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Research paper

Clinico-epidemiology and genetic diversity of Salivirus in acute gastroenteritis cases from Pune, Western India: 2007–2011

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A B S T R A C T

Acute gastroenteritis is a leading cause of mortality in children from developing countries. Recently, Salivirus has been frequently detected in acute gastroenteritis patients, suggesting its possible aetiological role. Conflicting reports available on disease association of Salivirus have made it difficult to ascertain their causative role. The overall epidemiology and clinical features of Salivirus infections are poorly understood. The present five year study was undertaken to investigate the presence and genetic diversity of Salivirus in acute gastroenteritis cases from Pune, Western India and to determine the clinico-epidemiological features of Salivirus infections. A total of 985 faecal samples (778 acute gastroenteritis and 207 asymptomatic controls), collected from three local hospitals (Jan2007–Dec2011) were examined for the presence of Salivirus by RT-PCR. Molecular characterization was performed by PCR amplification of the 3D and VP regions. Frequency of Salivirus detection in cases (2.6%) and controls (1.93%) was not significantly different ($p = 0.57$). Co-infection with other enteric viruses was seen in 50% of the cases. Comparison of clinical features between Salivirus mono and mixed infections revealed that Salivirus alone did not exacerbate gastroenteritis. The frequency of diarrhoea and overall clinical severity of mixed infections was significantly greater than mono infections ($p = 0.02$). Based on clinical findings, our study suggests that Salivirus does not cause severe gastroenteritis. Phylogenetic analysis indicated that study strains belonged to Salivirus A1 and formed 2 distinct clusters which shared nucleotide identities of 94.1–96.2% and 88.9–93.8% between themselves in 3D and VP regions, respectively. Interestingly, the more divergent Cluster2 strains shared a low nucleotide identity with the closest reference strain in both regions (~95% in 3D and ~92% in VP) suggesting that they could represent a variant type of Salivirus A1. The genetic diversity in strains detected from study region, emphasizes the need for Salivirus surveillance from other regions of India.

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1. Introduction

Diarrhoea, the primary symptom of acute gastroenteritis, is the second leading cause of death in South East Asian and African countries (Wardlaw et al., 2010). Approximately 70% cases of acute gastroenteritis are caused due to viruses (Chow et al., 2010). The aetiology of acute gastroenteritis with respect to rotavirus (RV), Caliciviruses (norovirus: NoV and sapovirus: SaV), enteric adenovirus (AdV) and astrovirus (AstV) has been well established worldwide (Oude Munnink and van der Hoek, 2016). Despite this, ~40% of the cases still remain undiagnosed for any causative agent (Knox et al., 2012). Several picornaviruses such as enterovirus, parechovirus, cosavirus, saffoldvirus and salivirus have been detected in faecal samples of children with acute gastroenteritis but, their causal association has not yet been clearly established (Nielsen et al., 2013; Van Der Sanden et al., 2013). Salivirus, a new member of the family *Picornaviridae*, has been detected in faecal samples of children with acute flaccid paralysis and acute gastroenteritis (Li et al., 2009). The frequent detection of Salivirus in faecal samples from diarrhoeic cases and subsequent detection in sewage suggests their possible aetiological association with acute gastroenteritis (Kitajima et al., 2014).

Like other members of the *Picornaviridae* family, *Salivirus virions* are ~30 nm in diameter enclosing a single stranded positive sense RNA genome which is ~7.4 kb in length and shows a typical picornaviral genome organization. Salivirus was first discovered as a novel virus in faecal samples from acute flaccid paralysis and gastroenteritis cases from Nigeria, USA and Nepal (Li et al., 2009). Comparison of this novel virus with other picornaviruses showed that it was most closely related to Aichivirus, a member of the *Kobuvirus* genus, and shared an amino acid identity of 42.6%, 35.2% and 44.6% in the P1, P2 and P3 region with it. Simultaneously, similar novel viruses (Klassevirus 1) sharing an amino acid identity of 38%, ~34% and ~43% in P1, P2 and P3 region with Aichivirus were detected in faecal samples from gastroenteritis cases and raw sewage samples from USA, Australia and Spain (Greninger et al., 2009; Holtz et al., 2009). As the Klassevirus 1 strains detected in these studies were observed to be ~90% identical with the previously detected Salivirus strains, they were considered as variant strains of the same species, Salivirus A (Li et al., 2009). According to the ICTV criteria, members of a new genus in the family *Picornaviridae*

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should share >40% amino acid identity in P1 and P2 region and >50% in P3 region (http://www.picornastudygroup.com/definitions/genus_definition.htm). Thus, the Salivirus A strains were classified under a new genus, Salivirus, in the family *Picornaviridae*. On the basis of variation in the capsid region, Salivirus A strains are further classified into Salivirus A1 and Salivirus A2 (Aldabbagh et al., 2015).

Salivirus has been detected in acute gastroenteritis cases at a frequency of 0.2 to 8.7% across USA, South America, Europe, Asia and Australia (Aldabbagh et al., 2015; Ng et al., 2016; Holtz et al., 2009; Santos et al., 2015). Higher prevalence rates have been reported (4–8.7%) in Asian countries such as China, Hong Kong, South Korea and Nepal (Greninger et al., 2010; Han et al., 2010; Li et al., 2009; Shan et al., 2010). In a lone study available from India, 4.2% of the children (age < 6 years) hospitalized with acute gastroenteritis from Vellore, South India were positive for Salivirus (Greninger et al., 2010). Further attempts to understand the genetic diversity of the strains detected in India have not been undertaken. Among the few available reports of Salivirus in gastroenteritis, limited number of studies have focused on finding a causal association (Li et al., 2009; Shan et al., 2010; Yu et al., 2015). Despite all the available reports, overall epidemiology and clinical features of this virus are poorly understood. Thus, the present 5 year study was undertaken to investigate the prevalence and genetic diversity of Saliviruses in acute gastroenteritis cases from Pune, Western India and to determine their clinico-epidemiological features.

