Characterization of Norovirus-Associated Traveler’s Diarrhea

N. Ajami,1,2 H. Koo,1,2 C. Darkoh,2 R. L. Atmar,1 P. C. Okhuysen,1,2,3 Z.-D. Jiang,2 J. Flores,2 and H. L. DuPont1,2,3,4

1Department of Molecular Virology and Microbiology and Department of Medicine, Baylor College of Medicine, 2Center for Infectious Diseases, School of Public Health, University of Texas Houston Health Science Center, 3Division of Infectious Diseases, University of Texas Medical School, and St Luke’s Episcopal Hospital, Houston, Texas

Background. Traveler’s diarrhea is the most common medical complaint of international visitors to developing regions. Previous findings suggested that noroviruses (NoVs) are an underappreciated cause of traveler’s diarrhea.

Methods. In the present study, we sought to define the presence of NoVs in 320 acute diarrheic stool samples collected from 299 US students who traveled to Guadalajara, Cuernavaca, or Puerto Vallarta, Mexico, during the period from 2007 through 2008. Conventional and quantitative real-time polymerase chain reaction assays were used to detect and determine NoV loads in stool samples. NoV strains were characterized by purification of viral RNA followed by sequencing of the viral capsid protein 1 gene. Sequences were compared using multiple sequence alignment, and phylogenetic trees were generated to evaluate the evolutionary relatedness of the viral strains associated with cases of traveler’s diarrhea.

Results. NoV RNA was detected in 30 (9.4%) of 320 samples. Twelve strains belonged to genogroup I, and 18 strains belonged to genogroup II. NoV prevalence was higher in the winter season than in the summer season (23% vs 7%, respectively; P = .001). The cDNA viral loads of genogroup I viruses were found to be 500-fold higher than those of genogroup II strains. Phylogenetic analysis revealed a diverse population of NoV strains over different locations and years.

Conclusions. NoV strains are important causes of traveler’s diarrhea in Mexico, especially during the winter-time, and US students in Mexico may represent a suitable group for future NoV vaccine efficacy trials.

Noroviruses (NoVs) are nonenveloped, single-stranded, positive-sense RNA viruses belonging to the family Caliciviridae; they are classified into 5 genogroups (GI–GV) on the basis of the sequence of the viral capsid protein VP1, of which GI, GII, and GIV affect humans [1–3]. NoVs are recognized as one of the most common causes of nonbacterial gastroenteritis affecting people of all ages worldwide and are transmitted through contaminated food and water, by person-to-person contact, or by mechanical transmission from environmental surfaces (ie, through hand or mouth contact) [4–9]. In industrialized nations, outbreaks are frequently reported to occur in restaurants, schools, cruise ships, and healthcare settings [10–13]; in developing countries, malnourished children or those without access to effective healthcare are at a higher risk of suffering significant morbidity and mortality [6].

Traveler’s diarrhea (TD) is the most common medical complaint of international visitors to tropical and semitropical regions [14–16], and although TD is most often caused by bacteria [17], travelers have been previously identified as a group at risk of contracting NoV-associated diarrhea [18–20]. NoVs have been shown to be the most commonly identified enteric pathogen (after diarrheagenic Escherichia coli) in studies on travelers to Mexico [19, 20].

Our research team monitors groups of US students traveling to Mexico as a model of TD, and previous findings suggested that NoVs were an underappreciated cause of TD marked by a continuous and diverse presence of viruses belonging to GI and GII [18–20]. An explanation as to why travelers are highly affected by GI strains is still missing, and little is known about naturally-occurring infection in locations where NoVs are endemic, including populations of international
travelers to endemic areas. The aims of this study were to determine the frequency of NoV-associated TD in US students traveling to Mexico, characterize the strains responsible for causing disease, determine the cDNA viral load, and characterize the clinical symptoms associated with NoV-associated TD.

