Rotavirus G2P[4] Detection in Fresh Vegetables and Oysters in Mexico City

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ABSTRACT

Rotaviruses are the principal cause of dehydration caused by diarrhea in children younger than 2 years of age. Although these viral infections have mainly been associated with ingestion of fecally contaminated food and water, few studies have addressed the presence of the virus in food that is consumed raw or slightly cooked. In this work, 30 oyster samples and 33 vegetable samples were examined for the presence of rotavirus genotypes to evaluate their potential to produce gastrointestinal infections. The rotaviruses were identified by reverse transcriptase PCR amplification of the VP7 gene. G and P genotyping was also performed by reverse transcriptase PCR, with a detection sensitivity of up to 15 PFU/ml. Rotaviruses were found in 17 (26.9%) of 63 samples (10 oysters and 7 vegetables). The G2 genotype was found in 11 (64.7%) of 17 of the rotavirus strains, and 16 (94.1%) of 17 had the P[4] genotype. The combined genotypes found most frequently were G2P[4] (10 [58.8%] of 17), G1P[8] (6 [35.29%] of 17), and G2P[NT] (1 [5.8%] of 17).

Group A of the Rotavirus genus (subfamily Sedoreovirinae, family Reoviridae) has the greatest clinical relevance and contains the best-described viruses of this genus. Enteric viruses are prevalent in the environment due to a variety of causes, but mainly to human fecal contamination; when viral particles in feces of affected individuals are dumped into the environment, they may reach shallow waters and become a sanitary hazard. There are few studies that directly assess rotavirus presence in foods; of these, most refer to rotavirus detection in shellfish and few refer to rotavirus detection in vegetables and other foods.

Due to the high rates of morbidity and mortality caused by rotavirus infections, these have become a concern worldwide. In Mexico, there have been reports in recent years of outbreaks in various regions of the country, including the states of Guerrero, Oaxaca, Tamaulipas, Hidalgo, Chiapas, and the State of Mexico. Nevertheless, there is a lack of regulation and of standardized techniques to investigate the presence of this virus in foods.

Classification as genotype G or P is determined by the presence of genes that code for the proteins in their external capsid, VP7 (glycoprotein) and VP4 (protease sensitive), respectively. Of the 15 G genotypes described currently, 10 infect humans (G1 to G6, G8 to G10, and G12); the most common are G1 to G4 (97.5%) and it accounts for over 50% of all human rotavirus genotypes.

Between January and March 2010, health authorities in Mexico reported an increase in the number of cases of gastroenteritis caused by rotavirus compared with the same period in previous years. Rotavirus strain G9P[4] was identified by the national surveillance system as the causative agent of these infections. This strain of rotavirus has been identified in several countries but is uncommon in Mexico, where the prevalent strains are G1P[8], G3P[8], G2, and G4 (63%). Among human rotaviruses, strain G1P[8] is considered the most common in worldwide circulation, and it accounts for over 50% of all human rotavirus genotypes. The strains G3P[8], G4P[8], G9P[8], and G2P[4], commonly implicated in cases of gastroenteritis worldwide, seem to behave cyclically in the human population, emerging as dominant every 3 to 4 years. The diversity of strains may be due to geographical differences: G8 strains have a special fondness for Africa, whereas G5 strains circulate widely in Latin America (23, 50). The present work focused on finding and identifying rotaviruses in foods in Mexico that are usually consumed raw or slightly cooked. Based on the epidemiological data published by various authors, rotaviruses are the main cause of diarrhea in children in Mexico; thus, the present work evaluated rotaviruses present in vegetables and oysters as vehicles of disease transmission.

MATERIALS AND METHODS

Virus and food samples. Samples were obtained from the Central Food Supply Station of Mexico City: 30 oyster
The One-Step RT-PCR with Platinum

Under aseptic conditions, 25 oyster tissue culture (38). Each vegetable sample weighed approximately 1 kg, whereas the oyster samples consisted of a bag or jar containing approximately 40 individual oysters.

