Characterization of rotavirus causing acute diarrhoea in children in Kathmandu, Nepal, showing the dominance of serotype G12

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Diarrhoeal diseases are a major problem in developing countries. Though precise data on childhood mortality associated with diarrhoeal diseases in Nepal are not available, it has been estimated that approximately 25% of child deaths are associated with diarrhoeal disease, particularly acute diarrhoea. The purpose of this study was to assess the incidence of rotavirus causing acute diarrhoea in children less than 5 years of age. A total of 525 children with acute diarrhoea in a children’s hospital of Kathmandu, Nepal, were enrolled between April and September 2011. The incidence of acute diarrhoea due to rotavirus was 25.9% (136/525) as determined by ELISA. The percentage of rotavirus-infected males was higher (64.5%) than females (35.5%). The frequency of rotavirus cases was higher in children less than 2 years of age, among which the majority of cases (80.2%) were in children between 6 and 24 months old (P<0.01). Genotypic characterization by RT-PCR revealed that the serotype G12 represented 55.9% of cases in this study associated with P-types of either P[6], P[4] or P[8]. Further to this, a total of eight G/P combinations were identified, G12P[6] being the most common strain type of rotavirus in Nepal, with a prevalence rate of 46.4%. The aim of this study was to find out the major genotypes of rotavirus causing acute diarrhoea in children.

INTRODUCTION

Diarrhoeal diseases are a major problem in Nepal as it is a developing country. Though precise data on childhood mortality associated with diarrhoeal diseases in Nepal are not available, it has been estimated that approximately 25% of child deaths are associated with diarrhoeal diseases, particularly acute diarrhoea (Maharjan et al., 2007). The WHO Child Health Epidemiology Reference Group estimates that 16% of deaths in African children younger than 5 years of age are directly attributable to diarrhoeal diseases (Klaus et al., 2004).

Diarrhoea can be caused by a wide range of bacteria (e.g. species of Shigella and Salmonella, as well as Escherichia coli and Vibrio cholerae), enteroparasites (e.g. species of Giardia and Entamoeba histolytica) and viruses (e.g. rotavirus, adenovirus and norovirus) (Vargas et al., 2004). Among the diarrhoea-causing viruses, rotavirus is the most important aetiological agent worldwide and is implicated in severe dehydrating diarrhoea requiring hospitalization. The annual burden of diarrhoeal disease is estimated as more than 110 million diarrhoeal episodes, 25 million clinic visits, 2 million hospitalizations and 600 000 childhood deaths per year (Cunliffe et al., 1998; Naghipour et al., 2008; Glass et al., 2006; Dennehy, 2007). In a 1-year study period in Nepal from October 2001 to November 2002, Sherchand & Haruki (2004) reported a 30.6% prevalence of rotavirus in hospitalized children with diarrhoea who were less than 5 years of age compared to a prevalence rate of 4.1% in the community in children of the same age. While 30.6% of the diarrhoeal stools were rotavirus-positive, only 0.87% of normal stools were positive for rotavirus.

Rotavirus infects not only children but also adults and rotavirus infection may occur repeatedly in humans from birth to old age. Young children are the most vulnerable and the prevalence of infection varies according to age. It has been observed that, generally, the prevalence of rotavirus
infection is significantly higher in groups less than 2 years of age than in older groups (P<0.01). The highest prevalence rate was seen in children from 13 to 24 months of age (57.6%), followed by those less than 1 year of age (46.3%) and the prevalence rate was lower in older children (Nguyen et al., 2004).

A study conducted in Nepal by Uchida et al. (2006) showed that ~70% of rotaviruses found in each of the three age groups belonged to serotype G1P[8]. Interestingly, there were 29 (20%) G12 rotaviruses carrying either P[8] or P[6] and one (0.7%) G11 rotavirus carrying an unusual P[25] genotype (Uchida et al., 2006).

People in developing countries are at particular risk due to contaminated drinking water, while in industrialized areas, these infections may occur in immigrants or people with a weakened immune system (Pearson, 2009).