METHODS

Study design and case definitions. Adults of at least 18 years of age who were experiencing TD acquired in Cuernavaca, Guadalajara, or Puerto Vallarta in the years of 2007 and 2008 were evaluated for the presence of NoVs in diarrheal stools. TD was defined as the passage of ≥3 unformed stools within a 24-h period associated with ≥1 of the following symptoms: abdominal pain or cramps, excess gas or flatulence, nausea, vomiting, fever (temperature, ≥100°F [≥37.8°C]), fecal urgency, blood and/or mucus in the stool, or tenesmus. Once TD was diagnosed, a stool sample was obtained; it was verified as being unformed stool by a clinic staff member and submitted to the microbiology laboratory for study. Exclusion criteria included previous enrollment in our study, a duration of diarrhea for more than 72 h, and the presence of a clinically important underlying illness other than diarrhea. For safety reasons, women who were pregnant or breast feeding were excluded from our study because most of the students enrolled in epidemiologic studies also participate in clinical trials. In addition, patients were excluded if they had taken any antimicrobial agent effective against diarrheal pathogens within the prestudy week or any antidiarrheal agent they had taken any antimicrobial agent effective against diarrheal pathogens within the prestudy week or any antidiarrheal agent within 12 hours of enrollment. All patients provided written informed consent. The study was approved by the University of Texas Health Science Center Committee for the Protection of Human Subjects.

Stool samples. All study participants were requested to submit stool samples to the local travel clinics within 48 h after onset of diarrhea. Additional stool samples were collected from participants during any subsequent acute diarrheal episodes. Stool samples were processed as reported elsewhere [17]. Aliquots of diarrheal stools were stored at −20°C and shipped on dry ice to our laboratory at the Center for Infectious Diseases, University of Texas, School of Public Health, Houston, Texas, for norovirus studies.

Collection of clinical information. Participants recorded on weekly diary cards the presence or absence of a number of gastrointestinal symptoms, along with the number, time, and characteristics of all stools passed during their stay in Mexico. Study subjects rated their signs and/or symptoms on the basis of the following severity scores: 0, no symptoms; 1, mild symptoms (tolerable, without interfering with normal activities); 2, moderate symptoms (distressing, forcing changes in normal activities); 3, severe symptoms (incapacitating, prohibiting performance of normal activities).

Detection and characterization of NoVs. A 10% (w/v) stool suspension was prepared with 0.01 mol/L phosphate-buffered saline and clarified by centrifugation at 3000 rpm (2000g) for 10 min. Viral RNA was extracted from 200 µL of the 10% stool suspension with a QIAamp viral RNA kit (Qiagen) according to the manufacturer’s instructions, and the RNA was stored at −80°C until used.

Reverse transcription (RT) was carried out in 50 µL of a reaction mixture containing 75 pmol of random hexamers (Thermo Fisher Scientific), 5 U of AMV-RT (Life Sciences), 1× AMV-RT buffer (Life Sciences), 10 mmol/L of dNTP mix, 10 U of Protector RNase Inhibitor (Promega), and 10 µL of RNA extract. The reaction was performed for 2 h at 43°C, and the enzyme was inactivated at 75°C for 5 min. The resulting cDNA was stored at −20°C until used in polymerase chain reaction (PCR) assays.

A set of degenerate primers [21] was used for initial detection of NoVs. The PCR reaction mixture contained 5 µL of template cDNA, 5 U of AmpliTaq (Applied Biosystems), 1× AmpliTaq buffer I (Applied Biosystems), 10 mmol/L of dNTP mix, and 200 mmol/L of each primer. A genotyping PCR assay was performed on positive samples using primers G1SKF, G1SKR, G2SKF, and G2SKR [22]. All PCR products were separated by 2% agarose gel electrophoresis and visualized under UV lamp after ethidium bromide staining.

Nucleotide sequence and phylogenetic analysis. PCR products resulting from the amplification with the GS primer set were used to sequence the ORF1-ORF2 junction region of all GI NoVs and GII NoVs identified. PCR products were purified using a MinElute PCR purification kit (Qiagen) according to the manufacturer’s instructions. Viral DNA was sent to SeqWright (Houston, TX) for sequencing. Sequences were analyzed using BioEdit, version 7.0.9.0, and aligned with Clustal W 2.0.10 (http://www.ebi.ac.uk/clustalw) with default parameters. Phylogenetic trees were constructed using the neighbor-joining and Poisson correction methods [23, 24] by use of MEGA, version 4.0 (http://www.megasoftware.net) [25]. The significance of the taxonomic relationships was obtained by bootstrap analysis (1000 replications) [26]. The reference strains described by Zheng et al [27] were used in the assignment of genotypes. The NoV sequences identified in the 2007–2008 Mexico cases were compared with those found in earlier studies in Mexico during 1998 and 2004 [19, 28] to determine relatedness of the viruses in 1 region over time.

Quantitative real-time PCR. The quantitative real-time PCR was carried out in triplicate on an ABI StepOne Real-Time PCR system (Applied Biosystems) using purified NoV GI or GII cDNA plasmids as standards and following the protocol described by Kageyama et al [29]. Amplification data were analyzed with StepOne Real-Time PCR System software, version 1.0 (Applied Biosystems).
### Statistical analysis.

