NORWALK-LIKE VIRUS AND BACTERIAL PATHOGENS ASSOCIATED WITH CASES OF GASTROENTERITIS ONBOARD A U.S. NAVY SHIP

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Abstract. Acute gastroenteritis is a potential cause of substantial morbidity in U.S. military personnel during deployment. This study investigated the microbial causes of diarrhea in U.S. troops on exercises in Southeast Asia aboard the U.S.S. Germantown from March through May 1996. A total of 49 (7%) patients with diarrhea reported to sick call during a 3-month deployment involving 721 personnel. Diarrheal samples from 49 patients were subjected to bacterial and parasitologic examination, but sufficient samples from only 47 of 49 were available for analysis of the presence of Norwalk-like virus (NLV). Of the 49 diarrhea cases, 10 (20.4%) appeared to be due to bacterial etiology alone, 10 (20.4%) due to bacteria and the prototype Taunton agent (TNA), 11 (22.4%) due to TNA only, and 4 (8.0%) due to parasites. Norwalk-like virus RNA was present in 21 (45%) of 47 stool samples from the diarrhea cases, 10 with bacterial etiologies and 11 without bacterial or parasitic etiologies. No pathogen was detected in 14 (29%) of the cases. Four of the controls showed the presence of parasitic organisms. Of the 11 cases in which enterotoxigenic Escherichia coli was isolated, 8 were positive for colonization factor antigen (CFA/IV), and 3 were CFA-negative. The bacterial pathogenic tests were all susceptible to gentamicin, and furadantin, but were resistant to ceftriaxone and norfloxacin, including 75% of the Campylobacter spp. These data support the view that the major cause of diarrhea for troops deployed in this geographic area is most likely NLVs.

Diarrheal disease has long been a major disease threat among deployed military forces, especially during combat. Studies have shown considerable loss of person-hours because of traveler’s diarrhea among U.S. military personnel during deployment. During Operation Desert Shield, 57% of the surveyed troops had 1 or more episodes of diarrhea, and 20% were unable to perform their duties due to diarrhea. In a study conducted aboard the U.S.S. John F. Kennedy in 1988, 21% of the surveyed personnel reported acute episodes of diarrhea during or shortly after shore leave. Of these, 70% had enterotoxigenic Escherichia coli (ETEC) isolated from their stools. This level of disease can have a significant impact on the combat readiness of deployed troops.

Viruses have also been implicated as the cause of several outbreaks of diarrhea onboard U.S. Navy ships. The association of viral pathogens such as Norwalk virus (NV) or Norwalk-like viruses (NLVs), which are small round-structured viruses (SRSVs), has been documented among travelers on cruise ships. Various morphologically indistinguishable but antigenically distinct NLVs have subsequently been identified. The grouping of these isolates has been based on nucleotide sequence homology and serologic typing results, which continue to be a subject of debate. Such analyses have provided an initial basis for grouping such isolates into 4 major groups: the prototype Norwalk virus (NV), the Taunton agent (TNA), the Hawaii agent (HWA), and the Snow Mountain agent (SMA). The documentation of outbreaks involving NV have been limited due to difficulties in the diagnosis of these viruses, which is secondary to the absence of convenient and well-defined diagnostic assays for this group of viruses. Lack of a diagnostic assay is believed to be due to the marked genetic diversity of NVs or NLVs, in addition to difficulty in identifying low concentrations of NLVs in fecal specimens by direct electron microscopy. The inability to propagate NV in tissue culture or induce infection in practical animal models has further limited the availability of biologic reagents needed to produce diagnostic assays. However, the recent description of the complete NV genomic sequence has facilitated the designing of 2 sets of primer pairs (G-1 and G-2). These primer pair sequences are based on the conserved nucleotide sequence within the RNA polymerase gene of a large number of NLVs. Such sequence analysis has allowed the grouping of these NLVs into 2 distinct genogroups represented by NV and SMA. In addition, based on sequence analysis of the amplified products of these 2 sets of primer pairs, 6 distinct oligonucleotide probes have been designed that allow classification of NV or NLV agents into 4 distinct groups. The availability of such sets of primers and probes has made it possible to predict the nucleotide sequence and classify the NLVs into groups associated with infection.

