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Noroviruses as a Cause of Diarrhea in Travelers to Guatemala, India, and Mexico

Hoonmo L. Koo[1,2*†], Nadim J. Ajami[1,2†], Zhi-Dong Jiang[2], Frederick H. Neill[1], Robert L. Atmar[1], Charles D. Ericsson[3], Pablo C. Okhuysen[3], David N. Taylor[4], A. Louis Bourgeois[5], Robert Steffen[6], Herbert L. DuPont[1,2,3]

[1]Baylor College of Medicine, [2]University of Texas – Houston School of Public Health, [3]University of Texas –Houston Medical School, [4]Salix Pharmaceuticals, Inc, [5]Johns Hopkins Bloomberg School of Public Health, [6]University of Zurich

†These authors contributed equally to the work presented.

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Communication:
Dr. H L Koo
Baylor College of Medicine
One Baylor Plaza
Houston, TX 77030
Phone 713/798-2900
Fax 713/798-0171
E-Mail: koo@bcm.tmc.edu

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Abstract

Noroviruses (NoVs) are increasingly being recognized as an important enteric pathogen of gastroenteritis worldwide. The prevalence of noroviruses as a cause of travelers' diarrhea acquired in developing countries is not well known. We examined the prevalence and importance of NoV infection in three international traveler cohorts with diarrhea acquired in three developing regions of the world, Mexico, Guatemala, or India. We also characterized the demographics and symptoms associated with NoV diarrhea in these travelers. Stool samples from 571 international travelers with diarrhea were evaluated for traditional enteropathogens. NoVs were identified using reverse transcription polymerase chain reactions and probe hybridization. Noroviruses were identified in 10.2% of cases of travelers' diarrhea, and overall was the second most common pathogen, following diarrheagenic *Escherichia coli.* The detection of norovirus diarrhea significantly varied over the 3 study time periods in Guadalajara, Mexico, ranging from 3 of 98 (3.0%) diarrheal stools to 12 of 100 (12.0%) fecal specimens (p = 0.03). The frequency of NoV diarrhea was also dependent upon the geographic region with 17 of 100 (17.0%) travelers to Guatemala, 23 of 194 (11.9%) travelers to India, and 3 of 79 (3.8%) travelers to Mexico positive for NoVs from 2002-2003 (p=0.02). Noroviruses are important pathogens of travelers' diarrhea in multiple regions of the world. Significant variation in the prevalence of norovirus diarrhea and in the predominant genogroup infecting travelers was demonstrated, dependent upon the specific geographic location and over time.

Introduction

Travelers' diarrhea (TD) is the most common illness experienced by travelers from industrialized nations to high risk developing regions (24, 28). Bacterial enteropathogens, such as enterotoxigenic *Escherichia coli* (ETEC) and enteroaggregative *Escherichia coli* (EAEC), are responsible for the majority of travelers' diarrhea cases (1, 27). However, up to 40% of TD cases never have any specific etiologic agent identified. These undiagnosed diarrheal illnesses are likely caused by undetected bacterial and non-bacterial enteropathogens (10-12, 27).

Noroviruses (NoVs) are important pathogens of gastroenteritis and are the most common cause of epidemic non-bacterial gastroenteritis outbreaks worldwide. NoVs are highly communicable with a low infectious dose and infect persons of all ages through fecal-oral transmission (4). NoVs are classified into five genogroups on the basis of phylogenetic analysis of the major capsid protein, with genogroup I (GI) and genogroup II (GII) strains most commonly associated with human infections (3). GII NoV strains are the predominant circulating genogroup worldwide (6).

The prevalence of NoV infection in travelers is not well known, with only two previous studies evaluating the frequency of NoV infection in travelers with diarrhea. The two reports demonstrated significant but variable prevalence rates of NoV diarrhea of 17% and 65% in persons visiting Mexico or Mexico and Guatemala, respectively. Interestingly, both studies identified GI NoVs as the most frequent genogroup detected (7, 16). The present study was designed to compare the prevalence of NoV infection in

international travelers with diarrhea acquired in Mexico over several time periods and in different developing regions of the world, including Mexico, Guatemala, and India and to characterize the symptoms associated with NoV diarrhea.

