Journal of Clinical Virology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

Non-polio enterovirus association with persistent diarrhea in children as revealed by a follow-up study of an Indian cohort during the first two years of life

Durga C. Rao[a,*], Harikrishna Reddy[a], K. Sudheendra[a], A. Raghavendra[a], Vembuli Varadharaj[a], Surekha Edula[a], Ramya Goparaju[a], Bharath Ratnakar[a], Arni S.R. Srinivasa Rao[b,c], Padmanabha P. Maiya[a,d,e], M. Ananda Babu[a,f,g]

[a] Department of Microbiology & Cell Biology, Indian Institute of Science, Bangalore, India
[b] Indian Statistical Institute, Kolkata, India
[c] Georgia Regents University, Augusta, GA, USA
[d] MS Ramaiah Teaching Hospital, Bangalore, India
[e] Agadi Hospital, Bangalore, India
[f] RMV Hospital, Bangalore, India
[g] Arpita Clinic, Bangalore, India

ARTICLE INFO

Article history:
Received 7 March 2014
Received in revised form 6 May 2014
Accepted 25 May 2014

Keywords:
Enterovirus (EV)
Non-polio enterovirus (NPEV)
Rotavirus
Acute diarrhea
Persistent diarrhea (PD)

ABSTRACT

Background: We recently reported significant association of non-polio enteroviruses (NPEVs) with acute diarrhea in children. Persistent diarrhea (PD) remains a major cause of morbidity and mortality in infants below two years of age in developing countries. Understanding age-dependent frequency and duration of NPEV infections is important to determine their association with persistent diarrhea and disease burden.
Objectives: A cohort of 140 infants was followed for 6 months to 2 years of age to determine the frequency, duration, and association with PD of NPEV infections in comparison with rotavirus and other agents.
Study design: Stool samples were collected every 14 days, and diarrheal episodes and their duration were recorded. Enteroviruses were characterized by RT-PCR and VP1 gene sequence analysis, rotavirus by electropherotyping, and other agents by PCR.
Results: Of 4545 samples, negative for oral polio vaccine strains, 3907 (85.96%) and 638 (14.04%) were NPEV-negative and NPEV-positive, respectively, representing 403 (8.87%) infection episodes. About 68% of NPEV infections occurred during the first year with every child having at least one episode lasting between four days and four months. Approximately 38% and 22% of total diarrheal episodes were positive for NPEV and RV, respectively. While about 18% of NPEV infection episodes were associated with diarrhea, 6% being persistent, 13% of total diarrheal episodes were persistent involving infections by monotype NPEV strains or sequential infections by multiple strains and other agents.
Conclusions: This is the first report revealing NPEVs as the single most frequently and persistently detected viral pathogen in every PD episode.

1. Background

Diarrheal diseases are a leading cause of morbidity and mortality in infants and young children, accounting for about a billion illness episodes and 1.6–2.5 million deaths annually [1–4]. However, the causative agent in approximately 40% diarrheal cases is still unknown [5,6]. Although only 3–20% of acute diarrheal episodes become persistent, persistent diarrhea (PD) contributes to approximately to one-half of total diarrhea-associated deaths in children [7–9]. Malnutrition, micronutrient deficiency, lack of breast feeding, transient impairment of cell-mediated immunity and lactose intolerance could be some of the risk factors for acute diarrhea becoming persistent [7,8,10,11], and PD often transforms marginal malnutrition to more severe forms [7]. Though a number of pathogens have been detected in some stool samples collected from children with persistent diarrhea, many of the agents have

* Corresponding author at: Department of Microbiology & Cell Biology, Indian Institute of Science, Bangalore 560012, India. Tel.: +91 80 23602149; fax: +91 80 23602697.
E-mail addresses: cdr@mcbl.iisc.ernet.in, cdrmcb@gmail.com (D.C. Rao).

http://dx.doi.org/10.1016/j.jcv.2014.05.015
1386-6532/

also been detected in children without diarrhea [10–17], suggesting a role for other or unknown factors/pathogens in the development of PD. Due to a paucity of systematic studies, information on the period of persistence of different pathogens in PD episodes is lacking. PD is often considered as a manifestation of sequential and/or simultaneous infections due to several agents during the episode [12,18].

Enteroviruses (EVs), belonging to the family *Picornaviridae*, are associated with a wide range of diseases especially in infants and young children [19,20]. Post-polio eradication, diseases caused by non-polio enteroviruses (NPEVs) are being recognized as significant public health concern in Asian countries [20–24]. A limited number of studies in India revealed significant association of NPEVs with non-polio acute flaccid paralysis, aseptic meningitis and encephalitis cases [21,23,24].

