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Research and Practice

Norovirus Epidemiology and Duration of Shedding in Michigan, 2007-2008

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Abstract

Background: In the United States, an estimated 23 million cases of norovirus (NoV) are reported each year, and although mortality is low, the morbidity and economic impact are substantial.

Methods: RT-PCR and sequencing were used for identification of NoV genotypes obtained from outbreak and sporadic cases. RT Quant PCR was used to determine the viral load in fecal specimens. In order to rule out bacterial infection as the cause for acute gastroenteritis (AGE), bacterial culture for *Salmonella, E.coli O157, Shigella, Campylobacter* and *Clostridium difficile* was performed by standard laboratory procedures. The duration of NV shedding was investigated with longitudinal sampling in the sporadic cases and an evaluation of the association between viral load and days since clinical onset in the outbreak-associated cases.

Results: We describe the epidemiology and strain identification for NoV circulating in Michigan during 2007-8 in concurrent sporadic and outbreak-associated cases. In 2007-8, 138 norovirus outbreaks (3,437 cases) were reported to the MDCH. Among the 47 outbreak specimens sequenced, GI was identified in 14 (29.8%) and GII in 33 (70.2%). The predominant type was GII.4, found in 23 of the 33 (69.6%) GII specimens. The statistical analysis of outbreak-associated cases showed that neither NoV type nor number of days post-onset were associated with NoV log concentration. Among the sporadic cases, the repeated measures analysis of variance showed that NoV type (I or II)

was not associated with log titer (P = 0.90), but that the number of weeks post-onset was statistically associated with declining log titer at p = 0.0005.

Conclusion: We found no predominant strain difference between concurrent sporadic and outbreak-associated cases. Prevalent strains of NoV were shed in high concentration for at least two weeks past disease onset, suggesting that current public health recommendations for 2-3 days home isolation following clinical recovery may need to be lengthened.

Keywords: public health, norovirus, epidemiology

Background

Noroviruses (NoVs) are the leading cause of acute gastroenteritis (AGE) in adults, causing numerous outbreaks worldwide (Frankhauser et al, 2002; Siebenga et al, 2009; Patel et al, 2009). In the United States, an estimated 23 million cases of NoV occur annually. Although mortality is low, the morbidity and economic impact are substantial (Mead et al, 1999; Marks et al, 2003). Cases are most commonly attributed to contaminated food and/or drinking water, exposure to aerosolized feces or vomitus, direct or indirect oral contact with contaminated surfaces and direct fecal-oral transmission (Harrington et al, 2004).

Noroviruses are a group of non-cultivable, genetically diverse single-stranded RNA viruses belonging to the family *Caliciviridae*. They are classified into five genogroups based on the sequences of the RNA polymerase and capsid region of the genome. The genomes have three open reading frames (ORF). ORF1 encodes the nonstructural polyprotein, while ORF2 encodes the capsid protein and ORF3 encodes a putative minor structural protein (Symes et al, 2007). Genogroups I, II, and IV are associated with infections in humans. Currently, 32 genetic clusters have been identified (8 from GI; 19 from GII; 2 from GIII; 2 from GIV and one from GV), demonstrating a high degree of genomic diversity among NV (Ando, Noel & Frankhauser, 2000; Green et al, 2000; Wang et al, 2006; Martella et al, 2007).

In the U.S. approximately 50% of outbreak cases of waterborne and food-borne enteric diseases are due to NoV, but there is less information about sporadic cases of NoV

related AGE. (CDC, 2001, 2003; Monroe, 2005). Information regarding sporadic NoV is lacking due to the difficulty in obtaining fecal specimens from patients sufficiently early in the clinical course of their illness, and also because specimens for laboratory analysis are rarely obtained in the absence of an outbreak. In Michigan, the state health department laboratory normally only accepts clinical specimens for NoV diagnosis from outbreaks but not from sporadic cases.

It may be possible that different norovirus strains predominate among the sporadic NoV cases, and that patterns of disease progression and viral shedding may be very different among these sporadic cases compared with cases from recognized outbreaks. Alternatively, sporadic NoV cases may result from low dose exposures to the same strains responsible for NoV outbreaks. Perhaps persons who have become ill by exposure to low doses of infectious particles may have a very different shedding pattern from individuals whose immune systems have been affected by massive doses of infectious particles as occurs in some outbreaks.

