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Short Communication

Retrospective Analyses of Norovirus Diversity during Gastroenteritis Illness Response in Milwaukee, Wisconsin for the Period of 2006- 2013

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Abstract

The City of Milwaukee Health Department Laboratory (MHDL) collected a total of 320 viral gastroenteritis outbreak suspected specimens during the period of January 2006 – August 2013. Gastroenteritis infection (GI) suspected cases originated from long term care facilities, local hospitals, area restaurants, social events, and child care facilities. Nucleic acid from stool samples was tested for the presence of Norovirus (NoV) RNA using a real time RT-PCR assay targeting the highly conserved ORF1-ORF2 junction region of the Capsid gene. PCR analysis identified Norovirus as the GI causing agent in 141 (44% of specimens) of these cases. The majority of identified specimens were associated with global pandemic GII.4 genotypes (88.7%), with additional outbreaks closely related to more diverse NoV groups that co-circulated with the pandemic strains (11.3%). Our findings highlight the role of local health department laboratories in monitoring the genetic diversity of the currently circulating NoV strains, emergence of novel variants, and determining their potential implications in gastrointestinal outbreak management.

INTRODUCTION

Norovirus has been estimated to cause approximately 21 million of Acute Gastroenteritis cases annually, with >56,000 hospitalization and 560 deaths in the United States [1]. Worldwide, norovirus accounts for 94% of reported outbreaks of non-bacterial gastroenteritis [2]. The infectious dose for norovirus is as low as 18 viral particles, while the amount of virus shed by infected individuals is often as high as 10^8 RNA copies per gram of stool [3]. Illness begins typically 24 – 48 hours after initial infection. Symptoms include low grade fever, vomiting, nausea, abdominal cramps, and diarrhea. Symptoms typically resolve without treatment between 1 to 3 days [4]. Humans are the only known reservoir of the virus, and transmission may be waterborne, food borne, or through person-to-person contact [4]. Outbreaks are often associated with institutional settings such as nursing homes, hospital, and schools, although they also occur at social events, restaurants, and in contaminated water and food sources [4]. Norovirus are non-enveloped, single-stranded RNA viruses classified into the genus Norovirus of the family *Caliciviridae*. There are six genogroups (GI – GVI), and only three genogroups (GI, GII, and GIV) affect humans. GI and GII can be further delineated into at least 22 genetic clusters or genotypes based on the complete sequence of the Open reading frame (ORF2) in the major capsid viral protein (VP1) [4,5]. Noroviruses positive- sense RNA genome is divided into three open reading frames (ORF) that encode the proteins necessary for virus replication and assembly [6]. The ORF 2 consisting of two regions (C and D) have been widely used for genotyping due to their relatively conserved sequence within an actively mutating region. The VP1 protein, which is divided into two major structural domains: the shell (S) and protruding (P) contains the highly antigenic sites and confers the virus the ability to attach to target cells [6,7]. Either region (C or D), or a combination of both have proven to be highly discriminating for

genotyping during GI surveillance in public health laboratories [8,9]. Norovirus outbreaks have been historically linked to the emergence of new genetic variants such as the rapidly evolving GII.4 genotypes [4,10]. GII.4 genotype has shown evolutionary genetic changes as often as every 2 to 3 years since its discovery in the mid 1990's [11]. The majority of the outbreaks have been associated with inadequate herd immunity towards the emerging viruses caused by the virus ability to rapidly mutate through changes in the major capsid viral protein (VP1) sequence which is associated with virus to host cell interaction and render herd immunity inefficient for evolving strains [4,10,12,13]. Studies have shown that several genotypes may co-circulate without one genotype completely overtaking the others [1,12]. Although there is no established seasonality, virus infection reports occur more frequently during the cooler temperature months (fall–spring) in the U. S. [1,14]. Worldwide, GII.4 has been the predominant outbreak genotype, with some lesser known non GII.4 strains linked to sporadic food borne and waterborne outbreaks [10,15]. The Centers for Disease Control and Prevention (CDC) Norovirus Outbreak Networks (CaliciNet) has recently reported the genotypic and epidemiologic trends of Norovirus outbreaks in the U. S., thus showed the role of public health laboratories in monitoring virus diversity during gastrointestinal enteric outbreaks. Our study reports the norovirus genotype diversity in the Milwaukee metropolitan area during past years. We have identified and genetically characterized local outbreaks associated with pandemic strains such as GII.4 2006b Minerva, GII.4 2009 New Orleans, and the most recently GII.4 Sydney AUS 12, along with outbreaks associated to non GII.4 genotypes. This study data was used to offer an insight in local public health response during epidemiological investigations of suspected viral food borne surveillance, and to highlight a highly sensitive and fast molecular based method that improves response to potential gastroenteritis outbreaks by streamlining laboratory analysis and improved turn-around-time.

