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Significant Prevalence and Genetic Diversity of Norovirus Infection in Irish Children

ALLISON WATERS, LINDA DUNFORD, GRÁINNE TUITE, JEFF CONNELL, SÉAMUS DOOLEY, BARBARA FOLEY, PAUL MCKEOWN, WILLIAM W. HALL, AND SUZIE COUGHLAN

National Virus Reference Laboratory (NVRL) [A.W., L.D., G.T., J.C., S.D., W.W.H., S.C.], University College Dublin, Dublin 4, Ireland; Health Protection Surveillance Centre (HPSC) [B.F., P.M.], Dublin 1, Ireland

ABSTRACT: Pediatric gastroenteritis places a considerable disease burden on children of the developed world. The national surveillance of gastroenteritis in Ireland is a combined virological and epidemiologic surveillance program. The objectives of this study were to characterize the norovirus (NoV) genotypes associated with viral gastroenteritis in children ≤5 y of age, and compare these strains with those detected in adult specimens. A total of five different NoV genotypes were associated with infection in Irish children [Genogroup II/type 2 (GII/2),GII/4,GII/6,GII/b,GII/14] whereas only GII/4 strains were identified in adults. This significant genotypic difference in the NoV strains associated with pediatric and adult infection was found in both community- and hospital-based infection. To assess the burden that NoV places on Irish children, the relative prevalence of norovirus, rotavirus, and adenovirus was determined in hospitalized symptomatic children ≤5 y old. Our results identified NoV as a major cause of gastroenteritis in children ≥4 mo of age and determined that NoV and adenovirus infection are equally significant in children in the first 5 y of life. This group of pediatric patients reported diarrhea as their most common symptom raising the question whether Kaplan criteria are the most effective method for clinically diagnosing outbreaks of enteric infection in pediatric patients. **(*Pediatr Res* 64: 312–316, 2008)**

Acute gastroenteritis remains a major cause of morbidity and mortality in children of both the developed and developing countries. Enteric viruses are collectively recognized as the most significant etiologic agents of pediatric gastroenteritis including, rotavirus (RoV), norovirus (NoV), adenovirus (AdV), and astrovirus. RoV has been considered the most common cause of childhood gastroenteritis in the developed countries. However, since the global NoV epidemic in 2002 (1), studies of pediatric gastroenteritis (children ≤5 y) have shown that NoV is prevalent and although usually second to RoV infection, can be the primary agent of childhood gastroenteritis (2). Recently, a large study (n = 1840) in the United States showed 8.5% of stools collected from hospitalized children were confirmed positive for a caliciviral infection, further implicating NoV as an important pediatric pathogen (3).

The NoV genus is composed of five genogroups, of which strains belonging to genogroup I (GI) and genogroup II (GII) are the predominant human pathogenic strains. Each genogroup is comprised of several genotypes and Vinjé and Koopmans have shown that strains belonging to the same genotype present more than 87 and 91% identities to each other for GI and GII strains, respectively (4). The genetic diversity is a result of both the error prone viral RNA dependent RNA polymerase and strain recombination. Kageyama *et al.* investigated the molecular epidemiology of 66 outbreaks that occurred in Japan, between 1997 and 2002 and found 31 NoV genotypes circulating during that period (5).

The Lordsdale- or Grimsby-like GII/4 NoV genotype accounts for the majority of infections globally. GII/4 viruses represented 92% of all adult associated NoV strains circulating in Ireland in 2003 and 2004 and infection was primarily associated with the healthcare setting (6). This finding was very similar to that observed in the United Kingdom where 93% of all outbreaks in 2000 occurred within the healthcare setting (1,7,8). The predominance of the GII/4 genotype is well documented and is endemic in many countries such as Ireland, the United kingdom, Spain, Netherlands, Continental Europe, New Zealand, and the United States (8–12).

