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Norovirus as the cause of medically attended gastroenteritis: a hospital-based experience

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SUMMARY

Gastroenteritis remains an important cause of morbidity and mortality worldwide. With the introduction of vaccines against rotavirus, interest has shifted to understanding the epidemiology of norovirus (NoV). While the importance of NoV in gastroenteritis outbreaks is well established, its role in sporadic gastroenteritis is less known. To better define the role of NoV as a cause of sporadic gastroenteritis we investigated its prevalence in the patients seen in our paediatric hospital with special emphasis on its seasonal and age distribution. Over a 12-month period discarded stool specimens submitted to our paediatric hospital for testing of an infectious aetiology were retrieved and additionally tested for NoV by real-time reverse transcriptase–polymerase chain reaction; demographical and clinical information were also obtained. Overall, NoV was the single most commonly identified pathogen and found in 68/892 (7·6%) total specimens or 68/258 (26%) of pathogen-positive specimens. The highest rates of NoV were detected in children aged 6 months to 4 years (50/332, 15·1%) and presenting between October and January (46/314, 14·7%). NoV has become the main cause of gastroenteritis in our paediatric population.

Key words: Gastroenteritis, norovirus, paediatrics, surveillance.

INTRODUCTION

Despite ongoing progress, diarrhoea remains an important worldwide cause of mortality in children aged <5 years accounting for 9% or 0·6 million of the deaths [1]. Until recently rotavirus (RV) accounted for ~50% of those diarrhoea-related deaths [2], but that is quickly changing now that RV vaccines are being introduced for childhood immunization. As expected, RV vaccines have been introduced more efficiently in developed countries and they are having a significant impact in the amount of RV disease. For example at our hospital, the rate of hospitalizations due to gastroenteritis in general and to RV-specific gastroenteritis decreased by ~50% and the number of gastroenteritis episodes seen at the doctor's office decreased by 27%, after only 55% utilization of the vaccine [3]. These findings have been extended and corroborated by national surveillance studies in the United States [4] and across European countries [5].

As RV diarrhoea decreases, the role of other viruses is becoming more evident. The second most common viral cause of diarrhoea is norovirus (NoV). NoV is a small (27 nm) human calicivirus; the prototype virus, Norwalk virus, was identified in 1972 by electron microscopy [6]. Detection of NoV particles, and hence understanding of its epidemiology, was rather difficult

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until the early 2000s when virus-specific conventional reverse transcriptase–polymerase chain reaction (RT–PCR) was developed. The introduction of RT–PCR soon identified NoV as the leading cause of epidemic (outbreak) gastroenteritis, affecting all age groups, and causing >90% of non-bacterial and ~50% of all-cause epidemic gastroenteritis worldwide [7, 8].

But, while the role of NoV in epidemic diarrhoea was soon established, its role in endemic (sporadic) diarrhoea is less well characterized – especially in children. A literature review in 2008 [9] identified 31 studies (published between 1997 and 2008) that met quality inclusion criteria (duration for >1 year and use of RT–PCR), and found that NoV accounted for 12% (5–36%) of cases of mild and moderate diarrhoea and 11% (3–31%) of severe diarrhoea. The studies corresponded to various countries and age groups. Only one study [10] was from the United States, which included children aged <4 years hospitalized for diarrhoea and found NoV in 7·1% of the cases. A more recent similar systematic review in 2014 [11] identified 175 studies (published between 2008 and 2014) from 48 countries and estimated a pooled NoV prevalence of 18% (95% confidence interval 17–20%) for all cases of acute gastroenteritis. In the United States specifically, investigators from the Centers for Disease Control and Prevention (CDC) analysed an insurance claims database including various public and private health plans (Medicaid and uninsured populations not included) covering about 30 million persons in all 50 states and estimated that NoV caused 13% of all gastroenteritis-associated visits [12]. However, these are estimates performed using sophisticated statistical models and need to be corroborated with more conventional epidemiological studies.

The dearth of information on the role of NoV as cause of sporadic gastroenteritis in the United States prompted us to investigate its prevalence in patients seen in our paediatric hospital (outpatient and inpatient departments) with special emphasis on its seasonal and age distribution.

METHODS

Study site

Children's Hospital New Orleans, a 247-bed university-affiliated hospital, is the largest single paediatric facility in the state of Louisiana, admitting 54% of paediatric patients in the New Orleans area. Annually, ~70 000 children receive care at our hospital and ~1200 stool specimens are submitted to the clinical laboratory for evaluation of a pathogen.

