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Approach to molecular characterization of partially and completely untyped samples in an Indian rotavirus surveillance program☆

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Abstract

Surveillance networks for rotavirus document the burden of the disease using the proportion of children hospitalized with gastroenteritis positive for rotavirus by enzyme immunoassay. They also describe genotypes of circulating viruses by polymerase chain reaction for the VP7 and VP4 genes, which determine G and P types, respectively. A proportion of samples cannot be genotyped based on initial testing and laboratories need to assess further testing strategies based on resources and feasibility. To 365 samples obtained from an Indian rotavirus strain surveillance program, we applied an approach to determine the G and P types in antigen positive samples that failed to type initially with the standard laboratory protocol. Fifty-eight samples (19%) were negative for the VP6 gene, indicating that the antigen test was likely to have been false positive. Alternative extraction and priming approaches resulted in the identification of G and P types for 264 strains. The identity of one strain was determined by sequencing the first-round amplicons. Thirty-five strains were partially typed and seven strains could not be typed at all. The distribution of G and P types among strains that had initially failed to type, except one strain, did not differ from that in strains that were typed using the standard laboratory protocol.

Keywords

Untyped Protocol; ELISA false positive; Specific priming

1. Introduction

Rotaviruses are an important cause of acute diarrhea in both humans and animals. The genus rotavirus belongs to the family Reoviridae and is further classified by three different specificities: group, subgroup and serotypes. Rotaviruses are classified based on the VP6

☆The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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protein into seven groups (A–G) [1]. Of these, Group A rotaviruses are an important cause of mortality and morbidity in children <5 years of age, especially in the developing world [2]. Group A rotaviruses are further classified into subgroupsbased on the VP6 proteins and into G and P sero-/genotypes based on two outer capsid proteins VP7 and VP4, respectively. Currently there are 27 G and 37 P genotypes characterized [3]. A wide variety of rotavirus types circulate in humans and animals. Rotavirus diversity is generated through three main mechanisms: mutation, reassortment and inter-species transmission [4,5].

Most surveillance networks now use polymerase chain reaction (PCR)-based approaches to determine VP7 (glycoprotein, G-) and VP4 (protease sensitive protein, P-) genotypes. These networks, largely coordinated by the World Health Organization (WHO) since 2008, have shown that there is a wide geographic and temporal diversity in circulating G and P types identified from children less than 5 years of age hospitalized with acute gastroenteritis, in whom rotavirus is detected by an enzyme immunoassay across the different regions of the world reporting as part of the WHO rotavirus surveillance network [6]. The understanding of genotype distribution has shown that two widely used vaccines appear to protect against homologous and heterologous viruses. But the long term effects on virus circulation exerted by the immune pressure of a vaccinated population are as yet unknown and warrant continued molecular surveillance at this time. Additionally, studies on virus diversity and evolution are important to understand the biology of transmission and circulation in the population. This knowledge propels the application of robust molecular methods to identify the prevalent genotypes and methods to track the emergence of novel viruses.

A WHO manual describes the methods used to perform initial identification and further characterize group A rotavirus isolates [7]. Although the methods and primer sets described in the manual and by other networks appear to identify the majority of strains based on updated WHO reports and network publications [6,8,9], a proportion of strains remain untyped and require further testing. As the referral laboratory for the Indian National Rotavirus Surveillance Network which collected >4000 stool samples from 11 hospitals in 4 regional centers [8,11], we have developed an approach to handling samples initially untyped by standard methods and describe its application to samples collected over five years from 2007 to 2012.

2. Materials and methods

2.1. Samples

Stool samples were received for VP7 and VP4 molecular characterization in the Wellcome Trust Research laboratory (WTRL) from 2007 to 2012, as part of the Indian Rotavirus Strain Surveillance Network (IRSSN) or as referrals. All samples were screened by enzyme immunoassay (Premier Rotaclone, Meridian Diagnostics, Cincinnati, OH) and the antigen positive samples were genotyped as previously described elsewhere [8]. Complementary DNA (cDNA) was synthesized by reverse transcription (RT) as previously described using random primers (Pd(N)6 hexamers; Pharmacia Biotech) and 400 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies) [8]. Briefly, a first-round RT-PCR targeting VP7 and VP4 consensus regions using primers (VP7F/R and Con3/Con2, respectively) described in Table 1 were performed. The first-round product was used as a

template to determine specific VP7 (G) types (G1, G2, G3, G4, G8, G9, G10 and G12) and VP4 (P) types (P[4], P[6], P[8], P[9], P[10], P[11]) in a semi-nested multiplex PCR format [8]. Of the 2226 rotavirus ELISA positive samples for which further molecular characterization was performed, 57 samples were partially genotyped and 308 samples were untyped for G and P types. These represent 2.5% partially genotyped and 13.5% completely untyped samples of the total samples forwarded for further analysis.

