Development of a Microtiter Plate Hybridization-Based PCR–Enzyme-Linked Immunosorbent Assay for Identification of Clinically Relevant Human Group A Rotavirus G and P Genotypes

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A microtiter plate hybridization-based PCR–enzyme-linked immunosorbent assay (PCR-ELISA) has been used for the detection and identification of a variety of microorganisms. Here, we report the development of a PCR-ELISA for the identification of clinically relevant human rotavirus VP7 (G1 to G6, G8 to G10, and G12) and VP4 (P[4], P[6], P[8], P[9], and P[14]) genotypes. The G and P types of reference human and animal rotavirus strains for which specific probes were available were correctly identified by the PCR-ELISA. In addition, reference strains bearing G or P genotypes for which specific probes were unavailable, such as G11, G14, P[3], P[10], and P[11], did not display any cross-reactivity to the probes. The usefulness of the assay was further evaluated by analyzing a total of 396 rotavirus-positive stool samples collected in four countries: Brazil, Ghana, Japan, and the United States. The results of this study showed that the PCR-ELISA was sensitive and easy to perform without the use of any expensive and sophisticated equipment, the reagents used are easy to obtain commercially and advantageous over multiplex PCR since more than one type-specific probe is used and the selection of probes is more flexible.

Rotaviruses are double-stranded RNA viruses comprising the genus Rotavirus in the family Reoviridae, which includes seven groups (A to G) (19). Group A rotavirus, the single most important cause of severe acute gastroenteritis among infants and young children worldwide (19, 59), has been estimated to be responsible for up to 611,000 deaths annually in children <5 years of age, predominantly in developing countries (59). In the United States, rotavirus diarrhea is estimated to cause approximately 60,000 hospitalizations and 37 deaths each year (22). Because of this significant disease burden, the development and implementation of a safe and efficacious rotavirus vaccine in childhood immunization programs has been an important global public health goal.

An infectious rotavirus particle consists of three concentric protein layers surrounding 11 segments of double-stranded RNA. The outermost layer is composed of two proteins, VP7 (which defines G serotypes/genotypes) and VP4 (which defines P serotypes/genotypes). Since VP7 and VP4 carry independent neutralization and protective antigens and since antibodies to either protein can confer resistance to virulent rotavirus in a type-specific manner in experimental animals (19), they are the primary targets of the rotavirus vaccines being developed. Fifteen G genotypes (19, 34, 70) and at least 27 different P genotypes (10, 53, 54, 55, 64, 70, 73) have been established thus far. The G-P combinations G1P[8], G2P[4], G3P[8], G4P[8], G9P[6], and G9P[8] are most commonly detected in humans (for a review, see reference 69). However, rotavirus strains bearing rare or unusual G and/or P genotypes (e.g., G5, G8, G10, G11, G12, P[11], P[14], and P[25]) associated with human infections have also been reported around the world with an increasing frequency (6, 7, 18, 32, 43, 56, 68, 78, 82). Observations made in various rotavirus vaccine trials have indicated that the induction of serotype-specific immunity may be important for optimal protection (12, 37, 44, 45, 67, 80, 81), which prompted the establishment of rotavirus strain surveillance programs in many countries. Such programs have generated a large amount of information on rotavirus epidemiology, which demonstrated the occurrence of a great variety of G-P combinations around the world (for a review, see reference 70). Moreover, because of the ubiquity of rotaviruses in the animal kingdom, which favors interspecies transmission and genetic reassortment between animal and human strains, the emergence of novel rotavirus strains is likely an expected phenomenon (25).

Several methodologies have been applied for rotavirus typing, such as an enzyme-linked immunosorbent assay (ELISA) using type-specific monoclonal antibodies, typing PCR, hybridization assay, and microarray analysis (11, 17, 20, 36, 49). Although ELISA using type-specific monoclonal antibodies is a simple assay and accessible to laboratories in less developed countries, it presents limitations: it relies on the presence of
TABLE 1. Prototype strains analyzed for VP7 and VP4 specificity