Viral recovery from food samples. The viral concentration in vegetables was determined using a filtration-extraction-precipitation procedure, as previously described (38). Vegetable samples (50 g) were purchased from local commercial sources (who, in turn, get their supplies from the Central Food Supply Station in Mexico City). The samples were weighed and, after the addition of 280 ml of 0.05 M glycine–0.14 N saline buffer (pH 9.0), were blended at maximum speed for 90 s in a Servall Omni-Mixer (Ivan Sorvall, Inc., Norwalk, CT). Sample homogenates were prefiltered through gauze and then through filter paper; the vegetable samples, except for lettuce, were further processed by extraction with a 60% volume of Freon (Fisher Scientific, Suwanee, GA). Clarification was achieved by centrifugation at 3,500 × g for 10 min. The resulting supernatants were collected and adjusted to pH 7.2 to 7.3 using 1 M HCl, they were supplemented with 5 M NaCl to reach a final concentration of 0.3 M, and they were precipitated with 6% polyethylene glycol 8000 (PEG) after overnight incubation at 4°C. The samples were centrifuged at 6,000 × g for 20 min at 4°C, and the pellets were resuspended in 50 mM Tris–0.2% Tween 20 (pH 9.0; 25 ml per food sample). Resuspended precipitates were kept at room temperature for 60 min and were vortexed every 5 min, followed by centrifugation at 14,000 × g for 15 min at room temperature. In the secondary PEG precipitation step, the pH of the eluent was adjusted to 7.2 to 7.3 and the NaCl concentration to 0.3 M, followed by supplementation with 12% PEG 8000 and incubation at 4°C for 2 to 3 h. The precipitates were recovered by centrifugation at 7,500 × g for 15 min at 4°C and were resuspended in 50 mM Tris–0.2% Tween 20 (pH 8.0; 2.5 ml per food sample). Afterward, concentrates were kept at −70°C until further analysis.

Virus concentration in oyster was performed according to the protocol of Atmar et al. (3). Under aseptic conditions, 25 oyster specimens were homogenized with a stomacher. From this homogenate, 20 g was weighed and supplemented with 135 ml of a phosphate-buffered saline solution (PBS; pH 7.4) and 80 ml of chloroform–butanol (1:1 in volume), mixing at 30-s intervals. This suspension was placed in a centrifuge tube, was mixed with 40 ml of PBS, and was gently homogenized for 5 min at 4°C. Then, after the suspension was centrifuged at 13,500 × g for 15 min at 4°C, the aqueous phase was collected and 86 ml of 24% PEG and 1.2 M NaCl was added. After the mixture was homogenized gently for 4 h at 4°C, and then at 11,000 rpm for 20 min, the supernatant was discarded and the pellet was resuspended in 3 ml of RNAse-free water. The solution was filtered through a 0.22-μm-pore-diameter nitrocellulose membrane; each filtered sample (1 ml) was placed in an Eppendorf tube (Eppendorf Scientific, Hamburg, Germany) and frozen at −70°C for further analysis. As a control for the procedure, 50 g of each vegetable to be analyzed (previously disinfected) was collected, as well as 20 g of oysters (the sample had been commercially disinfected); each sample was inoculated with 500 μl of the SA-11 rotavirus (1.4 × 10⁶ 50% tissue culture infective dose per ml); afterward, the viral concentration procedure was carried out. RNA was extracted, and the reverse transcriptase PCR (RT-PCR) reaction was performed.

RNA extraction and rotavirus detection by RT-PCR. Viral RNA was extracted from each sample using TRIzol LS (Invitrogen, Carlsbad, CA), followed by addition of 12.5 μl of lysis buffer per 500 μl of the viral concentrate and incubation for 2 h. Next, 500 μl of TRIzol reagent was added, a mild homogenization was performed, and the sample was incubated for 5 min at room temperature. Then, after addition of 100 μl of chloroform, the sample was incubated anew for 3 min at room temperature, followed by centrifugation at 12,000 × g for 15 min. The aqueous phase was transferred to an assay tube and was mixed with 500 μl of isopropanol alcohol; after incubation at room temperature for 10 min and centrifugation at 12,000 × g, the supernatant was removed and the pellet was washed with 500 μl of 75% ethanol. Afterward, samples were centrifuged at 7,500 × g for 5 min, and the RNA pellet was dried for 30 min. RNA was dissolved in 30 μl of RNAse-free water and was transferred to Eppendorf tubes in 5-μl aliquots. One of the aliquots was diluted 1:1,000, and the absorbance was obtained at a wavelength of 260/280 nm in a spectrophotometer. The remaining tubes were stored at −70°C. The VP7 protein gene was amplified with the primers listed in Table 1A, using the OneStep Sensiscript RT kit (Qiagen, GMH, Hilden, Germany). The PCR reaction was done with the Taq PCR Core kit (Qiagen), following the instructions provided by the manufacturer. PCR amplification was performed for 40 cycles (94°C for 30 s, 50°C for 30 s, and 72°C for 30 s) with a final extension step at 72°C for 7 min in a Mastercycler thermocycler (Eppendorf); 392-bp amplicons were separated by electrophoresis in 10% polyacrylamide gel and were visualized by ethidium bromide staining for 20 min.

