

# Homologous versus Heterologous Immune Responses to Norwalk-Like Viruses among Crew Members after Acute Gastroenteritis Outbreaks on 2 US Navy Vessels

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Host immune responses to human caliciviruses are difficult to study because of the lack of a clear definition of antigenic or serological types. This report describes antibody responses to several Norwalk-like viruses in large outbreaks of acute gastroenteritis on 2 US Navy ships. Enzyme immunoassays (EIAs) were used to measure antibody responses. To understand the antibody response to a homologous strain causing the outbreaks, the viral capsid gene of one isolate (C59) was expressed in baculovirus and included in the EIAs. Significantly greater seroresponses were detected in patients against the homologous strain than against the heterologous strains. Strains within genogroups reacted more strongly than did strains between genogroups. Significantly higher antibody titers against the outbreak strain were detected in acute serum samples from control subjects than in those from case patients. These results indicate that recombinant EIAs are useful for outbreak investigation and that the homologous antibody might be protective against reinfection.

“Norwalk-like viruses” (NLVs) are 1 of 2 genera—the NLVs genus and the “Sapporo-like viruses” (SLVs) genus—of human caliciviruses (HuCV) within the family *Caliciviridae* [1, 2]. NLVs also are known as small round-structured viruses. The genus contains many

members, and these viruses cause epidemic acute gastroenteritis in humans of all ages. Currently known NLVs are divided into 3 genogroups—GI, GII, and GIII—and each genogroup is further divided into genetic clusters. GI strains have been isolated from humans, GII strains have been isolated from humans and swine, and GIII strains have been isolated only from cattle [3, 4]. At least 15 genetic clusters of NLVs have been identified on the basis of capsid sequences [5]. New strains of HuCVs with unique genetic identities are continually being described.

Immunity to NLVs is poorly understood, because there is no cell culture or animal model available to measure neutralizing antibodies. Limited studies have demonstrated that volunteers who became ill after Norwalk virus (NV) challenge are usually resistant to rechallenge with the virus in a short period of time (6–14 weeks) but not to a different virus—for example, the Hawaii virus (HV) [6]. HV was later found to be genetically and antigenically distinct from NV [7–9]. However, other studies have shown that preexisting se-

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rum or jejunal fluid antibodies to NV did not provide protection against infection with NV [10]. Moreover, several studies repeatedly showed a reciprocal relationship between prechallenge anti-NV antibody levels and susceptibility to gastroenteritis among volunteers in the NV challenge studies [11–13].

The genetic and antigenic relationships of NLVs have been studied using recombinant EIAs since the molecular cloning of many HuCVs. By using EIAs with hyperimmune antiserum against recombinant capsid proteins of NLVs, individual genetic clusters of NLVs have been shown to be antigenically unique, with few cross-reactive antigenic epitopes among different genetic clusters [14, 15]. However, when the recombinant capsid antigens are used in EIAs to measure antibodies from patients involved in outbreaks of acute gastroenteritis, cross-reactive antibodies have been detected among different clusters [14]. Such cross-reactive antibodies could be reflected by a previous exposure to different antigenic types of NLVs that share antigenic epitopes. Whether these antibodies are neutralizing antibodies, and, if so, what the antigenic spectrum of these antibodies is, remains unknown.

In the present study, we characterized the antibody responses to several NLVs among crew members involved in large outbreaks of acute gastroenteritis on 2 US Navy ships. Both outbreaks occurred during deployments in the Pacific and Indian Oceans after a port visit in southeast Asia. Large numbers of cases of acute gastroenteritis were reported in a short period of time, and the ships continued their deployments without further stops before the outbreaks ended. Thus, each outbreak likely started with a single common source of infection without further exposure to new pathogens, providing an ideal situation for studying the host immune response to the etiologic pathogen(s). We also were able to clone, sequence, and express the viral capsid gene from 1 of the 2 outbreak strains, which allowed us to measure the homologous immune responses of crew members in this outbreak and compare them with heterologous immune responses to other strains of NLVs and to crew members in the other outbreak.