Most NoV outbreaks subside within 5-6 days, and the duration of symptoms in individuals is usually 48-72 hours (CDC, 2006). Health departments generally recommend that food handlers and health care providers stay at home for 2-3 days after clinical recovery. However, a recent report of an intentional NoV challenge showed that some people shed virus for as long as 2 or 3 weeks after clinical recovery, but it is unknown what percentage of naturally infected people will shed for these extended periods (Okhuysen et al, 1995; CDC, 2005; Atmar et al, 2008; Siebenga et al, 2008;

Kirkwood & Steitberg, 2008). Prolonged shedding of virus could increase the spread of NoV infection, thus it is possible that public health recommendations should be amended to increase the duration of home isolation of convalescing cases to reflect this prolonged shedding period.

Norovirus is one of the only enteric pathogen for which human challenge studies are ethically possible. While very helpful in elucidating NoV pathogenesis, these challenge studies were done with NoV from stored fecal specimens rather than from currently circulating strains. Challenge strains were derived from specimens collected before 1994 and from 1993-1996, respectively (Atmar et al, 2008; Phillips et al, 2009). Challenge studies may differ from naturally acquired infection in the challenge dose, method of transmission and the age and health status of the volunteers; challenge studies need to exclude volunteers with any underlying illnesses. It needs to be determined to what extent evidence of extended duration NoV shedding obtained from challenge studies can be generalized to naturally acquired infections.

Our objective in this study was to describe the epidemiology and strain identification for NoV circulating in Michigan during 2007-8 in concurrent sporadic and outbreak-associated NoV cases. We also sought to evaluate the duration of convalescent NoV shedding.

Materials and Methods

Outbreak Surveillance for NV in Michigan: The policy of the Michigan Department of Community Health (MDCH) is to conduct outbreak-associated NoV testing on fecal specimens from no more than 6 people per outbreak. Specimens are routinely classified by PCR as negative or positive for viral RNA, and strains are typically classified only as GI or GII. In 2008, a descriptive epidemiologic analysis was conducted for each outbreak setting for which a sufficient number of specimens had been obtained. Forty-seven NoV-positive specimens from outbreak-associated cases were selected for sequencing and quantitative assessment. Selection of specimens was based on the availability of individual onset dates and a sufficient quantity of specimen available for further laboratory testing. Specimens came from 14 outbreaks in 12 Michigan counties during 2007-08. These 47 specimens were analyzed using the quantitative NoV PCR assay described below.

Sporadic AGE Cases: Fecal specimens were obtained from 18 volunteers with AGE whose symptoms were compatible with norovirus infection and who were not part of any recognizable outbreak. Volunteers included some university students, but most were referred to our study by participating local outpatient clinics who assisted us in distributing sampling supplies to persons they suspected of having NV infection. In addition to not being part of an outbreak of more than 2 associated cases, our sporadic cases were over 18 years of age, had no more than a low-grade fever, did not have bloody diarrhea, and did not suffer from recurring episodes of gastroenteritis. Onset fecal specimens were verified as negative for *Salmonella, E.coli O157, Shigella,*

Campylobacter and *Clostridium difficile* by standard MDCH laboratory procedures (MacFaddin, 1980; Bopp, Brenner & Fields, 2003; Nachamkin, 2003). Food service and health care workers were not included in this study because identification of a reportable enteric pathogen may have required them to be excluded from their workplace pending negative stool culture results. Study subjects were from local primary care clinics (2), community volunteers (15), and a nursing home (1). Stool specimens were requested at the onset of vomiting or diarrhea, 1 week after onset and 2 weeks after onset. Cases were paid an honorarium for participation, as approved by the IRB Committees at both MSU and MDCH.

The sporadic cases were screened to verify the absence of *Salmonella, Campylobacter, Clostridium difficile, Shigella, and E.coli O157*. The initial specimens were submitted in Cary-Blair transport media for bacterial culture. Stool samples were inoculated onto MacConkey (MAC), MacConkey Sorbitol, MacConkey Sorbitol with cefizime and tellurite (CT-SMAC), and Hektoen Enteric (HEK) plates and a tetrathionate enrichment broth and incubated aerobically at 35˚C for 48 hrs. To demonstrate *Clostridium difficile*, an aliquot of stool was plated directly to a cycloserine cefoxitin fructose agar plate (CCFA) and incubated anerobically. An additional aliquot was treated with ethanol for one hour at room temperature and then plated on an anerobic blood agar plate. The plates were held for 96 hours before being discarded as negative. A *Campylobacter* blood agar plate was also inoculated and incubated microaerophilically at 42˚C for 72 hrs. The tetrathionate broth was subcultured to a MAC and HEK after 24 hours incubation. All plates were read at 24-hour intervals, but held 48 hours before being reported as negative. Suspect organisms were identified using Gram's stain, colony morphology and

conventional biochemical tests (MacFaddin, 1980; Bopp, Brenner & Fields, 2003; Nachamkin, 2003).