G genotyping. Food samples showing the 392-bp RT-PCR amplified product were G genotyped. To identify the G1, G2, G3, G4, G8, and G9 genotypes of VP7, the methods described by Gouvea et al. (27) were followed. Rotavirus double-stranded RNA segments were used as templates for RT to synthesize cDNA copies from both viral RNA strands suitable for PCR amplification. Both reactions were performed in a single, siliconized 600-μl Eppendorf tube containing 100 μl of reaction mixture. Extracted RNA (1 or 10 μl) was added to the reaction mixture, consisting of 10 mM Tris (pH 8.3), 40 mM KCl, 1.5 mM MgCl₂, 0.2 mM each deoxynucleoside triphosphate, 7% dimethyl sulfoxide, and 1 μM each of the desired oligonucleotide primers. The pair Beg9-End9 was used for the amplification of the full-length gene 9, or the primer mix containing all six serotype-specific primers: aBT1, aCT2, aET3, aDT4, aAT8, and aFT9; and the common primer RVG9 (Table 1B) was used for G genotyping. Before the enzymes were added, double-stranded RNA was denatured by heating at 97°C for 5 min and was quickly quenched in a dry ice–ethanol bath. The tubes were placed in an Eppendorf Mastercycler for an initial 30-min incubation at 42°C, followed by 25 cycles of PCR (94°C for 1 min, 42°C for 2 min, and 72°C for 1 min) and a final 7-min incubation at 72°C.

P genotyping. To identify the P[1], P[2], P[3], and P[4] genotypes of VP4, the method described by Gentsch et al. (22) was used. The One-Step RT-PCR with Platinum Taq kit (Invitrogen) was used, with the mixture prepared as indicated by the supplier and using the primers in Table 1C. Reverse transcription was performed at 45°C for 40 min, followed by a nested PCR, using denaturation at 95°C for 1 min, followed by 30 denaturation cycles at 94°C for 20 s, hybridization at 50°C for 2 min, and elongation at 72°C for 2 min, plus a final extension at 72°C for 8 min.

Rotavirus stability during frozen storage. To determine whether storing the viral concentrates obtained from the foods at −70°C affected viral stability, 900 μl of the concentrate of each of the analyzed vegetables and one oyster sample were supplemented with 100 μl of the SA-11 rotavirus. These samples were kept at...
−70°C for 3 months; each week, a sample of each food type was withdrawn, RNA was extracted, and RT-PCR amplification of the gene that codes for VP7 was performed.

### RESULTS

#### Detection of rotavirus in food samples.
Ten oyster samples (of 30 total) were positive for the VP7 rotavirus gene, as shown by the amplification of a 392-bp fragment. A similar amplicon was obtained for 7 (21.21%) of 33 vegetable samples (Fig. 1). Analysis of rotavirus strains revealed that 11 (64.70%) of 17 corresponded to genotype G2; however, in 6 (35%) of 17 of the rotaviruses, genotype G could not be determined (Fig. 2).

- P genotyping (VP4) showed that 16 (94.1%) of 17 of the rotaviruses were genotype P[4] (Fig. 3). We found that the most common genotype combination was G2 [P4], which was present in 10 (58.8%) of 17 of the samples. It was not possible to determine genotype G in six genotype P[4] samples, neither was it possible to determine genotype P in one sample with genotype G2 (Table 3).

This study also showed that it is possible to concentrate rotaviruses from vegetables and oysters and to maintain them at −70°C for up to 3 months without losing rotavirus detection sensitivity by RT-PCR.