## POPULATION, MATERIALS, AND METHODS

**Study population.** Detailed information about the outbreaks and onboard investigations is given elsewhere [16]. In brief, in 1999, we initiated a project to study HuCV-associated acute gastroenteritis outbreaks in US Navy ships in the Pacific Ocean. In total, 11 large ships were enrolled. For the present study, 2 vessels, the USS Peleliu and the USS Constellation, were involved. The ships had complements of ~2800 and 4500 personnel, respectively. Both ships departed from San Diego in June 1999 and headed to the Persian Gulf, with short port visits in southeast Asia. The ships were monitored for the entire course of the 6-month deployment. Medical departments on

each ship were responsible for monitoring gastroenteritis among crew members, reporting outbreaks, and initiating the outbreak investigation. In the case of outbreaks, a laboratory team from the Navy Environmental and Preventive Medicine Unit 5 (San Diego) was sent to the ships for on-site investigation. A case patient was defined as a person with nausea, abdominal cramps, and spontaneous vomiting (excluding seasickness), with or without diarrhea. An occurrence of  $\geq 25$  cases of similar illness within a week was defined as an outbreak.

**Specimen collection.** Diarrheal stools and vomitus were collected from case patients, tested aboard the ship, and frozen for additional assays. Paired serum specimens were collected from each case patient for measuring seroresponses to HuCVs. Acute-phase serum samples were collected within 3 days of the onset of illness, and convalescent-phase serum samples were collected ~14 days after the onset of illness. Paired serum samples from asymptomatic crew members were also collected at 14-day intervals during the outbreaks.

**Detection of NLVs by reverse-transcriptase polymerase chain reaction (RT-PCR).** The procedures of extraction of viral RNA and RT-PCR have been described elsewhere [17]. A broadly reactive primer set (p289/p290 and p289A/p290A) that was designed on the basis of the sequence of the RNA-dependent RNA polymerase region of the genome was used in the RT-PCR [18]. These primer pairs are expected to produce a 319-bp RT-PCR product for NLVs and a 331-bp product for SLVs. For amplification of the C59 capsid gene, an oligo-dT and a primer (p301) designed on the basis of the 5' end of the capsid gene were used [19].

**Cloning and sequencing of RT-PCR products.** RT-PCR products were cloned into the pGEM-T vector (Promega). The insert DNA of each clone was sequenced using the universal forward and reverse primers by the chain-termination method on an automated sequencer (ALFexpress; Pharmacia). For sequencing the 2.4-kb C59 cDNA, deletion clones from both orientations of the cDNA fragment were prepared (Kilo-Sequencing Deletion Kit; TaKaRa Biomedicals). Each clone was sequenced at least twice.

**Sequence and phylogenetic analysis.** The genetic identity of the isolates was determined by comparisons of the sequences with those in GenBank and our local sequence databases. Pairwise alignments were performed with the OMIGA 2.0 software package (Oxford Molecular). A dendrogram was constructed by the Molecular Evolutionary Genetics Analysis program (MEGA, version 2.1) [20], using the unweighted pair group method with arithmetic mean method with Jukes-Cantor correction for evolutionary rate. Reference strains used in the analysis were as follows: GI strains—Chiba/87/JP (AB042808), Desert Shield/90/Saudi Arabia (U04469), Norwalk/68/US (M87661), Potsdam/00/DE (AF439267), Southampton/91/UK (L07418), and Virginia/115/98/US (VA115 and AY038598); and GII strains—

Hawaii/71/US (U07611), Lordsdale/93/UK (X86557), Mexico/89/MX (U22498), MOH/99/HUN (AF397156), and Snow Mountain/76/US (U70059).

**Baculovirus expression of C59 capsid protein.** The procedures for generation of C59-baculovirus recombinant proteins were similar to those described elsewhere for expression of other NLV capsid proteins [15, 19]. Recombinant baculovirus clones with high expression of the capsid protein were selected and used for the production of the proteins by infecting the viruses in the Sf9 or H5 insect cells. The C59 recombinant capsid proteins were partially purified by discontinuous sucrose gradient centrifugation. The peak fractions containing the viral capsid protein with a molecular weight of ~60 kDa were pooled and characterized by Western blot analyses using serum samples from the outbreak (ECL Western blotting detection reagents; Amersham Pharmacia Biotech).