Among travelers to developing countries, one-third to one-half experience diarrhea. Enterotoxigenic E. coli is the predominant etiologic agent identified, although a variety of other enteropathogens have been isolated. Colonization factors in human ETEC strains are usually protein fimbriae, which are capable of agglutinating erythrocytes from different animal species in the presence of d-mannose. Colonization factor antigen (CFA/I) is a single fimbrial antigen, whereas CFA/II and CFA/IV consist of several subcomponents. These components are called E. coli surface antigens. Colonization factor/II is composed of the CS1, CS2, and CS3 antigens and CFA/IV is composed of the CS4, CS5, and CS6 antigens.

Travelers’ diarrhea due to bacterial pathogens may require treatment with antibiotics. Therapy with ampicillin, tetracycline, or trimethoprim/sulfamethoxazole has been shown to reduce the duration of illness compared with placebo. The emergence of drug-resistant bacteria in many parts of the world where antibiotics are commonly inappropriately used may eventually limit their effectiveness.

The 3-month deployment reported in this study presented an opportunity to study the incidence of diarrhea. The primary objective was to identify the etiologic agents of diarrhea by comparing isolates of pathogenic enteric bacteria or...
parasites from cases with those from controls. Secondary objectives were to examine the antimicrobial susceptibility patterns of the bacterial pathogens, the potential etiologic role of NV or NLV agents, and the symptoms associated with diarrhea in this population and setting.

**MATERIALS AND METHODS**

**Study site and subjects.** The shipboard population consisted of 721 persons in the Southeast Asia Cooperation and Readiness Afloat Training exercise, with port calls at Manila/Subic Bay, Surabaya, Jakarta, Singapore, Kuantan, and Sattahip from March 1 to May 31, 1996. The study was conducted by investigators of the U.S. Naval Medical Research Unit No. 2 (NAMRU-2) with at least 1 investigator present for the duration of the cruise.

**Selection of participants.** All patients presenting with diarrhea (both mild and severe) to the ship medical department were interviewed by investigators. Controls were seen in the same medical clinic for reasons other than gastrointestinal illness. They had not had diarrhea in the previous 2 weeks. A case of diarrhea was defined as a person with 3 or more loose or watery stools in a 24-hr period. The study protocol was reviewed and approved by the NAMRU-2 Committee for the Protection of Human Subjects. The purpose of the study was explained to the patients presenting with or without diarrhea, and written informed consent was obtained prior to enrollment into the study. A standard medical history was obtained, physical examinations were performed, and patients were asked to complete a 3-page questionnaire and provide a stool sample. The questionnaire was designed to determine the number of hours ashore, the source and type of food and beverage consumed, and the number of diarrheal episodes experienced. Stool specimens or rectal swabs were placed into tubes of Cary-Blair transport medium with 5% sheep blood and stored at 4°C onboard ship for approximately 7–10 days before being transported to NAMRU-2 for processing. Stool specimens or rectal swabs were processed immediately on site for the potential isolation of Campylobacter spp. and Shigella spp.

**Laboratory methods.** Although ETEC was the organism of primary interest, the study included standard microbiologic methods to identify Salmonella, Shigella, Campylobacter, and Vibrio cholerae. Laboratory procedures included streaking onto MacConkey (MC) agar, Salmonella-Shigella (SS) agar, thiosulfate citrate bile salts sucrose (TCBS) agar, and Campylobacter blood agar plates (CBAPs), as well as inoculation into alkaline peptone water (APW) and mannitol selenite broth (MSB) for enrichment. Isolates from MSB culture onto alkaline peptone water (APW) and mannitol streaking onto MacConkey (MC) agar, Salmonella CBAPs were incubated at 42°C for 72 hr in a microaerophilic atmosphere. Cultures were examined for the following agents: Salmonella, Shigella, Campylobacter, E. coli, and V. cholerae. Standard procedures were used, including conventional screening sets of Kligler iron agar, motility-indole-ornithine medium, and sucrose semi-solid agar. The presence of protozoa and helminthic parasites was determined by microscopic examination of fresh stools and specimens concentrated with 10% formalin.24

