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# Rotavirus genotype distribution during the pre-vaccine period in Bolivia: 2007–2008

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SUMMARY

*Objectives:* Rotavirus is the most important etiology of severe diarrhea in Bolivia. The monovalent attenuated human oral rotavirus vaccine Rotarix® was introduced in Bolivia in 2008. We describe the molecular epidemiology of circulating rotavirus strains before vaccine introduction.
*Methods:* Two thousand one hundred thirty-five diarrheal samples were collected from hospitals in four Bolivian cities during 2007–2008. Forty-three percent (445 of 1030 rotavirus-positive samples) were analyzed for G and P genotypes. Among those, 331 were electropherotyped by polyacrylamide gel electrophoresis. Disease severity was quantified using a modified Vesikari scale.
*Results:* Among the 445 samples, five genotypes were found to be prevalent: G9P[8] (33%), G1P[6] (17%), G2P[4] (13%), G9P[6] (12%), and G1P[8] (4%). Co-infections with two or more strains accounted for 14% of samples. The most prevalent strain, G9, showed greater electropherotype diversity compared to other serogroups. Strain G1P[6] generally infected younger children and peaked later in the year than other strains. No particular genotype was associated with a higher severity score, though there was a significant difference in the duration of diarrhea between genotypes.
*Conclusions:* During the 2-year pre-vaccine period, substantial diversity of rotavirus co-circulating strains was observed. These data constitute a baseline against which changes in circulating strains post-vaccine introduction can be monitored.


## 1. Introduction

Rotavirus is the leading cause of severe acute gastroenteritis in infants and young children worldwide.[1] In the pre-rotavirus vaccine era, nearly every child was infected with rotavirus before the age of five, and more than 85% of the mortality burden due to rotavirus occurred in the developing world, as poor access to medical care, malnutrition, and coexisting infections are frequent complicating factors.[2]

Among group A rotaviruses, two subgroups (I and II) can be found based on the reactivity of the VP6 protein. Two surface antigens to which neutralizing antibodies attach, known as VP7 (designated G) and VP4 (designated P), are used to further classify rotaviruses according to their serotype and genotype.[3] Ten G serotypes and 11 P genotypes have been identified in humans.[4] An additional method for distinguishing rotavirus strains is electropherotyping, in which migration patterns of 11 double-stranded (ds) RNA segments are compared between virus isolates.[5]

The clinical presentation of rotavirus encompasses a wide range of severity of symptoms, from asymptomatic infection to severe dehydrating diarrhea.[6] Little clear evidence has emerged regarding the host or viral factors that contribute to severe disease. Several studies have investigated associations between infection with rotavirus G types and P types and illness severity, but have not found any consistent patterns in terms of the particular genotypes associated with severe disease.[6–10]

A review of the epidemiology of rotavirus in Latin America pre-rotavirus vaccine revealed a need for consistent and comprehensive rotavirus surveillance by countries in the region, as the availability of long-term data was limited.[11] In South America, studies published between 1989 and 2004 found that the most common rotavirus genotypes included G1P[8] (34%), G2P[4] (23%), G9P[8] (15%), and G4P[8] (9%).[4]

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In 2009, the World Health Organization (WHO) recommended that the rotavirus vaccine be incorporated into routine immunization programs of all countries.[12] Results of rotavirus vaccine trials have shown that vaccine effectiveness is lower in developing countries than in developed countries.[13–17] The reasons for this disparity are not entirely clear, but understanding some of the factors that lead to disease severity in the absence of a vaccine may help provide insight into why vaccines are less effective in these settings.

Although the rotavirus vaccines are highly effective at preventing hospitalizations due to rotavirus diarrhea, they do not prevent rotavirus disease altogether. Thus, it will be important to monitor the vaccine's effects on rotavirus strain diversity in the years following its introduction.

In Bolivia, acute diarrheal diseases represent a major public health problem and are the leading cause of the high morbidity and mortality rates in young children.[18] Bolivia introduced the rotavirus vaccine into its routine immunization schedule in August of 2008. The goal of this study was to describe the molecular epidemiology of rotavirus in Bolivia prior to the introduction of the rotavirus vaccine and to identify some of the viral factors that may contribute to disease severity.

