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Correlation Between Rotavirus A Genotypes Detected in Hospitalized Children and Sewage Samples in 2006, Córdoba, Argentina

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Routine rotavirus A (RV-A) surveillance is based on clinical cases, so only symptomatic infections are reported. The objective of this study was to determine whether the RV-A genotypes and cold seasonal pattern described in patients with diarrhea is reflected by sewage surveillance, which could be representative of the RV-A genotypes circulating in the population. The genotype distribution of RV-A in effluent samples from a local sewage treatment plant was compared to those from local clinical cases. A total of 52 sewage samples and 70 stool specimens from children with acute non-bacterial diarrhea were collected from January to December 2006. The effluent specimens were concentrated and RNA extracts from concentrated sewage and clinical samples were genotyped for the rotavirus VP7 gene. The proportional distribution of the RV-A G-genotypes in sewage and clinical samples during the cold season was similar: G1 accounted for 26.6% of the typed sewage isolates and 28.8% of the clinical infections; G3 type accounted for 21.9% and 25.8%; G2 type 15.6% and 10.6%; G4 type 17.2% and 21.2%; G8 type 1.6% and 0%; and the G9 type 17.2% and 13.6%, respectively. A similar picture of RV-A genotype detection was obtained in sewage samples collected during the cold and warm seasons. The results indicate that there is a correlation between genotypes of RV-A isolates from human diarrheic patients and of those from sewage samples. In addition, sewage monitoring highlighted the uniform all-year RV-A circulation, which was in contrast to the peak incidence of RV-A infection in the community. ***J. Med. Virol. 82:1277–1281, 2010.*** © 2010 Wiley-Liss, Inc.

KEY WORDS: rotavirus; clinical surveillance; sewage surveillance; genotype; VP7

INTRODUCTION

Group A rotaviruses (RV-A) cause severe gastroenteritis in young children [Albert and Robert, 1996; Iturriza-Gómara et al., 2000]. Although almost all children experience RV-A infection by the age of 3–5 years [Glass et al., 1996], the majority of an estimated 527,000 rotavirus-associated deaths occur in developing countries [WHO, 2006].

Based on the diversity of the VP4 and VP7 outer capsid proteins, RV-A are classified into P (protease sensitive) and G (glycoprotein) genotypes, respectively [Greenberg et al., 1983; Hoshino et al., 1985; Offit and Blavat, 1986]. At least 31 P genotypes and 23 G genotypes have been identified [Matthijnssens et al., 2008; Trojnar et al., 2009]. Worldwide, the most common G genotypes associated with human infection are G1 to G4 and, owing to its global emergence, G9 is currently regarded as the fifth most important G genotype [Widdowson et al., 2004].

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Two RV-A vaccines administrable orally are licensed in many countries based on the results of large-scale clinical trials, each involving more than 60,000 children in both developing and developed countries [Ruiz-Palacios et al., 2006; Vesikari et al., 2006]. One of these vaccines is a pentavalent bovine-human RV-A reassortant vaccine that contains VP7 genes derived from human G1, G2, G3 and G4 strains and a VP4 gene derived from a human P[8] strain (RotaTeq®, Merck and Sanofi Pasteur MSD, Whitehouse Station, NJ). The second is a monovalent human G1P[8] RV-A strain attenuated by passage in cell culture (Rotarix®, GlaxoSmithKline Biologicals; Rixensart, Belgium). The immunity induced by vaccination attempts to mirror natural immunity derived from wild type RV-A infection. However, although clinical trials of both RV-A vaccines have shown high levels of protection against severe RV-A gastroenteritis [Matson, 2006; Ruiz-Palacios et al., 2006], the lack of data on heterotypic protection highlights the crucial role of strain surveillance in detecting antibody-escape mutants and changes in the local distribution of RV-A G genotypes circulating in the community.

Published studies on RV-A surveillance conducted in different regions of the world usually include symptomatic infections and samples collected from just one or only a few health centers [Pongsuwanna et al., 2002; Castello et al., 2006; Moe et al., 2009; Mukherjee et al., 2010]. The aim of the present study was to investigate whether the RV-A genotypes and the cold seasonal pattern described by clinical surveillance are representative of the RV-A genotypes circulating at the same time in the population. Therefore, we compared the distribution of frequencies of RV-A G types in clinical samples with that in sewage derived from the same population in Córdoba, Argentina, during the same period.