Air-dried, methanol-fixed smears prepared from stool specimens were examined for Cryptosporidium oocysts using a modified acid fast stain. Fecal leukocytes were detected by examination of fecal smears stained with methylene blue. Antibiotic resistance was determined by the disk susceptibility method for enteropathogens.25

**Ganglioside GM1-ELISA.** Fecal specimens cultured on MC plates were incubated overnight at 37°C and 5 E. coli lactose-fermenting colonies from each patient were isolated and subcultured on nutrient agar stab cultures until analyzed for heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST) by a ganglioside GM1-ELISA.26 Strains that were enterotoxin positive were further analyzed for CFAs by a dot-immunoblotting assay.7

**Detection of Rotavirus and NV.** Eighty-five stool specimens (47 cases and 38 controls) were examined for the presence of rotavirus by a commercial available ELISA (Rotazyme; Abbott Laboratories, North Chicago, IL). A reverse transcription–polymerase chain reaction (RT-PCR) assay was performed on the 85 stool samples for the presence of SRSVs. Briefly, RNA extracted from 85 stool samples were reverse transcribed and subjected to the PCR using primer pair sets G-1 and G-2, which amplify a predicted 123-basepair product (courtesy of Dr. Roger Glass, Centers for Disease Control and Prevention, Atlanta, GA). Six oligonucleotide probes (also obtained through the Courtesy of Dr. Roger Glass) were also used as probes. A mixture of 3 probes (SR63d, SR65d, and SR69d) constituted a probe set designated P1-A, whereas oligonucleotide SR67d served as probe P1-B, SR61d as probe P2-A, and SR47d as probe P2-B. Probe set P1-A was designed to hybridize with sequences encoded by the prototype SRSV isolates belonging to the UK-2 strains of NV (UK2-8, -9, -11, and -12), and for other previously reported strains. Probe P1-B was designed to hybridize with 2 of the UK-1 prototype strains (UK-1-1 and UK-1-4), probe P2-A with 4 of the UK-1 strains (UK-1-1 to UK-1-4) and 3 other strains of SRSV, and probe P2-B with 5 of the UK-3 strains, 4 of the UK-4 strains, and 2 reported SRSVs including 1 designated SMA. Southern hybridization was carried out in 2 steps. During the first step, an aliquot of each of the amplified products resulting from the RT-PCR was exposed to a mixture of all the 6 probes. The RT-PCR amplified products showing positive hybridization to this mixture of probes were subsequently tested for strain specificity by analysis of hybridization with individual P1-A, P1-B, P2-A, or P2-B probes.

**RESULTS**

Forty-nine (7%) of 721 Navy personnel reported to sick call during the 3-month study period aboard the U.S.S. Germantown. Diarrheal samples from these 49 patients were subjected to bacterial and parasitologic examination, but sufficient samples from only 47 of 49 were available for analysis of the presence of NLV. Symptoms associated with diarrhea included abdominal pain (73%), thirst (73%), fatigue (65%), vomiting (8%), and fever (27%). A diarrheal pathogen was found in 34 (72.3%) of 47 patients with diarrhea. The RNA samples isolated from the 47 diarrhea cases were subjected to the RT-PCR for NLV. As shown in Table 1, 21 (45%) of 47 stool samples from diarrhea cases were
positive for NLV. None of the 38 controls were positive for NLV. Identification of the RT-PCR-amplified products by electrophoresis and staining with ethidium bromide was confirmed by Southern hybridization. All 21 amplified products hybridized with the combined probes. To determine the specific antigenic type of the prototype virus, hybridizations with individual probes for the RT-PCR products were performed. All 21 RT-PCR products hybridized only with the SR61 (P2-A) probe set, which is similar to the UK-1 prototype of TNA (Tables 1 and 2 and Figure 1). Results of examinations of stool samples from all patients and controls for Rotavirus were negative.