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Strain(s) analyzed</th>
<th>Origin of isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>D, M37, Ku, Wa, K8</td>
<td>Human</td>
</tr>
<tr>
<td>G2</td>
<td>HN126, DS-1, S2, 1076, KUN</td>
<td>Human</td>
</tr>
<tr>
<td>G3</td>
<td>P, McN13, Ro1845, HCR3, M, AU-1, ST8, YO RRV, SA11</td>
<td>Simian</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VP4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P[3]</td>
<td>Ro1845, HRC3</td>
<td>Human</td>
</tr>
<tr>
<td>P[4]</td>
<td>HN126, DS-1, S2, KUN, L26, 1290</td>
<td>Canine</td>
</tr>
<tr>
<td>P[9]</td>
<td>K8, AU-1, Se584, PA151, Hun3, Hun4, Hun6, R44</td>
<td>Feline</td>
</tr>
<tr>
<td>P[10]</td>
<td>69M</td>
<td>Human</td>
</tr>
<tr>
<td>P[14]</td>
<td>HAL1166, PA169, Hun5</td>
<td>Human</td>
</tr>
</tbody>
</table>

TABLE 2. Rotavirus-positive stool samples analyzed in this study

<table>
<thead>
<tr>
<th>Country of origin of stool samples</th>
<th>Yr(s) of collection</th>
<th>Total no. of samples collected</th>
<th>No. of samples analyzed for gene by:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>PCR-ELISA&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>VP7(G)</td>
</tr>
<tr>
<td>Brazil</td>
<td>1996–2004</td>
<td>256</td>
<td>256</td>
</tr>
<tr>
<td>Ghana</td>
<td>2003–2004</td>
<td>45</td>
<td>28</td>
</tr>
<tr>
<td>United States</td>
<td>1997–2000</td>
<td>31</td>
<td>31</td>
</tr>
<tr>
<td>Japan</td>
<td>2003</td>
<td>64</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>396</td>
<td>315</td>
</tr>
</tbody>
</table>

<sup>a</sup> Analyzed in each country of origin.
<sup>b</sup> Analyzed in this study.

substantial numbers of triple-layered particles in stool samples, which is not always the case due to digestion or degradation of the particles; type-specific monoclonal antibodies are not widely available; and virus strains bearing point mutations at the antibody binding site cannot be detected in the assay (5, 20, 39, 40). Molecular methods are, in general, more sensitive and accurate than serological assays. Typing PCR for the detection and identification of human and animal rotavirus VP4 and VP7 genotypes that was developed in the early 1990s (15, 24, 27, 28, 29, 33, 38, 76) has become the method of choice for strain genotyping, which can now be regarded as the “gold standard” (20). However, this technique also suffers from inherent shortcomings: point mutation(s) on the VP4 or VP7 gene at the primer-binding site may lead to failure in the typing process, and cross-priming of primers between different genotypes may produce false-positive reactivity (1, 20, 21, 39, 42, 51, 52, 58, 60, 65, 69). Sensitive and reliable diagnostic techniques that do not bear such disadvantages (11, 17, 36, 49) are needed not only for accurate rotavirus strain surveillance but also for effective rotavirus vaccine development and evaluation (for example, analyses of homotypic versus heterotypic protection). In the present study, we developed a microtiter plate hybridization-based ELISA coupled with PCR (PCR-ELISA) and evaluated its usefulness for the identification of clinically relevant G (G1 to G6, G8 to G10, G12) and P (P[4], P[6], P[8], P[9], and P[14]) genotypes of group A rotavirus.

MATERIALS AND METHODS

Rotavirus strains. A total of 79 cell culture-adapted prototype human and animal rotavirus strains used in this study (Table 1) were obtained from the collections of the Laboratory of Infectious Diseases, National Institutes of Health. Each rotavirus strain was plaque purified three times on MA-104 cells and used as the source of genomic RNA.

Stool specimens. A total of 396 stool samples collected from children with diarrhea in Brazil, Ghana, Japan, and United States between 1996 and 2004 were analyzed (Table 2). Such specimens were previously determined to be rotavirus positive by either ELISA or polyclonal antibody detection in each country of origin. Three hundred fifteen of the 396 rotavirus-positive specimens were genotyped for VP7 and VP4, respectively, by typing PCR in each country of origin. Eighty-one of the 396 specimens (17 from Ghana and 64 from Japan) were previously not tested for rotavirus genotyping. In this study, all 396 rotavirus-positive samples were analyzed for G genotypes by PCR-ELISA; however, a subset of 246 of the 396 samples was analyzed for P genotypes by PCR-ELISA (Table 2). The study protocol was approved by the ethics committees of the Hospital Universitário Clementino Fraga Filho and the Instituto Evandro Chagas, Belém, Brazil; Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Ghana; and the University of Tokyo, Tokyo, Japan.
Primers and oligonucleotide probes. A complete list of primers and oligonucleotide probes used in the present study is shown in Tables 3 and 4. Positive-sense PCR primers were biotin labeled at the 5’ end (MWG Biotech, Germany). All oligonucleotide probes were labeled at the 3’ end with digoxigenin (MWG Biotech).

