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Burden of Norovirus and Rotavirus in Children after Rotavirus Vaccine Introduction, Cochabamba, Bolivia

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Abstract. The effectiveness of rotavirus vaccine in the field may set the stage for a changing landscape of diarrheal illness affecting children worldwide. Norovirus and rotavirus are the two major viral enteropathogens of childhood. This study describes the prevalence of norovirus and rotavirus 2 years after widespread rotavirus vaccination in Cochabamba, Bolivia. Stool samples from hospitalized children with acute gastroenteritis (AGE) and outpatients aged 5–24 months without AGE were recruited from an urban hospital serving Bolivia's third largest city. Both viruses were genotyped, and norovirus GII.4 was further sequenced. Norovirus was found much more frequently than rotavirus. Norovirus was detected in 69/201 (34.3%) of specimens from children with AGE and 13/71 (18.3%) of those without diarrhea. Rotavirus was detected in 38/201 (18.9%) of diarrheal specimens and 3/71 (4.2%) of non-diarrheal specimens. Norovirus GII was identified in 97.8% of norovirus-positive samples; GII.4 was the most common genotype (71.4% of typed specimens). Rotavirus G3P[8] was the most prevalent rotavirus genotype (44.0% of typed specimens) and G2P[4] was second most prevalent (16.0% of typed specimens). This community is likely part of a trend toward norovirus predominance over rotavirus in children after widespread vaccination against rotavirus.

INTRODUCTION

Norovirus is the leading cause of acute gastroenteritis (AGE) worldwide and has historically been the second most common cause of AGE in children, responsible for up to 200,000 pediatric deaths annually.[1] Before the global introduction of rotavirus vaccine, rotavirus was responsible for an estimated 453,000 deaths per year in children under age 5 mostly in the developing world.[2] Norovirus and rotavirus are the major causes of diarrheal illness in South American children.[3–9] Recent data suggest that the relative burden of norovirus versus rotavirus in children with AGE may be changing after widespread adoption of rotavirus vaccine. Relatively little is known of viral etiology of pediatric AGE in low income, high mortality settings after vaccine introduction.

In 2006, two rotavirus vaccines were approved that have been widely adopted into vaccination programs globally.[10,11] Rotarix™ (GlaxoSmithKline Biologicals, Wavre, Belgium) is a monovalent live-attenuated vaccine whose implementation has resulted in significant reductions in AGE hospitalization and mortality in Latin American countries.[6,12] Rotarix is included in vaccine schedules of 16 Latin American countries and one territory (Bolivia, Brazil, Columbia, Ecuador, Mexico, Cayman Islands territories, Panama, Peru, Paraguay, El Salvador, Guatemala, Honduras, Nicaragua, Guiana, the Dominican Republic, Haiti, and Venezuela) and it was introduced in Bolivia in August 2008.[13] Rotavirus vaccination uptake is high in Bolivia (> 85%, two-dose coverage), and the vaccine has demonstrated high efficacy against rotavirus infection despite fears that the vaccine may be less effective in resource poor settings.[14,15] Reductions in hospitalization due to all-cause AGE have been described in multiple Latin American countries with reductions as high as 18% after vaccine introduction.[6] Reductions in rotavirus prevalence after vaccine introduction has been previously demonstrated by Bolivian investigators.[16] These results may predict norovirus replacing rotavirus as the most prevalent enteropathogen affecting children in Bolivia and similar communities worldwide, where diarrheal disease remains a major cause of morbidity and mortality.