Continuous variables were performed by use of the t test and the Mann-Whitney U test. \( P < .05 \) was considered to be statistically significant.

### RESULTS

#### Sample collection.

Stool samples from 320 discrete episodes of TD acquired during a short-term stay in Guadalajara, Cuernavaca, or Puerto Vallarta, Mexico, during 2007 and 2008 were collected from 299 US students. Of the 320 stool samples, 165 were collected from 152 individuals with TD in Guadalajara in June–August 2007, and 152 stool samples were collected from 147 individuals with TD acquired in Cuernavaca in December–February 2007 and in March–May and June–August 2008 (Table 1); 3 cases were collected from travelers to Puerto Vallarta. Overall, 17 travelers experienced recurrent diarrhea, and the maximum number of samples corresponding to discrete episodes was 2 samples per subject. Travelers ranged in age from 19 to 45 years of age (mean age, 24 years; median age, 22 years); 72% of travelers were female, and 96% were white.

#### Prevalence of NoV among TD patients.

In the present study, 30 (9.4%) of 320 stool samples collected had detectable levels of NoV RNA, and 27 (9%) of 302 travelers were infected by NoV. Most travelers had single bouts of NoV diarrhea, and only 3 travelers experienced \( \geq 1 \) episode of diarrhea in which NoV was recovered (Table 1).

NoV infection was more prevalent among travelers to Cuernavaca than among travelers to Guadalajara (15.8% vs 3.6% of travelers; \( P < .001 \)). During the summer of 2007 in Guadalajara, 109 travelers experienced TD and provided 122 stool samples, of which 5 (4%) were positive for NoV. In 2008, a similar prevalence was observed in the same location, where only 1 (2.3%) of 43 samples collected were positive for NoV. One hundred and twenty-seven stool samples were collected from 126 ill travelers to Cuernavaca during 2007, of which 16 (12.6%) were positive for NoV. During 2008, 8 (32%) of 25 stool samples were positive for NoV, which resulted in the highest prevalence rate reported in our study. Overall, 24 (15.8%) of the 152 samples collected from Cuernavaca were positive for NoV, and the subset of samples collected from this location during the colder months (December–February 2007 and 2008) had the highest presence of NoV, in which 11 (22.9%) of 48 stool samples were positive. None of the 3 stool samples collected from Puerto Vallarta were positive for NoV RNA.

#### NoV single infections and NoV coinfections.

Information on the identification of bacterial pathogens was available for 17 of the 30 samples containing NoV. NoV single infection was identified in 6 samples, and coinfections with bacteria (ie, NoV coinfections) were identified in the remaining 11 samples. Enterotoxigenic E. coli was identified in 10 of the 11 NoV coinfection samples and 1 sample was positive for enteroaggregative E. coli. The overall prevalence of coinfections observed in the present study was 65% (11 of 17 samples), with enteroaggregative E. coli being the most prevalent copathogen identified (ie, present in 92% of cases of coinfection).

#### Clinical symptoms associated with NoV-associated TD.

Clinical data were available on 23 of the 27 travelers who experienced NoV-associated TD. The majority of travelers recorded clinical symptoms representing a typical TD episode. Data on 3 travelers who had second independent episodes of diarrhea were excluded from the analysis because most travelers received treatment drugs after initial episodes and because this patient population also takes part in pharmaceutical clinical trials. Among the recorded symptoms, median scores suggest that abdominal cramping, flatulence, and vomiting were experienced at a mild level, whereas moderate levels of nausea and fecal urgency were recorded. None of the subjects reported experiencing fever during the illness. NoV-associated TD in the NoV single infection group (6 subjects) was characterized by a mild level of flatulence and moderate-to-severe levels of nausea, abdominal cramping, vomiting, and fecal urgency, whereas

### Table 1. Data on Norovirus-Positive Stool Samples Obtained from Travelers with Diarrhea in Guadalajara or Cuernavaca, Mexico, 2007–2008