**Detection of anti-HuCV antibodies by EIAs.** Antibody detection EIAs derived from 5 recombinant (r) NLV capsid antigens were used, including NV and C59, the outbreak strain from the Constellation, in GI, and Mexico virus (MxV), HV, and Grimsby virus (GrV) in GII. All recombinant capsid proteins self-assembled into virus-like particle (VLPs), except C59. Purified recombinant capsid proteins were used to coat microtiter plates (100  $\mu$ L/well) at a concentration of 0.25  $\mu$ g/mL in 0.01 M PBS (pH, 7.4) overnight at 4°C. The antigen-coated plates were blocked with 5% Blotto (Carnation nonfat milk in PBS) for 1 h at 37°C. The plates were washed twice with 0.05% Tween 20–PBS, and 2-fold serial dilutions in 1% Blotto-PBS of acute- and convalescent-phase serum samples were added (100  $\mu$ L/well). After incubation for 2 h at 37°C, the plates were washed 5 times, horseradish peroxidase–conjugated goat anti-

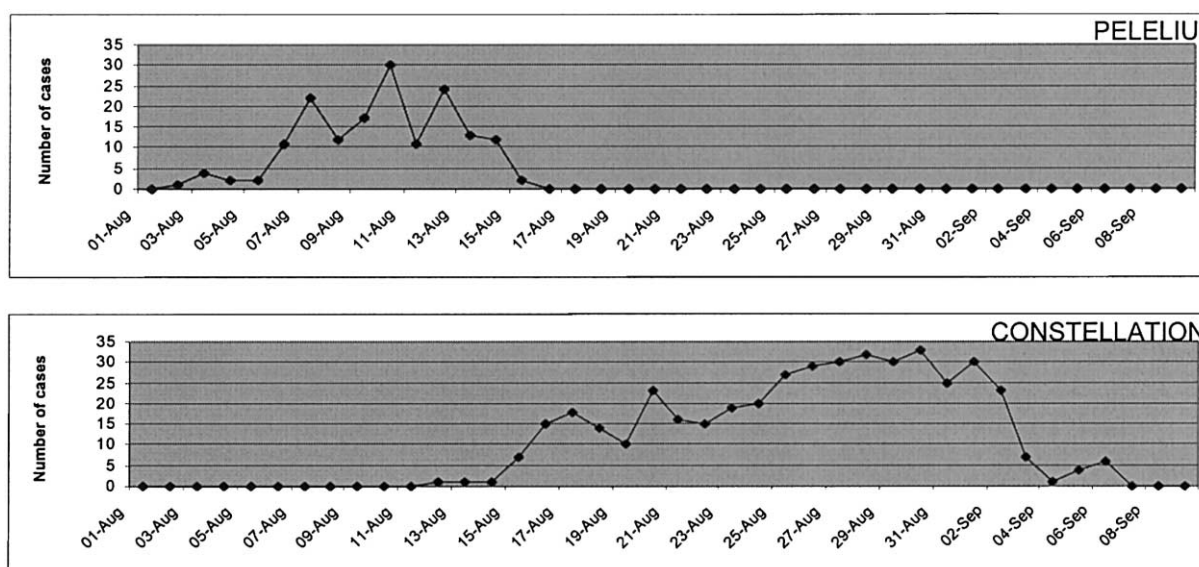
human immunoglobulin (IgA, IgG, and IgM, 1:5000 dilution in 1% Blotto-PBS [100  $\mu$ L/well]; Cappel Organon Teknika) was added, and the plates were incubated for 2 h at 37°C. After washing 5 times, TMB (3,3', 5,5'-tetramethyl-benzidine) substrate (100  $\mu$ L/well; Kirkegaard & Perry Laboratories) was added, and the plates were incubated for 10 min at room temperature. The color development was stopped by adding 100  $\mu$ L of a 1 M  $H_3PO_4$  solution to each well. Optical density was read by a Tecan Spectra II microtiter plate reader at 450 nm. Seroreponse against a specific antigen was measured as an increase of at least 4-fold between the acute- and convalescent-phase serum titer.

**Statistical analysis.** The  $\chi^2$  or Fisher's exact test was used to compare seroresponse and antibody levels between case patients and control subjects from the Constellation. Because antibody was measured at different dilution levels, the comparison between groups was made at each dilution level. To address the related multiple testing problems, statistical significance was determined at  $P < .01$ .