In this study, we measured the prevalence of norovirus and rotavirus in an urban Bolivian hospital after the introduction of rotavirus vaccine. Circulating genotypes of each virus are described and rotavirus data published from the same hospital before vaccine introduction are compared with current results.[16,17]

MATERIALS AND METHODS

Study design. The study was conducted from March 2010 to February 2011 at the Centro de Pediatría Albina R. de Patiño located in Cochabamba, Bolivia. Cochabamba is Bolivia's (gross domestic product $34B) third largest city with a population of 618,366.[18] Water sources for families within urban Cochabamba are: indoor plumbing (61%), water network outside of the home (22%), and no access to water network (17%). In the rural surroundings, water access is more limited: indoor plumbing (20%), water network outside of the home (36%), and no access to water network (44%).[19] Level of formal education in Bolivia is variable. High rates of secondary and college education (79%) are found in urban centers whereas only primary education (40%) or no formal education (18%) is more common in rural communities.[20]

Hospital Albina R. de Patiño is a private academic children's hospital serving the urban and rural communities of Cochabamba with outpatient clinics, emergency department (ED), and inpatient services. All AGE inpatient and outpatient care is covered 100% by national insurance. All

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inpatients are triaged at the ED or clinic. Inpatient criteria are based on the clinical judgment of a physician determining the degree of dehydration, nutrition status, and ability to tolerate oral feedings.

Study protocol and consent forms were approved by the institutional review boards of Universidad Peruana Cayetano Heredia (laboratory site), Asociación Benéfica PRISMA, CEADES Salud y Medio Ambiente, and Johns Hopkins Bloomberg School of Public Health. Written informed consent was attained for all children.

Children aged 5–24 months were recruited to include the age group eligible for rotavirus vaccination in the community at the time of recruitment. AGE cases were recruited from children admitted to the inpatient service responsible for all AGE admissions. AGE was defined as ≥ 3 liquid or semi-liquid stools within 24 hours. Asymptomatic children were those without history of loose stools or vomiting within 3 weeks of recruitment being seen for respiratory complaints at the hospital's outpatient clinic. A stool sample was collected within 48 hours of admission. Samples were stored at −20°C.

Rotavirus vaccination status was determined for all children by review of state-issued vaccination cards indicating full, partial, or no vaccination. Diarrhea severity was determined by Vesikari score.[21] The patient was measured on admission. Using World Health Organization Child Growth Standards, nutrition status was determined by calculating *z* scores for weight-for-height to determine wasting and height-for-age to determine stunting.[22] Degrees of severity were: normal (> −1), mild (−1 to −1.99), moderate (−2 to −2.99), and severe (≤ −3).

Viral testing. Norovirus was detected using real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) on RNA isolated from stool samples with the Qiagen QIAmp Viral RNA commercial kit (Qiagen, Hilden, Germany). Norovirus genogroup was detected with an in-house protocol utilizing TaqMan RT-PCR targeting the highly conserved open reading frame (ORF) 1–ORF2 junction region.[23] A sample was considered norovirus positive when cycle threshold (Ct) values were < 39 cycles. For genotyping, we applied conventional RT-PCR using primers described by Kojima and others[24] that target the highly variable N-terminal shell of the capsid protein VP1 (Region C). Positive GII.4 samples were sequenced at Macrogen Inc. (Rockville, MD). Sequences were edited by CAP3 program.[25] Genotypes and variants of GII.4 were identified using Noronet (http://www.noronet.nl).[26]

Because of the uncertain reliability of available enzyme immunoassay (EIA) in the field, rotavirus was detected and genotyped by two-amplification RT-PCR using the Qiagen OneStep RT-PCR Kit. Genotypes were determined with primers targeting VP7 (G) and VP4 (P) viral proteins corresponding to known rotavirus genotypes.[27,28] When a genotype combination could not be identified, strains were labeled as untypeable.

Statistical analysis. The comparison of categorical data was conducted with χ^2 test or Fisher's test and continuous data with *t* test or Wilcoxon rank sum test when appropriate. The effect of norovirus and/or rotavirus infections on diarrheal disease characteristics and severity were analyzed in a logistic multivariate regression. Each variable was evaluated in a univariate logistic regression and included in the multivariate analysis when $P < 0.2$. The covariables investigated were age, sex, rotavirus vaccination status, and chronic malnutrition status (stunting). The unadjusted attributable fraction for norovirus and rotavirus comparing the prevalence of either virus in cases and controls was calculated.[29] A significant result was considered if the *P* value was ≤ 0.05 and 95% confidence intervals (CIs) were estimated with standard methods based on the normal approximation to the binomial distribution. All data analysis was done with STATA (version 10.1; Stata Corp., College Station, TX).