The national surveillance of NoV in the Republic of Ireland is a combined virological [National Virus Reference Laboratory, NVRL] and epidemiologic [Health Protection Surveillance Centre, HPSC] surveillance program. The ''National Guidelines on the Management of Outbreaks of NoV Infection in the Healthcare Setting'' were introduced in Ireland in 2004 and state that specimens, from both adult and children, should be sent to the NVRL for viral examination, when an outbreak meets with the following amended Kaplan criteria: a) greater than 50% of patients have symptomatic vomiting, b) no bacterial or parasitic pathogen is detected (including *Clostridium difficile*), c) the average duration of illness is 12–60 h and d) the mean incubation period, from original exposure is 15–48 h and e) both staff and patients are affected (13). The NVRL is responsible for the detection and molecular characterization of NoV and the HPSC collates all national outbreak and epidemiologic data for infectious intestinal diseases. NoV became a notifiable disease in Ireland in 2004 and anonymized data collected by both institutions is shared to provide a comprehensive evaluation of NoV epidemiology, which is

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Correspondence: Allison Waters, Ph.D., National Virus Reference Laboratory, University College Dublin, Belfield, Dublin 4, Ireland; e-mail: allison.waters@ucd.ie

Abbreviations: AdV, Adenovirus; **HPSC,** Health Protection Surveillance Centre; **GI,** genogroup I; **GII,** genogroup II; **NVRL,** National Virus Reference Laboratory; **NoV,** Norovirus; **RoV,** rotavirus

further shared with European epidemiologic initiatives. However, the presence or absence of a bacterial or parasitic pathogen is not established by the NVRL.

At present, all faecal specimens received by the NVRL, from children ≤5 y of age are analyzed primarily by electron microscopy. Therefore, molecular characterization of NoV strains associated with children is rarely carried out in Ireland. However, recent molecular epidemiologic studies in both Spain and Sweden demonstrated a higher prevalence of the GII/b genotype in specimens received from children ≤15 y of age (14,15), rather than the globally predominant GII/4 NoV strain, indicating that different NoV genotypes may be associated with gastroenteritis in children.

The aims of this study were to: a) Characterize the NoV genotypes associated with viral gastroenteritis in Irish children ≤5 y of age (pediatric infection) comparing with those characterized for adult infection. b) Assess the relationship between NoV strains and the setting of infection from which they were isolated. c) Establish the relative prevalence of NoV, RoV, and AdV in children ≤5 y and evaluate the efficacy of the Kaplan criteria (13) at clinically diagnosing viral gastroenteritis outbreaks in children.

MATERIALS AND METHODS

Informed consent for this study was obtained by all authors and the study was approved by the NVRL and HPSC.

A NoV pediatric outbreak, in our study, was defined as a notified gastroenteritis outbreak that occurred in children ≤5 y of age, which was confirmed positive for NoV in the NVRL either by electron microscopy or a NoV specific reverse transcription polymerase chain reaction (RT-PCR).

Molecular characterization of NoV outbreaks that occurred between January 2006 and March 2007. Initially, we conducted a primary study that analyzed all pediatric outbreaks notified to the HPSC in the 15-mo period from January 2006 until March 2007. A total of 12 pediatric gastroenteritis outbreaks were reported, of which seven were confirmed as NoV positive by the NVRL during the study period. The number of specimens received per outbreak ranged from one to seven, and NoV RNA was detected in 29–100% of samples per outbreak. A total of 51 adult associated NoV outbreaks, notified to the HPSC, were confirmed positive for NoV RNA in the NVRL during the same 15-mo period.

Nucleic acid was extracted from faecal specimens (20% wt/vol or vol/vol suspensions) by the Roche MagNA pure LC™ automated extraction system, using the total nucleic acid isolation kit (Roche Diagnostics, Lewes, U.K.), according to the manufacturer's instructions. Adult and pediatric specimens were analyzed using a real-time RT-PCR from Kageyama *et al.* (17). A Brome Mosaic Virus was included as an internal control throughout (18).