Materials and Methods

Study Populations. Three cohorts of travelers from industrialized nations with diarrhea acquired in developing regions of the world were evaluated for the prevalence of NoVs as a cause of travelers' diarrhea. The first cohort consisted of 98 US travelers to Guadalajara, Mexico enrolled from June 1, 2007 to September 30, 2007. The second group of subjects included 100 US travelers with diarrhea acquired in Guadalajara, Mexico, enrolled from June 15, 2006 to August 15, 2006. A third cohort comprised of 373 US and European adults with diarrhea acquired in Guadalajara, Mexico; Antigua, Guatemala; or Calcutta or Goa, India from July 10, 2002 to May 14, 2003. Subjects from the third cohort were participants of a clinical trial evaluating therapy for TD (27). The third cohort was retrospectively evaluated as a comparison group, to determine if NoV diarrhea prevalence varied over time and in different geographic regions of the world. All subjects provided an illness stool specimen confirmed as unformed by team members if they developed TD. Travelers' diarrhea was defined as the passage of $\geq$3 unformed stools within a 24-hour period associated with $\geq$1 symptoms of abdominal pain or cramps, excessive gas/flatulence, nausea, vomiting, fever ($\geq$100°F or $\geq$37.8°C), fecal urgency, blood and/or mucus in the stool, or tenesmus. Exclusion criteria for all cohort groups included duration of diarrhea for more than 72 hours, moderate or severe dehydration, clinically important underlying illness other than diarrhea, and in women,

being pregnant or breast feeding. In addition, patients were excluded if they had taken any antimicrobial or antidiarrheal agent active against diarrheal pathogens. All patients provided written, informed consent. The Committee for the Protection of Human Subjects at the University of Texas—Houston Health Science Center approved the study protocol.

Microbiology. Stool samples were screened for enteric bacterial and parasitic pathogens by published methods (14). Five *E. coli*-like colonies were isolated from each stool specimen, transferred to peptone stabs for storage, and transported to the Houston laboratory. *E. coli* were tested by the probe-hybridization technique for ETEC (14) and by the HEp-2 cell adherence assay for EAEC (1). Samples were kept at -80°C prior to use.

RNA extraction. 100 µg of stool were weighed, diluted 1:10 in 0.01M PBS and vortexed for 30 seconds. Samples were clarified by centrifugation at 4,200 x *g* for 10 minutes at room temperature. Viral RNA was extracted from 140 µl of the supernatant with the QIAamp Viral RNA kit (QIAGEN) according to the column centrifugation procedure described by the manufacturer. The RNA extracts were kept at -80°C prior to use.

Reverse transcription polymerase chain reaction (RT-PCR). A two-step multiplex RT-PCR was performed for every sample using a set of previously described degenerate Region B primers (Table 1), designed to amplify NoV RNA with an expected PCR product size of 213 bp (2). The reverse transcription reaction was performed by adding 5

µl of extracted RNA to a reaction mixture consisting of 3 µl of GeneAmp 10X PCR Buffer (Applied Biosystems), 10 units of Optizyme Ribonuclease Inhibitor (Fisher BioReagents), 2 µl of 10 mM mix of deoxynucleoside triphosphates, 200 nanomoles of each reverse primer (Mon 433, Mon 434), 5 units of AMV RT (Life Sciences Inc), and 18.5 µl of DEPC treated, DNase and RNase-free deionized water (MP Biomedicals). Samples were incubated for 60 minutes at 43°C followed by 5 minutes at 94°C. After completion, samples were immediately quenched in ice water and 70 µl of PCR mix was added. The PCR mix included 200 nanomoles of each forward primer (mon 431, mon 432), 7 µl GeneAmp 10X PCR Buffer (Applied Biosystems), 5 units of AmpliTaq DNA polymerase (Applied Biosystems) and 60 ul of DEPC treated, DNase and RNase-free deionized water (MP Biomedicals). cDNA was amplified under the following conditions: initial denaturation for 2 minutes at 94°C; 40 cycles consisting of template denaturation for 15 seconds at 92°C, primer annealing for 30 seconds at 50°C, and primer extension for 30 seconds at 72°C; a final extension for 7 minutes at 72°C. PCR amplicons were analyzed on a 1.5% agarose gel.