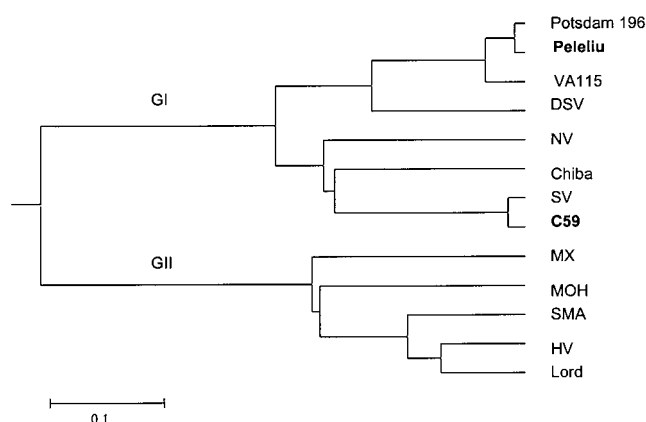
**Assays for other pathogens.** Stools were assayed for *Salmonella*, *Campylobacter*, and *Shigella* species by plate culturing. Methiolate-iodine-formalin wet-stool preps were observed for parasites. None of these tests were positive. No other viral pathogens were studied.

## RESULTS

**Outbreaks.** The ships left San Diego 1 week apart and sailed independent courses before making port visits in southeast Asia. The Peleliu made late July–early August port visits to Singapore and Phuket, Thailand, where symptoms began. The



**Figure 1.** Distribution of cases during outbreaks of acute gastroenteritis on 2 US Navy vessels



**Figure 2.** Phylogenetic relationship of the outbreak strains (*bold*) and reference strains, based on alignments of a 274-bp region of the RNA polymerase. The dendrogram was constructed by the Molecular Evolutionary Genetics Analysis program (version 2.1) [20], using the unweighted pair group method with arithmetic mean method with Jukes-Cantor correction for evolutionary rate. Chiba, Chiba/87/JP (AB042808); DSV, Desert Shield/90/Saudi Arabia; HV, Hawaii/71/US (U07611); Lord, Lordsdale/93/UK (X86557); MX, Mexico/89/MX; MOH, MOH/99/HUN; NV, Norwalk/68/US; Potsdam 196, Potsdam/00/DE; SMA, Snow Mountain/76/US; SV, Southampton/91/UK; VA 115 Virginia/115/98/US.

Constellation made August port visits to Singapore and Port Kelang, Malaysia, where the outbreak began. The Peleliu outbreak consisted of 162 cases (attack rate, 5.7%), and the Constellation outbreak consisted of 425 cases (attack rate, 9.5%) (figure 1) [16].

**Detection of NLVs in stool.** Two of 5 Peleliu and none of 27 Constellation stool samples gave positive RT-PCR results with p289/p290. However, 4 of 5 Peleliu and 9 of 27 Constellation stool samples resulted in amplicons of the expected size when p289A/p290A was used.

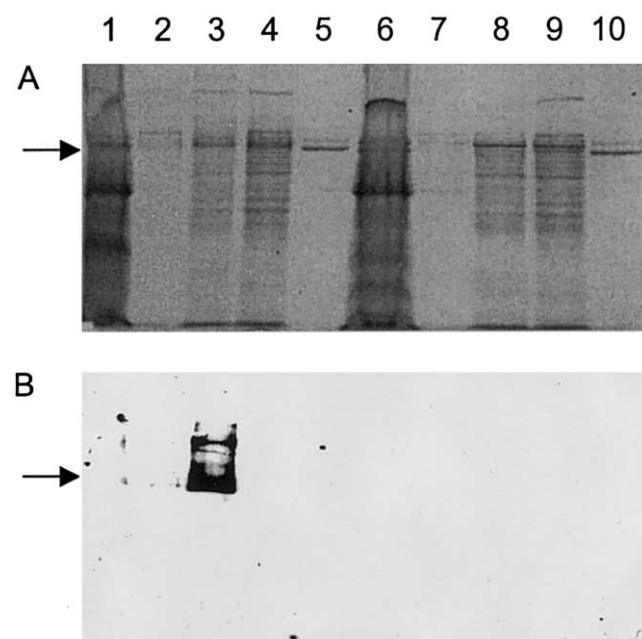
**Sequence analysis.** One amplicon from the Peleliu and 9 amplicons from the Constellation were cloned and sequenced. All 10 amplicons revealed typical HuCV sequences, and all 9 amplicons from the Constellation resulted in identical sequences, which suggests that a single HuCV strain likely was involved in the outbreak. The outbreak strain from the Peleliu shared 80% nt identity with Desert Shield virus (DSV), and the isolate from the Constellation (C59) had 97% nt identity with Southampton virus (SV) in the RNA-dependent RNA polymerase region as the most closely related cluster representative strains (figure 2).