METHODS AND MATERIALS

Specimen Collection and Nucleic Acid Extraction

The MHD laboratory tested 320 stool specimens collected over a 7 year period (2006 – August 2013). Specimens were submitted as either suspected outbreak (more than two related gastroenteritis cases within 24 hours from the same setting), or sporadic cases originating in local hospitals, long term care, child care facilities, area restaurants, and others social events. Raw stool specimens were transported in stool cups or and stored at 2 to 8° C until nucleic acid extraction. Stool (~0.5g) was re-suspended in PBS pH 7.4 and spun at 5000rpm for 5 minutes. 200µl of cleared supernatant was removed and nucleic acid extracted using the Easy MAG extraction system (BioMérieux, Durham, NC).

Real Time RT-PCR and Conventional PCR detection

Norovirus GI or GII molecular typing was performed using an adaptation of a Taqman RT-PCR protocol that targets the ORF1-ORF2 overlapping region between the polymerase and the capsid gene [8,9,16,17]. MHDL adopted and validated the CDC CaliciNet protocols for conventional PCR, PCR product purification, and cycle sequencing [8,9]. Qiagen One-Step RT-PCR kit (Qiagen, Valencia, CA) was used for the conventional RT-PCR. PCR products were visualized on 2% agarose gel in 1X TAE (Sigma, St. Louis, MO), and products of correct size (approximately 177bp for GI genotype, and 253bp for GII genotype) were purified using QIA quick PCR purification Kit (Qiagen, Valencia, CA).

Cycle Sequencing and Sequence Analysis

Cycle sequencing was carried out using the BigDye v1.1 Terminator Cycle Sequencing Ready reaction Kit (Life Technologies, Foster City, CA) [9]. Sequence products were purified using Centri Sep columns (Princeton Separations, Adelphia, NJ) and analyzed in a 3130xl Genetic Analyzer (Life Technologies, Foster City, CA). Sequence results were assembled and analyzed using BioNumerics version 5.10 software (Applied-Maths, Austin, TX). Multiple pairwise alignments as well as phylogenetic trees were created to compare global clustering of the VP1 gene D region for specimen genotype identification during this study. Sequence alignments were compared to CDC CaliciNet Norovirus GI and GII databases to determine phylogenetic relation amongst strains [4,9,18]. Norovirus genotype was determined depending of the percent relatedness to specimens in the database. Percent variation of less than 5% for GII.4 sequences or 10% for any non-GII.4 sequences were considered closely related genotypes.

RESULTS

During this study 320 cases were investigated, of which 133 (41.5%) were identified as NoV GII, and 8 (2.5%) were NoV GI, while the remaining suspected cases 179 (56%) remained unidentified or with unknown etiology based on the available conventional methods for the detection of other suspected pathogens. Subsequent sequence analysis of the Capsid gene region D of the NoV positive specimens indicates that from 2006 - 2009 the predominant genotype was GII.4 2006b Minerva (62/66 – 94%). During 2010 to 2012 the predominant outbreak genotype was GII.4 2009 New Orleans (48/75 –64%), and starting 2013 the predominant genotype has been the GII.4 Sydney AUS 12 genotype (10/13 – 77%). During the span of this study, additional genotypes were identified: GI.1 (1/141 – 1%), GI.6A (7/141 – 5%), GII.3 (4/141 – 3%), GII.6 (2/141 – 2%), and GII.12 (1/141 – 1%). A total of 18 different genotypes (9 GI & 9 GII) were reported to CDC CaliciNet during the study period [Table 1, 2].

Epidemiology, Seasonality, and Outbreak Settings

Milwaukee Health Department Laboratory reported local norovirus activity to CDC CaliciNet during the period of Jan 2006 to August 2013. Data analysis of Norovirus seasonal activity in metro Milwaukee area was illustrated based on number of cases detected per month over the span of the study. The MHDL findings indicate active norovirus infection periods coincide with the fall thru spring seasons in the Milwaukee metro area [Figure 1a]. The information was represented on a per year basis to demonstrate the norovirus activity reported after emergence of a new pandemic genotype [Figure 1b]. The second representation indicates there is a slight decrease in detection during the season after the initial genotype detection, but detection does not dramatically increase in subsequent seasons. Epidemiological data collected from local public health nurse investigations

Table 1: Diversity of non GII.4 genotypes observed during similar time span in different geographical regions.