Southern Blot. All NoV-positive samples were confirmed by hybridization at 55°C with digoxigenin-labeled oligoprobes (mon 458, mon 459) (Table 1) using the Genius Detection kit (Boehringer Mannheim) as described previously (5).

Statistical Analysis. Significant differences between proportions of NoV diarrhea were evaluated with Fisher's exact test. All analyses were conducted using STATA (version 9.2) software.

Results:

The overall prevalence of NoV infection in travelers with diarrhea was 10.2%. NoVs were identified in diarrheal stools from 3 of 98 (3.0%) travelers to Mexico in the 2007 summer, with 66.7% NoV strains belonging to GI (Table 2). During the summer of 2006, NoVs were recovered from 12 of 100 (12.0%) travelers who acquired diarrhea in Mexico, with 83.3% of NoVs detected belonging to GII. From 2002 to 2003, 3 of 79 (3.8%) travelers to Mexico experienced NoV diarrhea, with 66.7% of NoV strains identified as GII. The difference in proportions of NoV diarrhea in Guadalajara, Mexico over the three time periods was statistically significant (p = 0.03). NoV diarrhea was detected in 17 of 100 (17.0%) travelers to Guatemala, with 52.9% of NoV strains belonging to GI. Twenty-three of 194 (11.9%) travelers to India were positive for NoV, with 69.6% of NoVs identified as GII strains. The frequency of NoV diarrhea in the three developing countries was significantly different during the 2002-2003 study period (p=0.02).

The majority of NoV diarrhea cases presented as mixed infections with other enteric pathogens (Table 3). However, 21 of 58 (36.2%) of NoV diarrheal stools were infected with NoV alone. The most frequent co-pathogens identified were ETEC (23/58, 39.7%), EAEC (17/58, 29.3%), *Cryptosporidium* spp. (3/58, 5.2%), *Salmonella* spp. (3/58, 5.2%), *Giardia lamblia* (2/58, 3.4%), and *Shigella sonnei* (2/58, 3.4%). Multiple (≥3) enteric pathogens were found in 13/58 (22.4%) NoV diarrheal specimens, with ETEC and EAEC most commonly identified as co-pathogens in these infections.

To better characterize diarrheal disease attributable only to NoVs in travelers, rather than potential co-pathogens, we chose to examine travelers with NoVs as the sole enteric pathogen identified as the cause of their diarrhea (n = 21). We evaluated the demographic and clinical characteristics associated with NoV diarrhea (Table 4). There was a predominance of males (84.2%) infected with NoV. The mean number (± SD) of unformed stools passed by travelers with NoV diarrhea within the 24 hours prior to enrollment was 5.7 (± 2.3). The most common symptoms associated with NoV diarrhea were abdominal cramping (94.7%), nausea (73.7%), flatulence (63.2%), and fecal urgency (36.8%). Fever (21.1%), vomiting (21.1%), tenesmus (10.5%), and presence of blood or mucus in stools (10.5%) were less commonly reported.

Discussion:

This study demonstrates that NoVs are an important pathogen of travelers' diarrhea in multiple regions of the world. The overall prevalence of NoV diarrhea in travelers was 10.2%. Noroviruses were the second most common enteropathogen identified in travelers, following diarrheagenic *E. coli* (52.9%).