<table>
<thead>
<tr>
<th>Location, season</th>
<th>Samples obtained in 2007</th>
<th>Samples obtained in 2008</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guadalajara</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Summer</td>
<td>5/122 (4.1)</td>
<td>1/43 (2.3)</td>
<td>6/165 (3.6)</td>
</tr>
<tr>
<td>Cuernavaca</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spring</td>
<td>1/9 (11.1)</td>
<td></td>
<td>1/9 (11.1)</td>
</tr>
<tr>
<td>Summer</td>
<td>11/87 (12.6)</td>
<td>1/8 (12.5)</td>
<td>12/95 (12.6)</td>
</tr>
<tr>
<td>Winter</td>
<td>4/31 (12.9)</td>
<td>7/17 (41.1)</td>
<td>11/48 (22.9)</td>
</tr>
<tr>
<td>Total for</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cuernavaca</td>
<td>16/127 (12.6)</td>
<td>8/25 (32)</td>
<td>24/152 (15.8)</td>
</tr>
<tr>
<td>Total for both cities</td>
<td>21/249 (8.4)</td>
<td>9/68 (13.2)</td>
<td>30/317 (9.4)</td>
</tr>
</tbody>
</table>

**NOTE.** Data are proportion (% of samples. A total of 320 samples were collected; however, 3 norovirus-negative samples from Puerto Vallarta were obtained during the summer of 2007 and are not listed. Total calculations were based on 320 samples.
all symptoms were recorded to be at a mild level in the NoV coinfection group (11 subjects). We found that cases of NoV single infection were associated with more severe vomiting, compared with cases of NoV coinfection with more than 1 pathogen \((P = .047)\). Ninety percent of the stool samples collected from ill travelers with NoV-associated TD were watery; the remaining 10% had a soft consistency.

**Genetic diversity of NoV strains identified.** Of the 30 diarrheal stool samples that tested positive for NoV, 12 (40%) tested positive for GI NoV, and 18 (60%) tested positive for GII NoV. Results obtained from the analysis of the partial amino acid capsid sequence of the most conserved region of the capsid protein suggested a diverse population of NoV strains in the study area. As shown in Figure 1, NoVs matching 4 different GI clusters (GI.1, GI.3, GI.5, and GI.8) (Figure 1A) and 8 different GII clusters (GII.2, GII.3, GII.4, GII.5, GII.6, GII.7, GII.8, and GII.9) (Figure 1B) were identified.

Phylogenetic analysis of nucleotide sequences was carried out for NoV cases by season and location. All GI NoV-associated TD cases were identified during 2007. A single GI.8 strain was found to cause disease in 9 different travelers in 2 cities during the same season. Only 1 positive sample was recovered from a traveler during spring. Four different GII.4 strains were associated with 6 NoV-associated TD cases in travelers to Cuernavaca. In Guadalajara, 4 travelers had GI NoV-associated TD (3 GI.8 and 1 GI.3), and 1 traveler had a GII virus (GII.5). All 11 episodes of diarrhea observed during the winter months were associated with viruses belonging to GII, whereas all 12 of the GI strains identified were isolated from patients who had traveled during the summer months of 2007. All the NoV-

![Figure 1](https://academic.oup.com/cid/article-abstract/51/2/123/300978)

Figure 1. Phylogenetic analysis of (A) genogroup I and (B) genogroup II norovirus strains identified from subjects with traveler’s diarrhea in Mexico, based on partial amino acid sequences of the norovirus capsid gene. Samples are listed by accession number (GU339305–GU339334), followed by location (GUAD, Guadalajara; CNVA, Cuernavaca) and year of collection. Reference sequences are labeled by their classification, followed by their accession number in parenthesis, and were described by Zheng et al [27]. Percentage bootstrap values are shown at the branch nodes. The scale indicates nucleotide substitutions per site.
positive samples collected during 2008 belonged to GII. Furthermore, the GII.4 strains identified in the present study appeared to be related to the epidemic strain 2006b that peaked in 2006–2007, and another GII.4 sample was closely related to the other epidemic strain 2006a during 2006–2007 (data not shown).

The partial capsid sequence alignment of the GI.1 strain described in the present study showed a high degree of similarity with a subset of GI.1 viruses previously identified among a population of travelers to Guadalajara in 2004 [19]. Less similarity was observed with other GI.1 strains identified among a pediatric population living in a periurban location located along the eastern perimeter of Mexico City [28] (Figure 2), which shows strain diversity across time.