2.1.1. Approach to completely untyped samples—

RNA was re-extracted from 30% fecal suspensions using the QIAamp Viral Mini RNA kit (Qiagen, Hilden, Germany) as per the manufacturer's specifications for samples collected from 2007 to 2009 that were initially extracted using Trizol reagent (Invitrogen Life Technologies). Samples collected from 2010 to 2012 were initially subjected to RNA extraction using the Viral Mini RNA kit method; re-extraction was performed using the Trizol reagent.

Polymerase chain reaction amplifying the VP6 region was performed to determine the presence or absence of rotavirus using primers described in Table 1 and random primed cDNA [10]. For samples that were negative for the VP6 gene by PCR with random primed cDNA, cDNA was synthesized using specific priming and amplified with the VP6 primers using the OneStep RT-PCR kit (Qiagen, Hilden, Germany). Samples that were negative by this method were recorded as negative on VP6 PCR with false positive ELISA. The samples positive for the VP6 gene were subjected to G and P typing using the standard primer sets as previously described [11].

2.1.2. Approach to partially typed samples and VP6 PCR positive samples—

RNA from samples which were partially typed and VP6 PCR positive samples which remained untyped after re-extraction and application of the standard genotyping protocol were subjected to specific priming for reverse transcription and amplification using the VP7F/R and Con2/Con3 primers and the One Step RT-PCR kit (Qiagen, Hilden, Germany), followed by a second-round PCR with the standard primer set. Typing of samples that remained untyped was attempted using alternate primer sets targeting the consensus regions of the VP7 and VP4 genes (Table 1) [7]. If present, the first-round product was sequenced for strains that were still G and P untyped (Fig. 1).

2.2. Sequencing

Sequencing of the first-round amplicon was attempted for all VP6 positive, G- and P-untyped samples. Briefly, the amplicons were purified and sequenced in both directions with the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) using the same primer pairs as in the first-round PCR. The sequences were resolved in the automated DNA sequencer, the ABI PRISM 310 Genetic Analyzer (Applied Biosystems), and the electropherograms were analyzed using sequencing analysis software (Finch TV, version 1.4.0). Consensus sequences were compared with available rotavirus sequences in GenBank for genotype confirmation using the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3. Results

We explored an approach (Fig. 1) to further characterize partially and completely untyped samples for G and P typing of 57 partially typed and 308 untyped samples. Fifty-eight (58/308, 19%) of the untyped samples were negative for VP6 gene amplification after repeat extraction and VP6 PCR using both random and specific priming methods. These were considered ELISA false positives.

Of the 250 samples that were VP6 positive, we determined both G and P genotypes for 211 samples following re-extraction, reverse transcription (RT) using random hexamer priming and genotyping with the standard protocol. Inclusion of the remaining 39 untyped samples and 57 partially typed samples for reverse transcription and amplification with the One Step RT-PCR, using specific priming for VP7 and VP4, resulted in resolution of both G and P genotypes for an additional 45 samples. We subjected the remaining partially typed and untyped samples ($n = 51$) to specific priming for VP7 and VP4 RT using alternate primer sets (Table 1). This led to determination of both G and P types for 8 strains and partial typing for 35 strains (12 G untyped and 23 P untyped). Seven samples remained completely untyped (Fig. 2). Of the original 57 partially typed samples, 22 remained partially typed.

Only one sample which failed to type in the second-round PCR for either VP7 or VP4 had a first round product for both genes and these were sequenced and the strain identified as G11P[25].

The most common G and P types isolated were G1 ($n = 100/307$, 32%) and P[8] ($n = 157/307$, 51%), respectively (Table 2).