Bishop et al. (1973) first identified rotaviruses in humans when they observed characteristic particles in the cytoplasm of duodenal epithelial cells from young children admitted to hospital for treatment for acute diarrhoea. Rotaviruses are classified into serogroups A–G. However, only groups A–C have been shown to infect humans and animals, group A being the main cause of rotavirus disease (Nguyen et al., 2004). Group A rotaviruses are classified into serotypes on the basis of the outer capsid proteins VP7 (G serotypes) and VP4 (P serotypes), the G serotype being defined by a glycoprotein and the P serotype being defined by a protease-sensitive protein. On the basis of VP7, 19 different G serotypes have been identified so far. P serotypes, which are referred to by their numbers (e.g. P1 and P2), are difficult to characterize by traditional methods of virus neutralization; therefore, molecular methods based on sequencing are widely used and the resulting serotype designation is indicated by a number in square brackets (e.g. P[1] and P[2]). Currently, 29 P genotypes have been identified. G and P serotypes have been adopted to define rotavirus serotypes. G1, G3 and G4 serotypes associated with P[8] and G2 associated with P[4] represent over 88% of all rotavirus strains worldwide. G and P antigens segregate independently and interspecies transmission between humans and animals is common. Thus, unusual combinations of P and G serotypes have been observed (Pun, 2010).

There are differences in the distribution of rotavirus infections according to age in developing and developed countries. In the former, the highest rates occur during the first year of life. However, in developed countries, peak rates occur in the second year of life. This could lead to rotavirus vaccine being applied earlier in life in children in developing countries (Nguyen et al., 2004). It is generally believed that serotype-specific immunity plays a role in protection against disease, so the epidemiology of G and P serotypes of circulating strains forms a critical knowledge base for the development and implementation of rotavirus vaccines (Uchida et al., 2006).

Rotavirus is transmitted by the faecal–oral route, via contact with contaminated hands, surfaces and objects and possibly by the respiratory route. The faeces of an infected person can contain more than 10 trillion infectious particles per gram; only 10–100 infectious particles are required to transmit infection to another person.

**METHODS**

The present study was conducted by the Department of Microbiology and the Public Health Research Laboratory at Tribhuvan University Teaching Hospital, Kathmandu, Nepal. A total of 525 stool samples were collected from the children under 5 years of age visiting Kanti Children’s Hospital, Kathmandu, Nepal, with acute diarrhoea in the period between April and September 2011. Written informed consent was obtained from the children’s parents or guardians before enrolment. Ethical approval was taken from the Institutional Review Board (IRB), Institute of Medicine, Tribhuvan University Teaching Hospital, Kathmandu, Nepal. From each participating child, clinical data were obtained and a stool sample was collected in a sterile container. The collected stool samples were investigated for rotavirus infection using an antigen detection test by ELISA (ProSpecTTM Rotavirus Microplate Assay, Oxoid) according to the instructions of the manufacturer. Cases that were positive for rotavirus by ELISA were subsequently subjected to genotyping by RT-PCR.

For molecular typing, genomic RNA was extracted from all rotavirus-positive samples using the QIAamp viral RNA mini kit (Qiagen), according to the manufacturer’s instructions, and used to determine VP7 (G) and VP4 (P) genotypes by RT-PCR according to methods described previously (Uchida et al., 2006; Sherchand et al., 2009; Gouvea et al., 1990).

For rotavirus G genotyping, the VP7 gene was amplified by RT-PCR with VP7/F and VP7/R primers (Table 1). For rotavirus P genotyping, the VP4 gene was amplified by RT-PCR with con-2 and con-3 primers (Table 2).

**Data interpretation.**

**Gel electrophoresis and documentation.** The genotypes were identified based on the PCR amplicon size by using gel electrophoresis. PCR amplicons were resolved in 2% agarose gels stained with ethidium bromide (0.5 mg ml⁻¹) in Tris-boric acid-EDTA (TBE) buffer at constant voltage. Images were photographed under UV light using a gel documentation system.

**Validity and reliability.** To test the validity and reliability of this research, research proposal preparation was carried out. The clinical/microbiological profile sheet was approved and verified by the supervisors. Quality/reference procedures were followed with appropriate controls and standards.

**Analysis.** Differences in the proportions of each genotype were assessed by using the χ² test. P-values <0.05 were considered statistically significant.

**RESULTS**

Out of a total of 525 enrolled cases, 323 (61.5%) were from the inpatient department and 202 (38.5%) were from the outpatient department. Of the patients included in this study, 337 (64.2%) were male and 188 (35.8%) were female. The frequency of diarrhoea was higher in children...
less than 2 years of age, among which the majority of cases (367, 69.9%) were detected in the age group of 6–24 months followed by those less than 6 months of age (101, 19.2%). The smallest number of cases (14, 2.7%) was found in individuals between 49 and 60 months of age. Of the 525 paediatric patients, 136 (25.9%) tested positive for rotavirus antigen in their stools. The highest rate of rotavirus infection was found in children below 2 years of age, 109 (80.2%) were between 6 and 24 months of age (Fig. 1). The frequency of rotavirus infection in children less than 2 years of age was found to be significantly greater than in other age groups ($P<0.01$). The number of cases of rotavirus infection was higher in males (85, 62.5%) than in females.