Sequencing and phylogenetic analysis was subsequently carried out and was based on sequenced amplicons of the Orf 1/Orf 2 junction of the NoV viral genome (289 bp) as described previously by Waters *et al.* (6). A maximum likelihood phylogenetic tree was constructed using PAUP* version 4.0 Beta 10 (19) and Modeltest chose the most appropriate model of evolution using the hierarchal likelihood ratio test (20). The maximum likelihood phylogenetic tree was based on the K80 model of substitution and a gamma distribution. Bootstrap resampling was carried out for 1000 replicates of the sequence alignment using the neighbor-joining algorithm.

Relationship between NoV genotype and setting of pediatric infection. As a second study, the relationship between the NoV strains isolated from hospital acquired infection in adults and pediatric (children ≤5 y) specimens was evaluated. The study included NoV positive pediatric stool specimens, received from hospitalized children (≤5 yrs) with a suspected viral gastroenteritis infection (n = 24/220), during the 3-mo gastroenteritis outbreak peak of September to November 2004. Pediatric specimens were analyzed by a nested multiplex RT-PCR that was equally sensitive for RoV group A, NoV (GI and GII), and AdV group F, as described by O'Neill *et al.* (21). Hospital-derived adult specimens were included in this second study (n = 145) as a comparison group and were diagnostically analyzed for the presence of NoV RNA using the JV12Y or JV13I primer set (4,22). A representative number of NoV positive specimens received from hospitalized children were chosen for further molecular characterization (33%, 8/24) and represented a cross section of the pediatric NoV positive samples both by age and geographical distribution. Adult specimens (n = 24) and pediatric specimens (n = 8) were molecularly characterized and phylogenetically analyzed as outlined above. The GenBank accession numbers for sequences detected in our pediatric specimens are as follows: EU392248–EU392259.

Symptoms and viral agents associated with pediatric infection. The prevalence of AdV, RoV, and NoV in pediatric stool specimens (n = 309), received during the 3-mo period of September to November 2004 was determined using a multiplex RT-PCR. A proportion of these specimens (n = 89/309) had been previously tested at the requesting hospital for RoV and AdV using latex agglutination tests and only those with negative or equivocal results were submitted to the NVRL for further investigation. These specimens were removed from the analysis to alleviate any negative bias created by the pretested specimens. Therefore, the relative prevalence of AdV, RoV, and NoV was determined in 220/309 of specimens. Symptoms of infection were reported for 202/309 of the pediatric patients and were categorized as either diarrhea, or vomiting alone or vomiting with diarrhea.

RESULTS

Molecular characterization of NoV outbreaks that occurred between January 2006 and March 2007. Our primary aim, in this study, was to investigate the NoV genotypes associated with pediatric gastroenteritis. During the 15-mo period of January 2006 to March 2007, seven pediatric NoV outbreaks were laboratory confirmed and notified to the HPSC. Six outbreaks originated in crèches or the community setting, and a variety of NoV genotypes, including GII/2 (1/6), GII/4 (2/6), GII/6 (1/6), GII/b (1/6), and GII/14 (1/6) (Fig. 1) were detected. One outbreak was reported in a children's hospital ward and was caused by a GII/b NoV strain (07M