MATERIALS AND METHODS

Clinical Samples

Seventy stool specimens from children aged less than 3 years with a suspected acute viral gastroenteritis infection (general criterion: <20 polymorphonuclear leucocytes per field) at the Private Hospital in Córdoba City, Argentina, were collected over a 1-year period (from January to December 2006). Stool samples were collected within the first 24 hr of hospitalization to avoid the possibility of nosocomial RV-A infection. Voluntary informed consent was obtained from parents or guardians of children in accordance with the ethical principles outlined in the Declaration of Helsinki and the additional requirements of local and national authorities (National Normative 5330/97, National Administration of Food, Medicine and Technology). Of the 70 clinical samples collected, 6 were obtained during the warm months of the year (Summer and Spring seasons, January–March and October–December 2006, respectively) and 64 during the cold months (Winter and Autumn seasons, April–September 2006). Once in the laboratory, stool specimens were prepared as 10% homogenates in 0.02 M Tris–HCl (pH 7.2) (Baker®, New Brunswick, NJ/U.V.E.®, Buenos Aires, Argentina) and stored at −20°C until processed.

Rotavirus Detection

The fecal samples were first screened for RV-A infection by commercial enzyme linked immunosorbent assay (ELISA Pathfinder®) and/or polyacrylamide gel electrophoresis (PAGE) [Laemmli, 1970].

Sewage Samples

Samples (1.5 L) of untreated sewage entering the municipal sewage treatment plant in Córdoba City, Argentina, were taken once a week from January through December 2006 [Muller et al., 2009]. A total of 52 sewage samples were collected throughout the year, 26 were obtained during the warm months and the other 26 during the cold months of the year. The sewage system has a population coverage of 61%. Samples were collected between 9 and 11 a.m. to minimize the effects of diurnal variations and were transported within 12 hr at 4–8°C to the Virological Institute, Córdoba National University, for further processing and analysis.

Sewage Samples Concentration

Concentration of viruses in sewage specimens was performed using the method previously described by Lewis and Metcalf [1988] and Greening et al. [2002], modified by Huang et al. [2005]. The 1.5-L waste-water samples were concentrated 100-fold to 15 ml by high-speed centrifugation (two centrifugation steps, each of 8,300 rpm for 20–25 min), elution (two steps at room temperature for 1 hr), PEG precipitation (10% PEG 6000, Anedra®, San Fernando, Mexico/2% NaCl Anedra®, San Fernando, Mexico, ON at 4°C) and chloroform treatment (U.V.E.®).

Nucleic Acids Extraction

Viral double-stranded (ds)RNA was extracted from the RV-A ELISA- and/or PAGE-positive stool samples and sewage concentrated samples by the phenol-chlorophorm method, followed by alcohol precipitation according to standard procedures [Perry et al., 1972]. After drying, the pellets were diluted in 20 μl of sterile distilled water.

RT-PCR

Extracted dsRNA was first reverse-transcribed into gene 9 (VP7) full-length cDNA with the generic primers Beg9/End9 [Gouvea et al., 1990]. Then, in a second step, the cDNA product was used as a template for PCR VP7-amplification with the same Beg9/End9 pair of primers.

Genotyping

The VP7 full-length PCR products were used as templates in combination with two cocktails of

type-specific forward primers and the generic reverse primer End9 for G genotyping [Gouvea et al., 1990]. The cocktails were: G1 (aBT1), G2 (aCT2), and G3 (aET3) in one mixture; and G4 (aDT4), G8 (aAT8) and G9 (aFT9) in the second. The amplicons were analyzed by electrophoresis on 10% polyacrylamide gels and visualized after silver staining, as described elsewhere [Herring et al., 1982], to achieve high resolution of the products obtained.

Statistical Analysis

The Z-test for proportion comparison [Zar, 1996] was used to compare the detection frequencies of rotavirus G genotypes detected in clinical and sewage specimens.