RNA extraction and RT-PCR. The viral double-stranded RNA was extracted from stools or infected cell culture lysates using the TRIzol method (Invitrogen, Carlsbad, CA) and subjected to reverse transcription followed by PCR (RT-PCR). The cDNA of the rotavirus VP7 or VP4 gene was synthesized by RT-PCR using primers that were biotin labeled at their 5’ ends. Four different VP7 gene-specific primer pairs were selected to increase the efficiency of VP7 gene amplification (Table 3). Initially, the Beg9-End9 primer pair (27), which amplifies the entire VP7 segment of most of rotavirus strains, was used. When the VP7 gene was not amplified with this primer pair, one of the following three primer pairs was used: sBeg9-RVG9 (27, 28), 9Con2-G922 (11, 15), or LID1-G922 (11). The sBeg9-RVG9 primer pair was used as the first choice because it provided the best amplification. If we did not see an amplified cDNA in the agarose gel or the PCR-ELISA, we used one of the next two sets of primer pairs: sBeg9-RVG9 (27, 28), 9Con2-G922 (11, 15), or LID1-G922 (11).

PCR-ELISA. To detect the labeled PCR products and identify the genotypes of positive samples, 96-well microassay plates (Nunc-Immuno module; Nunc, Roskilde, Denmark) were coated with streptavidin (2 μg/ml in 50 mM carbonate buffer [pH 9.6]; Roche Diagnostic GmbH, Mannheim, Germany) at 37°C for 1 h or overnight and then washed three times with 200 μl/well of phosphate-buffered saline containing 0.05% Tween 20 (PBS-Tw). After the three washes, 95 μl of PBS-Tw was added to each well, and the biotin-labeled PCR product (5 μl) was distributed (one reaction per genotype). The plates were incubated at room temperature for 1 h or overnight. The plates were then washed three times with PBS-Tw, and 100 μl/well of denaturing solution (100 mM NaOH, 0.1% Tween 20) was added, followed by 10 min of incubation at room temperature. Plates were washed three times with PBS-Tw prewarmed at 52°C. One hundred microliters/well of the hybridization solution (300 mM NaCl, 100 mM Tris-Cl [pH 6.5], 10 mM EDTA, 0.1% Tween 20) containing 1 pmol/ml of the type-specific digoxigenin-labeled probe was added. Individual G- or P-type-specific probe mixtures (three type-specific probes/genotype/mixture) prepared in hybridization solution prewarmed at 52°C were distributed onto the plates. The plates were then incubated at 52°C for 1 h and washed three times, and 100 μl/well of anti-digoxigenin horseradish peroxidase conjugate (10 mU/ml in PBS-Tw) (Roche Diagnostic GmbH) was added, followed by incubation at 37°C for 30 min. After three washes, 100 μl/well of enzyme substrate (TMB peroxidase substrate system; KPL, Gaithersburg, MD) was added, the reaction was carried out at room temperature for up to 10 min and stopped by adding 100 μl/well of 2 M H2SO4 to the mixture, and the absorbance was measured at 450 nm by use of a spectrophotometer. Samples presenting the absorbance value for the reactivity with a genotype-specific probe(s) of twofold or above of that of the blank
were considered to be positive. Rotavirus-positive controls were included for each plate.