## 2. Materials and methods

### 2.1. Study sites and study population

The study took place from January 2007 to December 2008 in five tertiary hospitals of four Bolivian cities covering the major geographic regions of Bolivia: highlands (Hospital Materno-Infantil and Hospital Del Niño in La Paz, Hospital Boliviano Holandés in El Alto), valleys (Hospital Albina Patiño in Cochabamba), and tropical lowlands (Hospital Mario Ortiz in Santa Cruz).

Surveillance took place among children 0–59 months of age admitted to the hospitals as inpatients with a diagnosis of acute diarrhea. Diarrhea was diagnosed according to the WHO definition of three or more watery stools in 24 h. Data were collected on all children with a diagnosis of diarrhea. Those who had persistent diarrhea upon hospitalization or had a nosocomial diarrhea were excluded. Over the surveillance period, a total of 2135 samples were collected and laboratory tested for rotavirus; 849 samples were collected from the cities of La Paz and El Alto, 883 from Cochabamba, and 403 from Santa Cruz. The surveillance system was able to access approximately 87% of all fecal specimens from children identified as acute diarrheal cases.

### 2.2. Sample collection

Within 48 h after hospitalization, at least 5 ml of stool was directly collected and stored in a plastic vial or in the diaper. Samples were kept at 4 °C until they were transported to the laboratory at the Universidad Mayor de San Andrés where they were stored at −20 °C prior to analysis.

Clinical information was extracted from the medical chart of each child. Information included the child's sex, age at admission, symptoms, hydration status, height, weight, and length of hospital stay.

### 2.3. Rotavirus detection and strain characterization

Rotavirus status was ascertained by ELISA (IDEIA-Dako). G and P genotyping was performed by reverse transcription (RT)-PCR on 43% of randomly selected rotavirus-positive samples. Rotavirus RNA was extracted in accordance with previously described methods for nucleic acid purification.[19] The extracted RNA was used for RT-PCR as described to ascertain G, P, and VP6 genotypes.[20–22] Amplicons were analyzed on a 1.5% Tris–borate–EDTA (TBE) agarose gel and viewed under ultraviolet illumination after ethidium bromide staining.

### 2.4. Electropherotyping

Electropherotyping of viral RNA was available for 331 samples, using 7% polyacrylamide gels.[23] Long and short migration ds rotavirus RNA patterns were categorized as L and S respectively based on co-migration differences.[20,24]

### 2.5. Data entry and analysis

All data were collected by surveillance program staff and entered into Epi Info version 3.5.1 (CDC, Atlanta, GA, USA). Data were imported into and analyzed in SAS v 9.2 (SAS, Cary, NC, USA).

### 2.6. Evaluation of disease severity

A modified version of the previously described Vesikari scale was used to determine clinical severity of diarrhea episodes.[6] The following three modifications were made. A 17-point scale was used in lieu of a 20-point scale, as information on the duration of vomiting was not available. The original Vesikari scale categorizes dehydration according to the percentage of body weight lost. Because the surveillance program did not classify dehydration this way, we used the program's four-level scale categorized by the following: no dehydration, moderate dehydration, severe dehydration, and shock. The maximum number of vomiting episodes in 24 h was modified to the number of vomiting episodes in the 24 h preceding hospitalization, as the number of vomiting episodes was not recorded daily; the same was true for the number of diarrheal depositions.

### 2.7. Statistical analysis

Normality was assessed for all continuous variables. Several continuous variables, including age, duration of diarrhea, number of vomiting episodes in 24 h, and the number of diarrheal depositions did not follow a normal distribution. For these variables a $\log_{10}$ transformation was used, which resulted in a normal distribution. For all statistical analyses, a $p$-value of less than 0.05 was considered statistically significant. Analysis of variance (ANOVA) and Tukey pairwise comparisons were used to compare the severity score, duration of diarrhea, number of depositions, number of vomiting episodes, and age at infection among rotavirus genotypes. The Chi-square test and Fisher's exact test were used to make comparisons among categorical variables.