2. Materials and methods

2.1. Study subjects and clinical samples

A total of 985 retrospective faecal samples collected from three local hospitals in Pune, between January 2007 and December 2011, were examined for the presence of Salivirus. Among these, 778 were collected from children hospitalized with acute gastroenteritis and 207 from asymptomatic children. The age of cases and controls ranged from 0 to 5 years however; the cases and controls were not matched for age or gender. Informed consent was obtained from parents or legal guardians of all children enrolled for the study. Case reporting forms covering demographic and clinical details were filled for all of the cases and controls. A typical case of acute gastroenteritis was defined as passage of ≥3 loose/watery stools accompanied with or without any vomiting. In the control group, faecal samples were collected from patients without any gastrointestinal symptoms. Clinical severity was assessed in accordance with the 20 point Vesikari scoring system (Ruuska and Vesikari, 1990). The study was approved by the Institutional Human Ethics committee and the Institutional Bio-safety committee of the National Institute of Virology, Pune.

2.2. Sample processing

Faecal suspensions (30% w/v or v/v) were prepared in 0.01 M PBS (pH 7.4) by centrifuging at 15,000 rpm for 10 min. The supernatants were collected and 140 μl of each suspension was subjected to nucleic acid extraction using QIAGEN viral RNA mini kit as per manufacturers' protocol (QIAGEN, Hilden, Germany).

2.3. Salivirus detection and characterization

Detection of Salivirus in faecal samples was carried out by RT-PCR targeting partial 2C region using primers as described earlier (Greninger et al., 2010). RT-PCR reaction was performed using Superscript III RT-PCR kit (Invitrogen, CA, USA) under the following cycling conditions: 50 min RT step at 50 °C, 94 °C hold for 10 min followed by 40 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 60 s. PCR products were resolved on a 2% agarose gel and visualized under the UV transilluminator. PCR amplicons of expected size (345 bp) were excised from the gel and sequenced to confirm that they were Salivirus 2C sequences. Samples found to be positive for Salivirus were further subjected to genotypic characterization by amplification of the partial 3D (~688 bp) and VP0/VP3 (~852 bp) regions using Superscript III RT-PCR kit (Invitrogen, CA, USA). The outer and inner primers used for amplification of 3D were: LG0118 and LG0117 and KL3DF and KL3DR, respectively and VP0/V3 were: LG0119 and LG0136 and KLVPF and KLVPR, respectively. Nested PCR was carried out as described elsewhere (Han et al., 2010; Holtz et al., 2009). Positive amplicons were sequenced in both directions using BigDye terminator 3.1v cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). The sequencing products were resolved using an automated sequencer 3730 XL ABI PRISM DNA Analyzer (Applied Biosystems, Foster city, CA, USA).

2.4. Nucleotide sequence alignment and phylogenetic analysis

Nucleotide sequences of study strains along with prototype and reference strains obtained from GenBank were assembled and analyzed using ClustalW progressive alignment algorithm in the MEGA 6 software. Phylogenetic trees for 3D and VP sequences were constructed using Neighbour-Joining method in the MEGA 6 software (Tamura et al., 2013). To assess the reliability of the phylogenetic tree, bootstrap analysis with 1000 replicates was used. Pair wise distances were computed by the Kimura-2 parameter method. Nucleotide sequences detected in the study have been submitted in the GenBank under accession numbers KX091866–KX091903.

2.5. Statistical analysis

Statistical association between cases and controls was tested using the χ^2 test. Observed mean values of clinical symptoms between groups were compared using Student's *t*-test. *p* value of <0.05 was considered to be statistically significant.

3. Results

3.1. Detection of Salivirus in acute gastroenteritis cases

During the 5 year study period, at least one enteric virus was detected in 46% (360/778) of the cases while 54% (418/778) of the cases remained undiagnosed for a causative agent. Salivirus was detected in faecal samples of the acute gastroenteritis cases at an overall prevalence of 2.6% (20/778). The frequency of Salivirus detection in cases of unknown aetiology (2.4%, 10/418) and previously diagnosed cases in which at least one enteric virus was detected (2.8%, 10/360) was not found to

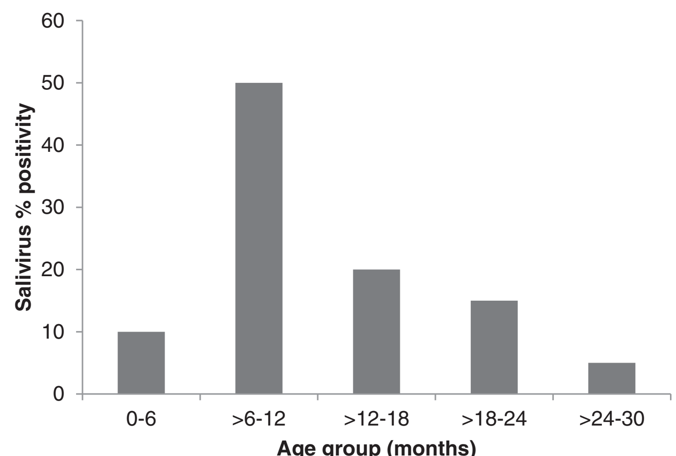

Fig. 1. Age-wise distribution of Salivirus positive acute gastroenteritis cases.

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