4. Discussion

Use of a standard protocol for genotyping had resulted in 308/2226 (13.5%) samples being untyped for G and P types and 57/2226 (2.5%) being partially typed for either G or P type. The approach we used, as shown in Fig. 1, is to sequence the first-round G and P amplification product, if available. If not present, the presence of rotavirus is confirmed by performing VP6 PCR using both random and specific priming approaches after re-extraction. If VP6 is positive, specific priming with standard G and P primers or alternate primer sets was carried out to attempt genotyping of these samples.

Application of the VP6 PCR for confirmation resulted in the identification of 58/2226 (2.6%) false positive ELISA results. A recent publication has indicated the sensitivity and specificity of the Premier Rotaclone kit to be 76% and 100%, respectively [12]. It is possible that the ELISA false positives identified in this study could be due to degradation of the nucleic acid in the samples, but it could also be due to variation in test performance characteristics depending on the laboratory and the types of samples included for evaluation.

In the remaining 307 untyped and partially typed samples, alternate extraction methods with the standard primer sets resulted in typing of both G and P types in 256 (83%) and partially typing in 43 (14%) samples. Hence, use of the standard primer sets resulted in G or P or both types in 97% of the samples obtained from India. The lack of initial typing may be

because of the inefficiency of the extraction followed by random priming or because PCR inhibitors may be carried over from extraction. A comparison of various available commercial manual and automated extraction methods reported that the QIAmp Viral Mini RNA kit may carry over RT-PCR inhibitors [13], which may have resulted in the lack of initial genotyping. The surveillance network uses Trizol or kit based extraction and a random priming approach for cDNA generation, because both G- and P-typing PCRs can then be set up using the same cDNA.

However, other kits, particularly the automated extraction methods and one-step RT-PCR kits, are expensive to use for the large numbers of samples in a surveillance program. Laboratories need to allocate resources for initial screening and genotyping followed by further characterization based on the level of detail necessary to meet surveillance objectives.

One inexpensive approach for controlling problems with extraction is to spike all samples with a non-competing internal control RNA virus to check for the efficiency of the extraction procedure performed, where PCR amplification for the control virus can be performed either along with the typing PCR or separately in samples that fail to genotype.

The use of additional primer sets typed an additional eight strains for both G and P types. Seven samples remained untyped and 35 were partially typed respectively after using additional primers [14]. Only for one sample from Delhi, sequencing of the first-round product led to the identification of G11P[25], a type previously reported infrequently from India and Bangladesh [15]. No new genotypes were isolated and the predominant G and P types identified were G1 and P[8], which were reflective of the types isolated previously from the various locations.

Using the approach detailed above, the number of samples fully or partially typed increased from 86% (1918/2226) to 97% (2161/2226). This approach shows that if a robust set of standard primers are available that genotype the bulk of specimens in initial testing, the unresolved genotypes are likely to be false positive ELISA samples or those which have had a problem with the efficiency of extraction. The use of additional primer sets resolves genotypes only in a very small fraction of the samples. Unlike in 2007, when an increase in the number of G-untyped strains resulted in the identification of a new genotype, G12, by sequencing of the first-round product [16], no new genotypes were detected in multiple untyped samples from the network. Future approaches to genotyping for untypable samples might also include next-generation sequencing, which has not been used for field surveillance so far.

While documenting genotypes has been a mainstay of rotavirus epidemiology in the past, the data emerging from the oral rotavirus vaccines indicate that real-time knowledge of genotypes may not be necessary to inform understanding of response to and protection afforded by vaccines. Since vaccines have only been in use for a few years and in limited geographic settings, it is possible that continued surveillance will provide data suitable for long term surveillance. Therefore, laboratories and networks need to decide the extent to which they will pursue genotyping of rotavirus strains and allocate appropriate resources.