Rotavirus G and P typing PCR. Three different sets of G-type-specific primer pools (69) and one P-type-specific primer pool (24) were used in this study to genotypen a subset of 55 of 256 samples collected in Brazil: the H2 pool, which contained G1, G2, G3, G4, G6, G8, and G9-specific primers (aBT1, aCT2, aCT3, aCT4, aCT5) (27, 76); the C pool reported previously by Das et al. (15), which consisted of G1-, G2-, G3-, G4-, G8-, and G9-specific primers (9T1, 9T2, 9T3, 9T4, and 9T9B); the A pool described previously by Gouvea et al. (28), which consisted of G1-, G2-, G3-, G4-, and G9-specific primers (9T1, 9T2, 9T3, 9T4, and 9T9B); and the P pool, which consisted of P4-, P6-, P8-, P9-, P14-, and P pool, which consisted of P4-, P6-, P8-, P9-, P14-, and P15 of 396 samples collected in four countries (Brazil, Ghana, Japan, and the United States) (Table 2). Three hundred ninety-four of the 396 (99.5%) samples were successfully G genotyped by the PCR-ELISA (Table 5). Three hundred twenty of the 394 G-genotyped samples gave visible bands after RT-PCR (indicating a large amount of amplified cDNA), whereas 74 (including 3 samples each from Brazil and Ghana that were nontypeable by typing PCR in each country of origin) did not produce any visible bands (suggesting a low amount of amplified cDNA) in an agarose gel, demonstrating the high sensitivity of the assay. Two previously untested samples from Japan were not amplified with any of the primer pairs used for the VP7 RT-PCR.

P genotype analysis by PCR-ELISA of rotavirus-positive human stool samples. Two hundred forty-six samples were analyzed for P genotypes by PCR-ELISA (Table 2). Two hundred forty-one of the 246 (98.0%) samples were successfully P genotyped; four of five PCR-ELISA-nontypeable samples noted above were previously not tested by typing PCR, and one sample was previously nontypeable by typing PCR (Table 5). One hundred thirty-six of the 241 P-genotyped samples gave visible bands in an agarose gel after RT-PCR, whereas 105 samples (including 11 samples that were previously nontypeable by typing PCR in each country of origin) did not produce visible bands. These samples, however, produced a colored reaction in the PCR-ELISA and therefore were successfully P genotyped, again indicating the high sensitivity of this assay.

Concordant and discordant results between typing PCR and PCR-ELISA. Concordance and discordance in G- and P-genotyping results determined by typing PCR and PCR-ELISA are summarized in Tables 5 and 6. Although the overall percentage of agreement between the two assays for the VP7 gene was 71.7% (226/315), it is noteworthy that the value varied widely from one country to another (i.e., 67.9% for Ghanaian samples, 68.8% for Brazilian samples, and 100% for U.S. samples).

**RESULTS**

G and P genotype analysis of rotavirus reference strains by PCR-ELISA. The usefulness of the PCR-ELISA was initially evaluated by testing 79 cell culture-adapted human and animal rotavirus reference strains with known G and P types. The G and P types of rotavirus strains for which genotype-specific probes were available (i.e., strains bearing G1, G2, G3, G4, G5, G6, G8, G9, G10, G12, P4, P6, P8, P9, or P14 specificity) were correctly identified by the PCR-ELISA, whereas strains for which genotype-specific probes were unavailable (i.e., strains bearing G11, G14, P3, P10, or P11 specificity) were not genotyped (data not shown), demonstrating the genotype specificity of G and P probes used in the assay.

G genotype analysis of rotavirus-positive human stool samples by PCR-ELISA. The usefulness of the assay was further evaluated by analyzing a total of 396 rotavirus-positive stool samples collected in four countries (Brazil, Ghana, Japan, and the United States) (Table 2). Three hundred ninety-four of the 396 (99.5%) samples were successfully G genotyped by the PCR-ELISA (Table 5). Three hundred twenty of the 394 G-genotyped samples gave visible bands after RT-PCR (indicating a large amount of amplified cDNA), whereas 74 (including 3 samples each from Brazil and Ghana that were nontypeable by typing PCR in each country of origin) did not produce any visible bands (suggesting a low amount of amplified cDNA) in an agarose gel, demonstrating the high sensitivity of the assay. Two previously untested samples from Japan were not amplified with any of the primer pairs used for the VP7 RT-PCR.

