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Circulating Genotypes of Rotavirus Prior to Rotarix®vaccine Introduction in Kathmandu, Nepal

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ABSTRACT

Background: In Nepal, it is estimated that about 3 million children under 5 years of age are prone to diarrhea and previous studies have shown rotavirus as the major etiological agent. Given the high burden of rotavirus, Rotarix® vaccine was introduced in the national immunization schedule in July 2020. This study was carried out in a tertiary health center from January- September 2018 to determine the burden of rotavirus diarrhea as well as genotypic variations in the circulating virus prior to vaccine introduction in Kathmandu, Nepal.

Methods: Hospital based cross sectional study was conducted among children less than 5 years of age attending Kanti Children's Hospital. Rotavirus antigen detection was performed by enzyme immunoassay using ProSpecT Rotavirus Microplate Assay. Rotavirus A positive samples were further confirmed by genotyping using Reverse-Transcription Polymerase Chain Reaction.

Results: A total of 530 children that included 184 males and 346 females were enrolled in this study. Rotavirus antigen was detected in 112 (21.1%) stool samples. Of the total 112 positive EIA stool samples that were genotyped, G12P[6] (30.3%) was found to be the most common type, followed by G3P[8] (26.8%), mixed type (14.3%), and G1P[6] (13.4%).

Conclusions: Continued surveillance should be carried out nationwide in Nepal to understand the effectiveness of the vaccination program and to report any new trends in the circulating genotypes.

Keywords: Children under five years of age; diarrhea; Nepal; rotavirus strains; RT_PCR

INTRODUCTION

Globally, diarrheal disease results in death of 525,000 children every year.[1] In context of Nepal, about 3 million children below 5 years of age are prone to diarrhea and a total of 1,1240,873 diarrheal cases and 63 diarrheal deaths were reported in 2017/18.[2] Rotavirus surveillance initiated from 2003 until the end of 2017 have shown rotavirus to be responsible for 22-33% of the total diarrheal cases in children under 5 years of age and G12P[6] as the predominating strain circulating in Nepal.[3-7] Given the high burden of rotavirus infection, live attenuated human monovalent Rotarix vaccine was launched in the national immunization schedule of Nepal in July 2020. 2 doses (1.5 ml oral) are recommended for all Nepalese children at 6 weeks and 10 weeks of age.[8]

However, pre-vaccination surveillance data on rotavirus diarrhea is necessary to compare and understand any new trend post vaccination programs.[9] So, the aim of this study was to determine the rotavirus disease burden in children according to host's age and gender as well as genotypic variations.

METHODS

The study was carried out in the Department of Clinical Microbiology, Public Health Research Laboratory, Institute of Medicine, Tribhuvan University Teaching Hospital (TUTH), Maharajgunj, Kathmandu in the period between January to September 2018. Ethical approval was obtained from the Institutional Review Board (IRB), Institute of medicine, Tribhuvan University Teaching Hospital, Kathmandu, Nepal. Written informed consent was obtained from the children's parents or guardians before enrolment. All children below 5 years of age visiting Kanti Children Hospital with chief complaint of diarrhea regardless of admission were included. After the recording of demographic and clinical details in a structured questionnaire, stool samples were collected

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in a sterile container and transported in ice packs to the laboratory.

In the laboratory, stool samples were aliquoted and tested for Rotavirus A infection using an enzyme immunoassay kit (EIA; ProSpecTM Rotavirus Microplate Assay, Oxoid Ltd., UK). The test detects the major inner capsid protein (VP6), present in Group A Rotaviruses. The reagents provided with the test kit were brought to room temperature before use. Sufficient number of wells for samples and controls were arranged in well holder. 100µl of diluted sample, positive and negative control were added to the separate wells. 100µl of enzyme conjugate was added to each well and mixed by rotating. It was incubated for one hour at room temperature. The liquid in the wells was poured out in a discard jar. The microtitre well holder was tapped vigorously against absorbent paper to ensure complete removal of liquid from the wells. The wells were washed out with deionized water for 5 times, tapped against absorbent paper each time. Then 100µl of substrate was added in each well and incubated for 10 minutes at room temperature. The substrate reaction was stopped by adding 100µl of stop solution in each well and result interpretation was done using ELISA reader capable of reading 450nm and compared with positive and negative controls.