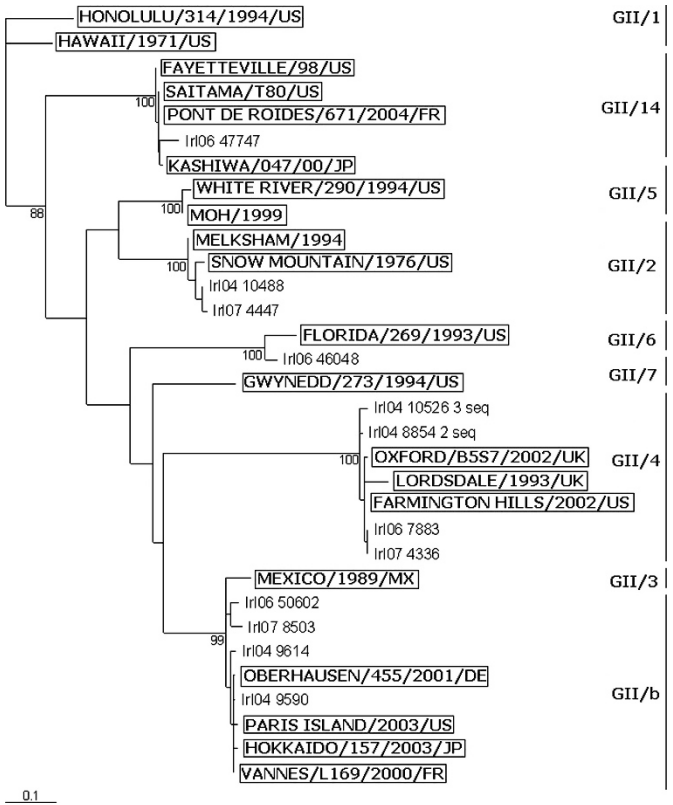

Figure 1. Maximum likelihood phylogenetic tree of NoV sequences amplified from specimens received from children ≤5 y during September to November 2004 and January 2006 to March 2007. The GenBank accession numbers for sequences detected in our pediatric specimens are as follows: EU392248–EU392259.

Table 1. *Pediatric and adult NoV outbreaks notified and laboratory confirmed in Ireland during January 2006 and March 2007. The number of characterized outbreaks, genotypes and the setting of infection are listed*

	Adult NoV infection outbreaks			Pediatric NoV infection outbreaks		
	No.	Genotype	Setting	No.	Genotype	Setting
Healthcare	43	GII/4	Hospital, residential care home	1	GII/b	Hospital
Others	8	GII/4	Restaurant, hotel, coach tour	6	GII/2, GII/4, GII/6, GII/b, GII/14	Crèche
Total	51		7			

8503, Fig. 1). Adult associated NoV outbreak strains, characterized during the same study period ($n = 51$), all clustered as the dominant GII/4 NoV genotype. The majority of adult specimens were derived from the hospital setting (84.3%, 43/51 specimens). However, community-based NoV outbreaks (15.7%, 8/51) were associated with restaurants, hotels, and coach tours (Table 1). No GI strains were detected in either specimen population.

The relationship between NoV genotype and setting of pediatric infection. Hospital-derived specimens, received from both pediatric and adult patients during the gastroenteritis outbreak peak of September 2004 to November 2004, were retrospectively analyzed in a second study. Three different genotypes were detected in these pediatric specimens ($n = 8$) and were characterized as GII/2 (1/8), GII/4 (5/8), and GII/b (2/8) (Fig. 1). The GII/4 NoV strain was detected in all adult specimens analyzed during the same period (24/24). As before no GI strains were detected in either the adult or pediatric specimens.

Symptoms and viral agents associated with pediatric infection. To assess the burden that NoV places on the Irish pediatric population, relative prevalence of three major viral agents of gastroenteritis, NoV, RoV group A, and AdV group F, was determined in specimens received from hospitalized children with diarrhea, vomiting or a suspected viral gastroenteritis infection, between September and November 2004 ($n = 309$). Some specimens ($n = 89$) had been previously tested at the source hospital for RoV and AdV by a latex agglutination test, and were removed from the analysis. Overall, AdV was the most prevalent agent and was detected in 11.3% (25/220) of specimens. NoV and RoV were present in 10.9% (24/220) and 5.5% (12/220) of specimens, respectively. The age-adjusted prevalence of each virus determined that AdV was the most prevalent agent of gastroenteritis in children of 0–4 mo. However, in all other age groups (4 mo–5 y) NoV was the most common cause of pediatric gastroenteritis (Fig. 2).