RNA extraction, RT PCR, sequencing and RT Quant PCR.

Stool specimens from both sporadic and outbreak-associated cases were suspended in sterile water (10 percent weight/volume). Specimens were centrifuged at 13000x*g* and the resulting supernatant was filtered through a 0.45 µM filter. NV RNA was extracted from 200 µl of the filtrate using QIAGEN QIAmp viral RNA mini kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. 5 µl of the eluted viral RNA suspension was used for RT PCR.

RT-PCR was performed using established primers and probes Cog 1F, Cog 1R, Ring 1A, Ring 1B, Cog 2F, Cog 2R and Ring 2 for region B as described previously (Kageyama et al, 2003).

Four primers that target the 3'-end of the RNA polymerase gene (region B) were used to detect strains of GI and GII genogroups. Primers, MON 432, MON 434 for detecting GI strains and MON 431, MON 433 for GII strains, yielding a 213 base pair amplicon (Anderson et al, 2001).

To analyze sequence information the biometrics applied MATH system was used. Sequences were blasted into a local database to provide similarities and dendrograms.

The RT Quant PCR was performed using the Roche LightCycler 1.5 with 4.0 analysis software with the following modifications of the procedure described above: GI probes (Ring1a and Ring1b) were used at 400nm each and the volume of H_2O in reaction mix was adjusted to allow for 5 µl of RNA (Trujillo et al, 2006). Standard curves for NV

quantification were generated using serial dilutions of GI and/or GII norovirus transcripts obtained from CDC and representing ORF1, 2 and 3 of the 3' region of G1.4 and GII.4 respectively.

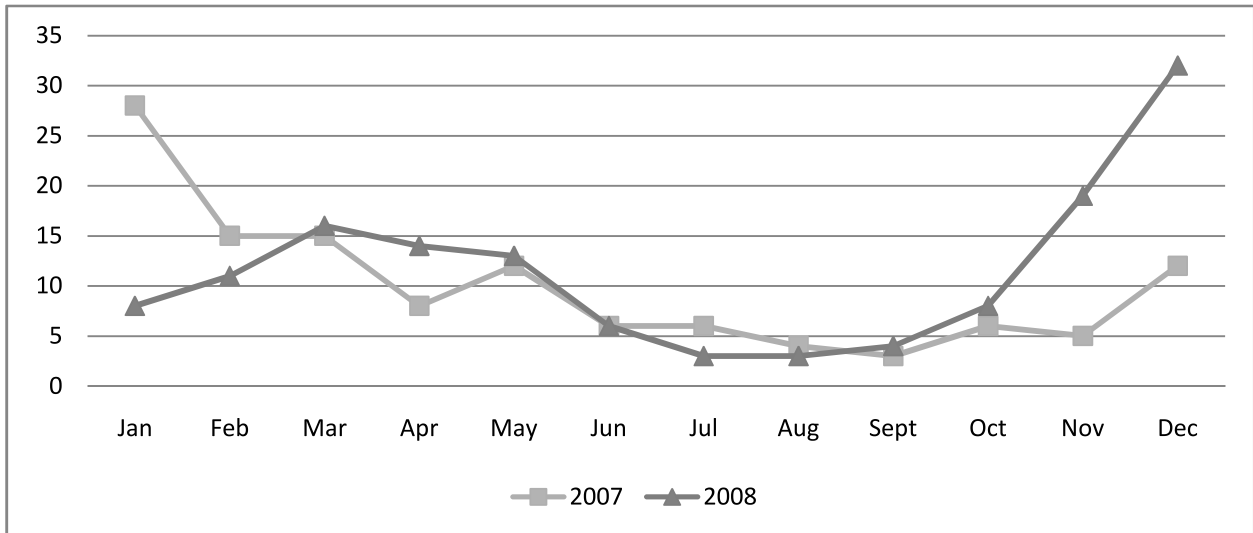
Statistical analysis: The outbreak-associated NoV concentrations were analyzed in a general linear model (SAS 9.1) with log concentration as the dependent variable and days post-onset and NoV group (I or II) as independent variables. The sporadic case data were analyzed as a mixed model with log titer as the repeated dependent variable and NoV type as the sole independent variable.

Results

Michigan NV Surveillance: In 2008, 138 norovirus outbreaks (3,437 cases) were reported to the MDCH (Figure 1). Of these, 30 were confirmed by laboratory analysis of 2 or more collected specimens; 26 (87%) outbreaks were related to GII and 4 (13%) outbreaks were related to GI. The remaining 108 outbreaks were judged to have been NoV based on clinical and epidemiologic presentation. Most outbreaks (n=63) were associated with healthcare services, followed by restaurants (26), schools (25) and other settings (24).