Genotype	Confirmed	Total (n=624)[a]	Confirmed	Total (n=141)[b]	Confirmed	Total (n=457)[c]
GII.1	13	2.1%			16	3.5%
GII.2	3	0.5%			4	0.9%
GII.3	**12**	**1.9%**	**4**	**2.8%**	**8**	**1.8%**
GII.6	**4**	**0.6%**	**3**	**2.1%**	**7**	**1.5%**
GII.7	1	0.2%			20	4.4%
GII.12	**16**	**2.6%**	**1**	**0.7%**	**24**	**5.3%**
GII.13	7	1.1%				
GII.14	5	0.8%				
GII.15	3	0.5%				
GI.1	3	0.5%			4	0.9%
GI.2					11	2.4%
GI.3	22	3.5%			20	4.4%
GI.4	5	0.8%			13	2.8%
GI.5					3	0.7%
GI.6	**24**	**3.8%**	**7**	**5.0%**	**27**	**5.9%**
GI.7	**14**	**2.2%**	**1**	**0.7%**	**4**	**0.9%**
GI.10					2	0.4%
Total (%)		**21.2%**		**11.3%**		**35.7%**

[a]Hasing et al (2013) study, [b]Current study, [c]Wisconsin State Lab of Hygiene Calicinet submitted sequences (2009 - Aug 2013).
Highlighted rows indicate NoV genotypes detected by all three studies

Table 2: Diversity of GII.4 genotypes observed in Wisconsin (2006 - 2013).

Genotype	Confirmed	Total (n=141)[a]	Confirmed	Total (n=457)[b]
GII.4 Yerseke	1	1%		
GII.4 Minerva	62	44%	5	1.1%
GII.4 New Orleans	49	35%	221	48%
GII.4 Sydney	13	9%	63	14%
GII.4 Osaka			1	0.2%
Total (%)		**88.7%**		**63.2%**

[a]Current study; [b]Wisconsin State Lab of Hygiene Calicinet submitted sequences (2009 – Aug 2013)

revealed the top three most common transmission settings for NoV outbreaks were Food borne, long term care facilities, and hospitals (36%, 35%, and 18% respectively) [Figure 2], with the most common associated transmission method reported during investigation being person-to-person contact.

Phylogenetic analysis of the genotypes

Phylogenetic data analysis of the NoV genotypes from outbreaks in Milwaukee area during 2006 – August 2013 shows the majority of outbreaks identified were associated with GII.4 genotypes. A phylogenetic tree was created to illustrate the diversity observed by our laboratory during the study based on 5% relatedness rule per CaliciNet data analysis guidelines for outbreaks [9]. Our findings show similarities in detection of outbreaks highly related to epidemic strain clusters [Figure 3]. The majority of our outbreaks were part of the GII.4 2009 New Orleans or GII.4 2006b Minerva clusters, but it also shows there were other GII genotypes circulating during the time period responsible for sporadic outbreaks. The tree shows the emergence of the current pandemic strain GII.4 Sydney AUS 2 within the GII.4 2009 New Orleans genogroup.

DISCUSSION

The data from this study highlights the trend that norovirus has experienced frequent and significant genetic changes in the last decades. Although GII.4 variants accounted for most of the cases reported (approximately 90% n=126/141), they did not completely outcompete the remaining variants. Recently published studies indicate that frequent and high norovirus activity (approximately every 2 to 3 years) has correlated to emergence of a new genetic variant within the GII.4 group [18–20]. During our study we found three emerging GII.4 genotypes circulated in Wisconsin out-competing their predecessor within a two to three year span [Figure 1b]. A visual representation

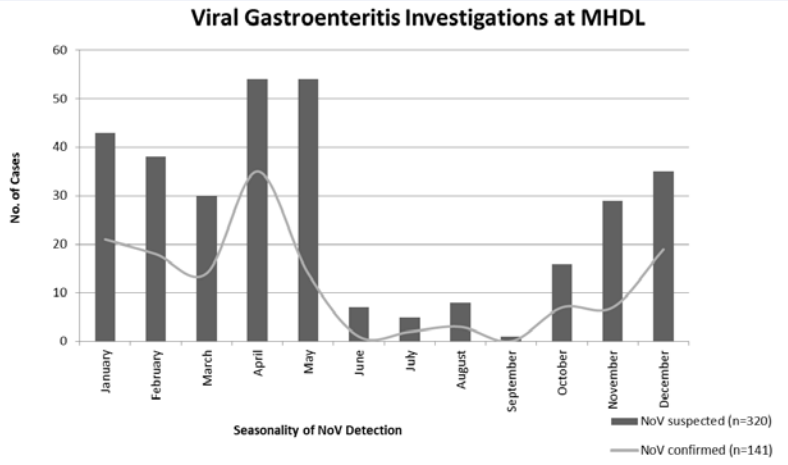

Figure 1a Seasonality of norovirus activity in Milwaukee during the period of 2006 to 340 August 2013. High virus activity observed during the fall to spring months.