RESULTS

A total of 206 children with AGE and 71 children without AGE were enrolled between March 2010 and February 2011 (Table 1). Stool samples were available for 272 (98.1%) patients. Of 201 samples from children with AGE aged 5–24 months, 69 (34.3%) had positive results for norovirus, 38 (18.9%) for rotavirus, and 9 (4.5%) for both viruses. Norovirus and rotavirus were detected in 13 (18.3%) and 3 (4.2%) of 71 non-diarrheal stools, respectively. Among infants with AGE aged 5–11 months, norovirus was detected at nearly three times the rate of rotavirus, present in 34 (42.5%) and 12 (15.0%) of 80 samples, respectively, with 3 (3.8%) mixed infections. Norovirus and rotavirus were detected in eight (23.5%) and two (5.9%) of 34 stool samples from non-AGE infants aged 5–11 months. Median Ct count for norovirus detected in diarrheal stools of all ages was 23.1 versus 28.5 in non-diarrheal stools, however, this difference was not statistically significant ($P = 0.086$). The unadjusted attributable fractions for norovirus and rotavirus were 25.6% and 16.2%, respectively. Both viruses were detected throughout the entire

TABLE 1

Characteristics of the study population with norovirus and rotavirus PCR results

Characteristic	Children		*P* value
	With AGE	Without AGE	
	n (%)	*n* (%)	
Total	206	71	
Male	133 (64.6)	43 (60.6)	0.569
Female	73 (35.4)	28 (39.4)	
Age in months (mean [SD])	12.91 (4.4)	13.07 (5.4)	0.804
Viral results*			
Norovirus positive			
Aged 5–24 months	69 (34.3)	13 (18.3)	0.01
Aged 5–11 months	34 (42.5)	8 (23.5)	0.13
Rotavirus positive			
Age 5-24 months	38 (18.9)	3 (4.2)	0.002
Age 5–11 months	12 (15.0)	2 (5.9)	0.224
Mixed norovirus/rotavirus	9 (4.5)	0	0.118
Nutrition Status			
No wasting	153 (74.3)	70 (98.6)	< 0.001
Moderate wasting	32 (15.5)	1 (1.4)	
Severe wasting	10 (4.9)	0	
No stunting	148 (71.8)	66 (93.0)	0.006
Moderately stunted	26 (12.6)	2 (2.8)	
Severely stunted	21 (10.2)	3 (4.2)	
Unmeasured	11 (5.3)	0	
Rotavirus vaccination			
Full vaccination (two doses)	148 (71.8)	69 (97.2)	< 0.001
Partial vaccination (one dose)	17 (8.3)	1 (0.01)	
Unvaccinated	35 (17.0)	1 (0.01)	
No data	6 (2.9)	0	

AGE = acute gastroenteritis; PCR = polymerase chain reaction; SD = standard deviation.
*Norovirus and rotavirus data available for 201 of 206 enrolled children with AGE.

TABLE 2
Clinical manifestations in children with AGE and detection of either norovirus or rotavirus

Clinical manifestation media (IQR)	Norovirus* $N = 69$	Rotavirus* $N = 38$	P value†
Vesikari score	15 (14–17)	15 (14–17)	0.836
Days with diarrhea	6 (4–9)	6.5 (5–8)	0.797
Diarrheal episodes/day	6 (4–9)	7 (6-9)	0.099
Days of emesis	3 (2–5)	2 (1–3)	0.006
Emesis episodes/day	3 (2–5)	3 (2–4)	0.131
Maximum temperature	38.5 (38.1–39.2)	38.6 (38–38.9)	0.615

AGE = acute gastroenteritis; IQR = interquartile range; SD = standard deviation.
*Mixed norovirus/rotavirus infections excluded.
†Wilcoxon rank sum test.

year of study with rotavirus showing peak detection rates between Bolivian winter months of May–August.

