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Non-polio enteroviruses and their association with acute diarrhea in children in India

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ABSTRACT

A causative agent in approximately 40% of diarrheal cases still remains unidentified. Though many enteroviruses (EVs) are transmitted through fecal-oral route and replicate in the intestinal cells, their association with acute diarrhea has not so far been recognized due to lack of detailed epidemiological investigations. This long-term, detailed molecular epidemiological study aims to conclusively determine the association of non-polio enteroviruses (NPEVs) with acute diarrhea in comaparison with rotavirus (RV) in children. Diarrheal stool specimens from 2161 children aged 0–2 years and 169 children between 2 and 9 years, and 1800 normal stool samples from age-matched healthy children between 0 and 9 years were examined during 2008–2012 for enterovirus (oral polio vaccine strains (OPVs) and NPEVs). Enterovirus serotypes were identified by complete VP1 gene sequence analysis. Enterovirus and rotavirus were detected in 19.01% (380/2330) and 13.82% (322/2330) diarrheal stools. During the study period, annual prevalence of EV- and RV-associated diarrhea ranged between 8% and 22%, but with contrasting seasonal prevalence with RV predominating during winter months and NPEV prevailing in other seasons. NPEVs are associated with epidemics-like outbreaks during which they are detected in up to 50% of diarrheic children, and in non-epidemic seasons in 0–10% of the patients. After subtraction of OPV-positive diarrheal cases (1.81%), while NPEVs are associated with about 17% of acute diarrhea, about 6% of healthy children showed asymptomatic NPEV excretion. Of 37 NPEV serotypes detected in diarrheal children, seven echovirus types 1, 7, 11, 13, 14, 30 and 33 are frequently observed, with E11 being more prevalent followed by E30. In conclusion, NPEVs are significantly associated with acute diarrhea, and NPEVs and rotavirus exhibit contrasting seasonal predominance. This study signifies the need for a new direction of research on enteroviruses involving systematic analysis of their contribution to diarrheal burden.

1. Introduction

Diarrheal diseases are a major cause of morbidity and mortality among infants, young children and the elderly. Acute gastroenteritis is the third leading cause of death in children <5 years of age, especially in developing countries, accounting for approximately 1.5 billion episodes and 1.5–2.5 million deaths annually worldwide (Parashar et al., 2003a; World Health Report, 2004; O'Ryan et al., 2005). Almost every child suffers several episodes of diarrhea during the first 7 years of life. A variety of agents, viral, bacterial, protozoan as well as noninfectious agents cause acute diarrhea. Among the viral agents, rotavirus, calicivirus, astrovirus and adenovirus account for significant burden of the disease (Clark and McKendrick, 2004; Dennehy, 2005; Parashar et al., 2003b). Torovirus (Jamieson et al., 1998), bocavirus (Jin et al., 2011), picobirnavirus (Gallimore et al., 1995) and a few picornaviruses (Harada et al., 2009; Harvala and Simmonds, 2009; Holtz et al., 2008, 2009; Kapoor et al., 2008; Nyangao et al., 2006; Patel et al., 1985; Patil et al., 2009; Phan et al., 2005; Rai et al., 2007; Scarcella et al., 2009; Silva et al., 2008; Yamashita et al., 1993) have also been recently reported from patients with diarrhea. Rotavirus (RV) accounts for about 20–50% of diarrheal infections in winter months, and >450,000 deaths annually (Parashar et al., 2003b). In spite of the identification of several gastroenteritis agents, approximately 30–40% of diarrheal cases are estimated to be of unknown

Abbreviations: EV, enterovirus; E, echovirus; CV-A, coxsackievirus A; CV-B, coxsackievirus B; AFP, acute flaccid paralysis; NP-AFP, non-polio-acute flaccid paralysis; RV, rotavirus; DEC, diarrheagenic *E. coli*; OPVs, oral polio vaccine strains; NPEVs, nonpolio enteroviruses (coxsackieviruses, echoviruses and newer enteroviruses); RD, rhabdomyosarcoma; HFMD, hand-foot-and-mouth disease; PAGE, polyacrylamide gel electrophoresis; RT-PCR, reverse transcription-polymerase chain reaction.

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etiology (Denno et al., 2007; Greniger et al., 2009; Holtz et al., 2008, 2009).

Enteroviruses, belonging to the family *Picornaviridae*, are associated with several acute and chronic diseases in humans (Pallansch and Roos, 2007). Majority of the enteroviral infections are considered to be asymptomatic, with about 1.0% of them leading to severe disease with high mortality and morbidity primarily in infants and young children (Pallansch and Roos, 2007). However, this predicted low rate of attack resulting in clinical illness is primarily based on known diseases such as acute flaccid paralysis (AFP), meningitis etc., and does not take into account acute diarrhea and other gastrointestinal symptoms which are not yet recognized to be associated with non-polio enterovirus (NPEV) infections. Though the genus enterovirus contains seven human enterovirus (HEV) species (HEV-A to D) and human rhinovirus (HRV-A to C) representing a large number of serotypes among echovirus (E), coxsackievirus A (CV-A) and B (CV-B), poliovirus and rhinovirus (Picornavirus Home, 2008) and constitute a major public health concern, this study deals only with non-HRV enteroviruses.