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These samples were reanalyzed in this study using three different primer pools and correctly typed as being G9, indicating again the mistyping those variant strains. A subset of 55 of the 77 G-genotype-dissident samples from Brazil were further analyzed in this study by typing PCR using three different sets of primer pools (i.e., H1 pool, C pool, and A pool). This was because the H1 primer pool used in typing PCR initially in Brazil typed each of the 55 samples as G4 plus G9, whereas PCR-ELISA typed all 55 samples as G9. As expected (69), the C primer pool correctly typed such samples as being G9, indicating again the importance of the primer pool(s) to be used in the assay. Representative strains characterized as being G9 by PCR-ELISA and G4 plus G9 by typing PCR were randomly selected to be further characterized by sequence analysis of the VP7 gene. Such strains shared 97.1 to 98.7% VP7 gene nucleotide identity with rotavirus G9 strains available in the GenBank database, which confirmed the G9 genotype of such strains (data not shown). Twelve samples that were typed as being G1 by typing PCR were identified as being G9 by microarray, in agreement with the PCR-ELISA results (36). In addition, all discordant Ghanaian samples were analyzed by microarray, the results of which corroborated the PCR-ELISA results (36).

Interestingly, stool rotavirus samples from Ghana and Brazil presented a remarkably high diversity of G genotypes (G1 to G5 and G8 to G10). In contrast, all the samples from the United States belonged to genotype G1 (Table 5). This high G-genotype diversity detected in Ghanaian and Brazilian samples may partly be responsible for the highly discordant values observed in such samples.

Eighty-three of 284 (29.2%) human rotavirus stool strains from Brazil and Ghana that were unequivocally G genotyped by PCR-ELISA, including three mixed infections, were demonstrated to be either nontypeable or mistyped by standard typing PCR assay in each country of origin (Tables 5 and 6). Of note was the finding that a total of 73 G9 strains and 2 G8 strains from Brazil and Ghana were wrongly typed as G1, G2, G3, or mixtures of G4 plus G9 or G6 plus G9 by typing PCR (Table 5), suggesting that (i) G8- and G9-specific primers used in the assay may be cross-reactive or (ii) such G8 and G9 strains may contain a mutation(s) capable of reacting with multiple G-type-specific primers. A previous study, for example, demonstrated the mistyping of rotavirus G8 strains as being G3 by typing PCR due to the cross-reactivity of the G3-specific primer with G8 strains (1). Moreover, the mistyping of G9 strains has been reported, particularly when the H1 pool is used, due to a cross-reactivity of the G4-specific primer with G9 strains (52, 69). The use of three different probes per genotype in the PCR-ELISA minimized the chances of mistyping those variant strains. A subset of 55 of the 77 G-genotype-dissident samples from Brazil were further analyzed in this study by typing PCR using three different sets of primer pools (i.e., H1 pool, C pool, and A pool). This was because the H1 primer pool used in typing PCR initially in Brazil typed each of the 55 samples as G4 plus G9, whereas PCR-ELISA typed all 55 samples as G9. As expected (69), the C primer pool correctly typed such samples as being G9, indicating again the importance of the primer pool(s) to be used in the assay. Representative strains characterized as being G9 by PCR-ELISA and G4 plus G9 by typing PCR were randomly selected to be further characterized by sequence analysis of the VP7 gene. Such strains shared 97.1 to 98.7% VP7 gene nucleotide identity with rotavirus G9 strains available in the GenBank database, which confirmed the G9 genotype of such strains (data not shown). Twelve samples that were typed as being G1 by typing PCR were identified as being G9 by microarray, in agreement with the PCR-ELISA results (36). In addition, all discordant Ghanaian samples were analyzed by microarray, the results of which corroborated the PCR-ELISA results (36).

Only 7 of 165 (4.2%) human rotavirus stool strains from Brazil, Ghana, and the United States that were unambiguously P genotyped by PCR-ELISA exhibited discrepant P genotypes as determined by typing PCR in each country of origin (Tables 5 and 6). The overall concordance between typing PCR and PCR-ELISA in P-genotyping results was 89.1% (147/165), which was much higher than that for G-genotyping results (Table 6). This may be due to the finding that, unlike a high diversity of G genotypes detected, a vast majority (149/165 [90.3%]) of the tested samples belonged to genotype P8, the most common human P genotype (Table 5). P genotypes determined by the PCR-ELISA were confirmed by microarray hybridization (data not shown).
**DISCUSSION**