Both norovirus and rotavirus were highly associated with diarrheal illness; odds ratio (OR) = 3.92 (95% CI: 1.76–8.71; $P = 0.001$) and OR = 6.92 (95% CI: 1.84–25.97; $P = 0.004$), respectively. There was no significant difference in Vesikari scores among children with AGE and norovirus infection compared with those with AGE and rotavirus (Table 2). Norovirus infection was associated with a longer duration of emesis versus rotavirus infection (median duration 3 days versus 2 days; $P = 0.006$). Children with severe stunting had significantly longer duration of diarrhea compared with children without severe stunting (median duration 8.6 days versus 6.8 days; $P = 0.037$). There was neither association between rotavirus vaccination status and severe rotavirus AGE as defined by a Vesikari score ≥ 11, nor was there association between vaccination and severe AGE of any etiology.

Children with AGE were less likely to be fully vaccinated (two doses) against rotavirus (71.8% of children with AGE versus 97.2% of those without; OR = 0.099, $P = 0.032$). Fully vaccinated children with AGE were less likely to have rotavirus detected in their stool versus unvaccinated children with AGE (14.02% versus 31.4%, OR = 0.31, $P = 0.006$).

Norovirus GII was identified in 97.8% of detected norovirus (Table 3). The most common genotypes were GII.4 (71.4%) and GII.6 (6.6%). GII.4/2006b was the most prevalent GII.4 variant found in 25.4% of all AGE cases, followed by GII.4/2008 and GII.4/2010. Three rotavirus G genotypes (G2, G3, and G9) and two P genotypes (P[8] and P[4]) were detected (Table 4). In patients with AGE, the most prevalent types were G3P[8] (44.0%) and G2P[4] (16.0%). Genotype data were available for only one of three non-diarrheal stool samples positive for rotavirus that showed G9P[4].

TABLE 3
Norovirus genotypes detected in specimens from children with and without AGE

Genotype	Total n (%)	Diarrheal n (%)	Non-diarrheal n (%)
GI.1	1 (1.1)	1 (1.3)	0
GII.1	1 (1.1)	1 (1.3)	0
GII.2	1 (1.1)	1 (1.3)	0
GII.3	1 (1.1)	1 (1.3)	0
GII.4	65 (71.4)	60 (76.9)	5 (38.5)
Den Haag 2006b	55 (60.4)	51 (65.4)	4 (30.8)
Apeldoorn 2007 (nonepidemic cluster)	5 (5.5)	4 (5.1)	1 (7.7)
New Orleans 2009	5 (5.5)	5 (6.4)	0
GII.6	6 (6.6)	6 (7.7)	0
GII.7	5 (5.5)	2 (2.6)	3 (23.1)
GII.22	3 (3.3)	2 (2.6)	1 (7.7)
Group I untypeable	1 (1.1)	0	1 (7.7)
Group II untypeable	7 (7.7)	4 (5.1)	3 (23.1)
Total	91 (100)	78 (100)	13 (100)

TABLE 4
G and [P] genotypes detected in rotavirus-positive stool samples

Genotype	n	%
G2P[4]	8	16.0
G3P[8]	22	44.0
G9P[4]	5	10.0
G9P[8]	6	12.0
Mixed genotypes	2	4.0
Untypeable	7	14.0
Total	50	100

DISCUSSION

Norovirus. Before the introduction of rotavirus vaccine, norovirus had emerged in the literature as a major cause of pediatric AGE in Latin American children.[8] Few studies have described norovirus prevalence relative to rotavirus after vaccine introduction.[4,7,30,31] To our knowledge, we are the first to describe norovirus infections in Bolivia. The effectiveness of available rotavirus vaccines in reducing hospital admissions because of AGE suggests that non-rotavirus pathogens will represent a greater proportion of enteropathogens detected in hospitalized children. Recently, norovirus was shown to be more prevalent than rotavirus in a large study of U.S. children with AGE.[30] Similar findings have been described in Latin American communities.[7,31]