Subsequently, all Rotavirus antigens positive samples were subjected to genotyping by RT-PCR. For molecular typing, genomic RNA was extracted using the QIAamp viral RNA mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. 560µl of AVL- carrier RNA mix was added in to labeled micro centrifuge tube. 140µl stool suspension was added to the micro centrifuge tube containing AVL-carrier RNA mix and incubated at room temperature for 10 minutes. The tubes were briefly centrifuged to remove drops from the inside of the lid. 560µl of ethanol was added and mixed by pulse vortexing for 15 seconds and the tube was centrifuged briefly. 630µl of the solution was carefully transferred to the spin column and centrifuged at 8000 rpm for 1 minute. The spin column was placed into a clean 2ml collection tube and filtrate containing tube was discarded. Again 630µl of the solution was carefully transferred to the spin column and centrifuged at 8000 rpm for 1 minute and spin column was placed into a clean 2ml collection tube and filtrate containing tube was discarded. 500µl of buffer AW1 was added to the column and centrifuged at 8000rpm for 1 minute. 500µl of buffer AW2 was added to the column and centrifuged at full speed for 3 minutes. The spin column was now placed in a clean 1.5 ml micro centrifuge tube. 60µl of buffer AVE was added and incubated at room temperature for 1 minute and centrifuged at 8000 rpm for 1 minute to elute the RNA. In this way RNA was extracted from the stool samples.

VP7 (G) and VP4 (P) genotypes were detected by RT-PCR according to methods described previously.[3,10,11] For Rotavirus G and P genotyping, the VP7 gene was amplified with VP7/F (ATGTATGGTATTGAATATACCAC) and VP7/R (AACTTGCCACCATTTTTTCC) primers and the VP4 gene with con-2 (ATTTCGGACCATTTATAACC) and con-3 (TGGCTTCGCCATTTTATAGACA) primers, respectively by RT-PCR.[12] Identification of Rotavirus genotypes were done on the basis of PCR amplicon size by using gel electrophoresis (PCR amplicons were resolved in 2% agarose gels stained with ethidium bromide (0.5 mg/ml) in Tris- Boric acid-EDTA (TBE) buffer at constant voltage) and images were photographed under UV light using a gel documentation system. Inactivated bovine Rotavirus and tris buffered saline solution was used as positive and negative control, respectively, for EIA. For genotyping PCR assays, genotype G1P[8] was used as a positive control.Data analysis was performed using Microsoft Excel 2016 (Redmond, WA, USA) and *p*-value <0.05 was considered significant.

RESULTS

From the beginning of January-September 2018, a total of 530 children that included 184 males and 346 females were enrolled in this study. Among the 530 stool samples tested, rotavirus antigen was detected in 112 (21.1%) samples (Table 1).

Table 1. Characteristics of children enrolled in our study.

Variable		Rotavirus positive [n (%)] (N =530)	Rotavirus negative [n (%)] (N =530)	p value
Sex	Male	37 (25.2)	147 (25.2)	0.67
	Female	75 (27.7)	271 (25.2)	
Age (months)	0-2	10 (20)	40 (80)	0.94
	3-5	16 (18.4)	71 (81.6)	
	6-11	42 (20.9)	159 (79.1)	
	12-24	33 (22.9)	111 (77.1)	
	>24	11 (22.9)	37 (77.1)	
Hospital admission	Yes	95 (21.3)	352 (78.7)	0.87
	No	17 (20.5)	66 (79.5)	

Out of the 112 positive cases, 37 (25.2%) were male and 75 (27.7%) were female. Overall, the highest number of enrollees was in age group 6- 11 months and the proportion of rotavirus positive cases were seen in age

groups 12-24 and >24 months. About two-thirds of the enrolled children were hospitalized. However, there was no significant difference in the gender-wise, age-wise, and hospital admission- wise proportion of rotavirus positivity [Odd's ratio; $p > 0.05$] (Table 1).

During the eight months of surveillance, rotavirus associated diarrhea was reported throughout the year with the highest positivity seen during February (63.8%) followed by January (55%). The rates of rotavirus detection were found to be comparatively lower during June and September as shown in Figure 1.

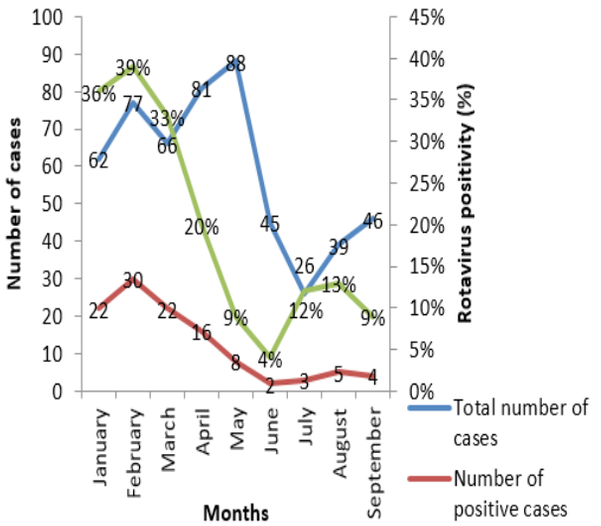

Figure 1. Month wise distribution of rotavirus cases from January to September 2018.