## 3. Results

### 3.1. Rotavirus strain dynamics in Bolivia

In order to investigate the dynamics of circulating strains of rotavirus, we analyzed rotavirus genotype data in association with demographic and temporal characteristics. 2135 diarrheal samples were collected from five hospitals in four Bolivian cities between January 2007 and December 2008. Four hundred forty-five (43%) of the 1030 rotavirus-positive samples were analyzed for G serotype and P genotype. Five strains representing common G–P types predominated (78%) during the study period: G9P[8] (33%), G1P[6] (17%), G2P[4] (13%), G9P[6] (12%), and G1P[8] (4%). We found that the strain proportions of G9P[8] and G9P[6] were consistent across the years, while the strain proportions of G1P[6], G1P[8], and G2P[4] varied from year to year (Figure 1). Specifically, G2P[4] exhibited considerable variation between years, ranging from 3% in

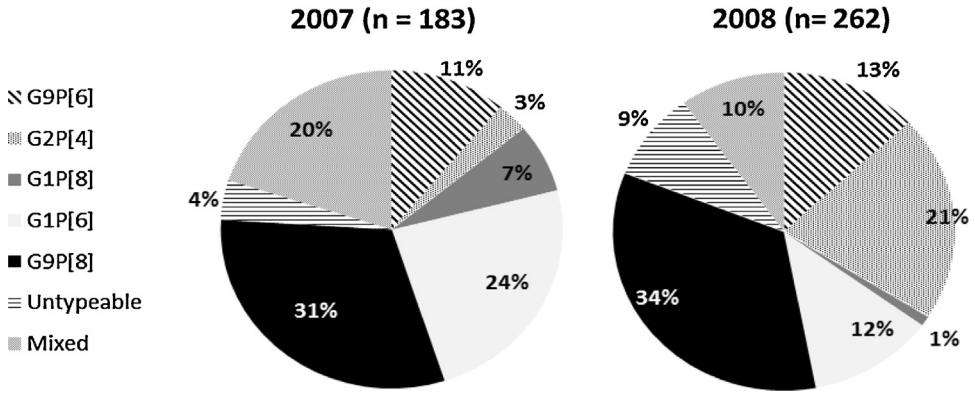


**Figure 1.** Genotype distribution 2007–2008. Five genotypes predominated during the surveillance period, and G1P[6], G1P[8], and G2P[4] showed considerable variation by year.

2007 to 21% in 2008. Co-infections accounted for 14% of samples. The majority of the mixed infections (92%) were associated with G9 and G1 strains (data not shown). Unconventional G and P associations, particularly G9 with P[4], or uncommon rotavirus genotypes, such as G4P[6], were observed infrequently (<1% of cases).

The strain proportions of all five strains were quite distinct across cities (data not shown). The distribution of rotavirus genotypes showed variation by season (Figure 2). The incidence of three strains, G1P[8], G2P[4], and G9P[8], all peaked at the same time coinciding with the primary peak of rotavirus from April to July, and then decreased to low levels. Interestingly, the majority of cases of G1P[6] were found later in the year, from August to October, with low levels of infection during the primary rotavirus peak from April to July and higher levels during the secondary peak between August and October. G9P[6] had two peaks, one at the latter end of the primary rotavirus peak and a second in September during the secondary rotavirus peak. The same patterns were observed when looking at data from each year individually (data not shown) as when examining aggregated data. In summary, five genotypes predominate in Bolivia, showing variation across cities and seasons.