We identify another urban community where norovirus was detected in a greater number of AGE cases than rotavirus after widespread rotavirus vaccination. This is reflective of near universal exposure to norovirus in the first 2 years of life coupled with high uptake of rotavirus vaccine throughout the same period.[4,32] Greater norovirus prevalence appeared to be more pronounced in infants aged less than 1 year, however, these differences did not reach statistical significance. Norovirus was detected in nearly three times as many patients with AGE than rotavirus in children aged 5–11 months. Asymptomatic norovirus infections were also higher in this age group, suggesting early exposure to the virus. Higher relative norovirus prevalence in this age group may be because of greater reductions in rotavirus infections in children aged < 12 months after vaccine introduction; however, rotavirus vaccine in Bolivia has demonstrated similar efficacy in both children under and above 12 months.[12,14]

Asymptomatic norovirus infections were very common. Children infected with norovirus are known to shed the virus for several weeks to months after both symptomatic and asymptomatic infections.[4] Such shedding poses a challenge in determining the relative incidence of norovirus versus other enteropathogens as asymptomatic shedding may increase the proportion of children with other-cause AGE found to have norovirus in their stool. We attempted to diminish the effect of recent norovirus infections by excluding children with AGE symptoms in the 3 weeks before presentation; however, the data presented by Saito and others[4] suggest that a period lasting as long as 3 months may be required to exclude asymptomatic shedding. This may not be practical, as exclusion

criteria requiring longer asymptomatic periods risks parental recall bias, and there is a high incidence of unique diarrheal illnesses in the first few years of life.

Although the difference fell below statistical significance ($P = 0.09$), median Ct count trended lower in samples from patients with diarrhea, 23.1 versus 28.5, suggesting an expected higher viral load in symptomatic patients. Some authors have suggested the use of qRT-PCR Ct counts analyzed with bacteriology and parasitology data to determine cutoffs for presumptive symptomatic infections.[33] This technique emphasizes the importance of excluding other enteropathogens in surveillance populations to determine accurate disease burdens.

Norovirus GII.4 is well known as the most prevalent genotype globally and in Latin America.[1,4,7] Norovirus Den Haag 2006b was the most prevalent variant detected in our population, representing 60% of all norovirus and a quarter of all diarrheal cases of any cause. This variant has shown similar predominance in neighboring Brazil and Argentina,[7,34] GII.4 was much more prevalent among children with symptomatic infection versus asymptomatic shedding in this study, suggesting unique pathogenicity and highlighting the importance of this genotype in future vaccine development for the prevention of clinically significant disease.

Rotavirus. The literature describing the burden of rotavirus in Bolivia has demonstrated the effectiveness of rotavirus vaccine in the region.[5,6,14,16,17] Seven years before vaccine introduction at this study site, rotavirus was detected by PCR in 24% of 317 children aged < 5 years hospitalized with AGE.[17] Rotavirus prevalence was 36% when children < 2 years were analyzed separately, nearly double the prevalence described here (19%). A study conducted at the same institution during peak rotavirus season in the year before and after vaccine introduction demonstrated a fall from 37% to 22% of rotavirus positivity in children < 12 months with AGE.[16] A recent case–control study showed rotavirus vaccine to be effective in Bolivia, reassuring against fears that rotavirus vaccine is less effective in low- versus high-income settings.[14]

Before widespread vaccination, rotavirus was the major viral enteropathogen affecting Latin American children.[5,35–37] Despite effective vaccination, rotavirus remains a major cause of pediatric diarrheal disease, and prevalence has remained high in Latin American countries that have not included rotavirus vaccine into national vaccine schedules.[38,39] Most children in the study received a full two-dose course of Rotarix before enrollment (78.3%). This is comparable to Bolivia's national rotavirus vaccination rate of 87% during the same period.[15] This uptake is similar to other Latin American countries that have experienced decreased rotavirus infections with a relative increase in norovirus infections after implementation of vaccine.[7,12,31,37]