The symptoms of intestinal infection were recorded for 67% (202/309) of pediatric samples received during the 3-mo period of September to November 2004. These were as follows: diarrhea only (95/202), vomiting only (47/202), and vomiting with diarrhea (60/202). Therefore, diarrhea was the most common symptom reported by children ≤5 y of age suffering from gastroenteritis.

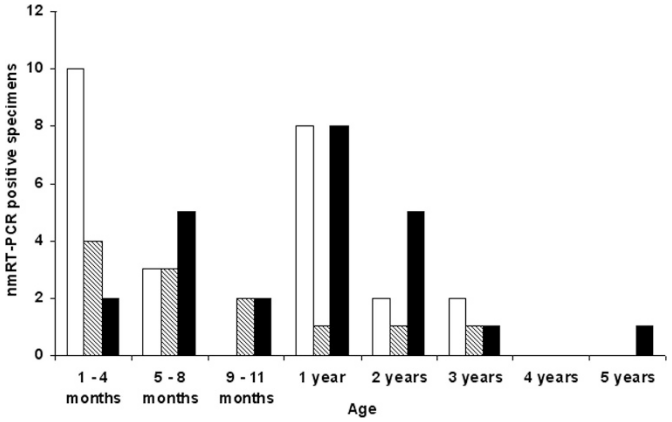

Figure 2. Age-adjusted distribution of norovirus, group A rotavirus and group F adenovirus infections detected in pediatric specimens received by the NVRL between September and November 2004. The figure key is as follows: NoV, *black bar*; AdV, *white bar*, RoV, *lined bar*.

DISCUSSION

In this article, we compared and contrasted the diversity of NoV genotypes detected in a pediatric and adult population in Ireland. From September to November 2004 and during the 15-mo period of January 2006 to March 2007, pediatric infection (children ≤5 y) was characterized by five different NoV genotypes (GII/2, GII/4, GII/6, GII/b, and GII/14). However, phylogenetic analysis determined that a range of genetically similar GII/4 viruses caused all adult infections during the same period. The GII/b strain was not detected in any specimens received from adults and this is in accordance with studies that observed the GII/b genotype was detected in a higher proportion of pediatric patients ≤15 y of age (14,15). The NoV genotypic difference between adults and children may be due to a number of influencing factors such as the setting of infection, age of the patient, susceptibility to infection, nonuniform sampling, viral virulence factors, and immunity.

Our initial study demonstrated that different NoV genotypes characterized gastroenteritis outbreaks in Irish adults and children over the same time period. However, the predominance of the GII/4 within adults may be overestimated due to increased sampling within the hospital setting. Therefore, we sought to determine whether the difference in NoV genotypes, characterized for adult and pediatric infection, was due to the setting in which the NoV outbreak occurred, *i.e.*, community or hospital-based infection. Community-based infection is usually characterized by a wide variety of NoV genotypes (9), whereas outbreaks within the hospital setting are primarily caused by the dominant GII/4 NoV genotype (7). When the two studies are analyzed together, it can be concluded that NoV infection in Irish children ≤5 y is characterized by a different set of NoV genotypes compared with those associated with adult NoV and this difference is evident regardless of whether the outbreak originates in the hospital or commu-

nity setting. It is particularly interesting that no GI strains were identified throughout the whole study. This is indicative of the molecular epidemiology of NoV in Ireland, where it has been shown previously that GII viruses predominate (6).