There was distinct variation in the prevalence of norovirus diarrhea and in the predominant genogroup infecting travelers, dependent upon the specific geographic location and over time, despite consistency in detection methods. In this study, the majority of NoV strains were identified as GII, except for the Guatemala cohort, which had nearly equal numbers of NoV GI and GII strains. Travelers who acquired diarrhea in Mexico in 2007, had more NoV GI than GII strains identified, although there were

164 relatively few NoV diarrhea cases in Mexico during this time period. Similarly, in a
165 previous surveillance study performed by our group in Mexico during the summer of
166 2004, 81% NoVs detected were NoV GI strains (16). In the present study, 66.7% and
167 83.3% of NoVs identified in travelers to Mexico in 2002-2003 and 2006, respectively,
168 were GII strains. Future studies are needed to investigate the potential underlying
169 determinants of variation in NoV diarrhea frequency, including the prevalence of NoV
170 infections in the indigenous communities, variable climate conditions such as increased
171 rainfall leading to more fecal contamination of the water supply, and host genetic and
172 immunologic susceptibility patterns among the indigenous populations and travelers.
173
174 Travelers likely acquire norovirus infections from local resident populations in
175 developing regions of the world, where sanitation and hygienic standards may be
176 inadequate, facilitating fecal-oral transmission of enteric pathogens. Unfortunately, the
177 current lack of defined molecular epidemiology of NoV infections in developing nations,
178 such as Guatemala, India, and Mexico, hinders correlation between the prevalence of
179 NoV strains endemic in indigenous populations and NoV diarrhea in travelers. Limited
180 epidemiologic surveillance has been conducted in these high risk regions for travelers'
181 diarrhea and primarily evaluate NoV diarrhea in pediatric populations. In a rural
182 Guatemalan community, 72% of screened children (n=522) between the ages of 6-36
183 months were shown to have been previously exposed to Norwalk viruses by ELISA,
184 using antibody to recombinant-expressed Norwalk capsid proteins. No evaluation for GII
185 NoVs was peformed in this study (25). Multiple epidemiologic investigations have been
186 performed in eastern, western, and southern India (8, 9, 15, 19, 21-23). The majority of

these studies examined outpatient or hospitalized children with acute gastroenteritis for NoV infection by RT-PCR. Overall, the prevalence of NoV diarrhea in these Indian children ranged from 10.9% to 18.9%, with a predominance of GII strains (90%-100%). One surveillance study in southern India detected a greater percentage of GI NoVs (25%), but a potentially less specific ELISA assay was used for detection (15). The prevalence of NoV diarrhea in our traveler cohort to India (11.9%) was similar to the detection of NoV diarrhea in these local pediatric populations, but the relative proportion of GII NoV strains identified in travelers (69.6%) was lower than rates reported in these studies. In Mexico, children < 5 years of age hospitalized for acute gastroenteritis have been evaluated for NoVs. GII NoVs were detected in 4.7% of stools tested, while no GI NoV strains were recognized (13). In our study, the prevalence of NoV diarrhea in travelers to Mexico varied from 3.0% to 12.0%, with a fluctuating predominant NoV genogroup. In the future, we plan to characterize NoV strains infecting Mexican children with diarrhea to determine whether they may serve as a reservoir for NoV strains infecting travelers to Mexico.

Mixed infections with other enteric pathogens were commonly found in travelers with NoV diarrhea. The most commonly isolated co-pathogens in the NoV diarrheal stools were ETEC and EAEC. The distribution of the rates of detection of these co-pathogens is not surprising because it is similar to the frequency with which these enteric pathogens cause travelers' diarrhea (1).

Travelers with NoV diarrhea in the present study passed an average of 6 unformed stools within 24 hours, which may be considered as moderate to severe diarrheal illness (18, 20). The most common symptoms associated with NoV diarrhea were abdominal cramping, nausea, flatulence, and fecal urgency. Interestingly, vomiting was less commonly noted by travelers, although infection with NoVs is classically described as a "winter-vomiting" disease (17). However, since diarrhea was an inclusion criterion for participation in this study, travelers with NoV infection manifesting only as vomiting may have been excluded, leading to decreased reports of vomiting associated with NoV diarrhea in this study.

Limitations of this study include the possible exclusion of travelers with NoV infection who may not have manifested diarrhea as part of their clinical illness. It is possible that NoV RNA degradation in stool specimens may have contributed to decreased rates of NoV detection. However, we feel that it is unlikely that significant RNA degradation led to lower NoV detection in fecal specimens from Mexico 2002-2003, since the highest NoV diarrhea frequency was identified in Guatemala during this time period. Subsequent testing of more recent stools from Mexico in 2007 confirmed a variable pattern of NoV detection with relatively lower frequency. Finally, tests for other viral pathogens were not performed; however, previous studies have shown that other enteric viruses play a much less significant role in the pathogenesis of travelers' diarrhea (14, 28).

