Molecular epidemiology of rotavirus in children and animals and characterization of an unusual G10P[15] strain associated with bovine diarrhea in south India

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ARTICLE INFO

Article history:

Keywords:
Rotavirus
Epidemiology
Diarrhea

ABSTRACT

Rotaviruses are enteric pathogens causing acute, watery, dehydrating diarrhea in various host species, including birds and mammals. This study collected data on the disease burden and strain prevalence of group A rotavirus in animals and humans in Vellore and investigated interspecies transmission by comparison of circulating genotypes. Stool samples from children aged less than 5 years, admitted to the hospital between January 2003 and May 2006 for diarrhea and diarrheal samples from animals that were collected from a veterinary clinic and several dairy farms near Vellore between February 2007 and May 2008 were processed and subjected to RNA extraction and reverse-transcription PCR for genotyping of VP7 and VP4. Of 394 children with diarrhea, 158 (40%) were positive for rotavirus and the common G types identified were G1 (47, 29.7%), G2 (43, 27.2%), G9 (22, 13.9%), G10 (2, 1.2%), G12 (1, 0.6%) and mixed infections (27, 17.8%). The common P types were P[4] accounting for 57 (36%) samples, P[6] 57 (36%), P[11] 3 (1.8%) and P[6]2 (1.2%). Of 627 animals, 35 (1 bullock, 2 goats, 32 cows) were found to be infected with rotavirus (5.5%). The common G types identified in order of frequency were G6 (17, 48.5%), G2 (10, 28%), G10 (4, 11%), G8 (2, 5.7%) and mixed infections (2, 5.7%). The common P types were P[6] accounting for 16 (46%) samples, P[4] 7 (20%), P[1] 3 (8.5%) and P[8] 3 (8.5%). An unusual P type P[15] was seen in one sample in combination with G10. The finding of G2 infections which are rarely identified in animals implies anwbranopoxonic transmission since this genotype is predominantly associated with infection in humans.

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1. Introduction

Rotaviruses are enteric pathogens causing acute, watery, dehydrating diarrhea in various host species, including birds and mammals. Rotavirus is the cause for approximately 500,000 child deaths each year, mainly in developing countries [1]. Likewise, rotavirus-associated enteritis is a major problem in young calves [2]. Besides affecting cattle and buffalo calves, rotaviruses also affect pigs, foals, lambs, and young ones of pet animals and poultry [3–5]. The rotavirus genome consists of 11 double-stranded RNA segments and each genome segment encodes at least one protein (VP1–VP4, VP6, and VP7, NSP1 to NSP6) [6]. Traditionally, the rotavirus classification scheme has been based on a dual nomenclature to differentiate the VP4 (P) and VP7 (G) type specificities encoded by two genome segments, 4 (for VP4) and 7, 8 or 9 (for VP7, depending on the strain). At least 27 G genotypes and 35 P genotypes based on the sequence analysis of the VP7 and VP4 genes have been identified [7]. Recently, the 11 rotavirus gene segments (VP1, VP2, VP3, VP4, VP6, VP7, NSP1, NSP2, NSP3, NSP4 and NSP5 genes) have been assigned letter codes for each gene and classified into at least 6 R, 6 C, 7 M, 35 P, 13 I, 27 G, 16 A, 6 N, 8 T, 12 E and 8 H genotypes, respectively, based on specific nucleotide sequence identity cut-off percentages for each gene segment [8,9].

Human rotaviruses most commonly belong to G types 1–4, 9 and P types [4] and [8] [10] whereas bovine rotaviruses most commonly belong to G types 6, 8 and 10 and P types [1], [5], [6] or [11] [11]. G10 strains commonly occur in combination with P[1], P[5] and P[1] [2]. A G10 P[15] strain was reported for the first time in a lamb infected with rotavirus in China [12]. In India, so far, bovine diarrhea associated with G10 rotavirus has been seen in combination with P[1] [13–15], P[6] [16], P[14] [17] and P[3] [18]. Despite clear evidence of host range restriction, a number of animal gene segments, mostly those encoding the neutralizing antigens (defining
G and P types), have been identified repeatedly in humans in different parts of the world during surveillance studies [19,20], providing evidence that animals may act as a source of virus and/or of genetic material for evolutionary diversification of human rotaviruses. For example, strains such as G3 (found commonly in species such as cats, dogs, monkeys, pigs, mice, rabbits, and horses), G5 (pigs and lambs), G9 (pigs and lambs), and G10 (cattle) have been isolated from the human population throughout the world [10]. The G9 strains reported in humans [21] which are found in lambs and pigs [22], may have been become established in humans during the past two decades through transfer from animals.