### 3.2. Genotype and severity

In order to investigate the relationship between the rotavirus strain and disease severity in this population, we used clinical data to calculate the Vesikari score quantifying disease severity. Clinical data were not complete for all observations. Of 2135 observations, only 516 included all of the required clinical information for a calculable severity score; 228 of these were rotavirus-positive. Of the 228 rotavirus-positive cases with calculable Vesikari scores, 83 had genotype data. We found no differences in the mean severity score among the five most common genotypes (Table 1). Scores ranged from 10 to 16 and the median score was 13. In addition to the overall severity score, we examined each of the clinical symptoms individually for associations with genotype. The duration of diarrhea showed significant differences by genotype (Table 1) and there were no significant differences in strain distribution by gender (data not shown). Additionally, children infected with the G1P[6] strain were of a significantly lower average age at the time of infection than children infected with the G1P[8], G2P[4], and G9P[8] strains (Table 1). In addition we performed separate analyses looking at associations between severity and G type and P type discretely. No significant differences were found between G types or P types for overall severity score (data not shown). These data suggest that in Bolivia, in general, all common strains of rotavirus are equally severe, and thus it is important that vaccines show high efficacy and effectiveness against all of these strains.

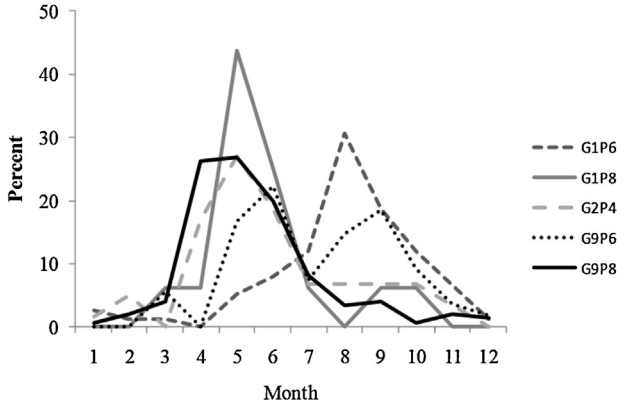


**Figure 2.** Two genotypes, G1P[6] and G9P[6], had peaks outside the primary rotavirus peak (April to July). Lines represent the number of cases per month (month 1 = January, 2 = February, etc.) for each genotype as a percentage of the total number for that genotype for the period 2007–2008.

### 3.3. Electropherotype diversity

Fourteen electropherotypes were identified between 2007 and 2008 by examination of RNA migration patterns in polyacrylamide gels. Strains with long electropherotypes (L) were predominant (85%) and showed more variation (12 discrete variants), while short electropherotypes (S) were less frequent (15%) and showed only two variants (S1 and S2). Electropherotypes showed considerable temporal diversity. Electropherotypes S1 and variant forms of L3 (L3-1, L3-2) were somewhat rare in 2007, accounting for approximately 25% of the total electropherotypes identified in that year, while in 2008 those three electropherotypes emerged to be predominant, accounting for almost 85% of total electropherotypes in that year (Table 2). Conversely L1 and L5 were relatively common in 2007 (39%) but virtually disappeared in 2008 (2%). Strains with electropherotypes L1, L3, and L5 were dominant in 2007, and electropherotypes L3-1, L3-2, and S1 were dominant in 2008 (Table 2). Co-circulation of more than one type was observed annually, particularly during the peak months of rotavirus infection where the highest frequency of co-infections was recorded.

Some genotypes were frequently associated with a particular electropherotype. G2P[4], as part of the DS-1-like genogroup, was almost exclusively found in conjunction with electropherotype S, G1P[8] was always observed with the L5 electropherotype