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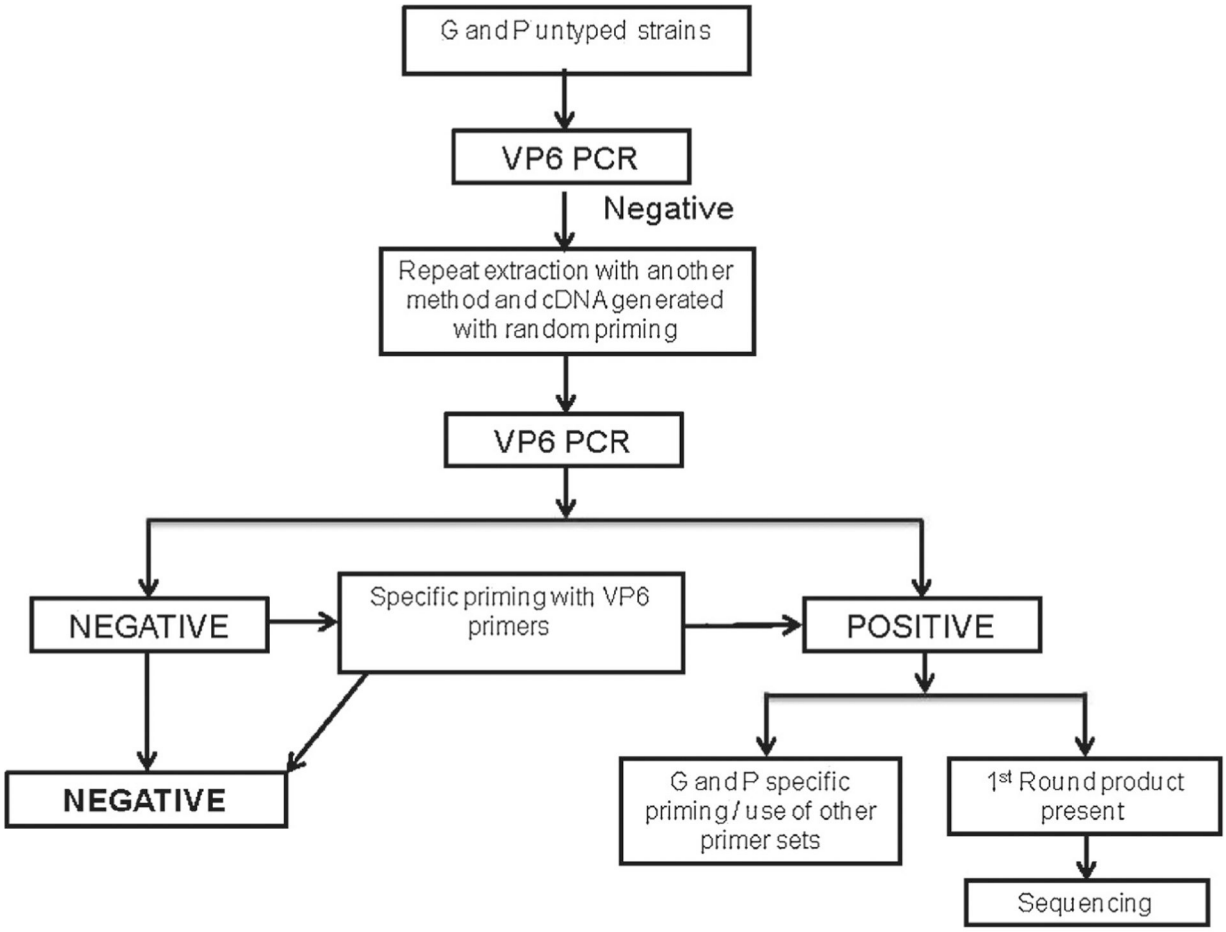

Fig. 1.

Testing strategy for untyped rotavirus ELISA positive samples obtained from the Indian Rotavirus Strain Surveillance Network sites, 2007–2012.