Data collection

The list of all stool samples submitted for microbiological analysis was reviewed three times a week by one of the authors (R.E.B.) and patient-identifying information was extracted. By regulation, the laboratory saves specimens for 72 h before being discarded. After all clinical tests had been completed and before discarding, the specimen was retrieved for further NoV analysis. Stool specimens were stored at +4 °C in the laboratory until retrieved and then stored at −70 °C until tested for NoV.

Using the identifiers, the clinical records corresponding to the gastroenteritis encounter were retrieved and information collected regarding duration and severity of diarrhoea, associated symptoms, need for hospitalization and laboratory results. Full information was available for children seen in the emergency department or hospitalized. For children seen in the outpatient clinics, full demographical information was available but only limited information on symptoms or clinical findings.

NoV testing

NoV testing was performed by real-time RT–PCR following a protocol previously described by Kageyama *et al*. [13] and adapted by the CDC (kindly provided by Dr Pengbo Liu, Emory University, Atlanta, GA). Briefly, stool specimens were thawed, a 10–20% suspension prepared in DNAse/RNase-free water, centrifuged and the supernatant used to purify RNA using QIAamp Viral RNA Mini kit (Qiagen, USA) and stored at −70 °C. The resulting viral RNA concentration was measured and 1 µg subjected to reverse transcription (Superscript II Reverse Transcriptase; Invitrogen, USA) with random primers (Random Primers, Invitrogen). The resulting cDNA was included in the PCR reaction (Taqman Universal PCR Mastermix, Applied Biosystems, USA) and amplified in a thermocycler (QuantStudio 12Kplex, Applied Biosystems). Amplification reactions were run in duplicate and with positive and negative controls (provided by CDC). For NoV Genogroup I (GI), the primers COG1 F and COG1R and the probes RingG1(a) and RingG2(b) were used; for NoV GII, the primers COG2 F and COG2R and the probe RingG2-TP were used [13]. The PCR activation step used 95 °C for 15 min, followed by

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45 amplification cycles at 95 °C for 15 s for denaturing and 56 °C for 1 min for annealing/extension. Any cycle threshold signal indicated a possible positive result.

Specimens with a positive signal by real-time RT-PCR were also subjected to conventional RT–PCR [14], the resulting amplicon run in an agarose gel (for confirmation of correct size), purified (QIAquick PCR Purification kit, Qiagen) and sequenced (Stanley S. Scott Cancer Center's Translational Genomic Core, LSUHSC, New Orleans, LA) for identification as NoV and subtyping by comparing the DNA sequence to databases at NIH's GenBank (http://www.ncbi.nlm.nih.gov/genbank) and the Norovirus Genotyping Tool (http://www.rivm.nl/mpf/norovirus/typingtool).

Data analysis

In the event that multiple stool specimens were submitted for the same patient, only the first specimen was retrieved and tested. All specimens submitted within a 14-day period were considered as part of the same gastroenteritis episode (and only one tested). Cases were classified as community-acquired gastroenteritis if the specimen was obtained from an outpatient or within the first 3 days of admission, or hospital-acquired gastroenteritis if the specimen was collected >72 h after admission and the patient had not been admitted for acute gastroenteritis. Specimens from cases of hospital-acquired gastroenteritis were collected and tested for NoV but are presented separately since they represent a different clinical entity.

The frequency and seasonality of NoV infection (monthly occurrence) was calculated and compared; as well as frequency for age subgroups. Tables depicting absolute and percent frequency of the clinical and epidemiological variables were constructed. Proportion variables were compared with χ^2 with Yates' correction or Fisher's exact test, as appropriate (Statcalc, Epi-Info v. 3.5; CDC, USA); continuous variables were compared with the non-parametric Mann–Whitney *U* test (Prism v. 5.04; GraphPad, USA).

Ethical standards

The study was reviewed and approved by the Institutional Review Board of Louisiana State University Health Sciences Center and Children's Hospital, both in New Orleans, LA, USA. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

RESULTS

During the 12-month period (1 June 2011 to 31 May 2012), 1136 stool specimens were submitted to the hospital's laboratory that met the study inclusion criteria; and of these, 975 (85·8%) specimens from 791 patients were available for testing.