Figure 1

Reported Michigan Norovirus Outbreaks, 2007-2008



Outbreak-associated Cases:

Among the 47 outbreak specimens sequenced, GI was identified in 14 (29.8%) and GII in 33 (70.2%) (Table1). The predominant type was GII.4, found in 23 of the 33 GII specimens (69.6%). The statistical analysis showed that neither NV type (I or II) nor days post-onset were associated with RNA log concentration, at $p > 0.8$ in both instances.

Table 1

Sequencing and Quantitative Assessment of Selected Michigan Norovirus Outbreak-associated Cases

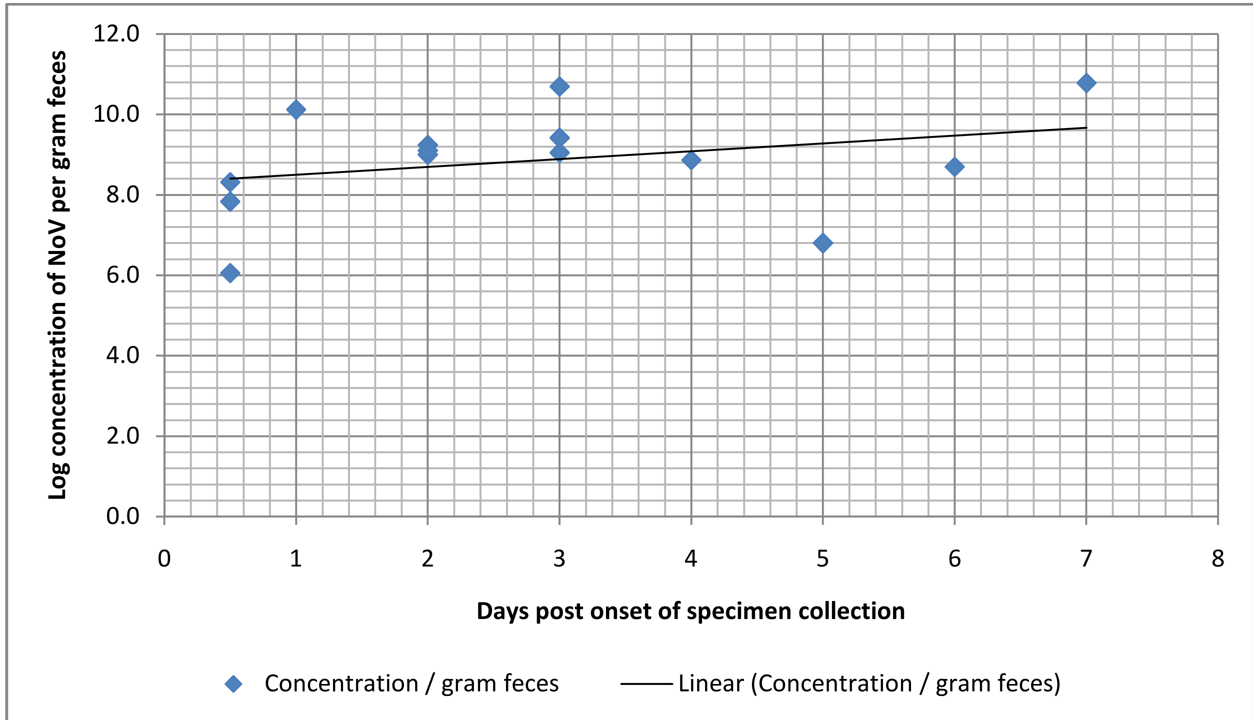
County	Date	Genotype	Specimen Number	Concentration / gram feces	Days Post Onset	Log Concentration / gram feces
Eaton	10/27/07	SaitamaT13-JPN_GI.2	O-1A	2.07×10^{8}	0.5	8.3
			O-1B	1.12×10^{9}	3	9.0
			O-1C	1.72×10^{9}	2	9.2
			O-1D	6.81×10^{7}	0.5	7.8
			O-1E	1.14×10^{6}	0.5	6.1
			O-1F	7.29×10^{8}	4	8.9
Berrien	11/13/07	Lit-USA94_GI.3B	O-2A	9.94×10^{8}	2	9.0
			O-2B	6.02×10^{10}	7	10.8
			O-2C	4.97×10^{8}	6	8.7
			O-2D	2.61×10^{9}	3	9.4
			O-2E	4.94×10^{10}	3	10.7
Jackson	12/13/07	SaitamaT13-JPN_GI.2	O-3A	1.31×10^{10}	1	10.1
Calhoun	12/18/07	FMHill-USA_GII.4	O-4A	9.76×10^{7}	4	8.0
			O-4B	1.58×10^{8}	5	8.2
			O-4C	1.14×10^{8}	4	8.1
Kalamazoo	12/20/07	Minerva2006B (GII.4)	O-5A	3.76×10^{8}	1	8.6
			O-5B	3.17×10^{8}	2	8.5
			O-5C	3.67×10^{10}	1	10.6
Genesee	12/21/07	SaitamaT13-JPN_GI.2	O-6A	1.33×10^{9}	2	9.1
			O-6B	6.27×10^{6}	5	6.8
Kent	1/11/08	FMHill-USA_GII.4	O-7A	7.77×10^{8}	6	8.9
			O-7B	1.83×10^{9}	6	9.3
			O-7C	2.08×10^{9}	6	9.3
			O-7D	4.70×10^{8}	5	8.7
Genesee	2/15/08	GN273-USA94_GII.7	O-8A	2.70×10^{7}	1	7.4
			O-8B*	1.53×10^{7}	3	7.2
			O-8C	6.57×10^{8}	1	8.8
Mason	2/19/08	FMHill-USA_GII.4	O-9A	1.84×10^{9}	5	9.3
			O-9B	6.33×10^{11}	5	11.8
			O-9C	2.33×10^{8}	1	8.4
			O-9D	2.37×10^{8}	2	8.4