RESULTS

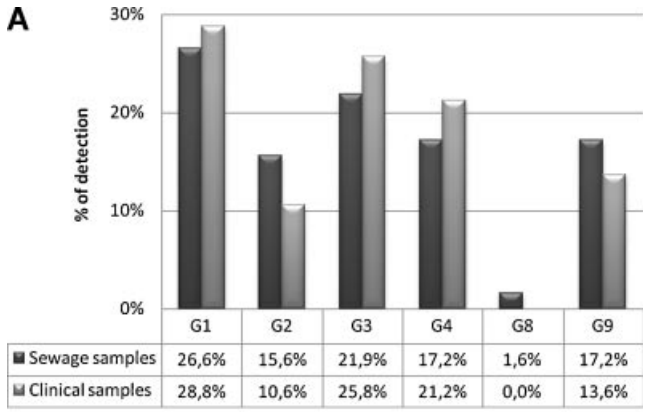
Frequency of Rotavirus G Genotype Detection

Of the 6 clinical samples collected during the warm months of the year (Summer and Spring 2006) and the 64 collected during the cold months (Winter and Autumn 2006), 3 (50%) and 47 (73.4%) were positive for RV-A infection by ELISA and/or PAGE, respectively. All sewage samples (n = 52) tested for RV-A detection by RT-PCR followed by heminested PCR were positive for RV-A. Due to the low number of RV-A clinical samples obtained during the warm months of the year, the comparison of genotypes detected by both surveillance systems was performed during the cold months. Also RV-A G type detection was analyzed in sewage samples during the cold and warm seasonal periods.

The total of the RV-A positive stool specimens collected during the cold months (n = 47) and the sewage samples collected during the year (n = 52, 26 collected during the cold months and 26 during the warm months) could be G genotyped. Almost 32% of the RV-A positive clinical samples (15/47) were RV-A mixed G-type infections, giving a total of 66 G types detected. Similarly, more than one G type was detected in 77% (20/26) and 58% (15/26) of the individual sewage samples collected during the cold and warm months, accounting for a total of 64 and 55 G types, respectively.

Proportional Distribution of Rotavirus G Genotypes

The proportional distribution of the RV-A G genotypes detected in clinical and sewage samples during the cold months (April–September) is shown in Figure 1A. The results show that there is no significant difference ($P > 0.05$) between the analysis of RV-A G genotypes detected in clinical specimens and those detected in sewage samples. On the other hand, there was no significant difference ($P > 0.05$) in the proportional distribution of the RV-A G genotypes detected in sewage samples collected during the cold and warm months. The results are shown in Figure 1B.



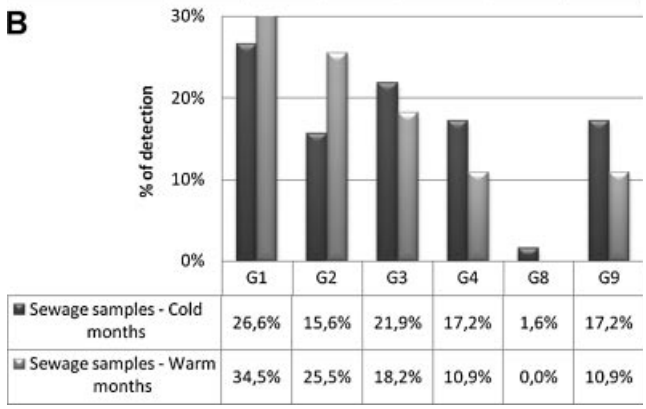


Fig. 1. **A**: Proportional distribution of rotavirus G genotypes detected in clinical (n = 66) and sewage (n = 64) samples during the cold season. **B**: Proportional distribution of rotavirus G genotypes detected in sewage samples during the cold (n = 64) and warm (n = 55) seasons.

DISCUSSION

The present study examined the epidemiological relationships between the RV-A genotypes involved in human clinical infections and those found in sewage samples. The Córdoba sewage treatment plant services approximately 61% of the local population; therefore, it is reasonable to assume that testing of RV-A genotypes in the sewage could reflect the RV-A local diversity, including viral strains excreted by both symptomatic and asymptomatic individuals. In contrast, clinical samples represent individuals and are usually collected from patients with acute gastroenteritis symptoms. Therefore, strains excreted from asymptomatic infections are not represented when analyzing clinical samples.