There are a number of studies reporting rotavirus strain distribution in animals or humans in India but they do not provide any geographic or temporal comparisons of distribution among animals and humans [14,18,23,24]. This is also similar to the lack of such reports worldwide with only a few studies that have compared the strains isolated from animals and humans simultaneously in the same region [25,26]. In this study, we aimed to provide data on the disease burden and strain prevalence of rotavirus in animals and humans in our region and investigate interspecies transmission by comparison of circulating genotypes using hemi-nested PCR typing for common human G- and P-types. In addition, a G10 rotavirus strain isolated for the first time with combination of P[15] in India was characterized by partial genome sequence analysis.

2. Materials and methods

2.1. Patient and animal recruitment

Stool samples were collected from children aged less than five years, admitted to the hospital between January 2003 and May 2006 for diarrhea, defined as the passage of three or more watery stools in a 24-h period [27]. The severity of diarrhea was assessed using the Vesikari scoring system [28]. Information was collected on duration of diarrhea, maximum number of stools passed per day, duration and peak frequency of vomiting, degree of fever, presence and severity of dehydration and treatment. An episode was considered mild for scores 0–5, moderate for a score of 6–10, severe for a score of 11–15 and very severe for scores 16–20. Diarrheal samples from animals were collected from a veterinary clinic and several dairy farms near Vellore between February 2007 and May 2008. At the dairy farms, diarrheal samples from cows alone were collected, while from the veterinary clinic, samples from cows, buffaloes, bullocks and goats were collected.

2.2. RNA extraction, PCR and sequencing

Animal stool samples were subjected to proteinase K (2 μg/ml in 20 mM Tris, pH 7.5, 10 mM EDTA, and 0.1% SDS) treatment for 1 h followed by CC41 extraction [29]. From the stool samples of hospitalized children, RNA was extracted using Trizol™ reagent [30].

cDNA was synthesized from the extracted viral RNA through reverse transcription in the presence of random hexamers. Amplification of the VP6 gene was performed using primers described previously [31]. G and P typing were performed using VP7 and VP4 specific multiplex hemi-nested RT-PCRs for common human genotypes, as described previously [32–34]. Forward and reverse primers for the amplification of each segment other than VP7, VP6, VP4 and NSP4 to characterize G10P[15] strain were obtained from a published protocol [35]. PCR cycling conditions were determined based on the melting temperatures (Tm) of the primer pairs used for each PCR.

When strains failed to genotype or genotypes needed to be confirmed, the first round PCR products generated through the use of consensus primers were sequenced and the genotype determined by sequence and phylogenetic analysis. Sequencing was done using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) after purification of amplified products in an automated DNA sequencer (ABI PRISM 310 Genetic Analyzer, Applied Biosystems, Foster City, CA). The electropherograms obtained were analyzed using the sequencing analysis software (Sequence Navigator, version 1.01, Applied Biosystems). The nt and deduced aa sequences were compared with sequences available in the NCBI (National Center for Biotechnology Information) GenBank database using the BLAST (Basic Local Alignment Search Tool) program. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4.0 [36]. Dendrograms constructed were confirmed by two different methods, neighbor joining and maximum parsimony.

2.3. Statistical analysis

The data were analyzed using Epi Info 2002 and Statat 10.0. Chi square and Mann Whitney U tests were performed to determine the significance of differences observed between groups.

2.4. Accession numbers

Partial nucleotide sequences of VP1, VP2, VP3, VP4, VP6, VP7, NSP1, NSP2, NSP3, NSP4 and NSP5 of the G10P[15] strains were submitted to the GenBank database and their accession numbers are HQ660637, HQ660638, HQ660639, FJ798615, FJ798616, FJ798617, HQ660640, HQ660641, HQ660642, FJ798618, HQ660643 respectively.

3. Results

3.1. In children

The median (interquartile range [IQR]) age of the 394 children enrolled in the study was 10 (7) months, with >90% of children less than 2 years of age. The median Vesikari score of diarrheal severity was 11.0 and the children required admission for a mean duration of 2.8 days. Of 394 children screened, we found that 158 children were infected with rotavirus (40%).

3.2. Genotype distribution

The common G types identified in order of frequency were G1 (47/158, 29.7%), G2 (43/158, 27.2%), G9 (22/158, 13.9%), G10 (2/158, 1.2%), G12 (1/158, 0.6%) and mixed infections (27/158, 17.8%). The common P types were P[4] accounting for 57/158 (36%) samples, P[8] 57/158 (36%), P[11] 3/158 (1.8%) and P[6] 2/158 (1.2%). Mixed infections with varied P types were seen in 5 (3.2%). G typing alone was possible in 23 samples (14.4%), only P typing in 5 samples (3.6%) and 11 samples were completely untypeable (6.5%). The common G:P combinations seen in children were, G2P[4] in 39/158 (24.6%) samples, G1P[8] in 29/158 (18.3%) samples, G9P[8] in 21/158 (13.2%) samples, G1P[4] in 4/158 (2.5%) samples and G10P[11] in 1/158 sample (0.6%) (Fig. 1a).