Enteroviruses are primarily transmitted through fecal-oral and nasopharyngeal routes and have been reported in a few sporadic diarrheal cases and epidemiological studies (Harada et al., 2009; Patel et al., 1985; Patil et al., 2009; Phan et al., 2005; Nyangao et al., 2006; Rai et al., 2007; Scarcella et al., 2009; Silva et al., 2008), but their association with the disease has not been well-grounded due to lack of detailed investigations. Further, in great majority of the epidemiological studies on acute diarrhea, NPEVs remained unexamined.

Since the etiology of approximately 40% diarrheal cases is unknown, this long-term molecular epidemiological study spanning more than four years from 2008-to-date was undertaken to conclusively determine the association of NPEVs with acute diarrhea, burden of the NPEV-associated disease in children and identify NPEV serotypes frequently associated with the disease. This study demonstrates for the first time that NPEV association with acute diarrhea is highly significant, and that RV and NPEV exhibit contrasting seasonal incidence.

2. Material and methods

2.1. Definitions

An episode of acute diarrhea is defined as 3 or more watery stools per day. An infection episode is considered as asymptomatic if the child did not have diarrhea and passed 1 or 2 normal stools per day during the week before and after the detection of the virus in stool, and that as symptomatic if the virus is detected in a stool during the diarrheal episode and/or during the 48 h period before or after the diarrheal episode. A new diarrheal episode is considered if the first episode ended at least two days prior to the onset of the second episode.

2.2. Diarrheal stool samples

A total of 2330 fecal specimens from 2028 children with acute diarrhea in the age group 0 to 9 years, with 70 samples collected from 30 children during more than one diarrheal episode, reporting as outpatients, and 232 admitted to hospitals and private clinics in four different widely separated (3–12 km) regions in Bangalore, India from January 2008 to October 2012 were examined for enterovirus and rotavirus. Of the total patients, 2161 are in the age group of 0–2 years and 169 are between 2 and 9 years. Four stool samples from adult volunteers (18–25 years) with acute diarrhea were also examined for enterovirus, rotavirus and diarrheagenic *Escherichia coli*. All children with acute gastroenteritis are enrolled and followed until the end of the episode. Samples were collected on alternate days during an acute diarrheal episode, but only one sample is counted to represent the episode. Supernatants of 10% suspension of stool specimens in PBS, post centrifugation, were stored at −80 °C until further use. Stool specimens from diarrheal patients were collected at M.S. Ramaiah Hospital, K. C. General Hospital, R. M. V. Hospital, Mamatha Hospital, Agadi Hospital, Arpita Clinic, Vani Vilas Hospital or patient's residence.

2.3. Healthy children

In India, almost every child receives OPV on the average 6–7 times during the first 2 years of life. During our recent study on NPEVs in AFP (Rao et al., 2012), examination of 750 fecal samples selected from among 2700 total samples collected every 14 days during 2008–2010 from a follow-up cohort of 110 children, aged 0–2 years, revealed that a significant number of OPV recipients excreted the vaccine strains for about 2 weeks and even up to a few weeks, though majority of the children excreted OPV strains for less than a week. If OPV recipients and children with diarrhea and other clinical symptoms are not excluded, about 36% (37/104) of randomly selected stool samples in 2008–2009 became positive for enterovirus by reverse transcription (RT)-PCR for VP1 and by culture in rhabdomyosarcoma (RD) cells. Sequence analysis of VP1gene from 36 of these RT-PCR-positive isolates from children without prior knowledge on OPV administration or status of healthiness, revealed that 28/36 isolates represented OPV strains accounting for about 78% of the total isolates, and non-polio enteroviruses (NPEVs) for about 22%. The percent positivity of NPEVs widely varied during winter and non-winter months.

Because of the intensive OPV vaccination programme being implemented in India, we have excluded OPV recipients for 14 days post-vaccination from being considered as healthy subjects as the data based on only RT-PCR and cell culture, in the absence of VP1 sequence information, lead to erroneous interpretation on the incidence of NPEV infections in healthy children. OPV administration schedules of all the children who participated in the study have been maintained by the pediatricians and in the laboratory which was possible only with the follow-up cohort. Because of the extensive polio vaccination and endemic circulation of OPV strains, it becomes necessary to screen at least 4 times the number of diarrheic children to select the OPV-negative healthy controls which was not practical under the conditions in India.

By having a complete knowledge on OPV vaccination and health status of children, which is difficult to have from those reporting as outpatients, in this study, we selected 1700 stool samples from a follow-up cohort of 152 children, 0–2 years of age, recruited over 4 years from 2008 to 2011, and samples collected during 2008–2012 when they were considered healthy by the pediatricians, amounting to approximately 5–6 samples/child in a year spaced evenly during the follow-up period. We have also examined stool specimens from 100 age-matched healthy children between 3 and 9 years. Thus a total of 1800 stool samples from healthy children were examined by culture in RD cells of which 300 randomly selected samples were also analyzed by RT-PCR using RNA isolated directly from the stool specimens.

The work was carried out after obtaining the necessary approvals from Institutional Biosafety and Ethics committees. Written informed consent for participation in the study was obtained from either the mother or father for each participating child by the pediatricians.

2.4. Exclusion criteria

Several exclusion criteria were applied for a stool sample to be considered as that of a healthy child. Stool samples collected from