There were 11 isolations of ETEC (22%), 6 of Campylobacter spp. (12%), 1 of a Salmonella group D (2%), 1 of S. enteritidis (2%) and 1 of V. parahaemolyticus (2%). Concomitant Campylobacter and Salmonella group D were detected in 1 patient (Table 1). Of the 20 patients with bacterial pathogens, 10 (20.4%) had only a bacterial infection and 10 (20.4%) had mix infections with both bacteria and NLV (Table 1). Four of the 38 stool specimens showed the presence of Blastocystis hominis. Of the 11 cases with ETEC, 1 (9.0%) produced LT and 10 (91%) produced ST. A study of the CFAs present in the 11 ETEC isolates indicated that 8 were reactive with CS6 (CFA/IV), and 3 were CFA-negative.

Parasites (B. hominis) were found in stool samples of 2 patients with diarrhea. Two other patients had Giardia lamblia and B. hominis with concomitant ETEC and Campylobacter detected (Table 1). Cryptosporidium and Cyclospora were not isolated from any cases or controls. Four of the 38 stool samples from the control patients had readily detectable B. hominis. Stool samples from 18 of the diarrhea patients had fecal leukocytes.

Antibiograms showed that all isolates were susceptible to gentamicin, furadantin, ceftriaxone, and norfloxacin, with the exception of Campylobacter spp., which were resistant to trimethoprim-sulfamethoxazole, norfloxacin, and cephalothin. V. parahaemolyticus isolates showed resistance to ampicillin and colistin, while S. enteritidis was resistant to tetracycline and colistin.

**DISCUSSION**

Diarrheal disease due to NLV of the TNA prototype and bacteria, specifically ETEC, Campylobacter spp., Salmonella group D, and V. parahaemolyticus, were documented in this study of Navy personnel in Southeast Asia. Seven percent of 721 personnel reported to sick call with diarrhea. All had mild or no dehydration and the severity of illness did not differ according to the pathogen found. Of interest was the use of Campylobacter and Salmonella group D as concomitant pathogens in patients with diarrhea. Of the 11 patients with ETEC, 1 (9.0%) produced LT and 10 (91%) produced ST. A study of the CFAs present in the 11 ETEC isolates indicated that 8 were reactive with CS6 (CFA/IV), and 3 were CFA-negative.

**TABLE 1**

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>Pathogens</th>
<th>RT-PCR (NLVs)</th>
<th>Southern hybridization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>P1-A</td>
<td>P2-A</td>
</tr>
<tr>
<td>2</td>
<td>ETEC</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>1</td>
<td>ETEC and Giardia lamblia</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>8</td>
<td>ETEC</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>1</td>
<td>Campylobacter and Blastocystis hominis</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>Campylobacter spp.</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>1</td>
<td>Campylobacter and Salmonella group D</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>1</td>
<td>Salmonella group D</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>1</td>
<td>S. enteritidis</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>1</td>
<td>Vibrio parahaemolyticus</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>11</td>
<td>NPF</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>14</td>
<td>NPF</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>B. hominis</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>NPF</td>
<td>NSA</td>
<td>ND</td>
</tr>
<tr>
<td>38 controls</td>
<td>B. hominis and NPF</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Total 87</td>
<td></td>
<td>20</td>
<td>21</td>
</tr>
</tbody>
</table>

*RT-PCR = reverse transcription–polymerase chain reaction; NLVs = Norwalk-like viruses; ETEC = enterotoxigenic Escherichia coli; NPF = no pathogen found; NSA = no stool available; ND = not determined.