Of the 975 specimens, 83 (8·5%) (from 68 unique patients) were classified as hospital-acquired gastroenteritis. Sixty-five (78·3%) specimens were tested for *C. difficile* toxin (ten found positive), 63 (75·9%) were cultured for bacteria (one found positive for *Shigella sonnei*), 56 (67·5%) were tested for RV/adenovirus antigen (one found positive for adenovirus) and one was tested for *Giardia*/*Cryptosporidium* antigen (found negative). All 83 hospital-acquired gastroenteritis specimens were tested for NoV and none was positive.

The remaining 892 specimens represented community-acquired gastroenteritis, 552 (61·9%) seen on an outpatient basis only (either clinics or emergency department and discharged) and 340 (38·1%) requiring hospitalization. All 892 specimens were tested for NoV, while 748 (83·9%), 534 (59·9%), 312 (35·0%) and 163 (18·3%) were additionally tested for bacterial culture, RV/adenovirus antigen, *C. difficile* toxin or parasites, respectively. At least one pathogen was identified in 258 (28·9%) specimens, corresponding to 240 distinct patients. NoV was detected in 68 cases, accounting for 7·6% of all the specimens or 26·4% of the pathogen-positive ones. Numerically, NoV was the most commonly identified pathogen (68) followed by *C. difficile* (62), *Salmonella* spp. (46) and *Shigella* spp. (45); others were detected in much lower numbers (Table 1). RV was isolated from only 16 (2·9%) of 534 specimens. Since testing was selective for pathogens other than NoV, the percent positivity varied by pathogen and was highest for *C. difficile* (19·9%) followed by NoV (7·6%) (Table 1).

The most common NoV genogroup was GII, seen in 56 (82·3%) specimens followed by GI, seen in 11 (16·2%) specimens, with one (1·5%) specimen testing positive for both GI and GII. The most common genotype was GII.4 seen in 44 (64·7%) specimens. The 68 NoV-positive specimens corresponded to

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Table 1. *Results of microbiological studies*

Group	Subgroup	No. tested	No. positive	% positive
Bacteria	*Clostridium difficile* toxin*	312	62	19·9
	Salmonella spp.†	748	46	6·2
	Shigella spp.†	748	45	6·0
	Campylobacter spp.†	748	4	0·53
	Escherichia coli O157†	748	1	0·13
	Cronobacter sakazakii†	748	1	0·13
	Aeromonas caviae†	748	1	0·13
	Edwardsiella tarda†	748	1	0·13
Viruses	Norovirus‡	892	68	7·6
	Adenovirus antigen§	534	25	4·7
	Rotavirus antigen‖	534	16	3·0
Parasites	*Cryptosporidium* antigen¶	163	5	3·1
	Giardia antigen¶	163	2	1·2
	Blastocystis hominis#	163	2	1·2

* By nucleic acid amplification (Illumigene *C. difficile*; Meridian Bioscience Inc., USA).
† By in-house culture.
‡ By RT–qPCR (as described in Methods section).
§ By enzyme immunoassay (Premier Rotaclone; Meridian Bioscience Inc.).
‖ By enzyme immunoassay (Premier Adenoclone; Meridian Bioscience Inc.).
¶ By rapid immunoassay (ImmocoCardSTAT! Crypto/Giardia; Meridian Bioscience Inc.).
# By direct smear.

65 unique patients. One patient had two positive specimens 3 weeks apart (both GI.6) and another patient had three specimens 3 weeks and 3 months apart (GII.7, GII.4 and GII.7).

NoV showed a distinct seasonal distribution with 46 (67·6%) of the cases presenting between October and January (Fig. 1). NoV cases appeared later in the year compared to the peak season for *Shigella* (July–September, $P < 0·0001$) or *Salmonella* (July–October, $P = 0·003$) and earlier than RV (March–April, $P = 0·02$); adenovirus, *C. difficile* and pathogen-negative cases had no clear seasonality or a bi-modal pattern. Similarly, there was a distinct age distribution for NoV with 50 (73·5%) of the cases presenting in children aged 6–48 months (Fig. 2). Children aged <4 years had NoV detected about four times more frequently than older children (11·6% *vs* 2·9%, $P < 0·0001$). The age of children affected by NoV [median 1·8, interquartile range (IQR) 0·9–3·1 years] was significantly younger than those affected by *C. difficile* (6·97, IQR 1·6–15·0 years, $P < 0·0001$), *Shigella* (5·0, IQR 3·3–6·8 years, $P < 0·0001$) or pathogen-negative (3·61, IQR 0·6–12·8 years, $P = 0·008$) diarrhoea.