Though the majority of NPEVs are transmitted through fecal–oral route, replicate in cells of gastrointestinal system, and have been reported in a few case studies and small-scale epidemiological studies on acute diarrhea [25–37], they remained unrecognized and neglected as etiological agents of diarrhea due to lack of detailed investigations. Our recent report represents the first detailed long-term molecular epidemiological study revealing significant association of NPEVs with acute diarrhea in children in Bangalore, India, as well as an interesting and contrasting seasonal predominance between rotavirus (RV) and NPEV infections, with rotavirus diarrhea prevailing during winter months and NPEV-associated diarrhea predominating during April to October [22]. Further, detection of NPEVs in sequential samples, collected at 15-day intervals during our previous study on rotavirus diarrhea [38], from two rotavirus-negative children suffering from PD for more than 2 months suggested their association with the disease.

To our knowledge, no long-term follow-up study exists either on age-dependent incidence of NPEV infections or their association with persistent diarrhea in children during the initial two years after birth. Many epidemiological studies on diarrhea either did not examine NPEVs, or failed to detect them in significant numbers in rotavirus-centric studies focused during winter months. The majority of persistent diarrheal episodes occur during the first two years of life [3,18,39]. Since a systematic analysis of frequency of NPEV infections and their association with persistent diarrhea from children randomly reporting to pediatricians is difficult, we sought to address the problem through a detailed follow-up study of a cohort of infants from birth to two years of age.

2. Objectives

A cohort of 140 children was followed for 6 months to 2 years from birth to determine the age-dependent frequency of NPEV infections, duration of the episodes and their association with PD and acute diarrhea in the community setting in comparison to infections involving rotavirus and other agents through analysis of sequentially-collected stool samples.

3. Study design

3.1. Definitions

Acute diarrhea is defined as 3 or more watery stools per day, with majority of episodes lasting for 1–7 days but some extending for up to 14 days [3,18,39]. Diarrhea extending for more than 14 days is considered as persistent. PD is an episode of diarrhea of infectious etiology often involving sequential infection by more than one agent, with or without a gap of less than two days between successive infections [3,18,39].

A non-diarrheal infection episode is one that did not result in diarrhea and the child passed 1 or more normal stools per day during the week before and after detection of virus in stool, and a diarrheal episode is that in which virus is observed in stool during the episode and/or the 48-h period before or after the episode. A diarrheal episode is considered new if the first one ended at least two days before onset of the second episode.

3.2. The cohort of follow-up study and stool samples

Infants born in hospitals and clinics were recruited from day one of birth during 2009 and 2010 with written and signed consent from either of the parents. All the children were normal at birth and no underlying condition such as immunodeficiency, malnutrition etc. was observed during the study period. A major criterion for selection was parental commitment to participate in the long-term study. Although 152 infants were recruited, 12 children dropped out within 2–4 months and were not considered for analysis. Due to further unexpected dropout, the follow-up period varied between six months and two years among the remaining 140 infants. Mothers recorded the frequency and type of stools (diarrheal or non-diarrheal) daily, and collected in sterile containers every 14 days. Samples were also collected on alternate days during diarrheal and non-diarrheal NPEV episodes to determine their duration. But these repeat samples were excluded from total sample count per child or infection episode. Children were regularly monitored by the pediatricians. R.M.V. Hospital, M.S. Ramaiah Teaching Hospital, Agadi Hospital and Arpita Clinic served as nodal centers for the study.

3.3. Detection of enterovirus, rotavirus and other agents

Enterovirus in stool specimens was detected by growth in Rhabdomyosarcoma and HeLa cells and reverse transcription-PCR of complete VP1 gene as described [21,22,40]. All NPEV- and RV-negative diarrheal samples were examined for Aichi/Kobuvirus, Klassevirus, cardiovirus and H-cosavirus as described [21,22].

Rotavirus was detected by native polyacrylamide gel electrophoresis of viral double-stranded RNA segments [41,42], which was as efficient as VP6-RT-PCR [43]. Norovirus and sapovirus were examined by RT-PCR [44] and diarrheagenic *Escherichia coli* (DEC) by PCR as previously described [45]. Details of methods, primers and GenBank accession numbers are given in Supplementary Material.

3.4. The oral polio vaccine (OPV)-period, and exclusion criteria for NPEV detection

Since a significant number of OPV recipients excreted vaccine strains for up to 14 days [22], the 14-day period post-vaccination was considered as OPV-period, and only 100 random samples collected during this period were examined by VP1 RT-PCR and sequence analysis to reduce the burden of sequencing a large number of OPV isolates. A record of OPV-administered dates was maintained by the pediatricians. Since the goal of this study is to determine the incidence and burden of NPEV-associated PD, OPV-associated events were not consistently monitored.

3.5. Serotype analysis of NPEVs

NPEV serotypes were identified by phylogenetic analysis of VP1 sequences (Macrogen, Korea) as described [21,22,40,46]. A list of VP1 accession numbers of diarrheal and non-diarrheal NPEV isolates are provided in Supplementary material.

Please cite this article in press as: Rao DC, et al. Non-polio enterovirus association with persistent diarrhea in children as revealed by a follow-up study of an Indian cohort during the first two years of life. J Clin Virol (2014), http://dx.doi.org/10.1016/j.jcv.2014.05.015