A 2.4-kb amplicon was obtained from one patient (C59) on the Constellation by RT-PCR with p301 and an oligo-dT. This cDNA revealed a typical NLV genomic structure: a complete capsid gene (open-reading frame [ORF] 2; 1632 nt), a small ORF (ORF3; 633 nt) that encodes a minor structural protein [21], and an untranslated region (81 nt) between ORF3 and the poly-A tail. Analysis of the C59 capsid sequence revealed

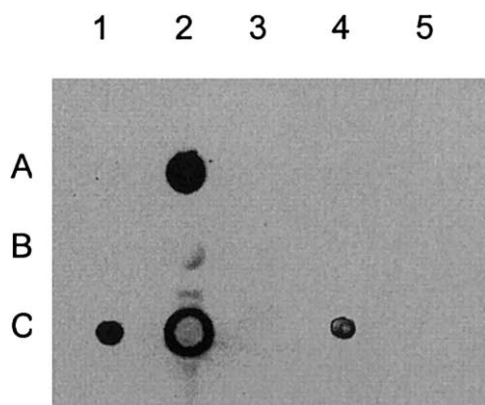
a 96% nt and 98% aa identity with SV. The C59 and Peleliu partial RNA-dependent RNA polymerase sequences were submitted to GenBank under accession numbers AF435808 and AY149297, respectively. The C59 capsid sequence was submitted under accession number AF435807.

**Expression of C59 capsid protein in baculovirus.** A unique protein with an apparent molecular weight of 60 kDa was detected in the insect cell culture infected with the C59 recombinant baculovirus (data not shown). Sucrose gradient separation of the proteins from the insect cell culture showed no VLP formation, because the proteins failed to migrate into the lower portion of the gradient that is usually found for VLP-forming proteins (data not shown). However, the rC59 capsid protein reacted with convalescent-phase serum samples of case patients from the outbreak in Western blot and dot blot analyses (figures 3 and 4).

**Seroresponses against rNLV capsid antigens.** Of the 29 acute- and convalescent-phase serum sample pairs collected from the Peleliu, 16 (55%), 18 (62%), 10 (34%), 14 (48%),



**Figure 3.** Western blot immunostaining of baculovirus-expressed C59 capsid protein. *A*, Coomassie blue-stained 10% SDS-PAGE. Lanes 1 and 6, Wild-type baculovirus-infected Sf9 cell lysate; lanes 2 and 7, sucrose gradient-purified Norwalk virus (NV) virus-like particles (VLPs); lanes 3 and 8, sucrose gradient-purified C59 recombinant capsid proteins; lanes 4 and 9, sucrose gradient-purified VA115 recombinant capsid proteins; and lanes 5 and 10, sucrose gradient-purified Hawaii virus VLPs. The samples were boiled (lanes 6–10) or not (lanes 1–5) before loading into the gel in a loading buffer that contained SDS and 2-mercaptoethanol. *B*, Western blot analysis of a duplicate of panel *A*. A convalescent-phase serum sample from a patient from the Constellation outbreak, who had a high seroresponse (32-fold) in the NV EIA, was used in the immunostaining. The arrow indicates the position of the 60-kDa capsid proteins.



**Figure 4.** Dot blot immunostaining of baculovirus-expressed C59 capsid protein. Lane 1, Sucrose gradient-purified Norwalk virus virus-like particles (VLPs); lane 2, sucrose gradient-purified C59 recombinant capsid proteins; lane 3, sucrose gradient-purified VA115 recombinant capsid proteins; lane 4, sucrose gradient-purified Hawaii virus VLP; lane 5, wild-type baculovirus-infected Sf9 cell lysate. The samples were dotted onto the membrane in a buffer that contained SDS and 2-mercaptoethanol (row A) or in PBS with (row B) or without (row C) boiling. The same convalescent-phase serum sample used in figure 3B was used in the immunostaining.

and 10 (34%) showed at least 4-fold increases in titers against rC59, rNV, rMxV, rHV, and rGrV antigens, respectively. Eighty-nine (87%), 66 (65%), 27 (26%), 31 (30%), and 25 (25%) of 102 serum pairs collected from the Constellation responded to rC59, rNV, rMxV, rHV, and rGrV antigens, respectively. From the 28 asymptomatic crew members on the Constellation, 2 (7%) and 1 (3%) had low-level (4–8-fold) seroresponses to rC59 and rNV, but none had seroresponses to rMxV, rHV, or rGrV (table 1).