Of the 68 NoV-positive patients from whom clinical data was available, diarrhoea was present in 90·7% (49/54), vomiting in 86·8% (46/53), fever in 58·8% (30/51), and dehydration in 48·7% (19/39). Twenty-one of the 68 (30·9%) NoV-positive patients were admitted; 52·2% (12/23) in those with underlying illness and 25·0% (5/20) in those without underlying illness ($P = 0·13$). Faecal occult blood was investigated in 36 patients and six (16·7%) were positive, with two having an alternative pathogen detected (one *Salmonella* sp. and one *Shigella sonnei*).

DISCUSSION

The main objectives of our study were to determine the prevalence of NoV infection in children attending for gastroenteritis at our hospital, as well as its seasonal and age distribution.

We found an overall NoV prevalence of 7·6% (68/892). Few other studies have looked into endemic NoV infection in paediatric US populations. Similar to ours, all these studies were hospital-based, spanned at least 1 year and evaluated specimens submitted for stool pathogens using RT–PCR. One study [10] restricted to children aged 15 days to 5 years found NoV in 7·1% (131/1840) of specimens; and two other studies evaluating all paediatric age groups found NoV in 11% (352 of/3222) and 10% (98/941) of specimens [15, 16]. In contrast, two other studies reported a somewhat higher NoV prevalence. One study [17] was restricted to children aged <5 years and found NoV in 21% (278/1295), and the other study [18] included all paediatric age groups and found 17% (30/172) of the specimens positive for NoV. The difference in NoV prevalence in the studies is likely a reflection of the differences in the populations studied, the selection criteria used (for the specimens and subjects to be tested) and the local and year-to-year variation in NoV activity.

While hospital-based surveillance may not be the best methodology to study the full spectrum of NoV illness, it has proven most convenient for specimen collection and testing. Community-based studies are not practical at the present time, mainly because of lack of a simple and accurate test to detect NoV; currently, conventional RT–PCR or real-time RT–PCR

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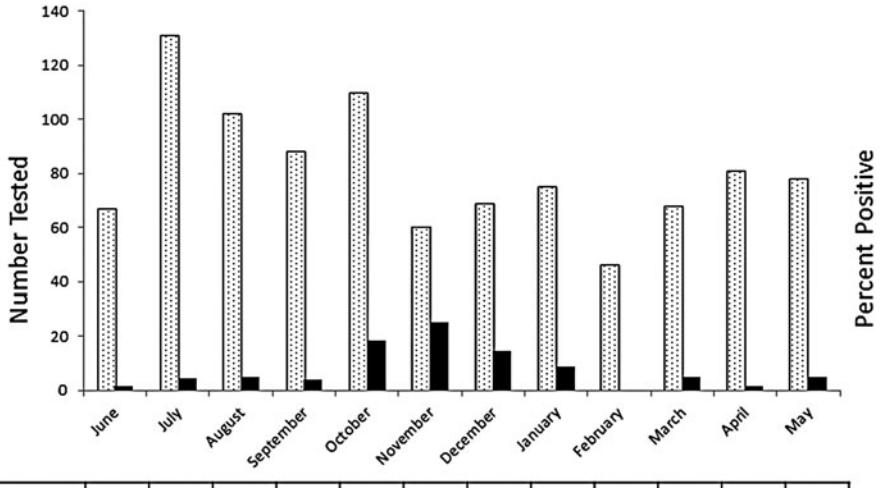
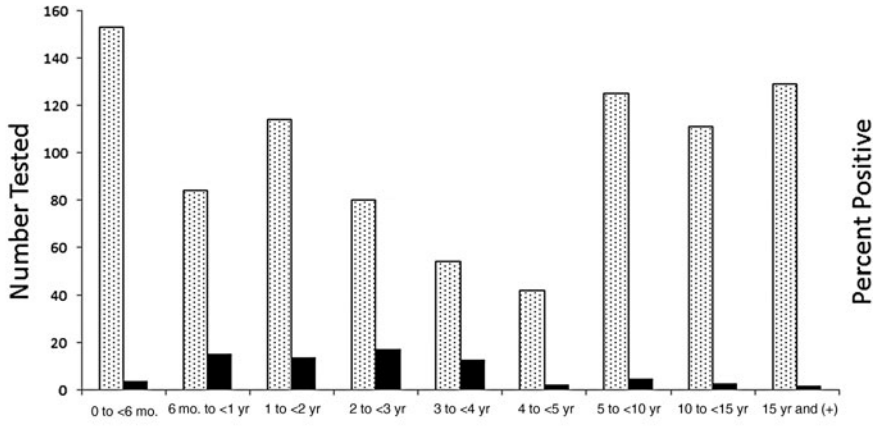


No. tested	67	131	102	88	110	60	69	75	46	68	81	78
Percent positive	1·5	4·6	4·8	3.8	18·2	25·0	14·5	8·6	0·0	4·7	1·5	5·1

Fig. 1. Number of specimens tested for norovirus (light columns) and percent positivity rate (dark columns) by month, 2011–2012.