			O-9E	7.53 x 10^9	4	9.9
			O-9F	1.50 x 10^8	4	8.2
Genesee	3/7/08	SU1-JPN_GII.12	O-10A	8.67 x 10^9	0.5	9.9
Menominee	3/19/08	FMHill-USA_GII.4	O-11A	6.45 x 10^8	3	8.8
			O-11B	1.71 x 10^9	3	9.2
Mecosta (DHD#10)	3/20/08	FMHill-USA_GII.4	O-12A	6.69 x 10^{10}	1	10.8
			O-12B	3.63 x 10^9	0.5	9.6
			O-12C	3.19 x 10^8	1	8.5
Wayne	4/1/08	IDA-USA96_GII.8B	O-13A	5.33 x 10^7	8	7.7
			O-13B	5.74 x 10^8	6	8.8
			O-13C	5.46 x 10^8	6	8.7
			O-13D	9.10 x 10^7	6	8.0
			O-13E	3.98 x 10^7	7	7.6
			O-13F	1.71 x 10^8	5	8.2
Western UP - Gogebic	5/15/08	FMHill-USA_GII.4	O-14A	2.71 x 10^8	2	8.4
			O-14B	1.49 x 10^8	4	8.2

* Specimen did not sequence

Figure 2

Concentration of Norovirus GI Particles per gram of Feces in Outbreak-associated Cases