The DIVINE-NET project of the European Foodborne Virus Network (http://www.eufoodborneviruses.co.uk), recently published NoV epidemiologic data on the 13 participating European countries, including Ireland, for the period of June 2005 to June 2006. This collated European data concluded that 69% of all NoV outbreaks occurred within the healthcare setting and the GII/4 virus strain was implicated in 90% of these outbreaks [EFVN, 2006, Prevention of emerging (foodborne) enteric viral infections: diagnoses, viability testing, networking and epidemiology. second annual progress report]. This is consistent with NoV infection in Ireland (6,10,11). However, an inherent sampling bias exists within the Irish adult population as the majority of specimens received are from hospitals and long-stay residential care facilities and it is widely recognized that gastroenteritis is underestimated in the community setting. A telephone survey of infectious intestinal disease in Ireland estimated that the rate of acute gastroenteritis was 0.60 episodes per person per year and that a general practitioner was consulted by 29.2% of affected individuals only (23). Under-investigation of community infection may contribute to the detection of a single NoV genotype associated with Irish adults, as opposed to the variety of genotypes detected in our pediatric specimens, and a true reflection of both healthcare-derived and community-based NoV infection in adults remains difficult to test. As the NoV epidemiology of Irish adults is similar to that reported for most of Europe, it is likely that the diversity of NoV strains characterizing pediatric infection may be found throughout Europe also.

Pediatric gastroenteritis places a considerable disease burden on children of the developed countries and we describe for the first time the relative prevalence of three major agents of pediatric viral gastroenteritis in an Irish symptomatic pediatric group. NoV was identified as a major cause of childhood gastroenteritis in children $\geq$4 mo of age and the current national pediatric specimen diagnostic algorithm must evolve to ensure that NoV is considered as a major childhood pathogen. The low level of RoV detected in our sample population may be due to the RoV season in Ireland predominantly occurring in March or April, which is outside the study period.

Enteric AdV infection is not a notifiable disease in Ireland and national incidence figures are unavailable, it is therefore likely that AdV is probably underestimated as a cause of pediatric gastroenteritis. AdV was the most prevalent agent of gastroenteritis in children from 0 to 4 mo of age, demonstrating AdV as a significant pediatric pathogen and AdV specific maternal antibodies are not protecting neonates against infection. A comparable study in Italy, analyzed stools from 215 hospitalized children with symptomatic gastroenteritis from January to December 2003. RoV, NoV, and AdV were present in 25.1, 18.6, and 6% of specimens, respectively. This study further implicates all three viruses as major childhood pathogens (24).

At present, the amended Kaplan criteria, outlined in the national NoV guidelines (13, HPSC 2003) (http://www.hpsc.ie/hpsc/A-Z/Gastroenteric/Norovirus/Publications/File,2109,en.pdf) are used to clinically diagnose a viral gastroenteritis outbreak. Our results indicate that pediatric patients do not always meet these criteria, as diarrhea was reported as the most common symptom of infection. Recent reports on NoV infection in adults are describing more prolonged diarrhea resulting from NoV infection (25,26). This shift in the most distinguishing symptom of viral gastroenteritis may correlate with strain type, setting of infection or other unknown criteria. A recent study in the United States reviewed 4050 gastroenteritis outbreaks, reported between 1998 and 2000, and examined the ability of the different clinical and epidemiologic profiles to discriminate between outbreaks of viral gastroenteritis and those because of bacterial agents. The study concluded that until more sensitive and broader spectrum NoV diagnostic tests become widely available, the Kaplan criteria remain the most useful and discriminating diagnostic test of outbreaks of viral associated gastroenteritis (27).

In conclusion, our study has demonstrated that NoV is an important childhood pathogen and that within both, the community and hospital setting, NoV infection in children $\leq$5 y is characterized by a variety of different genotypes. This is in contrast to the NoV strains associated with Irish adults, where the GII/4 genotype predominates. We have highlighted significant reporting of diarrhea as the most common symptom of pediatric viral gastroenteritis. This may impact at the initial clinical diagnostic stage and raises questions regarding the efficacy of the Kaplan criteria at diagnosing outbreaks of viral gastroenteritis in children. In addition, the diversity of genotypes in children $\leq$5 y suggests that a multivalent approach is necessary for NoV vaccine development.

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