Of the total 112 positive EIA stool samples that were genotyped, 3 samples (2.7%) could not be typed (Figure 2). G12P[6] (30.3%) was found to be the most common type, followed by G3P[8] (26.8%), mixed type (14.3%), and G1P[6] (13.4%).

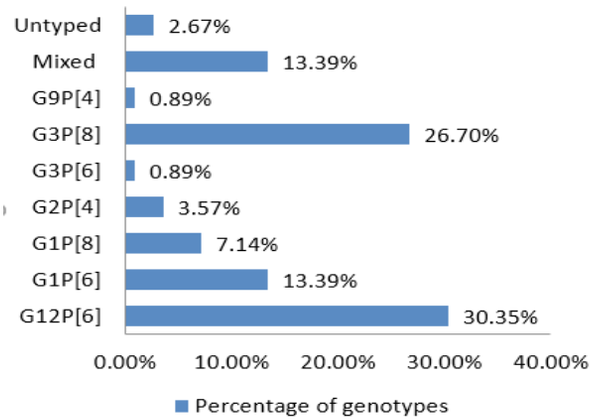

Figure. 2. Circulation of rotavirus genotypes.

DISCUSSION

This cross- sectional study reports the burden of rotavirus among children visiting a tertiary pediatric hospital with chief complaint of diarrhea. The overall prevalence of rotavirus diarrhea during the eight months of study was found to be 21.1% which is similar to previously conducted studies in the same geographical area.[5, 13-15] Although, a recent study,[16] has reported 91% of the total stool samples to be positive for rotavirus in the same study area in children below 15 years of age, that study used highly sensitive PCR assays as compared to EIA. These findings suggest rotavirus as the major cause of childhood diarrhea despite attempts to improve hygiene and sanitation. The same study also detected rotavirus in drinking water used by the family of children visiting the hospital suggesting drinking water as the major mode of transmission in the Kathmandu valley.In addition to the treatment cost of diarrhea, loss of wages by parents due to work leave to look after their children results in significant financial burden for an average Nepali household.[17]

Different variables like gender, age group, and hospitalization were found to be insignificantly associated with rotavirus. But one particular finding that merits attention in this study and previous other studies in Nepal [4,6,13,14] is the higher rate of rotavirus infection after 6 months of age. Since, rotavirus vaccine is given in two doses at 6 and 10 weeks of age we expect rotavirus vaccine to have significant impact in reducing childhood morbidity associated with rotavirus in Nepal similarly as in other countries where Rotavirus vaccination has been adopted.[18] However, it is paramount to continue surveillance of rotavirus and circulating genotypes nationwide post vaccine introduction to study the true impact of mass vaccination programs.

Rotavirus associated diarrhea was observed throughout the study period but peaked during the winter, months of February and January. Similar observation has been reported in almost all of the studies conducted in Nepal.[4,19,20] This could be explained by cooler environment with low humidity favoring the survival of rotavirus[21] and poor personal hygiene during winter.

Among the 27 G and 37 P genotypes that has been reported, G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8] are the predominant strain combinations responsible for majority of rotavirus infections globally.[21, 22] In our study, G12P[6], G3P[8], and mixed type was found to be the predominant strain which is in agreement with previous studies conducted in Nepal.[6-9,13,14,18] Circulation

of a different rotavirus strain can have implications for effective vaccine implementation.As per the recommendation of the World Health Organization, more than 100 countries have included rotavirus vaccine in their national immunization schedule.[23] Since, the vaccine that was recently introduced in Nepal is derived from a monovalent G1P[8] strain, we can anticipate that the vaccine will provide homotypic protection against G3P[8] and G1P[6] strains. But in Malawi, the effectiveness of vaccine was lowest against fully heterotypic strains such as G12P[6].[24] The efficacy of vaccine can also be affected by several factors including maternal rotavirus-specific antibodies, enteric co-infections, vaccine schedule, oral polio vaccine use, malnutrition, household sanitation and gut microbiome.[25]Future studies should be carried out nation-wide and focus not only on strain specific effectiveness but incorporate all other possible factors affecting effectiveness of vaccine in Nepal. Also, the World Health Organization recommends the surveillance of intussusceptions post vaccination for the adverse effect of the vaccine. Currently, only one study has been carried out to evaluate the epidemiology of intussusception among children in Nepal and additional studies are needed for post vaccine safety monitoring.[26]

Surveillance was carried out in a tertiary health center in Kathmandu and therfore may not reflect the national scenario. Other enteropathogens responsible for pediatric diarrhea were not monitored in this study. Such data would have provided information regarding coinfection with other enteropathogens prevaccination.

CONCLUSIONS

This study demonstrated G12P[6] as the predominant strain circulating in the Kathmandu valley. Since, rotavirus vaccine is considered less potent in developing countries,[27] continuous surveillance is necessary to assess the true impact of the vaccination program.

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