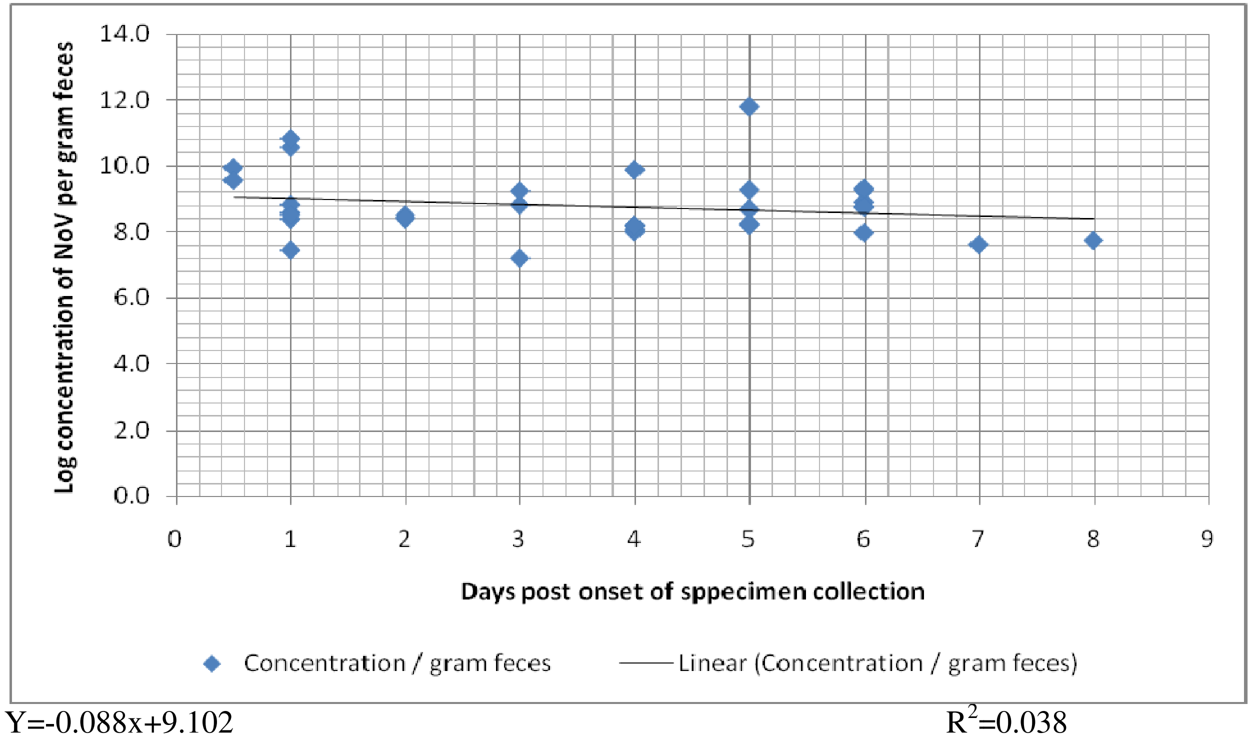


Y=0.193x+8.306 R^2=0.091

Convalescent shedding was observed in both GI and GII genotypes (Figures 2, 3). Our analysis of the outbreak-associated cases showed that viral shedding was detected in specimens collected as long as 8 days post-onset. The finding that days post-onset was not statistically associated with viral load means that we did not see any decrease in shedding in the days after disease onset. No information was available on duration of illness for these cases, but since NoV symptoms normally last 12-60 hours (CDC, 2001) it is likely that shedding continued several days past clinical recovery.

Figure 3

Concentration of Norovirus GII particles per gram of Feces in Outbreak-associated Cases



Y=-0.088x+9.102 R^2=0.038

Sporadic Cases:

Culture results for *Salmonella, Shigella, Campylobacter, E.coli* O157, and *C.difficile* were negative for all sporadic cases. Ten of the 18 sporadic cases of AGE were positive for norovirus (Table 2). Specimens at onset were collected a mean of 1.2 days (range 0-3 days) after the first manifestation of diarrhea or vomiting. Norovirus GI were detected in 3/10 (30%) and GII sequences were detected in 7/10 (70%) of cases. Strain GII.4 was predominant, having been found in 4/7 (57%) of the GII cases. Duration of illness was a mean of 1.7 days (range 1 to 2) for the 7 cases where this information was available. The repeated measures analysis of variance showed that NoV type (I or II) was not associated

with RNA log titer (P=0.90) and that the number of weeks post onset of stool collection was statistically associated with declining RNA log titer at p=0.0005.

Table 2

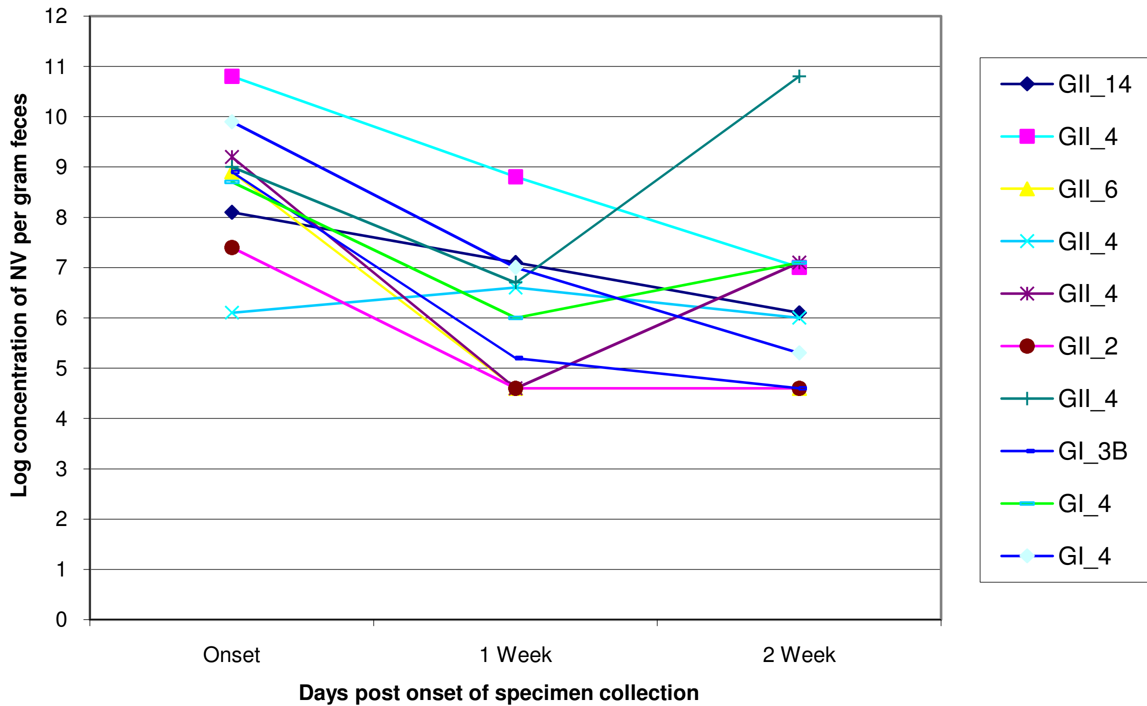
Sequencing and Quantitative Assessment of Michigan Sporadic Norovirus Cases