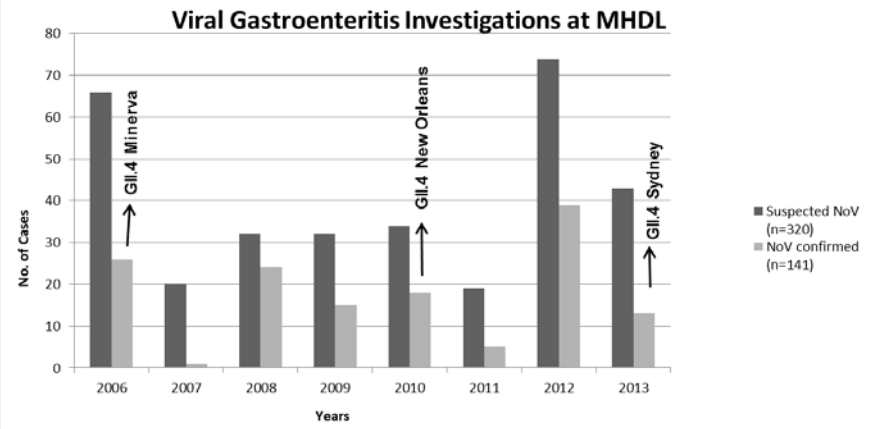

Figure 1b Norovirus activity reported to CDC CaliciNet by Milwaukee Health 351 Department Laboratory for the period of Jan 2006 to August 2013.

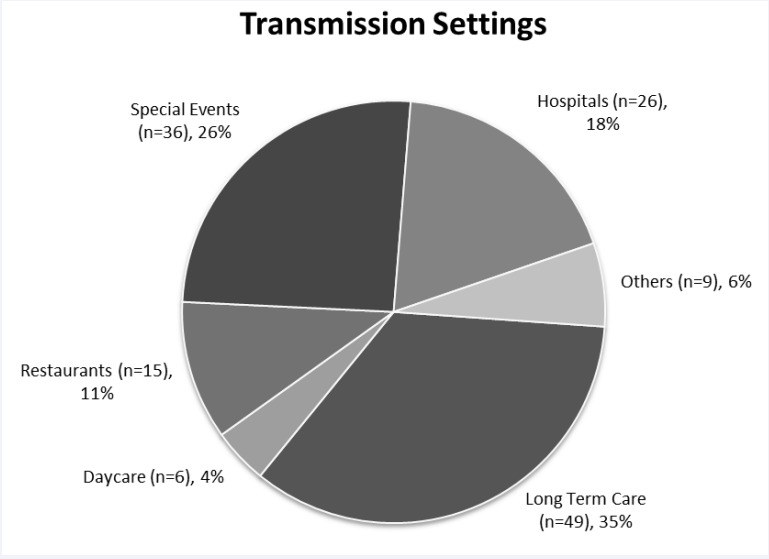

Figure 2 Norovirus outbreaks transmission settings: Over half of transmission (57%) of 366 NoV occurred in health care settings, daycares, and long term residential facilities.