**TABLE 2**

<table>
<thead>
<tr>
<th>PCR primer</th>
<th>Probe</th>
<th>UK type (prototype)?</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>P1-A (SR63, SR65, SR69)</td>
<td>UK2 (NV)</td>
</tr>
<tr>
<td>G-2</td>
<td>P2-B (SR67)</td>
<td>UK1 (?)</td>
</tr>
<tr>
<td>G2</td>
<td>P1-B (SR67)</td>
<td>UK1 (TNA)</td>
</tr>
<tr>
<td>P2-A (SR61)</td>
<td>UK3/4 (HWA/SMA)</td>
<td></td>
</tr>
</tbody>
</table>

* Ando and others.*
† NV = Norwalk virus; TNA = Taunton agent; HWA/SMA = Hawaii agent/Snow Mountain agent.
detected in 10 diarrhea samples that also contained pathogenic bacteria. However, this finding makes it difficult to define the primary cause of diarrhea in these patients. In the 14 cases in which no pathogen was detected, delays in processing may have increased the yield of pathogens, the level of virus excreted may have become undetectable in some cases by the time of examination, and/or prolonged freezing or thawing of samples or nucleosidase enzymes could have destroyed the NLVs. Although the number of diarrheal samples that contained parasites were few (4 of 49, 8%), it is interesting that none showed evidence of NLV RNA sequences. It is possible that cytokines with antiviral potential, such as interferon, induced by such parasites may play a role in the decreased incidence and/or elimination of NLV infections. This clearly requires further study. None of the 38 stool samples from the control subjects had NLV, suggesting that asymptomatic carriage is uncommon in this population, unlike an earlier challenge study in which NLV was found in the placebo group. The finding in the present study that 11 (22%) of the cases were attributed to ETEC is similar to the findings of other studies that have found ETEC to be the most common diarrheal pathogen identified on cruise ships and Navy ships. Data obtained from questionnaires indicate that the illness usually began within a 24-hr period after visiting a port city. The most common activities associated with diarrhea were drinking non-bottled water with ice (60%) and eating in restaurants (60%). This finding is in agreement with those of a previous study. Outbreaks have been attributed to food handlers in other settings. A high ETEC attack rate with a reported incidence of 1.1 per 1,000 was noted in this study, corroborating a previous study involving cruise ships in 1986–1993, in which the attack rate was 1.4 per 1,000. Enteric bacteria possess adhesive structures composed of protein surface antigens arranged into hair-like structures called fimbriae. These express a variety of CFAs depending on the strain of ETEC. Colonization factor antigens I, II, III, and IV have been shown to cause disease in challenge studies in humans. In this study, ETEC were isolated from 11 patients, of which only 1 had LT and 10 had ST. Heat-stable enterotoxin is the predominant ETEC toxin found in isolates from China and Australia. The fact that no CFA was identified in ETEC isolates from 3 patients suggests that there may be unique CFAs in this region that have not been previously reported. The differences in CFAs can affect the regional, i.e., Southeast Asian, efficacy of ETEC vaccines. A large-scale investigation would elucidate the relationships of CFAs, ETEC toxins, and diarrheal disease in this region. Parasitic diseases caused by G. lamblia and Cryptosporidium may be seasonally associated. Blastocystis hominis is not normally implicated with a disease, but a mild, persistent diarrhea can occur with heavy infection. All of the bacterial enteropathogens isolated were susceptible to norfloxacin, furadantin, and gentamicin, with the exception of 3 strains of Campylobacter spp., which were resistant to norfloxacin. The antimicrobial susceptibility data confirm the emergence of quinolone-resistant Campylobacter spp. in this region and can guide the choice of therapy for traveler’s diarrhea. This study applied standard bacteriologic methods and molecular-based viral diagnostics to detect pathogens associated with gastroenteritis on board a U.S. Navy ship. Data obtained indicate that NLV, bacteria, and parasitic pathogens are important causes of gastroenteritis in this military population. The results of this investigation support prior data documenting the common polymicrobial etiology of traveler’s diarrhea, but more importantly indicate that a substantial number of patients were positive for NLVs of the TNA prototype.

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