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Genetic diversity of cosaviruses in nonpolio acute flaccid paralysis cases of undefined etiology, Northern India, 2010–2011

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ABSTRACT

Background: No cases of wild poliovirus have been reported for more than one and a half years from India. Cases of acute flaccid paralysis (AFP) of undefined etiology continue to occur in the region. Despite the recent discovery of the human *Cosavirus* (HCoSV) in the feces of children from developing countries, there have been no studies of cosavirus infection in India.
Objectives: To detect and characterize HCoSVs in stool specimens of nonpolio AFP cases by RT-PCR followed by sequencing.
Study design: A total of 387 fecal samples collected from AFP cases in Uttar Pradesh, India, between May 2010 and April 2011, tested negative on cell culture according to WHO algorithm, were subjected to 5′-UTR region specific RT-PCR followed by sequencing to detect HCoSV. Molecular characterization of HCoSV strains was done by sequencing followed by phylogenetic analysis.
Result: 123 (32%) samples tested positive for cosaviruses and 87 (70.7%) were identified for genetic variants by sequencing a 316-nucleotide interval in the partial 5′-UTR region. Cosavirus strains were characterized as putative species HCoSV-A ($n = 70$; 82%), HCoSV-B ($n = 7$; 8%), HCoSV-C ($n = 1$; 1.1) and HCoSV-D ($n = 4$; 4.5%) while 5 (5%) strains remain uncharacterized. The cosavirus infection appeared highest (63.5%) in younger children, and showed a distinct seasonality, with a late summer peak and winter low.
Conclusion: This study demonstrates a diversity of cosavirus strains in circulation, and reports the first investigation of HCoSV infection in children with nonpolio acute flaccid paralysis in India. Currently, this study provides baseline data for further studies of HCoSV infections in children with common enteric infections in India.

1. Introduction

Acute flaccid paralysis (AFP), characterized by the rapid onset of asymmetric paralysis, can be caused by a variety of viral infections or coinfections.[1] Its worldwide occurrence makes it globally a major health concern, especially prevalent in children. AFP is a hallmark symptom of acute poliomyelitis caused by wild polioviruses (genus – *Enterovirus* (EV); family *Picornaviridae*). Beside polioviruses other picornaviruses that can cause AFP-like illness include nonpolio enteroviruses (NPEVs) and human parechoviruses (HPeVs).[2–4] Rarely flaviviruses are also found to have associated with cases of AFP.[5]

Since 1988 WHO's, efforts have contributed to a significant reduction in the global incidence of poliomyelitis.[6] Under active AFP surveillance in developing countries stool specimens from numerous AFP cases have been tested by the Global Polio Laboratory Network (GPLN) and reported. Since 1997 the detection of polioviruses in ~6.5% and NPEVs in 15–30% of AFP cases,[7] while ~68–77% of the AFP cases remained virus negative.[8–11]

Despite of the fact that no cases of wild poliovirus have been reported from India since January 2011[6]; nonpolio AFP cases of unknown etiology continue to present an important neurological presentation in polio endemic areas, are of serious concern.[12] NPEV are detected in less than half of the nonpolio AFP cases[2,3,8] whereas over half of the remaining cases of nonpolio AFP remains without a potential etiological agent.[8,13] Therefore, at this time when a new virus in nonpolio AFP has been discovered, there is a need to monitor its circulation and prevalence to elucidate their role in infections.

Abbreviations: HCoSV, human cosavirus; AFP, acute flaccid paralysis; UTR, untranslated region; NPEV's, nonpolio enteroviruses; WHO, World Health Organization; UP, Uttar Pradesh; RT-PCR, reverse-transcriptase-polymerase chain reaction; RD, human rhabdomyosarcoma cells; CPE, cytopathic effect.

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Recently a new viral group, human *Cosavirus* (HCoSV), a new genus in the family *Picornaviridae*, was recently characterized and tentatively classified into four distinct species labeled as HCoSV-A to -D.[14] HCoSV have been identified from stool samples of nonpolio AFP cases in developing countries other than India, like Pakistan, Afghanistan, Nigeria, Tunisia and Nepal.[14,15] In addition, there are also reports of cosavirus detection in raw sewage and stool samples from Australia, United Kingdom, China, Thailand and Brazil in adults as well as in children with and without diarrhea and gastroenteritis, confirms the geographical widespread occurrence of their infection.[14,16–19]

As Pakistan and Nepal are two adjoining countries, share similar socioeconomic strata as India from where the human cosavirus has been detected. Therefore, this is the foremost requirement to study these new circulating picornaviruses. All countries have nonpolio AFP of various causes, moreover, India is endemic for nonpolio AFP like illness and an active surveillance sponsored by WHO is underway; this provided us the opportunity to prospectively investigate the stool samples of nonpolio AFP cases of unknown etiology for HCoSV. Therefore, here we first time report the detection of HCoSV in children with nonpolio AFP in India.