**Genotypic distribution of rotaviruses**

Molecular analysis of rotavirus genotypes G and P revealed that the predominant genotype was G12 (55.9%) followed by G2, G1 and G9 with 9.6% of cases having a mixed type and 2.2% being non-typable (Fig. 2). These genotypes were associated with different P-types representing P[6] (51.5%), P[4] (19.2%) and P[8] (9.6%) with 11.0% of cases having a mixed type and 8.8% being non-typable (Fig. 3).

In addition, a total of eight G/P type combinations were identified, including G12P[6] (46.4%), which was the most commonly detected rotavirus strain type, followed by G2P[4] (15.4%), G12P[8] (5.2%), G1P[6] (4.4%), G1P[8]...

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**Table 1. PCR primers and cycling conditions used for VP7 genotyping of rotavirus strains**

<table>
<thead>
<tr>
<th>PCR</th>
<th>Cycling conditions</th>
<th>Primer</th>
<th>Primer sequence (5’→3’)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP7</td>
<td>94 °C for 2 min</td>
<td>VP7/F</td>
<td>ATGTATGGTATGAAATATACCAC</td>
<td>881</td>
</tr>
<tr>
<td></td>
<td>94 °C for 1 min</td>
<td>VP7/R</td>
<td>AACTTGCCACCATTTTTTC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>52 °C for 1 min</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>72 °C for 1 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>72 °C for 7 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>15 °C – hold</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VP7</td>
<td>94 °C for 4 min</td>
<td>G1</td>
<td>CAAGTACTCAATCAATTGATG</td>
<td>618</td>
</tr>
<tr>
<td></td>
<td>94 °C for 1 min</td>
<td>G2</td>
<td>CAAGTACTCAACACGGAGG</td>
<td>521</td>
</tr>
<tr>
<td></td>
<td>42 °C for 2 min</td>
<td>G3</td>
<td>ACGAACAATCACAGGAGG</td>
<td>682</td>
</tr>
<tr>
<td></td>
<td>72 °C for 1 min</td>
<td>G4</td>
<td>CTGTTCTGGTGAGGATG</td>
<td>452</td>
</tr>
<tr>
<td></td>
<td>72 °C for 7 min</td>
<td>G5</td>
<td>GTCACACATTGTGAAATTCG</td>
<td>754</td>
</tr>
<tr>
<td></td>
<td>15 °C – hold</td>
<td>G9</td>
<td>CTGATGTGACTAAYAATAC</td>
<td>179</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G10</td>
<td>ATGTACAGTAARACTG</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>G12</td>
<td>CGATGGAGTGAACTG</td>
<td>387</td>
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**Table 2. PCR primers and cycling conditions used for VP4 genotyping of rotavirus strains**

<table>
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<tr>
<th>PCR</th>
<th>Cycling conditions</th>
<th>Primer</th>
<th>Primer sequence (5’→3’)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP4</td>
<td>94 °C for 2 min</td>
<td>Con 3</td>
<td>TGGCTTCGCCCATTTTTATAGAC</td>
<td>876</td>
</tr>
<tr>
<td></td>
<td>94 °C for 1 min</td>
<td>Con 2</td>
<td>ATTCGGACCATTATAACC</td>
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</tr>
<tr>
<td></td>
<td>50 °C for 1 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>72 °C for 1 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>72 °C for 7 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>15 °C – hold</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VP4</td>
<td>94 °C for 2 min</td>
<td>P[4]</td>
<td>CTATTTGTAAGGTTAGAGTC</td>
<td>483</td>
</tr>
<tr>
<td></td>
<td>94 °C for 1 min</td>
<td>P[6]</td>
<td>TGGTGATTAAATGAGTTCA</td>
<td>267</td>
</tr>
<tr>
<td></td>
<td>45 °C for 2 min</td>
<td>P[8]</td>
<td>TCTACTGGGATRNTG</td>
<td>345</td>
</tr>
<tr>
<td></td>
<td>72 °C for 1 min</td>
<td>P[9]</td>
<td>TGGACGTATGCAATTGGC</td>
<td>391</td>
</tr>
<tr>
<td></td>
<td>72 °C for 7 min</td>
<td>P[10]</td>
<td>ATCATAGTAGTAGTGC</td>
<td>583</td>
</tr>
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</table>
DISCUSSION

Diarrhoeal disease remains one of the largest health problems in many parts of the world. The disease is often mild and self-limiting but, particularly in the elderly and young children, the symptoms may be very severe. Studies in developing countries have shown that in the first 2 years of the life, children may have up to 10 episodes of diarrhoeal disease, often resulting in significant mortality rates (Black, 1993). Among the top ten diseases in Nepal, diarrhoeal disease occupies second place (Ministry of health and population, 2007).