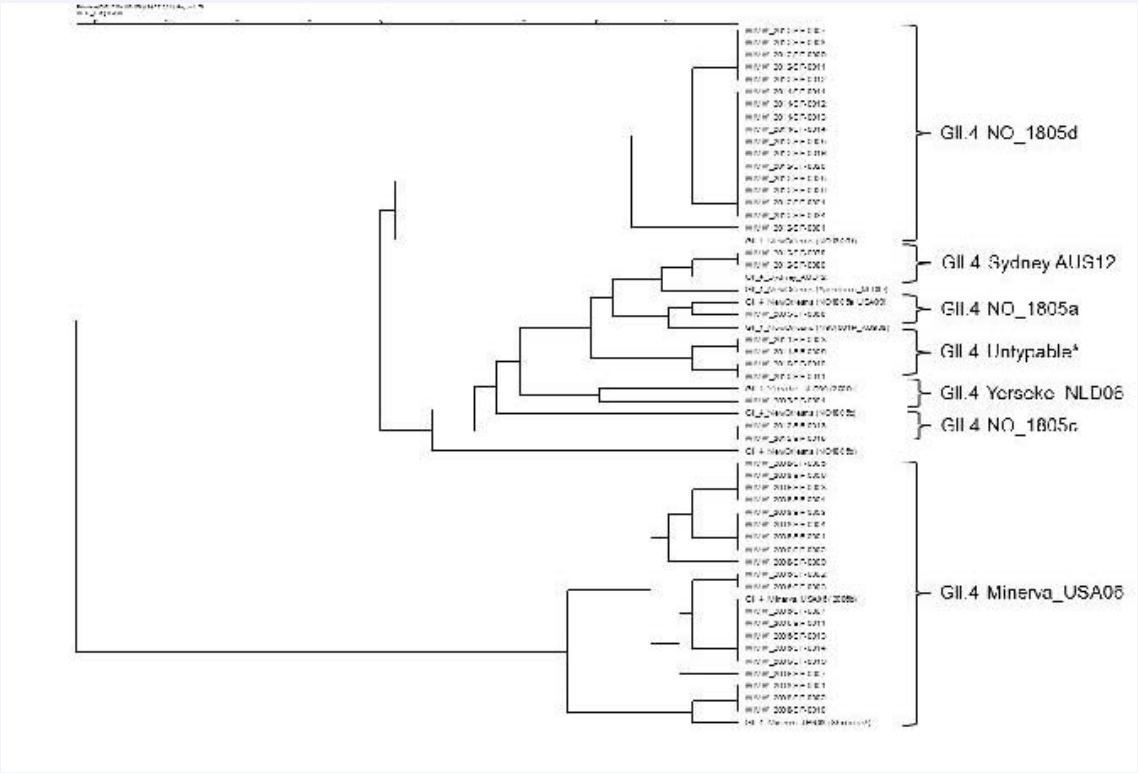

Figure 3 Phylogenetic analysis of NoV outbreaks reported by MHDL to CDC during the 379period of 2006 – August 2013.

in Figure 3 of genotypes identified by our laboratory shows how mutations within the Capsid gene of the GII.4 subgroup are indicative of a highly variable genetic group with likely tendency of introducing random mutations leading to new virus genotypes. High mutation frequency within this genogroup is evidence of how evolving strain drifts under selective immune pressure until mutations have accumulated to the point where a novel genetic variant phenotype becomes established and evades pre-established host immunity [10,21-23]. Interestingly, the emergence of new GII.4 outbreak did not out-compete other genetically diverse groups. Successful co-circulation of the non-GII.4 strains indicates the possibility that evolutionary pressures might not affect all genotypes similarly and that some particular genotypes could be linked to outbreak settings and/or transmission settings as well. We compared our data with CDC CaliciNet reported outbreaks data from the state of Wisconsin and a comprehensive study by researchers in Canada to determine diversity within the non GII.4 genotypes during our reporting season [24]. Although our study reported 11.3% non GII.4 detection; a published study from Canada and Wisconsin data submitted to CaliciNet demonstrated greater diversity (approximately 21% and 36% respectively) (Tables 1, 2). The sustained diversity of norovirus cases as observed in these three regions highlights the theory of outbreak settings and transmission modes association with particular genotypes. GII.4 infections often are associated with person-to-person transmission in clinical settings such as long term care centers, hospitals, and residential facilities, while non-GII.4 strains were found to be linked to food borne and waterborne outbreaks in restaurant and social events settings [19,24-28]. Our findings were consistent with these studies, as the top transmission settings and transmission methods with other regional areas as depicted in tables 1 and 2 helped us open a discussion in support of an active local public health role in approaching norovirus outbreaks. Prompt recognition of new genetic variants and increase in awareness of norovirus diversity associated with transmission settings allows for more adequate outbreak response and aids in determining trends that lead to evolutionary mutations. The molecular surveillance at the local PHLs like ours allows virus identification within 4 hours and genotype phylogenetic analysis within 6 hours after virus identification. While targeted detection of gastrointestinal viruses like NoV is currently in place during symptomatic surveillance, molecular approaches to multiple pathogen screening may improve outbreak investigation algorithm by shortening the turn-around time and improving the sensitivity of detection [29]. Our study highlights the importance to implement efficient and real- time PHL response during suspected GI outbreaks and to timely share epidemiology data between local, regional and national networks that aid in determining the future of this fast evolving pathogen.

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