#### DISCUSSION

In Mexico, diarrheal diseases are one of the main public health issues. In 2011, 5,283,896 cases of infection were...
TABLE 2. Vegetable samples positive for rotavirus

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. positive/no. of samples (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coriander</td>
<td>2/6 (6.06)</td>
</tr>
<tr>
<td>Parsley</td>
<td>1/5 (3.03)</td>
</tr>
<tr>
<td>Celery</td>
<td>1/5 (3.03)</td>
</tr>
<tr>
<td>Spinach</td>
<td>2/6 (6.06)</td>
</tr>
<tr>
<td>Papaloquelite</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td>Lettuce</td>
<td>1/6 (3.03)</td>
</tr>
<tr>
<td>Total</td>
<td>7/33 (21.2)</td>
</tr>
</tbody>
</table>

reported (1,386,702 [26.2%] of them in children younger than 5 years). These illnesses are second among the 20 most common causes of disease in all ages, with a rate of 4,837.8 cases per 100,000.

Children younger than 5 years of age suffer from a median of two to four episodes of diarrhea per year, i.e., diarrheal illnesses are the cause of 20.8% of the doctor visits at health clinics and account for 10% of the pediatric hospitalizations (15).

All the information generated in Mexico regarding rotavirus comes from our clinics. In this study, we found that 3.0% of the lettuce samples contained rotaviruses (Fig. 1). Monge and Arias (45) found evidence for rotavirus presence in 6.0% of lettuce samples. In our study, rotaviruses were found in 33.3% of the analyzed oyster samples (Fig. 2), which is within the range (22.7 and 42%) reported for oyster samples in Italy (20) and Japan (30), respectively.

Enteric viruses, especially rotaviruses, are present in oysters because they are filter feeders and as much as 20 liters of water per h passes through their digestive tract. In the process, they retain all sorts of solid particles, many of which are sedimentary floccules that can be transported or viral particles that may either be adhered or free (48), leading to passive bioaccumulation.

Whereas type 1 poliovirus in seawater has been detected at 10^5 PFU/ml, the viral concentration in oysters after 1 h of exposure could be 27 times greater (44). Bivalve mollusks can be treated to remove pathogenic organisms, but the techniques that are most effective at eliminating bacteria do not entirely remove viral particles. Thus, it is very important to include virological criteria in the sanitary control of mollusks (37).

The fact that many mollusks are consumed raw or slightly cooked is one reason why these viral particles reach consumers in a viable and infectious condition (5). This explains why filter-feeding organisms are vectors in the transmission of diseases, such as infectious hepatitis or gastroenteritis. The public health risk is even greater because mollusks thrive in polluted areas (39).

Of the 33 vegetable samples analyzed in this study, 10 (21.21%) (Fig. 2) were positive for rotavirus. No other data, however, have been published to date on the presence of rotaviruses in the other vegetables studied in the present work. Studies in the United States (1971, 1977, 1986), England (1983), and Peru (1990) showed that irrigation of vegetables with untreated water leads to the greatest loss of sanitary quality of these foodstuffs (7, 10, 18, 21, 58, 61).

Fruit and vegetable contamination can also occur by filtration of residual waters into fields, the presence of farm animals on cultivated land, the use of inappropriate fertilizers, or through the handling of crops during harvesting and packaging, as well as distribution and marketing (52). The concern about microbial contamination of these foods becomes even greater when we consider the survival span of pathogenic microorganisms, often weeks or months (31), particularly when they are found in moist areas of the vegetable or in parts that are protected from the sun, as occurs in lettuce, cauliflower, carrots, and radishes (11).

Rotaviruses in foodstuffs can be monitored by RT-PCR of the G and P genes that code for VP7 and VP4, respectively, the two most external viral proteins. To date, there is no information on genotyping of rotavirus strains obtained from foods. Data on rotavirus genotypes are mainly from viral strains associated with pediatric gastroenteritis. Rotavirus strains with genotype G2P[4] were found in 58.82% of the vegetables and oysters in the present work (Table 3). This is the second most frequent strain reported in clinical samples worldwide, after G1P[8] (13, 22, 24, 26, 46, 50, 62). Of rotavirus strains characterized in clinical samples in Mexico, genotypes G1P[1], G1P[3], G2P[2], G3, G4, G9, and G12 were the most prevalent (41, 47); these studies did not report the presence of genotype G2P[4], which was found in the foods studied in the present work. Genotype G2P[4] has been detected in clinical samples from Colombian cities, such as Bogotá (16.9%), Barranquilla (21.6%), and Cali (25%) (8), as well as from Argentina in 1995 and 1998 (19.8%) (2, 4, 17).