**Table 1**
Associations between genotypes, disease severity and clinical characteristics

| | G1P[6] Mean ± SD, column % (*n* = 75) | G1P[8] Mean ± SD, column % (*n* = 16) | G2P[4] Mean ± SD, column % (*n* = 59) | G9P[6] Mean ± SD, column % (*n* = 54) | G9P[8] Mean ± SD, column % (*n* = 145) | Total *N*[a] |
|---|---|---|---|---|---|---|
| Average age at infection (months) | 8.0 ± 6.9 | 12.8 ± 5.6[b] | 11.8 ± 5.4[b] | 9.5 ± 6.2 | 11.4 ± 5.7[b] | 349 |
| Modified Vesikari score | 13.5 ± 1.5 | 13.0 ± 1.4 | 12.7 ± 1.2 | 13.8 ± 1.5 | 13.7 ± 1.5 | 83 |
| Duration of diarrhea | 7.1 ± 4.1[c] | 6.3 ± 3.3 | 5.4 ± 2.8 | 7.8 ± 2.9[c] | 7.1 ± 3.4[c] | 254 |
| Number of depositions | 8.2 ± 3.5 | 7.2 ± 2.9 | 7.1 ± 3.1 | 7.4 ± 3.2 | 8.2 ± 4.1 | 347 |
| Number of vomiting episodes | 5.6 ± 3.6 | 4.5 ± 2.7 | 5.4 ± 4.1 | 4.6 ± 2.7 | 5.3 ± 3.0 | 309 |
| Incidence of vomiting (%) | 92 | 94 | 93 | 93 | 94 | 349 |
| Hydration status (%) | | | | | | |
| None | 2.7 | 0 | 1.7 | 1.9 | 2.1 | 7 |
| Moderate | 64 | 62.5 | 74.6 | 48.2 | 68.3 | 227 |
| Severe | 32 | 37.5 | 22 | 48.2 | 28.3 | 110 |
| Shock | 1.3 | 0 | 1.7 | 1.9 | 1.4 | 5 |
| Metabolic acidosis (%) | 79 | 100 | 81 | 83 | 76 | 163 |

[a] Sample size varies by category, based on available clinical information.
[b] $p < 0.05$ compared to G1P[6].
[c] $p < 0.05$ compared to G2P[4].

(Figure 3), while the highly prevalent G9 strains showed a high diversity of long electropherotypes.

### 3.4. Association between VP6 subgroup genotypes and electropherotypes

In addition to the VP6 genogroup determined by RT-PCR, the G types, P types, and electropherotypes of the rotavirus strains were determined, and the associations between genogroups, genotypes, and electropherotypes were analyzed. We found that G2P[4] strains were mostly associated with subgroup I (83%) and with short electropherotypes (100%), while non-G2P[4] genotypes (G9P[8], G1P[6], G9P[6]) were associated with subgroup II (98%) and with long electropherotypes (100%). A number of strains with unusual associations were identified, such as G2P[4] sgII S, G9P[4] sgI L, G4P[6] sgI L, G2P[6] sgII L, G4P[6] sgII L, and G9P[8] sgI-II L. The unusual combination of G2P[4] with subgroup II and short electropherotypes (17%), suggests the occurrence of inter-genogroup reassortment.

## 4. Discussion

The goal of this study was to describe the molecular epidemiology of rotavirus in Bolivia prior to the introduction of the rotavirus vaccine and to identify viral factors that may contribute to the severity of rotavirus illness. The present study confirms that, in the same country, in different regions, and from year to year, circulating rotavirus genotypes and electropherotypes may be highly variable. Five rotavirus genotypes predominated, accounting for 99% of the total genotyped samples. Considerable electropherotype diversity was observed, with 14 distinct RNA migration patterns detected. Genotypes G1P[6], G1P[8], and G2P[4] showed little electropherotype diversity, associating overwhelmingly with one particular electropherotype, while genotypes G9P[6] and G9P[8] showed greater electropherotype diversity, associating consistently and concurrently with several electropherotypes. No particular genotype was clearly associated with more severe disease.

Although mixed infections between different G and P genotypes and different electropherotypes were observed, they were commonly associated with the most prevalent genotypes and electropherotypes co-circulating at the time of the study, providing opportunity for potential reassortment events.

Of the five most common strains worldwide, three are among Bolivia's five most common (G1P[8], G2P[4], and G9P[8]). A summary of worldwide rotavirus surveillance data for 1973–2003 indicated that circulating strains showed considerable variation by region and by year.[4] In the South American region, G1 was the most common serotype, accounting for 57.5% of infections, followed by G2 (18.3%), G4 (8.8%), and G9 (8.5%).[4] In our study, G9 (47.7%) was the predominant serotype, followed by G1 (32.5%) and G2 (19.4%). G9 has been an important emerging serotype in the past two decades,[4] and our study confirms the importance of G9 for the under-five population in Bolivia. Interestingly, since 2006, the prevalence of G9P[8] and G9P[6] has been increasing, while G1P[8] has been decreasing. In contrast, other studies in Ecuador documented a decrease in G9 from 2005 to 2008 showing that this genotype was replaced by the G1 and G2 types.[25] Similarly, G1P[8] genotype was considered one of the most prevalent genotypes worldwide.[26,27] Together, these data suggest country-specific rotavirus genotype changes each year.