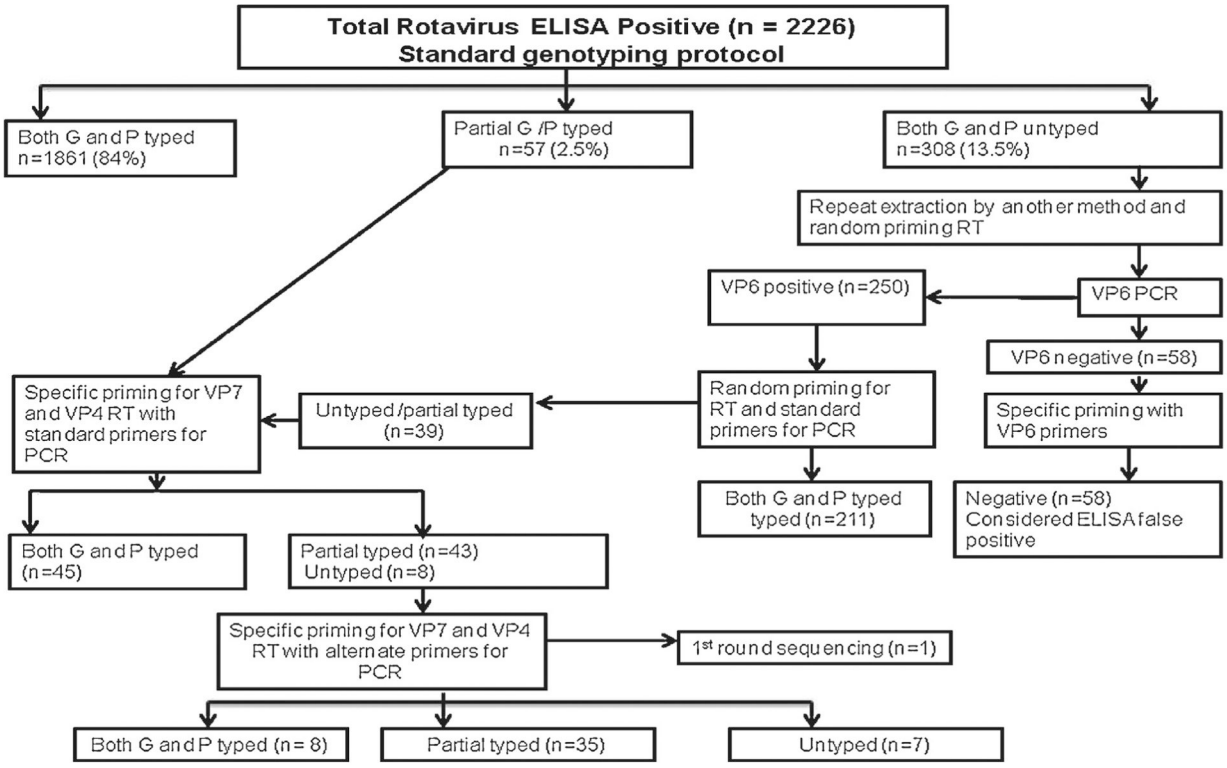

Fig. 2.

Number of rotavirus strains typed by the approach to genotyping used in this study.

Table 1:

VP6, VP7 and VP4 primers used in this study [7].

VP6 primers		
VP6-F	GACGGVGCRACTACATGGT	nt 747–766
VP6-R	GTCCAATTCATNCCTGGTGG	nt 1126–1106
VP7 consensus primers		
Primers used in the standard protocol		
VP7-F	ATG TAT GGT ATT GAA TAT ACC AC	nt 51–71
VP7–R	AAC TTG CCA CCA TTT TTT CC	nt 914–932
Alternate consensus VP7 primers		
9con1-L	TAG CTC CTT TTA ATG TAT GGT AT	nt 37–59
VP7-R	AAC TTG CCA CCA TTT TTT CC	nt 914–933
VP4 consensus primers		
Primers used in the standard protocol		
Con3	TGG CTT CGC TCA TTT ATA GAC A	nt 11–32
Con2	ATT TCG GAC CAT TTA TAA CC	nt 868–887
Alternate consensus VP4 primers		
VP4-F	TAT GCT CCA GTN AAT TGG	nt 132–149
VP4-R	ATT GCA TTT CTT TCC ATA ATG	nt 775–795

Table 2:

Combination of G and P types of rotavirus strains obtained by following the approach to typing of initially untypable samples obtained from the Indian surveillance network, 2007–2012. Note that there was no circulation of G3 and G4 strains at the surveillance sites during this period.

	P[4]	P[6]	P[8]	Other P types	P Untyped	Mixed P types	Total
G1	12	0	73	0	12	3	100
G2	68	0	0	0	2	0	70
G9	8	0	48	11	7	0	74
G12	0	10	28	0	2	0	40
Other G types	0	0	0	1	0	0	1
G Untyped	4	0	8	0	7	0	19
Mixed G types	2	0	0	0	0	1	3
Total	94	10	157	12	30	4	