**Antibody titers in acute-phase serum samples.** A wide range of antibody titers against the 5 rNLV capsid antigens were detected in patients and control subjects on the Constellation (table 2). At low dilutions (1:400–1:3200) of the serum samples, significant differences in the numbers of control subjects and case patients with certain antibody titers were observed for only 2 antigens (NV and HV) and at only 2 levels of antibody dilution (1:400 and 1:3200, respectively). However, at high dilutions of the serum samples, significantly higher numbers of control subjects with antibodies against rC59 at all levels of antibody dilutions >1:6400 but not against other strains (rNV, rMxV, rHV, and rGrV), except for rHV at dilution 1:6400, were observed than of case patients (table 2). This result indicates that a high level of preexisting antibody against a homologous strain may be protective against reinfection with the same strain.

## DISCUSSION

The present study is a part of a larger study to investigate outbreaks of acute gastroenteritis on US Navy ships. Each of these 2 outbreaks started after a port visit in southeast Asia

and lasted for weeks. The distribution of cases during the outbreaks on both ships was characteristic of person-to-person transmission, which likely started with index case patients who contracted the diseases during the port visit (figure 1). Crowded conditions on the ships enhanced the spread of the disease by either direct or indirect contact among crew members.

The outbreaks likely were caused by NLVs, because stool specimens from patients in both outbreaks were positive for NLVs by RT-PCR. Sequence analysis of the RT-PCR products showed that both outbreak strains belonged to GI but were in different genetic clusters, the DSV and SV clusters (figure 2). These 2 clusters are found circulating in other parts of the world [22–26]. Additional indicators that NLVs caused these outbreaks include the significantly higher antibody responses to NLVs, particularly to GI NLVs, among sick crew members, compared with the nonsick control subjects, and the high attack rates of diseases among embarked crew members in certain Marine units [16].

We successfully cloned and expressed the capsid protein of one outbreak strain (C59) in baculovirus. Although the expressed C59 capsid protein did not form VLPs, it was antigenically competent, providing useful tools for the outbreak investigations. In particular, we studied the antibody responses to recombinant capsid antigens representing additional genetic clusters in different NLV genogroups, which allowed us to assess the homologous versus heterologous immune responses among the crew members. We also compared antibody levels in the serum samples collected at acute and convalescent phases of illness and between sick and nonsick crew members, which allowed us to address type-specific immune responses and the role of preexisting antibody against infection.

Our data showed that a broad spectrum of antibody responses was observed against all 5 NLVs studied. Although both outbreaks were caused by GI viruses, low levels of responses to GII viruses also were detected in both outbreaks. Fewer

**Table 1.** Seroresponses against 5 recombinant (r) human calicivirus (HuCV) antigens among subjects from the Peleliu ( $n = 29$  case patients) and the Constellation ( $n = 102$  case patients and  $n = 28$  control subjects).

Subject groups	Antigen				
	rC59	rNV	rMxV	rHV	rGrV
Peleliu	16 (55)	18 (62)	10 (34)	14 (48)	10 (34)
Constellation	89 (87) <sup>a</sup>	66 (65)	27 (26)	14 (48)	25 (25)
Control	2 (7)	1 (3)	0	0	0

**NOTE.** Data are no. (%) of crew members who had seroresponses to the specified antigens. Seroresponses were measured against rHuCV antigens on the basis of  $\geq 4$ -fold increases of antibody titers in the convalescent- vs. acute-phase serum samples. C59, Constellation; GrV, Grimsby virus; HV, Hawaii virus; MxV, Mexico virus; NV, Norwalk virus.

<sup>a</sup>  $P < .05$  vs. rNV, and  $P < .001$  vs. rMxV, rHV, and rGrV (McNemar  $\chi^2$  test for both).