Noroviruses are an important cause of endemic travelers' diarrhea in at least two regions of Latin America and one Asian country. It is likely that this enteric pathogen has long

been underestimated as a cause of travelers' diarrhea due to limitations of detection methods. We believe that future studies of travelers' diarrhea will demonstrate that NoVs are an important cause of diarrhea in travelers to other high risk developing regions across the world. Future studies will be important to characterize the genetic diversity of the circulating NoV strains, which may impact future therapeutic and preventative strategies targeting NoV infection. More studies are necessary to help us to better understand the human immunological response to NoVs, which may help in vaccine development. International travelers to developing regions of the world may represent one of the few populations with relatively high rates of NoV endemicity, making them valuable for evaluation of NoV vaccines in the pipeline for development (26).

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Table 1. Primer and Probe Sequences of RT-PCR and Southern Blot

NoV genogroup	Name	Use	Sequence (5' to 3')	Sense
I	MON 434	Primer	GAASCGCATCCARCGGAACAT	Reverse
	MON 432	Primer	TGGACICCYGGICCYAAYCA	Forward
II	MON 433	Primer	AAYCTCATCCAYCTGAAYCAT	Reverse
	MON 431	Primer	TGGACIAGRGGICCYAAYCA	Forward
I	MON 458	Probe	ATGTATGTRCCACGATGGCARGCC	Forward
II	MON 459	Probe	ATGGATTTTTACGTGCCCAGGCAA	Forward

Note. I: inosine; R: purine (A/G); Y: pyrimidine (C/T); S: Strong (C/G)

Table 2. Prevalence of Norovirus Diarrhea in International Travelers, by Genogroup and Geographic Location

Location	Norovirus Diarrhea (%)	Genogroup I (%)	Genogroup II (%)	Genogroup Indeterminate
Guatemala	17/100 (17.0)	9/17 (52.9)	8/17 (47.1)	0
India	23/194 (11.9)	7/23 (30.4)	16/23 (69.6)	0
Mexico (2002-2003)	3/79 (3.8)	1/3 (33.3)*	2/3 (66.7)*	1/3 (33.3)
Mexico (2006)	12/100 (12.0)	1/12 (8.3)	10/12 (83.3)	1/12 (8.3)
Mexico (2007)	3/98 (3.0)	2/3 (66.7)	1/3 (33.3)	0

Note. *One specimen tested positive for both genogroups

Table 3. Co-Pathogens of Norovirus Infection

Co-Pathogen	Co-Infection (%)
Norovirus alone	21/58 (36.2)
Enterotoxigenic *E. coli*	23/58 (39.7)
Enteroaggregative *E. coli*	17/58 (29.3)
Cryptosporidium spp.	3/58 (5.2)
Salmonella spp.	3/58 (5.2)
Giardia lamblia	2/58 (3.4)
Shigella sonnei	2/58 (3.4)
Aeromonas spp.	1/58 (1.7)
Plesiomonas shigelloides	1/58 (1.7)
Multiple ($\geq$3) pathogens	13/58 (22.4)

Table 4. Demographic and Clinical Characteristics of International Travelers with Norovirus Diarrhea (Excluding Travelers with Co-pathogens)

Variable	Travelers with Norovirus Diarrhea (n = 21)*
Age, mean years ± SD	28.8 ± 12.1
Male sex	84.2
Number of unformed stools, mean ± SD**	5.7 ± 2.3
Abdominal cramping	94.7
Nausea	73.7
Fecal urgency	63.2
Flatulence	36.8
Fever	21.1
Vomiting	21.1
Tenesmus	10.5
Presence of blood/mucus	10.5

Note. Data are percentage of subjects, unless otherwise indicated
*Data missing for one patient from Guatemala and India
**Within 24 hour prior to enrollment