3.3. In animals

We collected total of 627 samples from animals with diarrhea, including 589 cows (25 were calves), 2 buffaloes, 11 bullocks and 25 goats (11 were kids). The mean duration of diarrhea was 4.5 days for adult animals, 4 days for calves and 3 days for goat kids. Out of 627 animals we found 35 (1 bull, 2 goats, 32 cows) infected with rotavirus (5.5%). The common G types identified in order of frequency were G6 (17/35, 48.5%), G2 (10/35, 28%), G10 (4/35, 11%),
G8 (2/35, 5.7%) and mixed infections (2/35, 5.7%). The common P types were P[6] accounting for 16/35 (46%) samples, P[4] 7/35 (20%), P[1] 3/35 (8.5%) and P[8] 3/35 (8.5%). We observed an unusual P type, P[15], in one sample in combination with G10. G typing alone was possible in five samples (1.2%).

The common G:P combinations seen among 35 infected animals were G6P[6] in 15 (42.8%), G2P[4] in 7 (20%), G2P[8] and G10P[15] in 3 (8.5%) each, G6P[1] in 2 (5.7%) animals and G8P[6], G8P[1] and G10P[15] in 1 animal each (2.8%) (Fig. 1b). The distribution of genotypes in animals showed G6 infections as the predominant cause of symptomatic rotavirus infection, followed by G2. Since G2 strains that are commonly reported in humans were found in animals, the G2P4 and G2P8 strains isolated from animals and humans were sequenced to investigate the possibility of anthropontic transmission. By phylogenetic analysis, the animal strains showed >95% similarity at nt level and deduced aa level with human rotavirus sequences.

3.4. Strain characterization of G10P[15]

Since P typing was not possible for a G10 strain after the second round of multiplex PCR using type specific primers, we sequenced a fragment of the 876 bp first round product. This strain was isolated from an adult cow in a dairy farm on 27th July 2007. The cow was five years old and had endured diarrhea for five days. The partial nucleotide sequence of the VP4 gene and deduced amino acid sequence were determined and compared with VP4 sequences of prototype strains belonging to P1 to P35 genotypes using maximum parsimony. Phylogenetic and sequence analysis of the VP4 gene of AD63 showed maximum identity to the prototype ovine P[15] strain isolated in China [12] (91% identity at nt and 93% at the deduced aa level) (Fig. 2).

We also sequenced amplified products of VP6, VP7 and NSP4 genes using the respective oligonucleotide primers and we constructed phylogenetic trees. Sequence analysis of G10 genotype showed maximum identity to the bovine G10 genotypes (99% at nt level and 98% at aa level) (Fig. 3). VP6 gene analysis indicated that the G10P[15] strain was of subgroup I and clustered with animal strains. The NSP4 gene analysis identified it as genogroup A of human origin with 95% identity at nt and aa level (Fig. 4). Taken together, the data indicated that genetic reassortment could have occurred. Therefore all other genes of this strain were analyzed by sequencing.
Fig. 3. Phylogenetic tree constructed from sequences of the VP7 gene of the G2 and G10 rotavirus strains from our study with other representative G types using the neighbor joining method. The animal G2 (dashed box) strains has >95% identity at nt level with human G2 strains from Vellore (solid box).

Fig. 4. Phylogenetic tree constructed from sequence of the NSP4 gene of prototype genogroup A and other genogroups using Maximum parsimony method. AD63 is shown in a dashed box.

Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Nt. size</th>
<th>Accession no.</th>
<th>Identity</th>
<th>Origin</th>
<th>Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP1</td>
<td>804</td>
<td>GU937877</td>
<td>97%</td>
<td>Caprine</td>
<td>R2</td>
</tr>
<tr>
<td>VP2</td>
<td>587</td>
<td>GU937878</td>
<td>95%</td>
<td>Caprine</td>
<td>C2</td>
</tr>
<tr>
<td>VP3</td>
<td>218</td>
<td>GU937879</td>
<td>94%</td>
<td>Caprine</td>
<td>M2</td>
</tr>
<tr>
<td>VP4</td>
<td>471</td>
<td>L11599</td>
<td>91%</td>
<td>Ovine</td>
<td>P15</td>
</tr>
<tr>
<td>VP6</td>
<td>377</td>
<td>EF200566</td>
<td>96%</td>
<td>Animal</td>
<td>I2</td>
</tr>
<tr>
<td>VP7</td>
<td>788</td>
<td>AF386916</td>
<td>95%</td>
<td>Bovine</td>
<td>G10</td>
</tr>
<tr>
<td>NSP1</td>
<td>1381</td>
<td>GU937883</td>
<td>94%</td>
<td>Caprine</td>
<td>A11</td>
</tr>
<tr>
<td>NSP2</td>
<td>657</td>
<td>GU937884</td>
<td>94%</td>
<td>Caprine</td>
<td>N2</td>
</tr>
<tr>
<td>NSP3</td>
<td>947</td>
<td>EU708964</td>
<td>95%</td>
<td>Feline</td>
<td>T6</td>
</tr>
<tr>
<td>NSP4</td>
<td>616</td>
<td>AJ427316</td>
<td>94%</td>
<td>Human</td>
<td>E2</td>
</tr>
<tr>
<td>NSP5</td>
<td>716</td>
<td>GU937887</td>
<td>97%</td>
<td>Caprine</td>
<td>H3</td>
</tr>
</tbody>
</table>

Sequence analysis of VP1, VP2, VP3, NSP1, NSP2 and NSP5 genes of AD63 showed 97%, 95%, 94%, 95%, 94%, and 97% identity respectively to the genes of caprine G03 strain isolated from Bangladesh [37] (Table 1). The NSP3 gene showed 95% similarity to the feline rotavirus G2/G3P[9] [38]. This VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5 genes of strain G10P[15] to the G10-P[15]-I2-R2-C2-M2-A11-N2-T6-E2-H3 genotypes, respectively.

4. Discussion

The study found that in children, 40% of diarrhea was associated with rotavirus, which is in agreement with multi-site Indian data from 2005 to 2007 [39] and similar to global data [40]. However an earlier review of studies carried out between 1990 and 2005 from India, estimated the burden of rotavirus disease in hospitalized children with diarrhea to be 20.8% [27]. The studies used a number of different protocols such as LA, ELISA, EM, PAGE and PCR. The burden of rotavirus disease among hospitalized children is higher when molecular methods are incorporated.

The most prevalent rotavirus strains causing childhood diarrhea globally are G1–G4 and G9 [40]. Significant diversity of circulating rotavirus strains exists in India though G1, G2 and G9 are currently the most common strains followed by G12 [39,41]. Studies on rotavirus epidemiology have been carried out at Vellore for a number of years [23,42–44], and demonstrate the differences in strain circulation over time. Data from 2002 to 2003 showed that G1 was the most common genotype followed by G9 and G2 strains (46.8%, 19.1% and 8.5% respectively) [42]. The present study (2003–2006) showed that G1 was predominant followed by G9 and G2 (11.9%, 10.5% and 5.6% respectively). Another surveillance study in an overlapping time period (2005–2009) showed similar findings, with G1 being the most common genotype followed by G2, G9 and G12 (25%, 21%, 13% and 10% respectively) [39]. G3 and G4 rotavirus strains that are described as common genotypes across the world [20] and in previous studies from Vellore [43,44] were not seen in the present study. When we examined G:P combinations, G2[P4] strains were predominant (9.9%) followed by G1[P8] (7.4%) and G9[P8] (5.3%). This pattern is in agreement with findings from different regions of India but with a lower prevalence [41]. G10P[11] viruses are also seen in children in Vellore, but mainly in neonates, where both symptomatic and asymptomatic infections were documented [34,35].

In animals, we documented a prevalence of 5.5% (35/627) rotavirus infection which is low when compared with a study from Kolkata that reported a prevalence of 10.52% (10/95) [24], but comparable to a study in Haryana [18] which had a prevalence of 4.61% (21/455). Studies from animals in different regions of India have reported G6[P1], G6[P11], G3[P3], G10[P1] and G10[P11] genotypes of group A rotavirus [14,15,45,46]. Our study found G:P
combinations of G6P[6], G2P[4] and G2P[8]. With G2 infections rarely identified in animals, this finding implies anthropopropic transmission since this genotype is predominantly associated with infection in humans. Additionally, we isolated G6P[1] genotype from only two animals in our region: a genotype commonly reported from cattle in other parts of the country [14,46] and the world [47]. Moreover this study failed to identify G10P[11], which has been found in asymptomatic infections in children and neonates in our region and from animals in other parts of the country, indicating that the strain is now well adapted to human neonates in our setting.