NoV cDNA viral loads among affected travelers. The median cDNA viral load of NoV GI (for 11 subjects) detected in the fecal samples was $1.73 \times 10^{10}$ genome copies per gram (range, $3.76 \times 10^7–1.18 \times 10^{11}$ genome copies per gram) (Figure 3). The median cDNA viral load of NoV GII (for 12 subjects) detected in the fecal samples was $3.43 \times 10^7$ genome copies per gram (range, $3.26 \times 10^5–8.93 \times 10^9$ genome copies per gram) (Figure 3). The median GI NoV fecal shedding was found to be 500-fold higher than that of GII ($P = .003$). Similar results were observed when NoV single infection GI cases (3 subjects) were compared with NoV single infection GII cases (5 subjects) ($P = .036$) (data not shown). No statistical difference was observed when cases of GI and GII NoV single infection (8 subjects) and cases of GI and GII NoV coinfection (9 subjects)
were compared (P = .962). In addition, individual analysis by NoV genogroup and the presence or absence of copathogens did not show any statistical difference (cases of GI NoV single infection vs cases of GI NoV coinfection [P = .721]; cases of GII NoV single infection vs cases of GII NoV coinfection [P = .691]). These results suggest that NoVs may play a role in the development of TD. Furthermore, we found no association between severity of clinical symptoms and intensity of cDNA viral load (data not shown).

**DISCUSSION**

In line with our previous results showing that NoVs are a significant agent of TD [19, 20], the study reported herein has shown a prevalence rate of 9% for travelers to Mexico experiencing NoV-associated diarrhea. Although the prevalence rate observed here is lower than that reported in previous years [18–20], the present study represents a multiseasonal analysis of travelers during 2 consecutive years in 3 different locations. The role of NoVs in travelers’ illnesses could be even greater, because stool samples collected and analyzed in the present study were only from travelers with acute diarrhea and not from travelers who had a vomiting illness and no diarrhea in the presentation of their gastroenteritis. This observation should be taken into consideration for further studies of the true NoV prevalence among ill travelers, because the onset of vomiting and the absence of diarrhea can be seen after NoV infection [31].

Consistent with previous reports in which NoV infection had been referred to as a “winter-vomiting disease” [32], the highest prevalence of NoV infections occurred from December to February, which is considered the winter season in Mexico, and GII.4 strains were responsible for 55% of the cases, which correlates with previously published data showing that winter outbreaks were primarily due to GII.4 strains [33, 34]. However, it is important to mention that it was only during the winter of 2008 in Cuernavaca that we observed a prevalence rate of NoV that was higher than the prevalence rates observed in other seasons. Interestingly, all the NoV-associated TD episodes observed during the winter months were associated with viruses belonging to GII, whereas all the GI strains identified were isolated from patients who had traveled during the summer months of 2007.

As observed in the past (unpublished data) and in this present study, travelers to Cuernavaca are usually more commonly affected by NoV-associated diarrhea than those who travel to Guadalajara. A possible explanation for this finding might be that Cuernavaca is closer to Mexico City, the most populated city in Mexico, and it is occupied by a large population of people who commute to Mexico City on a daily basis. Guadalajara, on the other hand, is a larger urban city inhabited by people who usually do not commute to other cities to work, thus decreasing the possibility of introducing new pathogens into the community.

In line with previous reports [18, 19], we observed a strong and diverse presence of GI viruses despite the predominance of GII.4 viruses in most outbreaks [35–42]. Although our results demonstrate a great diversity of NoV strains affecting travelers (12 [40%] of 30 samples positive for GI NoV and 18 [60%] of 30 samples positive for GII NoV), an outbreak caused by a single G1.8 virus affected 9 different travelers during the summer months of 2007 in both Cuernavaca and Guadalajara, which means that 75% of the NoV-associated TD cases were attributed to G1.8 viruses.

In our study, we show that the median cDNA viral load of NoV GI is 500-fold higher than that of GII in fecal samples of travelers with NoV-associated TD. Differences in viral loads may be caused by experimental and host variables, including time of sample collection after infection, affinity of annealing of primers and probes to diverse viral genome sequences, age of the affected person, host immune response, and course of infection. However, on the basis of our results, we speculate that the increased cDNA viral load observed in NoV-GI samples had an impact on the transmission of these strains among susceptible hosts by the fecal-oral route, partially explaining the continuous presence of GI strains in travelers to these locations, as previously reported by our group and others [18, 19].

The majority of clinical symptoms reported by patients with NoV-associated TD did not differ from the symptoms that have been reported by patients with TD caused by other common bacterial pathogens [19, 43, 44]. Nonetheless, vomiting was more often associated with NoV single infections.

Our study demonstrates that the vast diversity of NoVs found among travelers to Mexico play an important role in travelers’ health, posing a challenge for vaccine development. Prevention methods such as vaccination should focus on eliciting cross-reacting antibodies that will protect individuals against a diverse population of NoVs, because natural infection could indicate the presence of cocirculating strains, as shown in our study.

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