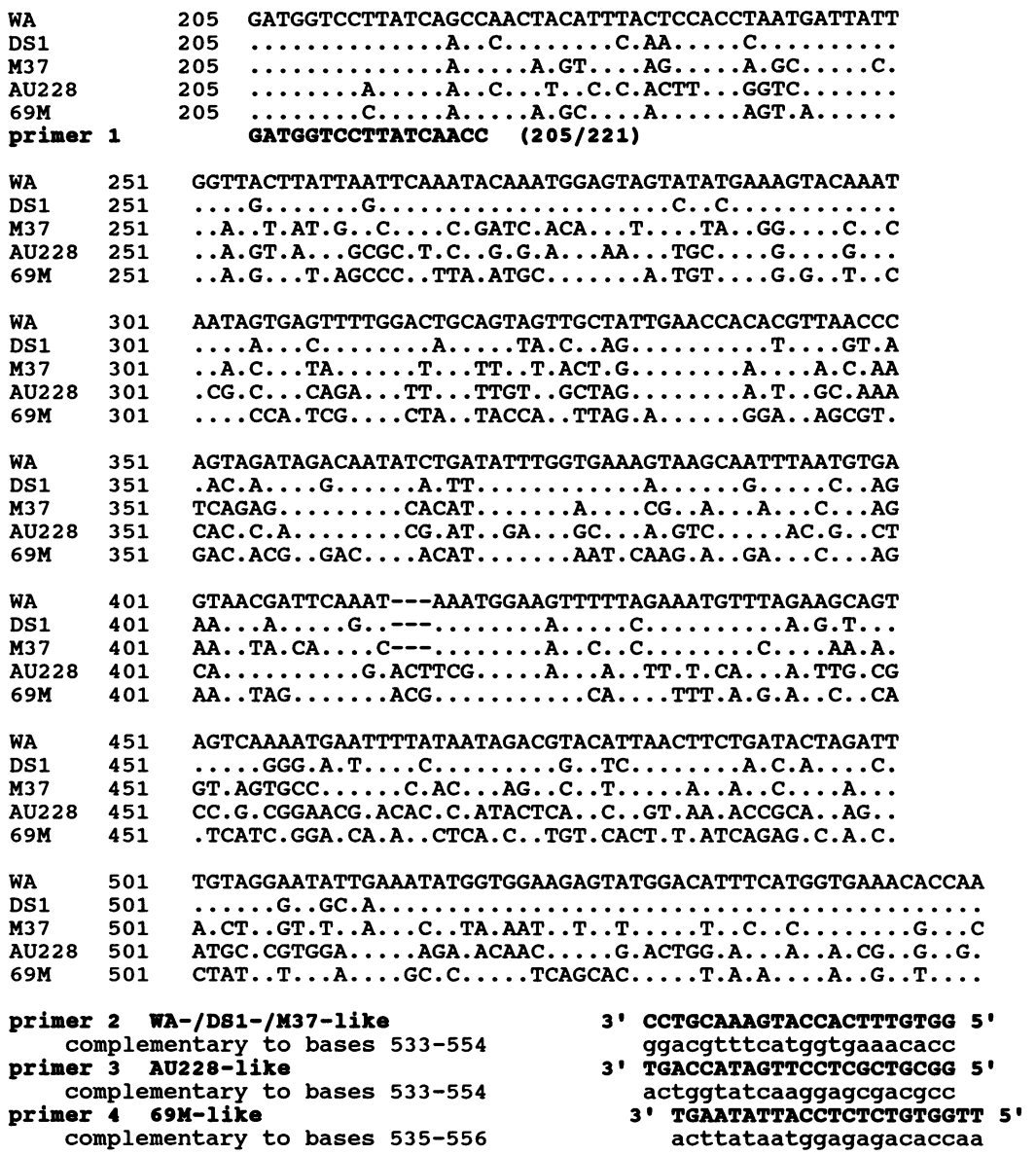

FIG. 1. Comparison of nucleotide sequences in the hyperdivergent region of the fourth gene used as a probe among the five gene 4 alleles tested. Differences between the sequence of each allele (DS1, M37, AU228, and 69M) and Wa are noted. The sequences of the four primers used to amplify the region are noted in boldface type.