Note that not all strains could be genotyped in this work (Table 3). Also, in a recent study from Croatia and Slovenia, 18.7% of the rotavirus strains were not typeable (14, 60);
and, in a revision of G and P typing by RT-PCR in Latin American countries in 1995 and 2004, 20% of the strains were nontypeable (9). This may be the result of reassociation among human rotavirus strains and animal strains, as has recently been shown to occur among G5 (from pigs), G8, G10, and P[1] strains from cows, and P[9] from cats and dogs. Genetic variability is, thus, increased and, consequently, nontypeable strains appear (12, 29, 51). Fischer et al. (19) found that in 20% of nontypeable P strains, the VP4 gene showed point mutations in the P[8] primer when it was amplified and sequenced. Iturriza-Gómez et al. (34) found that the recognition site for the P[8] primers shows variability and, therefore, the previously designed primers (22) do not amplify these genes. Consequently, we suggest that nontypeable strains must be sequenced to achieve their identification. Also note that comparison of the genotypes of rotavirus strains found in foods with those isolated from cases of gastroenteritis, within the same time span, would allow the potential risk posed by virus strains to human health to be assessed, in addition to determining the differences in their persistence in the environment and/or their virulence.

The presence of G2P[4] rotavirus genotype in foods might be caused by a change in the distribution of the various rotavirus genotypes that cause pediatric gastroenteritis. This has occurred worldwide: in Ecuador, the new genotype G9 P[8] was found in 62% of clinical samples in 2007 (16); in Korea, G4P[6] was found in 26.4% of samples (35, 36); and, in the United States, G1P[4] was identified in 41% of samples (1).

Selective pressures from the use of rotavirus vaccines could have influenced these changes in the distribution of rotavirus genotypes. For example, the Rotarix vaccine, used in Mexico since 2006, is a monovalent vaccine made of attenuated human rotavirus (G1P[8]); it may contribute to an increase in the selection of new strains (49) because it does not provide efficient protection against genotypes G2P[4] and G9P[4] (20, 25, 28).

In Mexico, cases of gastroenteritis due to rotavirus have been reported since 2008; in that year, 2,640 cases were reported, with an incidence rate of 2.4 per 100,000. Numbers of cases have increased steadily, up to 4,529 cases in 2011, with an incidence rate of 4.1 per 100,000. To monitor the epidemiology of diarrhea caused by rotaviruses, operational definitions have been established for the detection, notification, study, and classification of the cases, to determine, based on clinical, laboratory, and epidemiological criteria, whether the infectious condition is caused by rotavirus (15).

Because Mexico lacks regulations to require monitoring of rotaviruses in foods that are consumed raw or only slightly cooked or that might be important vehicles for transmission of rotavirus, the data from this study contribute useful information on the incidence of rotaviruses and the potential risk in the consumption of the studied foods.

### TABLE 3. G and P genotypes present in rotaviruses found in vegetables and oysters

<table>
<thead>
<tr>
<th>Positive sample</th>
<th>G (VP7)</th>
<th>P (VP4)</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coriander</td>
<td>2</td>
<td>4</td>
<td>G2P[4]</td>
</tr>
<tr>
<td>Coriander</td>
<td>2</td>
<td>4</td>
<td>G2P[4]</td>
</tr>
<tr>
<td>Parsley</td>
<td>2</td>
<td>NT</td>
<td>G2P[NT]</td>
</tr>
<tr>
<td>Lettuce</td>
<td>NT</td>
<td>4</td>
<td>GNTP[4]</td>
</tr>
<tr>
<td>Celery</td>
<td>2</td>
<td>4</td>
<td>G2P[4]</td>
</tr>
<tr>
<td>Spinach</td>
<td>2</td>
<td>4</td>
<td>G2P[4]</td>
</tr>
<tr>
<td>Spinach</td>
<td>NT</td>
<td>4</td>
<td>GNTP[4]</td>
</tr>
<tr>
<td>Oyster</td>
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<td>4</td>
<td>G2P[4]</td>
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<tr>
<td>Oyster</td>
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<td>G2P[4]</td>
</tr>
<tr>
<td>Oyster</td>
<td>2</td>
<td>4</td>
<td>G2P[4]</td>
</tr>
</tbody>
</table>

a NT, not typeable.