2. Study design

2.1. Patients and clinical samples

Nonpolio AFP cases showing signs of acute onset of paralysis characterized as flaccid (reduced muscle tone) preceded by fever were considered for HCoSV analysis. 387 fecal specimens collected from Uttar Pradesh (UP), India, during May 2010–April 2011 as a part of the polio eradication program from children aged <15 years who had symptoms of AFP were analyzed for HCoSV by 5′-untranslated region (UTR) semi-nested reverse-transcriptase-polymerase chain reaction (RT-PCR) assay and sequencing.[14] All stool specimens from AFP cases considered for HCoSV screening presented negative virus isolation when inoculated in L20B and RD cell as described in WHO-prescribed protocol.[20] Mock infected cells were used as controls. Stool specimens were processed and suspensions were prepared with chloroform treatment and centrifugation as described in WHO manual.[20]

2.2. Virus detection and reverse transcription PCR assay

Stool samples considered negative for virus isolation on RD and L20B cell lines, screened for HCoSV. A total of 140 μL of centrifuged stool supernatant was filtered through a 0.45-μm filter to remove eukaryotic and bacterial-sized particles. HCoSV positive samples were also tested for the presence of enteroviruses (EV's) using pan-enterovirus (Pan-EV) primers as described in WHO manual[20] to determine mixed enterovirus infection. Co-infection was also analyzed for Aichi virus, parechovirus and klasseviruses and for cardioviruses using RT-PCR with the primers previously described.[21–24]

Viral RNA was extracted from stool suspensions (140 μL per sample) by using a Viral RNA Mini Kit (QIAamp, QIAGEN, Hilden, Germany). cDNA was synthesized using random hexamers (Fermentas, cat. no. S0142, USA) and SuperScript III reverse transcriptase (Invitrogen, cat. no. 18080-044 Carlsbad, CA, USA) in a 20-μL reaction volume following manufacturer's instructions. A nested RT-PCR targeting a 316-nucleotide interval in the 5′-UTR was used to test for HCoSV. Primers DKV-N5U-F1 and DKVN5U-R2 were used in the first round and primers DKV-N5U-F2 and DKV-N5U-R3 were used in the second round as previously described.[14] Amplification products were analyzed by electrophoresis in 2% agarose gel stained with ethidium bromide. All positive PCR products were purified by using a Gel Extraction Kit (QIAGEN Co., Hilden, Germany). Sequencing of the purified PCR products was performed in an automated sequencer (3130/3130xl Genetic Analyzer, 3730/3130xl DNA Analyzer; Applied Biosystems) with the two primers used in the second round of PCR.

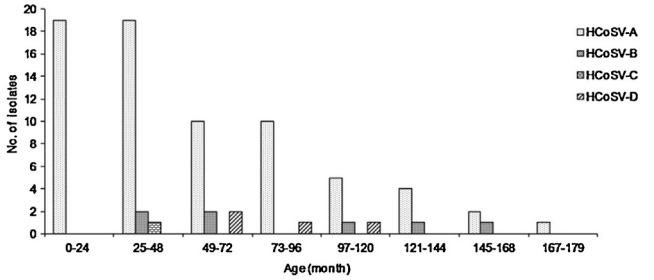

Fig. 1. Age distribution of HCoSV positive nonpolio AFP-cases.

2.3. Phylogenetic analysis

The 5′-UTR nucleotide sequences of positive strains were compared with the sequences of cosaviruses reference strains available in the NCBI GenBank database using BLAST server (http://www.ncbi.nlm.nih.gov/blast). MEGA 5.05 software[25] (http://www.megasoftware.net) was used for phylogenetic analysis. Phylogenetic tree was generated by using the neighbor-joining method and nucleotide percentage distance substitution model with bootstrap 1000 replicates.

2.4. Nucleotide sequence accession numbers

The nucleotide sequences of HCoSV strain described in the present study were deposited in the GenBank under accession numbers: JX074707–JX074736, JX313068–JX313124 and KC222026.

3. Results

3.1. Virus detection in clinical specimens

Of 387 stool samples analyzed, 123 (32%) tested positive by 5′-UTR semi-nested RT-PCR assay for human cosavirus (HCoSV) and subsequently identified for cosavirus variants by sequence analysis. The HCoSV positive samples were further checked for presence of EV's using WHO approved 5′-UTR directed RT-PCR primers. The two cosavirus positive nonpolio AFP cases shared a co-infection with EV's. The two EV-PCR positive samples with HCoSV infection were identified as HEV-B, following criteria of Oberste et al.[26] However, none of the HCoSV positive samples showed amplification with any of the primers specific for Aichi virus/kobuvirus, parechovirus, klasseviruses and cardioviruses.

3.2. Clinical findings and epidemiology

The ages of the HCoSV-positive children varied from 3 months to 14.5 years (average, 5.2 years). The highest rate of HCoSV infection (65.3%) occurred in children younger than 6 years of age (Fig. 1). The positivity of HCoSV infection decreases with increase in age. Of the 123 total cosavirus positive nonpolio AFP cases, 73 were from males and 50 were from females resulting in a sex ratio of 1.4:1.

Clinical findings showed that 14 (11%) of the 123 patients had residual paralysis, while one cosavirus positive nonpolio AFP patient (0.8%) died during the 60 days follow-up period.

However, HCoSV infection was reported throughout the year during the study period, variation in the rate of detection (Fig. 2).