TABLE 1. Differences in nucleotide sequences among the gene 4 probes used in the present study

Strain	No. (%) nucleotide difference[a]			
	DS1	M37	AU228	69M
WA	58 (17)	114 (33)	168 (48)	159 (45)
DS1		120 (34)	154 (44)	151 (43)
M37			165 (47)	153 (44)
AU228				180 (51)

[a] Nucleotide differences in pairwise comparison between intersecting strains. All of the probes except 69M consisted of 350 nucleotides; probe 69M had 352 nucleotides.

from Europe. The presence of the AU228-like gene 4 in these strains was confirmed by Northern blot hybridization and PCR amplification of the hyperdivergent region by using AU228-specific primers (data not shown).

Twenty-two percent of the field strains (89 of 402) could not be typed by the probes. Most of these specimens did not contain enough nucleic acid to react with the probes (59 specimens), as indicated by the lack of visible RNA bands on gel electrophoresis. Six specimens reacted with a high background, which did not allow for the identification of the allele that was present. Fourteen specimens (12 of them from Germany) which did have sufficient RNA (i.e., visible RNA bands in an agarose gel) did not hybridize to any of the probes used in the present study and may constitute a new, as yet unrecognized, VP4 allele.

Eleven strains reacted equally well with more than one of

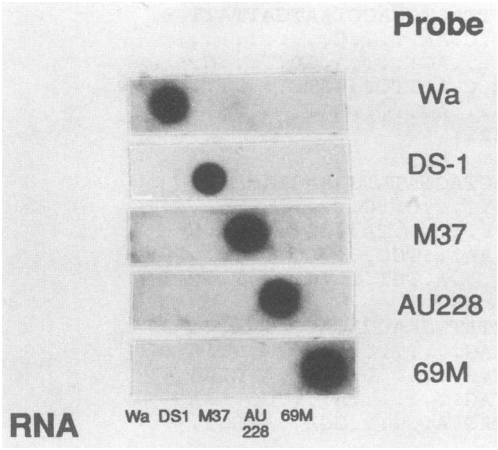

FIG. 2. Hybridization of allele-specific gene 4, PCR-generated probes to rotavirus RNA dotted onto four similar membrane strips. The membranes were exposed to X-ray film with an intensifying screen for 12 h.

the VP4 probes used in the present study; seven of these were subsequently determined to be dual infections with two virus strains when examined by monoclonal antibodies directed to the subgroup (VP6) and serotype (VP7) antigens. Three strains from Venezuela reacted with both the 69M and DS1 probes; they were subgroup 1, VP7 serotype 2, and had a short RNA electropherotype; however, they could not be adapted to growth in tissue culture, and the lack of sufficient stool material did not allow clarification of their VP4 specificities.

Further studies of the untyped strains revealed that five strains (three from Finland and two from Italy) carried a similar, although unique, gene 4 (prototype strain PA169) which was distinct from those described above (this study) and from a battery of animal strains described elsewhere (10). RNA transcription probes made from strain PA169 failed to hybridize to the VP4 gene of the 12 untyped strains from Germany (data not shown).

DISCUSSION

In the present study, we obtained full-length clones representing the entire sequence of the VP4 gene or partial clones from the VP4 region corresponding to VP8 (one of its cleavage products) (13, 25, 29). The clones were confirmed by nucleotide sequence analysis to be similar to the parent strains and were used as templates for the derivation of VP4 allele-specific probes by PCR. The VP4 probes were determined to be both sensitive and specific for the detection of the VP4 gene allele present in rotavirus field specimens. As little as 10 ng of RNA could be detected.