Of the few studies that compare human and animal rotavirus simultaneously in a particular setting [25,26], a study in the Netherlands did not show any evidence of interspecies transmission [26], while a study from Slovenia documented evidence of zoonotic transmission in one human rotavirus strain, where the VP6, VP7, VP8* and NSP4 genes of G3P[6] strains had identity with porcine strains. In addition, two porcine rotavirus strains carried VP7 of probable human origin, suggesting an interspecies reassortment event [25]. In this study although we did not find any animal strains in human infection, the finding of human G2P[4] and G2P[8] strains in 10/35 rotavirus positive animal diarrheal samples suggests the possibility of anthropopropic transmission.

The genetic analysis of the strain G10P[15] (AD63) provides interesting insights into the origin and evolution of rotaviruses and may suggest that the strain has arisen through reassortment between strains of different animal species or humans. G10 genotypes are predominantly bovine strains. Although G10 strains are common in human neonates in this region, phylogenetic analysis did not show a relationship between AD63 and G10 human neonatal strains, indicating that the VP7 gene more likely came from a bovine source [34]. Characterization of the VP4 gene of the AD63 strain revealed identity with the ovine strain LP14 from China [12], which is the only available P[15] sequence. Given the original ovine report of P[15], isolation of this genotype from a cow may indicate interspecies transmission, but there are seven aa mismatches between P[15] of AD63 and LP14 protein sequences. Analysis of the whole genome rather than partial gene sequences may better explain the origin of this strain.

Characterization of the VP6 (SG1) and NSP4 (genogroup A) genes of AD63 revealed animal and human origin, respectively. To further confirm human origin of NSP4 gene, we compared two representative NSP4 genogroup A sequences of human origin (RV5 – accession number US9103) and bovine origin (B223 – accession number AF144803) strains with AD63. The percentage identity of the NSP4 sequence of AD63 was 90% and 82% with RV5 and B223 strains respectively. Analysis of gene linkages indicates that usually rotaviruses possess either SG1/NSP4A or SG1/NSP4B specificities in both human and animal strains [48]. In AD63, the VP6 sequence clustered with SG1 strains of animal origin, while the NSP4 clustered with genogroup A sequence of human origin. This indicates the possibility of a reassortment between rotaviruses of animal and human origin, while maintaining the VP6-NSP4 linkage, and suggesting that this genetic linkage is not host restricted, but VP6/NSP4 genogroup restricted.

The NSP3 gene of G10P[15] strain showed maximum identity with that of Ca2t G3P[9] strain from USA isolated from a cat [38], but interestingly is believed to be of bovine origin based on phylogeny. Therefore, the NSP3 of AD63 is likely of bovine origin, maintaining its host specificity.

The other six genes VP1, VP2, VP3, NSP1, NSP2 and NSP5 of G10P[15] strain showed maximum identity with that of a caprine GO34 RV strain isolated from Bangladesh [37]. However, the VP1, VP3 and NSP2 of this GO34 RV strain showed maximum identity with human strains indicating that these genes, as well as the NSP4, in AD63 may be of human rather than caprine or bovine origin. Additionally, the VP2 and NSP1 genes of GO34 RV showed maximum identity with ovine strains and NSP5 genes with bovine strains, indicating that even though these genes of AD63 are closely related to GO34, they are unlikely to be of caprine origin. Taken together the data indicate that the G10P[15] strain in this study is a result of one or more reassortment events between human, bovine and possibly ovine strains. There is phylogenetic evidence regarding possible inter-species reassortment. For example, the finding that most zoonosis detected in humans involve DS-1 like genomes, which may have a common ancestry with bovine strains suggest that reassortment may occur in humans or animals after cross-species transmission. The unusual African G8P[6] and G8P[8] strains could have emerged through several reassortment events involving human G2P[4] strains, which are DS-1-like, and strains carrying the G8, P[6] and P[8] genotypes. Similarly G5 rotavirus strains detected in children in Brazil since the early 1980s and subsequently in Argentina and Paraguay that were shown to be naturally occurring reassortants between Wa-like human and porcine viruses [49].

In this study, the predominant cause of symptomatic rotavirus infection in animals was G6 followed by G2, while in children G1, G2 and G9 strains were common. With G2 infections identified in animals, reverse zoonotic transmission should be considered since this genotype is predominantly associated with infection in humans. This report highlights the genomic diversity of such circulating rotavirus strains and underlines the need for frequent surveillance of domestic animals as they may be potential reservoirs for future rotavirus outbreaks in the human population.

Conflicts of interest

None.

Acknowledgement

PR was supported by the Global Infectious Disease Research Training grant to GK (D43 TW007392).

References

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