G1 genotypes were most prevalent in Bolivia and Latin America before vaccine introduction.[9] In the 2007 and 2008 seasons immediately before vaccine implementation, G1 genotypes represented 21% of circulating rotavirus in Bolivia.[5] G1 genotypes were non-detected in this study. These findings fit within a regional trend of decreased G1 prevalence in bordering Brazil and Argentina during the same period.[3,37] As seen in Argentina, we saw high prevalence of G3P[8] and G2P[4] genotypes.[3] Similar to Brazilian and Argentine populations, Bolivia experienced an increase in G2P[4] genotypes immediately before vaccine introduction, suggesting year-to-year variability toward this genotype.[5,6,37]

The G1P[8]-derived vaccine is effective against genotypes heterotypic to the vaccine type, including the G2P[4] genotype that some have suggested is favored after vaccination.[40–43] Waning immunity to G2P[4] in the second year of life has been suggested as a means by which the genotype may persist at higher levels in vaccinated populations.[3] Despite any theoretical influence by vaccine, our data suggest that regional circulation patterns remain highly influential on detected genotypes. As mentioned, the most common genotype detected, G3P[8] was also highly prevalent in neighboring Argentina during the same period.[3] As is the case with other partially homotypic genotypes, Rotarix is highly effective against G3P[8].[10] Extensive analysis has not demonstrated any genotype-specific variability in rotavirus gastroenteritis because of vaccine.[44] Finally, the monovalent vaccine is known to be effective against heterotypic genotypes specifically in Bolivia.[14]

There were several limitations to this study. Because of insufficient supply of reliable EIA in the field, we used RT-PCR to detect rotavirus. Typically, EIA is used for rotavirus detection. EIA has a sensitivity of ~80% compared with RT-PCR, therefore our description of rotavirus prevalence may be higher than studies that use EIA.[45] The clinical significance of rotavirus detected by RT-PCR has been questioned as PCR may detect subclinical rotavirus shedding in patients with other-cause AGE.[46] The influence of such cases is likely to be small in this study; however, as there was low rotavirus detection in stool samples from non-diarrheal patients (4.2%). Considering the lower sensitivity of EIA compared with RT-PCR, it is possible that the deployment of EIA in this study would have resulted in an even larger detected gap between norovirus and rotavirus prevalence than that presented here. Finally, as highly sensitive PCR is the current standard for detecting norovirus in stool, comparing relative prevalence using the same technology may be appropriate.

Patients presented with relatively severe AGE (median Vesikari score = 15). As hospitalization itself selects for more severe disease, this study is not representative of all children with AGE in the community, but rather of those most severely affected by AGE. The effect of vaccine on AGE within the greater community should include future study of outpatients.

Although both norovirus and rotavirus were strongly associated with diarrheal disease, we cannot rule out other viral, bacterial, or parasitic coinfections. This is of particular importance considering the high prevalence of asymptomatic norovirus infections. Further study should include all common enteropathogens with consideration of Ct value cutoffs for true infections.[33] Although our results are consistent with larger regional surveillance studies conducted during the same period, the study is limited to a single year of surveillance, thus year-to-year seasonal variability of viral prevalence and genotypes must be considered.[3,4,6–9,12,37] Finally, as previous norovirus data are unavailable for Bolivia, comparison to historical norms is unavailable.

CONCLUSION

This study highlights the importance of norovirus infections along with rotavirus infections in the developing world after rotavirus vaccination. Knowledge of circulating norovirus genotypes in resource-poor settings such as Bolivia, where mortality because of AGE is highest will be of critical importance as we move forward in the development of vaccine against what is now likely the major cause of viral AGE in children.

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