Rotaviruses in nature have been demonstrated to undergo constant genetic variation via sequential point mutations, genetic reassortment, genomic rearrangement, or intragenic recombination (3, 16, 30, 41, 74). Thus, the emergence of strains that cannot be G and/or P typed by the currently available methodologies is not surprising. Moreover, because rotaviruses infect a wide variety of animal species, interspecies transmission of animal virus to humans or reassortment between human and animal viruses during mixed infections can occur, which may lead to the emergence of novel strains that could become of epidemiologic significance. In this regard, of note are the findings that rotavirus strains bearing unusual genotypes such as G6, G8, G10, G11, P[11], and P[14] that are commonly found in animals have recently been detected in humans with diarrhea (6, 7, 43, 56, 78, 82). In addition, it is noteworthy that genotype G12, which has been detected at an increasing frequency in recent years in some parts of the world, especially in India, Nepal, and Bangladesh (9, 48, 61, 66, 72, 78), was isolated from pigs in India in 2006 (26). Consequently, the methodologies used for rotavirus typing need to be monitored closely and updated accordingly to improve the capability in detecting and typing such strains. Indeed, a number of studies published lately have reported problems in genotyping certain human rotavirus field strains using the currently available methodologies such as typing PCR or monoclonal antibody-based ELISA (1, 13, 20, 21, 31, 42, 52, 60, 65, 69, 79).

Molecular typing methodologies such as typing PCR, probe hybridization, and microarray hybridization have been valuable tools for rotavirus genotyping, allowing the detection and characterization of strains not commonly found in humans and animals (4, 11, 15, 17, 24, 27, 28, 29, 49). Observations made in such studies have contributed much to our understanding of rotavirus epidemiology and evolution. However, such techniques also present limitations. For instance, in the case of typing PCR, the most widely used method for rotavirus typing, the genotype of a rotavirus strain is determined by the size of the amplified genome fragment. If the amplified fragment size of one genotype is very close to that of another genotype, it is sometimes difficult to differentiate the two in the agarose gel, which may lead to a misdiagnosis of the result. In addition, spurious bands sometimes seen in the gel can be wrongly interpreted as being mixed infections or false-positive reactions. Moreover, cross-reactivity of a certain primer(s) in the typing primer pool can also occur (69). In fact, this problem was observed in the present study with a subset of 55 Brazilian samples that were mistyped as a dual G4-plus-G9 infection by using the H1 primer pool but were correctly identified as being G9 by using the C primer pool in the typing PCR. Furthermore, the constant need for updating primers in the typing primer pool due to the emergence of mutant strains is hampered by the difficulty in selecting specific primers that fit into the assay design (42, 52, 65, 69).

Many PCR-ELISAs have been developed for the detection, identification, and quantification of a large variety of viruses (2, 8, 14, 23, 35, 46, 47, 50, 57, 62, 63, 71, 75, 77, 83). In the present study, we have developed and evaluated a PCR-ELISA protocol for the detection and identification of clinically relevant human group A rotavirus G (G1 to G6, G8 to G10, and G12) and P (P[4], P[6], P[8], P[9], and P[14]) genotypes. This assay is easy to perform and highly sensitive. The simplicity of the assay allows the methodology to be used in less endowed laboratories in less developed countries since there is no need for expensive or sophisticated equipment, and the reagents used are easy to obtain commercially. This is particularly important since various laboratories in less developed countries have recently participated in an evaluation of phase 3 clinical trials of selected rotavirus vaccines conducted in such countries (67, 81). With the introduction of these vaccines in the immunization programs of countries, there will be a need to monitor any changes in circulating strains as well as the emergence of new strains. Obviously, it is critically important to identify G and P genotypes of prevailing rotavirus strains as accurately as possible in such situations to evaluate the G- and P-type-specific efficacies of these rotavirus vaccines. The PCR-ELISA developed in this study is an ideal assay for such a purpose to be used in less developed countries. This assay clearly has some advantages over the traditional typing PCR since more than one type-specific probe is used in the same reaction, which increases sensitivity; the selection of probes is more flexible since the nucleotide position of the probe is not critical for the interpretation of the results; and it is easy to include additional probes for newly discovered or emerging genotypes in the assay. Although the cost of the labeled probes is more expensive than those of the ones regularly used for typing PCR, these probes are used at a very low concentration, and therefore, the overall cost will be approximately the same as that of the regularly used PCR assay.
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REFERENCES