Significant year-to-year variations in strain circulation appear to be common globally.[28] For example, an Argentinean study from 2006 revealed an increase in the frequency of G9, while G1 was

**Table 2**
Electropherotype counts during peak rotavirus periods

| | 2007 | | | | | | 2008 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | April | May | June | July | August | Total (%) | April | May | June | July | August[a] | Total (%) |
| S1 | 1 | 2 | 3 | 0 | 0 | 6 (3.5) | 15 | 16 | 9 | 1 | | 41 (25.9) |
| L1 | 7 | 20 | 11 | 1 | 1 | 40 (23.1) | 0 | 0 | 0 | 0 | | 0 (0) |
| L2 | 1 | 4 | 3 | 1 | 13 | 22 (12.7) | 5 | 2 | 2 | 8 | | 17 (10.8) |
| L3 | 5 | 13 | 21 | 1 | 0 | 40 (23.1) | 3 | 0 | 0 | 1 | | 4 (2.5) |
| L3-1 | 7 | 10 | 3 | 1 | 5 | 26 (15.0) | 16 | 4 | 4 | 1 | | 25 (15.8) |
| L3-2 | 4 | 5 | 3 | 0 | 0 | 12 (6.9) | 19 | 27 | 18 | 4 | | 68 (43.0) |
| L5 | 3 | 15 | 7 | 0 | 2 | 27 (15.6) | 2 | 0 | 0 | 1 | | 3 (1.9) |
| Total | 28 | 69 | 51 | 4 | 21 | 173 (100) | 60 | 49 | 33 | 16 | | 158 (100) |

[a] Electropherotype data not available for August 2008.

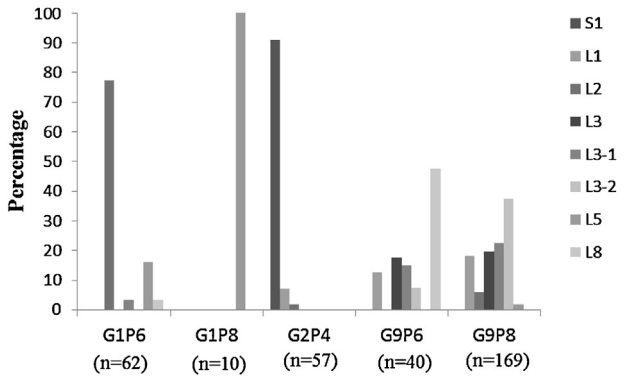


**Figure 3.** G9P[6] and G9P[8] showed greater electropherotype diversity than other genotypes. Bars represent individual electropherotypes as a percentage of the total electropherotypes detected for each genotype (i.e., total bars for each genotype should add up to 100).

nearly absent.[29] This was in contrast to previous surveillance data from 2004, where G1 accounted for 40% of circulating strains.[28] In Bolivia, the G2P[4] strain increased in frequency in 2008, indicating a cyclic pattern for this strain (Figure 1). In 2006, G2P[4] accounted for 23% of total genotyped strains (data not shown), while in 2007 it decreased considerably, accounting for only 4% of the total. In 2008 it reappeared as an important strain, accounting for 26% of the total. We found that G2P[4] was strongly associated with subgroup I, while the remaining non G2P[4] genotypes (G1P[6], G1P[8], G9P[6], and G9P[8]) were associated with subgroup II. This result is in agreement with other studies that found that subgroups segregate according to the P type (P[4] associated with sgI and P[8] with sgII).[30] In this study we found unusual G2P[4] strains associated with sgII and short electropherotypes; this result suggests that upon mixed infections, P[4] and VP6-encoding genes can segregate independently in reassorted strains. Reassortment is considered an important evolutionary mechanism among prevalent circulating rotavirus strains.[31]