In an assessment of the distribution of the different VP4 alleles in rotavirus field specimens from patients with diarrhea, 402 specimens from Europe and Latin America were analyzed. The Wa-like allele was observed to occur most frequently in all areas examined, occurring in 73% of those specimens that were successfully typed. The DS1-like allele was the second most frequently detected VP4 gene allele (18% of those typed). Thus, by the VP4 serotype classification system proposed by Gorziglia et al. (15), in which the WA- and DS1-like alleles are classified as VP4 serotype P1A or P1B, respectively, VP4 serotype P1 strains represent over 90% of all VP4 alleles successfully typed by the five VP4-specific probes used in the present study. Overall, serotype P1 strains comprised 70% of all the specimens examined. This has important implications for future vaccine strategies should the VP4 antigen be shown to strongly influence the success or failure of candidate vaccine strains (7).

The M37-like VP4 allele was detected in 18 specimens (6%). Previously, the M37-like VP4 gene had been reported to occur only in strains of the virus recovered from asymptomatically infected neonates (5). However, in the present study, four of the M37-like strains were recovered from neonates symptomatically infected as described previously (9). The remaining 14 cases of infection (detected in Venezuela and Peru) were all observed in older infants with symptomatic infection with rotavirus. No information was available on the relative severity of diarrhea among the children tested. Further studies on these M37-like strains are under way.

Strains carrying the AU228-like VP4 allele were identified both in Europe (three strains) and in Latin America (six strains), indicating that the global distribution of this VP4 allele is more widespread than was previously thought. Strains possessing this VP4 allele (strains K8 and AU228) had only been detected in Japan (29).

Seven specimens which reacted with more than one probe were found on further examination to contain more than one strain of virus. The most common dual infection was found to involve strains bearing a Wa and a DS1 VP4 gene (four specimens); specimens with this combination of dual infection have been reported previously, although not often, on the basis of analysis of the VP7 serotype or by RNA electrophoresis (1, 8, 23, 26). However, dual infections with strains bearing the AU228- and Wa-like VP4 genes (two

TABLE 2. Distribution of VP4 alleles among rotaviruses from various geographic locations

Country of origin	No. of strains tested	No. of strains with VP4 gene allele:				No. of untyped strains	
		WA	DS1	M37	AU228	Failure to type	Insufficient RNA
Guatemala	31	6	17			2	6
Peru	69	49	14	6			
Venezuela	198	113	25	8	6	6	40
Finland	3					3	
Germany	38	14		4		15	5
Italy	63	47	1		3	5	7
Total	402	229 (73)[a]	57 (18)	18 (6)	9 (3)	31	58

[a] Values in parentheses are in percent with respect to the total number of strains successfully typed (n = 313). None of the 69M strains has the VP4 gene allele.

specimens) and the AU228- and M37-like VP4 genes (one specimen) were also observed. Three specimens reacted with both the DS1- and 69M-like VP4 probes; however, the presence of a 69M VP4 gene could not be confirmed.

Some of the rotavirus strains from Europe could not be typed with the VP4 probes used in the present study. At least five of these strains, which were isolated in Italy (two specimens) and Finland (three specimens), when analyzed by Northern blot hybridization with a battery of human (this study) and animal (10) rotaviruses, showed that they bore a VP4 gene which was distinct and unique to this group and may well represent a new human VP4 gene allele. The three Finnish strains (HAL1166, HAL1271, HAL8590) were reported previously to be VP7 serotype 8 strains with a long RNA electropherotype (12). The two Italian strains (PA169 and PA710) have also been previously described as having unusual antigenic characteristics. PA169 is one of two rotavirus strains in Italy with VP7 serotype 6 (the other rotavirus strain has an AU228-like VP4 gene) (10), and PA710 is one of three strains described as having VP7 serotype 3 and subgroup I specificity (the other two strains have an AU228-like VP4 allele) (11). The VP4 gene allele associated with the prototype strain PA169 is under further investigation to determine its VP4 gene sequence.

The development of PCR probes capable of distinguishing the five VP4 gene alleles recognized thus far has allowed the examination of field isolates of rotavirus in an attempt to determine the distributions of the VP4 gene alleles in nature. The findings that the majority of rotaviruses belong to either the Wa or the DS1 gene 4 allele has important implications for future vaccine development.

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