**Table 2. Preexisting Norwalk-like virus antibody titers in acute-phase serum samples from 102 case patients and 28 control subjects from the Constellation.**

Dilution	Antigen, subject group									
	C59		NV		HV		MxV		GrV	
	Case	Control	Case	Control	Case	Control	Case	Control	Case	Control
≥400	84 (82)	19 (68)	35 (34)	16 (57) <sup>a</sup>	80 (78)	24 (86)	80 (78)	24 (86)	64 (63)	14 (50)
≥800	70 (69)	16 (57)	21 (20)	10 (36)	63 (62)	21 (20)	63 (62)	17 (61)	47 (46)	11 (39)
≥1600	52 (51)	14 (50)	11 (11)	5 (18)	38 (37)	13 (46)	44 (43)	11 (39)	23 (22)	7 (25)
≥3200	28 (27)	10 (36)	6 (5)	3 (11)	16 (15)	11 (39) <sup>a</sup>	27 (26)	8 (28)	12 (12)	4 (14)
≥6400	9 (9)	7 (25) <sup>a</sup>	1 (1)	2 (7)	5 (5)	6 (21) <sup>a</sup>	10 (10)	4 (14)	5 (5)	1 (3.5)
≥12,800	2 (2)	5 (18) <sup>a</sup>	1 (1)	1 (3.5)	2 (2)	3 (11)	4 (4)	0	1 (1)	1 (3.5)
≥25,600	2 (2)	5 (18) <sup>a</sup>	0	0	0	1 (3.5)	0	0	0	0
≥51,200	1 (1)	3 (11) <sup>a</sup>	0	0	0	1 (3.5)	0	0	0	0

**NOTE.** Data are no. (%) of crew members who had antibody against the antigens. C59, Constellation; GrV, Grimsby virus; HV, Hawaii virus; MxV, Mexico virus; NV, Norwalk virus.

<sup>a</sup>  $\alpha < .05$ , case patients vs. control subjects (Fisher's exact test).

patients had antibody responses to GII than GI NLVs. The antibody responses correlated with the genetic relationships among the strains studied—for example, higher antibody responses to strains within genetic clusters, rather than between genetic clusters, were detected. These results are consistent with previous reports describing different formats of assays with different recombinant capsid antigens [27]. Our conclusion is that the antigen-coating EIAs are useful for outbreak investigation if recombinant capsid antigens representing proper antigenic types of NLVs are used.

When antibody titers against C59 in the acute-phase serum samples of case patients and control subjects from the Constellation outbreak were analyzed, significantly higher antibody titers were detected in control subjects than in patients at a titer of  $\geq 1:6400$  (table 2). Although the number of subjects with responses at each titer was small, the trend revealed a significant association between preexisting antibody and protection against NLV infection. This result challenges the observation that preexisting antibody is not protective against NV infection, which was made in the 1970s and 1980s in volunteer studies. Our explanation is that the preexisting antibody in those volunteers could have been induced by different NLVs but not by NV. NLVs recently have been found that recognize human histo-blood group antigens as receptors for infection [28], and different strains of NLVs recognize different receptors defined by Lewis, secretor, and ABO types [29]. Thus, disease in volunteers who had higher antibody levels but were more susceptible to NV than volunteers who did not have the antibody could have been due to a cross-reactive antibody induced by antigenically related strains that share common receptors, but not common neutralization epitopes, with NV.

The rC59 capsid protein was characterized in Western and dot blot analyses. Homologous convalescent-phase serum samples from patients in the outbreak recognized only nondena-

tured C59, not VA115, NV, and HV recombinant capsid proteins or Sf9 cell proteins in Western blots (figure 3). However, the same serum samples gave high titers (1:12,800 dilution of the serum samples) in the NV EIA. To investigate this controversy, dot blot experiments were used in which proteins were dotted in conditions that mimicked those in EIA (in PBS) and Western blot (in  $2\times$  loading buffer) analyses. Of interest, NV and HV were recognized by the outbreak serum samples in the EIA conditions but not in the Western blot conditions (either denatured or nondenatured conditions; figure 4). The loading buffer used in the gel electrophoresis before the Western blot analysis contained SDS and 2-mercaptoethanol, which may significantly denature the capsid protein structure. Therefore, we concluded that both continuous (linear) and discontinuous (conformational) antigenic epitopes exist in C59 but that only conformational epitopes are shared among C59, NV, and HV.

The 2 ships described in the present study were the first 2 of 11 Pacific Fleet ships recruited in our study. Since then, 3 other study ships have experienced outbreaks. The investigations of these outbreaks are ongoing, and preliminary results have shown that NLVs were the major causes of these outbreaks. Similar large outbreaks of gastroenteritis caused by NLVs in cruise ships also have been reported elsewhere [30–34]. In summary, NLVs are an important cause of large outbreaks of acute gastroenteritis in such settings. More studies to understand the nature of the virus transmission in these settings and to develop strategies to control and prevent the transmission are necessary.

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