STUDY NUMBER	GENOTYPE	AGE	GENDER	ONSET	~ 1 WK POST ONSET*	~ 2 WK POST ONSET*
S-11	BerlinBA-DEU_GI.4	57	M	8.73×10^9	1.04×10^7	1.93×10^5
S-12	BerlinBA-DEU_GI.4	56	F	4.95×10^8	1.04×10^6	1.34×10^7
S-104	GII.14_0732OH_USA00	19	F	1.20×10^8	1.13×10^7	1.37×10^6
S-111	GII.4_FMHill_USA	49	F	6.85×10^{10}	5.99×10^8	1.12×10^7
S-112	GII.6_FLO_USA93	25	F	8.02×10^8	$\leq 3.86 \times 10^4$	$\leq 3.86 \times 10^4$
S-113	GII.4_FMHill_USA	24	F	1.28×10^6	3.68×10^6	1.00×10^6
S-116	GI.3B-LR316_USA94	31	F	7.29×10^8	1.47×10^6	$\leq 3.86 \times 10^4$
S-121	GII.4_FMHill_USA	50	F	1.61×10^9	$\leq 3.86 \times 10^4$	1.20×10^7
S-123	682_USA_GII.2	37	F	2.39×10^7	$\leq 3.86 \times 10^4$	$\leq 3.86 \times 10^4$
S-124	GII.4_FMHill_USA	54	F	1.08×10^9	4.85×10^6	6.33×10^{10}

*Concentration of NoV particles per gm feces at onset and 1 and 2 week post-onset. Specimens with concentrations listed as $\leq 3.86 \times 10^4$ did not amplify, and therefore this concentration is a minimum level of detection for the analysis and indicates that there were less than 64 transcripts (target sequences) for amplification in the 5 µl sample.

Figure 4

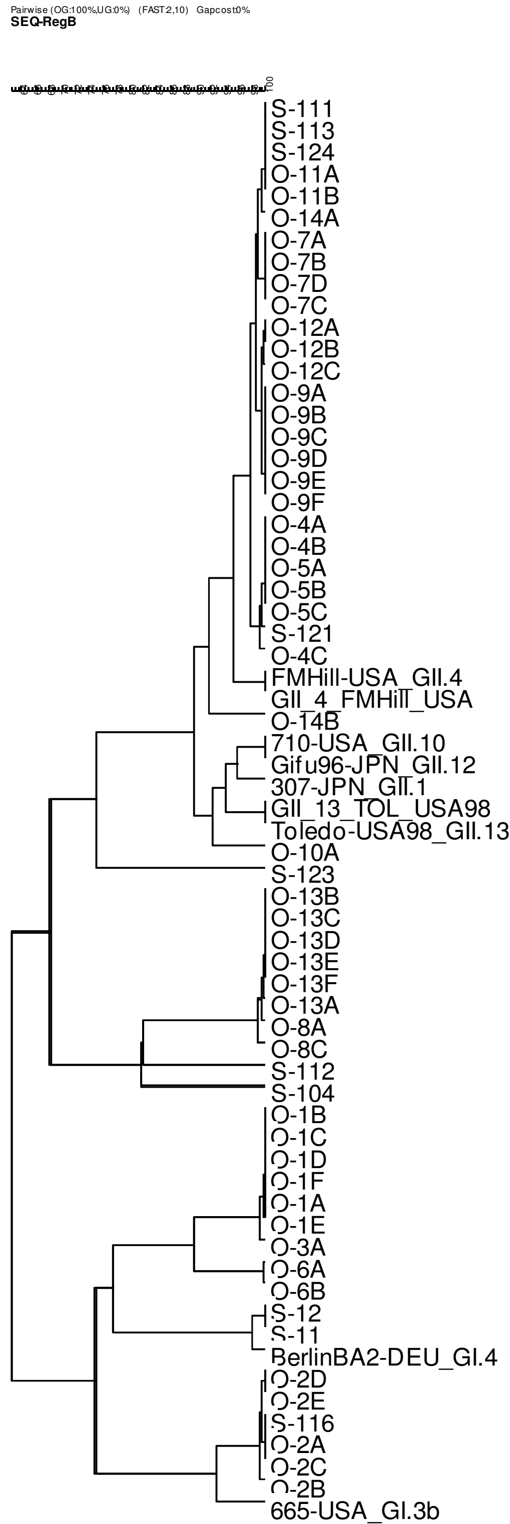
Concentration of Norovirus RNA Versus the Number of Days Post Onset of Specimen Collection for 10 Michigan Sporadic Cases.