Our study indicated that a higher frequency of diarrhoea was seen in males (64.2 %, 337/525) than in females (35.8 %, 188/525). Similar results were also shown by Shariff et al. (2003) in children less than 5 years of age in eastern Nepal with 67.5 % cases of diarrhoea being in male patients. Higher prevalence rates of diarrhoea in males have also been reported in Nepal (56.4 %; Sherchand et al., 2009), Missouri (57 %; Klein et al., 2006) and Tanzania (61.4 %; Moyo et al., 2011).

It appears that infants less than 6 months of age were initially protected against severe diarrhoea, to some extent, by maternal antibodies and they seem to have acquired adequate immunity between 12 and 16 months of age. Infants and young children aged 6–12 months appear to be at greater risk, declined levels of maternal antibodies to rotavirus infection having been documented by Sherchand et al. (2009).
Age-wise distribution of the diarrhoeal cases revealed that diarrhoea was more prevalent in children less than 2 years of age, among which the age group of 6–24 months showed the highest prevalence (367/525, 69.9 %) compared with that of the age group of less than 6 months (101/525, 19.2 %). The prevalence of diarrhoea in children less than 2 years of age was found to be statistically significantly higher than other age groups \((P<0.01)\). In a similar study conducted by Shariff et al. (2003) in children less than 5 years of age in eastern Nepal, patients between 6 months and 2 years of age represented the majority of diarrhoeal cases (70.9 %). Similar results were reported by Rerksuppaphol & Rerksuppaphol (2011) who showed that a higher proportion of males were infected with rotavirus. Among the 136 rotavirus-positive cases in the present study, the majority of cases were present in children less than 2 years of age: 80.2 % (109/136) were between 6 and 24 months of age and 15.4 % (21/136) were aged less than 6 months.

According to Odimayo et al. (2008), in Nigeria, rotavirus is an important causative agent of diarrhoea in children under the age of 5 years, with 80 % of rotavirus-positive cases being in children under 24 months of age (Odimayo et al., 2008). A similar study carried out by Saravanan et al. (2004) with 745 children under 3 years of age in Chennai, South India, also revealed that a greater proportion (62.5 %) of rotavirus-positive cases were in children between 7 and 18 months of age. Another study carried out by Ahmed et al. (2009) at the Institute of Child and Mother Health between July 2005 and June 2006 in Bangladesh showed that 92 % of cases of rotaviral diarrhoea were identified in children in the age group of less than 2 years. These results are comparable to the findings of our study. A similar study carried out by Nimri & Hijazi (1996) on children in a refugee camp in Jordan also showed the overall prevalence of rotavirus was significantly higher (62.0 %) in children aged less than 24 months.

The lower incidence rates of rotavirus infection in children who were less than 6 months old can probably be attributed to the high rate of breast feeding during these months. Breast milk contains rotavirus antibodies and other components that reduce the risk of rotavirus infection in babies. However, many children over 6 months of age were receiving some breast milk. The apparent lack of protection against rotavirus infection in this age group may be partly due to reduced total daily intake of breast milk or to the reduced amount of rotavirus antibody in mature breast milk or to both.

Among the total 525 cases of diarrhoea, rotavirus accounted for 25.9 % (136/525). This finding is accordance with other studies that detected prevalence levels of 31.2 % in Nepal (Sherchan et al., 2011), 22.6 % in South India (Saravanan et al., 2004), 26.0 % in Bangladesh (Hoque et al., 1994), 24 % in Africa (Cunliffe et al., 1998) and 28 % in Ghaza, Palastine (Abu-Elamreen et al., 2008). Genotyping of the rotaviruses showed that 13.2 % (18/136) of rotavirus diarrhoeal cases were due to mixed genotypes with the viruses carrying more than one G type (G1, G2, G12) and/or P type (P[4], P[6], P[8]); 1.5 % (2/136) were non-typable cases, whereas 8.1 % (11/136) of cases were partially t ypable with an absence of either G or P types, and 77.2 % of cases were completely t ypable. Viruses carrying G12 with either P[4], P[6] or P[8] were the most common causes of diarrhoea in the children studied, together with less common genotypes such as G1, G2 or G9 with either P[4], P[6] or P[8]. Genotype G12P[6] was the most prevalent at 46.4 % followed by G2P[4] (15.4 %) and G12P[8] (5.2 %), whereas genotype G1P[8] was detected in only 2.2 % of cases and G12P[4] and G9P[4] were the least prevalent, both accounting for only one case (0.7 %) each.