No. tested	153	84	114	80	54	42	125	111	129
Percent positive	3·9	15·5	14·0	17·5	13·0	2·4	4·8	2·7	1·6

Fig. 2. Number of specimens tested for norovirus (light columns) and percent positivity rate (dark columns) by age group.

are the most accurate tests but they are rather laborious [19]. Community-based, active surveillance, population studies of NoV have been undertaken in developing countries. For example, in Peru 220 children were followed from birth to 2 years and NoV was found in 23% of 1495 diarrhoea specimens, resulting in 38% and 71% of children with at least one episode of NoV-associated diarrhoea by age 1 and 2 years, respectively [20]. However, these results may not be applicable to the United States.

In our study NoV was the most common virus detected, surpassing RV (68/892 *vs.* 16/534). However, it should be noted, that 2011–2012 was a particularly low year for RV activity, and the relative proportions may vary from year to year. Still, the finding of NoV preponderance over RV has also been described by others [15–17] and is a direct consequence of RV vaccination. Yet, even though NoV has become one of the main causes of diarrhoea in children in our area, it still does not have the levels of morbidity and severity that RV used to have. As an example, before the introduction of RV vaccines in our hospital we used to see about 200 RV-positive stool specimens every year and about 57% of these

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required hospitalization [3]; whereas in this study there were 68 NoV-positive patients and 21 (31%) were hospitalized. It also should be noted that percent-wise *C. difficile* was the most prevalent pathogen (19·9%) surpassing NoV (7·6%). This finding may reflect the result of selective testing and the more widespread use of nuclear amplification assays, which tend to overestimate the importance of *C. difficile* as a pathogen [21].

NoV infections showed a clear seasonal distribution (Fig. 1); the average positivity rate was 14·6% (46/314) for October–January while it was 3·3% (22/661) for the rest of the year; this seasonality has also been described by others [17, 18]. NoV was more common in specimens obtained from children aged <4 years (56/485, 11·6%) and especially in those aged 6 months to <4 years (50/332, 15·1%) (Fig. 2). The age distribution suggests partial protection from maternal antibodies in the first 6 months of life that subsequently wanes and is followed by natural infection and development of own immunity. Yet the presence of NoV infections in children aged <6 months or >4 years, without much of a significant change in the detection rates with increasing age would indicate incomplete protection and suggest that a potential NoV vaccine would need to confer long-term protection to have an impact on the epidemiology of NoV disease. In fact, initial studies based on human challenge experiments suggested immunity lasted only between 6 months and 2 years [22, 23], while a more recent study, based on clinical transmission models has suggested a somewhat longer but still limited duration of immunity of 4·1–8·7 years [24].

A number of limitations of the present study must be recognized. First, this was a hospital-based surveillance, and as such it captures medically attended gastroenteritis but not cases of lesser severity attended in the community, and the lack of a denominator (catchment population) does not allow for population incidence estimates. Second, our data applies to paediatric patients only and to sporadic (not outbreak) diarrhoea (while we cannot be entirely certain, to our knowledge none of our cases was epidemiologically linked to an outbreak). Third, the study includes children seen at a single centre and for a single 12-month period; so the applicability of the findings for other locales or other years cannot be determined. Fourth, while the collection of stool specimens was prospective, the collection of clinical data was retrospective and not standardized; so, we had no control over data documentation or procedures, resulting in some variables being missing or imprecise (and hence, not used for analysis). Fifth, the study used specimens originally obtained for testing of other pathogens, so a selection bias (based on what clinicians thought likely aetiologies) may have been introduced. Indeed, not all specimens were tested for all pathogens making comparisons difficult; for example all specimens were tested for NoV (making its number higher), while others (e.g. *C. difficile* toxin) were tested more selectively based on clinical presumption (and possibly making its rates higher).

Despite these limitations, we believe our data shows that NoV has become one of the main causes of community-acquired gastroenteritis in the United States and the number 1 cause of viral diarrhoea, especially in young children. These findings may also be applicable to other countries with widespread immunization against RV.

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DECLARATION OF INTEREST

None.

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