The issue of shedding duration is better addressed in our longitudinal database of sporadic NoV cases where specimens were collected from each person at 0, 1 and 2 weeks post onset. A downward sloping trend was detected for all but one case, and no significant difference in slope was seen between GI and GII strains (Figure 4). While showing a statistically decreasing trend in viral load, titers of log 4 to10 were still being shed after 2 weeks.

Figure 5

Phylogenetic Tree of Sporadic (S) and Outbreak-associated Cases (O) Based on Similarity to CDC Reference Strains.



We saw no distinctive strain differences between the sporadic and outbreak-associated NoV cases (Figure 5). Our findings agree with other reports that both GI and GII were circulating in Michigan and other U.S. states during 2008 (CDC, 2009).

Discussion

Duration of Shedding:

Our study of outbreak-associated and sporadic cases showed that viral shedding was observed in the specimens obtained up to 8 days post-onset and after 2 weeks, respectively. As such this finding is inconsistent with the theory that shedding rapidly subsides within a couple of days of disease onset.

Determining the duration of shedding is of paramount importance in establishing public health policy regarding the prevention of transmission from NoV convalescent carriers. Public health isolation precautions may need to be extended beyond 2-3 days post recovery given the reportedly small infectious dose of < 100 NoV particles, the high environmental stability, and the current information regarding the lengthy and high-titer NoV convalescent shedding of NoV (Kapikian, Estes & Chanock, 1996; LeBaron et al, 1990; CDC, 2003; Widdowson et al, 2005).

However, it has been suggested that some post infection detection may be non-infectious soluble viral antigen without public health significance (CDC,2001). Recent studies show that NoV genome can survive in water at 4^0, 25^0 and -20^0C for up to 2 months. Viral degradation was lower at 4^0 and -20^0C compared to 25^0C. Complete degradation of the genome was observed after 100 days (Ngazoa, Fliss & Jean, 2008). Infectivity studies are needed to determine how much of the shedding from a convalescent carrier is infectious, however, such studies are problematic for a disease agent without an established animal model.

Norovirus infection is somewhat unique among etiologies of food-borne disease in that the benign nature of the disease allows researchers to purposefully challenge human volunteers in order to study the disease syndrome. Only one NoV strain was used for each challenge study, but this may not have been the strain that was currently circulating among the human population. Persons made ill by naturally acquired infectious particles at low doses may have very different shedding patterns from individuals whose immune systems were overwhelmed by massive doses of infectious particles. Additionally, human volunteers for challenge studies cannot ethically include the very young, old and chronically ill (Atmar et al, 2008). Challenge volunteers, who are institutionalized for the duration of the challenge study, could have a very different standard of supportive care from self-treated cases occurring in the community. For all these reasons, field studies are still necessary for studying the epidemiology and ecology of sporadic and outbreak-associated NoV cases under natural conditions of exposure, transmission and host susceptibility.

The lowest infectious doses for norovirus are estimated to be 10 to 100 virus particles (FDA, 2007). Recent studies showed that the infectious dose depended on the genetics of the volunteers; 50 to 62% of the volunteers who had an epithelial binding gene (Se+), developed diarrhea when they were given doses from 10^4 to 10^8 viral particles and whereas volunteers who did not have the gene (S-) remained well with doses up to 10^8 viral particles (Lindesmith et al, 2003; Hutson et al, 2005). Also, the viral RNA load of

GII was found to be 100-fold higher than that of GI in fecal specimens of patients with norovirus-associated gastroenteritis (Chan et al, 2006).

In conclusion, we found no dramatic strain differences between sporadic and outbreak-associated cases of NoV. The prevalent strains of NoV in both the sporadic and outbreak-associated cases were shed in high concentration for at least two weeks past disease onset, suggesting that current public health recommendations regarding hygienic precautions for convalescent carriers may need to be re-evaluated.

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