The changing rotavirus RNA electropherotype dynamics observed from year to year in our study were similar to results that have been demonstrated elsewhere in the world.[5] During each seasonal peak of rotavirus, several rotavirus RNA electropherotypes circulated, with a few high frequency strains predominating, and several less common strains exhibiting lower levels of activity (Table 2). The rotavirus strains occurred in a periodic manner in which some strains disappeared and were replaced by new strains. It was also shown that, whereas some strains circulated for short periods (L1, L3), other persisted for longer periods of time (L2, L3-1, Table 2). Two groups of RNA patterns were detected, designated long and short profiles. The predominant electrophoretic pattern detected in children from Bolivia was the long electropherotype (85%), which has more variation in comparison to the short pattern. The predominance of the long electropherotype appears to be common globally.[32,33] The short patterns showed less diversity and a cyclic pattern of emergence, therefore we hypothesize that these strains are undergoing antigenic drift, as this would explain their distribution in time and the reduced accumulation of mutations. It is interesting to note that strains G9P[6] and G9P[8], the two most prevalent strains throughout the study period, showed greater electropherotype diversity than other strains (Figure 3). We also hypothesize that their success and growth over time can be partially attributed to this diversity.

Few studies have found differences in strain distribution by age group. To our knowledge, this is the first study to suggest that G1P[6] infects younger children (Table 2). An Italian study found that G2P[4] infected older children;[34] in our study G2P[4] had the highest mean age at infection, but was not itself significantly different from any genotype other than G1P[6] (Table 2). The Italian authors hypothesized that the higher mean age for G2 infections was attributable to the fact that the G2 infections may have been re-infections of children previously infected with another rotavirus genotype. However, this hypothesis would not serve to explain our finding of a significantly younger mean age for G1P[6]. Maternal antibodies against rotavirus are thought to provide protection to young infants against rotavirus infection.[27] G1P[6] is an uncommon genotype globally, and perhaps maternal antibodies to this genotype are less common. G1P[6] was also anomalous in that it peaked later in the season than other genotypes (Figure 2). A study in Colombia found that the three most common strains (G1P[8], G2P[4], and G3P[8]) showed distinct seasonal patterns that were consistent across all three cities studied in spite of different climatological factors.[35] In our study, G1P[6] and G9P[6] showed consistently later peaks, and the pattern was consistent over both years of surveillance. Though the reason for this is not clear, one hypothesis that we might propose is that these two strains are less infective in our population than other strains and thus cannot compete efficiently during the rotavirus peak when other strains show high circulation.

Our study did not identify an association between genotype and overall disease severity (Table 1). Numerous studies have examined severity in relation to virus type, but no consistent patterns have emerged. One study that came out of the placebo arm of a Latin American vaccine trial found that G9 caused more severe disease in comparison to G1 (a more common serotype globally).[7] Despite a relatively small sample size ($n = 45$), they found that G9 had a higher severity score, longer duration of diarrhea, more frequent hospitalization, and more severe dehydration as compared to G1.[7] In contrast, a study among French children hospitalized for rotavirus found no difference in severity, length of hospitalization, or the necessity for intravenous rehydration between G1 and G9 strains.[9] A third study of hospitalized rotavirus patients in India found that G1 caused more severe disease and more severe dehydration than G9 strains.[8] Several theories have been put forward to explain these inconsistencies. One plausible suggestion is that strains newly introduced into a community may cause more severe disease due to the lack of pre-existing immunity.[7] Also, there may be year-to-year variations in virulence of particular serotypes or genotypes.[8]

This study demonstrates the importance of rotavirus as an agent of severe diarrhea in Bolivia, and did not identify a strong association between rotavirus strain and severity of disease, emphasizing the importance that the vaccine is effective against all rotavirus genotypes.

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*Ethical review:* Ethical approval was obtained from the Bolivian Bioethical Committee prior to data and sample collection. This study was exempt from Emory University institutional review board approval, as it involved secondary analysis of de-identified data and did not qualify as human subject research.

*Conflict of interest:* None of the authors have financial or other relationships that might lead to a conflict of interest.

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