In several studies, genotypes G1 and G4 were found to be the most prevalent. Gerna et al. (1990) reported that 84 % of all strains investigated between 1981 and 1988 were of type G1 and G4. Gentsch et al. (1996) reviewed the prevalence of G serotypes and found that 71–97 % of the strains characterized belonged to these serotypes. In past years, Gentsch et al. (1996) and Parashar et al. (1998) published data from a global collection, which included specimens from the United States, Costa Rica, Korea, Israel, China, Mexico, Bolivia, India and Bangladesh. These data have shown that G1P[8] was the most common genotype (53 %). Similarly, a study by (Naficy et al., 1999) in Egypt on characterization of rotavirus strains in circulation showed that 76 % of the strains were of the commonly encountered genotypes P[4]G2 and P[8]G1; Samajdar et al. (2006) in eastern India also reported the distribution of genotypes among strains with G1 (53.8 %) being the most predominant, followed by G2 (22.5 %), G12 (17.1 %) and G9 (2.1 %).

In the present study, strains with genotype G12 accounted for 56 % of cases, followed by G1 (7.4 %), G2 (20.6 %) and G9 (4.4 %). Various combinations of G12 with either P[4], P[6] or P[8] were also detected. Sherchan et al. (2011) reported 48 % of strains as having genotype G12 in Nepal. A study by Castello et al. (2006) on strains collected in Argentina from 1999 to 2003 reported 6.7 % of the strains as having the G12 genotype. Uchida et al. (2006) reported that in Nepal there was a greater dominance of G1P[8] and that the emergence of G12 in combination with P[8] or P[6] was the second most common G type (20 %). Similarly, combinations of G12 with either P[6], P[8] or P[4] have been reported by Samajdar et al. (2006) and Pongsuwanna et al. (2002). In a 3-year study from November 2005 to October 2008 in Nepal, Sherchand et al. (2011) found that the G12 genotype was predominant in all three time periods, 2005–2006 (50 %), 2006–2007 (29 %) and 2007–2008 (33.7 %), with P[6] constituting 37 %, 33 % and 27.7 % of cases in these time periods, respectively, and G12P[6] was the most prevalent combined genotype, accounting for 34 % of strains in 2005–2006, 24 % in 2006–2007 and 47.5 % in 2007–2008.

In the present study, the G9 serotype was recovered from 4.4 % of cases. Carvalho-Costa et al. (2006), studying strains from Rio de Janeiro, Brazil, in 2004, suggested that
G9 was more frequent (40%) than some classical genotypes. The incidence of G9 with either P[8] or P[6] was also reported by Jain et al. (2001) to be 17% in stool samples collected from hospitals in Bhopal, New Delhi, Davengere, Lucknow, Nagpur and Shimla from 1996 to 1998. The incidence of infection with particular group A rotavirus serotypes and genotypes varies between geographical areas during a rotavirus season and from one season to the next. Globally, viruses carrying either G1, G2, G3, G4, G9 and P[4] or P[8] genotypes are the most common causes of rotavirus disease in humans. G12 is also recognized as an emergent serotype that may become important in human disease (Khoury et al., 2011).

Rotaviruses are ubiquitous in the animal kingdom; therefore, interspecies transmission and, more importantly, exchange of genetic material between animal and human strains through gene reassortment can lead to the emergence of novel rotavirus strains of epidemiological significance. It is speculated that G12 strains have evolved so that they are capable of spreading more effectively between humans by gaining VP4 protein genes P[4], P[8] or P[6], which are common P genotypes found in human rotaviruses (Uchida et al., 2006).

Conclusions

The present study indicates that rotavirus was the cause of 25.9% of the diarrhoeal cases studied and that rotavirus is a major public health problem in children under 5 years of age. These children can subsequently become sources of outbreaks. Genotyping analysis revealed that G12 and P[6] were the major genotypes causing rotavirus diarrhea in Nepal. The study was conducted in a tertiary care government Children’s Hospital of Kathmandu. As these findings do not represent the complete scenario nationwide, further analysis is necessary to determine the prevalence of rotaviral infection in Nepal.

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REFERENCES