However, it is important to acknowledge the limitations of sewage sample collection. Daily fluctuation in the abundance of the virus strains is expected in raw sewage [Hejkal et al., 1984]. Therefore, it would be possible that the RV-A circulation could vary if samples were collected at different times during the day. To minimize daily fluctuations, in the present study the sewage samples were collected at a single time point (9–11 a.m.) that is likely to correspond to maximum fecal excretion by the population (based on discussion

with the head of the Córdoba sewage treatment plant, and as shown by the results of total and fecal coliphorm analysis). Also, the volume received by the sewage system could change during the year, particularly in the dry and wet seasons. The volume of sewage received by the treatment station is important and could interfere with the results of analysis. Anyway, RV-A was detected in all the samples collected throughout the period studied.

By comparison, characterization of RV-A during the cold months from sewage and clinical samples showed a similar G genotype circulation profile. Both systems of monitoring detected G1 and G3 as the most predominant G genotypes, followed by genotypes G4, G9 and G2. Genotype G8 was only detected in sewage specimens; this is expected because it is a common genotype in cattle [Garaicoechea et al., 2006; Nataraju et al., 2009] but an atypical type in humans in most parts of the world, except for some African countries [Barril et al., 2006; Matthijnssens et al., 2006; Bányai et al., 2009]. The discrepancy in G8 detection may simply be due to the small number of RV-A isolations from the small group of pediatric patients, whereas environmental surveillance represented composite samples from a large catchment population. Mixed RV-A genotype detection accounted for a total of 31.9% of clinical samples, which is in agreement with previous results obtained in Córdoba [Barril et al., 2009]. The totality of the mixed infections was confirmed by re-amplification with individual specific primers.

These results indicate that clinical surveillance could mirror circulation of RV-A in the population, although it could also represent a small part of the visible tip of an otherwise unseen iceberg of inapparent and mild RV-A infections in the community. It should be noted that few studies have compared sewage and clinical RV-A genotype surveillance. A recent study conducted in Greater Cairo established a correlation between rotaviral gastroenteritis and the concomitant presence of RV-A in the environment [Kamel et al., in press]. An epidemiological study of enteric viruses in France showed that some RV-A genotypes detected in environmental samples were very similar to those of clinical strains [Arraj et al., 2008]. Other short-term comparative clinical and sewage studies conducted in enterovirus surveillance [Sellwood et al., 1981; Payment et al., 1993; Sedmak et al., 2003] have also demonstrated similar virus detection rates. However, in the present study, the clinical-sewage correlation was not absolute because G8 sewage genotype was not detected in clinical infections. The present study provides the first data from the Americas about RV-A environmental epidemiology and its correlation with RV-A isolates from human patients.

An unexpected result was that sewage surveillance detected RV-A in all samples tested throughout the year, and that similar G-type distributions were detected during the two seasonal periods. Therefore, environmental monitoring highlights the uniform year-round RV-A circulation, which is in agreement with data from studies conducted in Cairo and Barcelona [Villena et al., 2003] in which seasonal distribution of rotavirus could not be detected. However, the results obtained in the present study are in contrast to those data reported by Mehnert and Stewien [1993] and Hejkal et al. [1984], who detected RV-A seasonal variations in Brazil and USA, respectively. Therefore, the lack of a variation in detection of RV-A positivity was not in agreement with the incidence of RV-A diarrhea in clinical patients, which increases significantly during the cold period. This poses the question of how to explain RV-A clinical seasonality? The currently available data do not yet provide an explanation. However, a quantitative assessment of RV-A genotype detection in the sewage would have been useful to interpret the discrepancy between the lack of seasonal variation in the environment and the peak incidence of RV-A diarrhea during the cold months.

Our results indicate a genotype correlation between RV-A isolates from human diarrhea patients and sewage samples. Therefore, if sewage is sampled weekly and the isolates are compared to clinical cases, a similar picture of RV-A genotype circulation is clear for the community. Therefore, both clinical and sewage surveillances would be feasible systems to monitor the circulation of community rotavirus G types.

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