Circulation of multiple enterovirus serotypes causing hand, foot and mouth disease in India

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INTRODUCTION

Hand, foot and mouth disease (HFMD), a common contagious disease that usually affects children, can be caused by enteroviruses. Coxsackievirus A16 (CV-A16) and enterovirus 71 (EV-71) are the major aetiopathological agents of HFMD. Other EV serotypes, CV-A4-7, CV-A9-10, CV-B1-3, CV-B5, E-4 and E-19, have also been found associated with both sporadic infections and outbreaks of HFMD. In India, outbreaks of HFMD have been documented; however, molecular characterization of the aetiological agents has rarely been reported. Cases of HFMD were identified during 2009–2010 on the basis of clinical features in southern and eastern parts of India. The aim of the present study was to detect and characterize the aetiological agents associated with the disease. A total of 89 specimens consisting of 41 sera, 24 vesicular fluids, 18 stools and 6 throat swabs were collected from 61 clinically diagnosed HFMD cases from southern and eastern parts of India. RT-PCR followed by sequencing of PCR amplicons and phylogenetic analyses were performed on all specimens for detection of EV RNA and identification of EV types. EV RNA was detected in 47.1% (42/89) of the specimens collected from 57.4% (35/61) of the HFMD cases. Thirty-six of 42 EV strains showed amplification of the VP1/2A junction or VP1 regions. Sequence analysis of the amplicons identified the presence of CV-A16 (54.8%), CV-A6 (38.1%), EV-71 (2.4%), CV-A10 (2.4%) and E-9 (2.4%) serotypes in the HFMD cases. The study documents CV-A16 and CV-A6 as major and CV-A10, EV-71 and E-9 as rare viral pathogens of HFMD in India.

Investigations of two outbreaks of HFMD that occurred in the Kerala and West Bengal states of India in 2003 and 2007, respectively, have been reported (Sasidharan et al., 2005; Sarma et al., 2009). These investigations identified 119 cases of HFMD on the basis of clinical examination and serological tests. No fatal cases were reported during these outbreaks. Using a molecular approach, the present study reports the investigation of EV aetiology in cases of HFMD that occurred during June to October 2009 and January to September 2010, respectively, in southern (Kerala)/eastern (West Bengal and Orissa) and southern (Tamil Nadu and Kerala) states of India.

METHODS

Clinical specimens. A total of 89 specimens consisting of 41 sera, 24 vesicular fluids, 18 stools and 6 throat swabs were collected from 61 HFMD cases identified in the Allapuzha and Pathaam thitta districts, central Kerala ($n=37$, 30 from 2009 and 7 from 2010), Kolkata, West Bengal ($n=8$, 2009), Bhubaneswar, Orissa ($n=7$, 2009) and Ooty, Tamil Nadu ($n=9$, 2010). The patients belonged to the paediatric age group, ranging from 4 months to 10 years, with a median age of 5.1 years. Multiple specimens were collected from 23 cases and at least one sample was collected from the remaining cases. Clinical features of the patients included rashes on the face, hands, feet and buttocks, with mouth ulcerations. All specimens were transported to the

Abbreviations: CV-A6, coxsackievirus A6; CV-A16, coxsackievirus A16; EV, enterovirus; EV-71, enterovirus 71; HFMD, hand, foot and mouth disease; 5’NCR, 5’ non-coding region.

The GenBank/DDBJ/EMBL accession numbers for the VP1 sequences of CV-A6 and CV-A16 are HM190268–HM190277 and HM190278–HM190298, respectively.

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National Institute of Virology, Pune, on ice (+4 °C) and stored at −20 °C until tested.

Detection of EV RNA. Viral nucleic acids were extracted from neat serum and vesicular fluid samples and from 30 % (w/v) stool samples and throat swabs suspended in 0.01 M PBS pH 7.5, using the QIAamp viral RNA mini kit (Qiagen) according to the manufacturer’s instructions. The presence of EV in clinical specimens was determined by RT-PCR using primers S1 (5’-CGGTACCTTTTGATAGCCGTGTTG-3’) and AS1 (5’-GGTTCAACACACAAGGCAC-3’), targeting 537 bp of the 5’ non-coding region (5’NCR) followed by nested PCR amplifying a 400 bp region by using primers S2 (5’-CAAGCACTCTGTTTCCCCGGAAG-3’, 166–186) and AS2 (5’-GAAACACGGACACCAAGG-3’, 566–546) as described previously (Sapkal et al., 2009). The amplicons were sequenced and sequence identity was determined by BLAST (www.ncbi.nlm.nih.gov/blast).

Molecular typing of EV. To identify the EV type in the specimens, an RT-PCR-based amplification of the VP1/2A junction region was performed by using the forward primers 011 (5’-ATGTAYTGTCATGCAG-3’) and reverse primer 011 (5’-GCICGGTGTGTCAGCGR-3’) or of the VP1 gene using primers specific for CV-A16 (CA16F, 5’-ATGTAYTGTCATGCAG-3’, 2456–2475) and CA16R (5’-GAGTGATGGTCAACACACA-3’, 2666–1685) and EV-71 [71F (5’-GGTTGGCGAGTGATGGTGAAGG-3’, 2448–2467) and 71R (5’-GTGATGTCATGGTCAACACACA-3’, 2779–2760)] as described previously (Oberste et al., 1999; Yan et al., 2001). PCR conditions involved an initial reverse transcription step of 1 h at 42 °C, followed by PCR activation at 95 °C for 15 min, 35 cycles of amplification (94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min) and a final extension of 5 min at 72 °C. The PCR products of ~434 bp (for CV-A6, CV-A10 and EV-71) followed by HEV-B (2.8 %, E-9). The amplicons were sequenced and sequence identity was determined by BLAST (www.ncbi.nlm.nih.gov/blast).

Nucleotide sequencing. PCR products were purified by using the QIAquick Gel Extraction kit (Qiagen) and both the strands were sequenced using Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) in an ABI PRISM 3130XL Genetic Analyzer (Applied Biosystems).

Phylogenetic analysis. Sequence identity was determined by BLAST (www.ncbi.nlm.nih.gov/blast). Sequences were curated from both ends and aligned by Clustal W. Phylogenetic trees were constructed using the Kimura two-parameter algorithm and neighbour-joining method using MEGA4 software, and reliability of the analysis was tested by applying bootstrap analysis with 1000 bootstrap replications (Tamura et al., 2007).

RESULTS

Circulating virus strains

Of the 89 specimens, 42 (47.1 %), which included 18 vesicular swabs, 8 sera, 2 throat swabs and 14 stool samples from 35 of 61 (57.3 %) HFMD patients, showed presence of EV RNA by RT-PCR targeted against the 5’NCR (400 bp) (Table 1). EV RNA positivity detected in patients from Kerala, West Bengal and Orissa states during 2009 was 53.3 % (16/30), 62.5 % (5/8) and 71.4 % (5/7), respectively, while it was 42.8 % (3/7) and 66.6 % (6/9), respectively, during 2010 from Kerala and Tamil Nadu states. All of the amplicons obtained in RT-PCR of a single or multiple specimens from individual patients were sequenced. BLAST analysis of the sequences indicated homology of the Kerala strains to CV-A6 (n=16), CV-A16 (n=2), CV-A10 (n=1), EV-71 (n=1), E-9 (n=1) and of the West Bengal (n=9), Orissa (n=5) and Tamil Nadu (n=7) strains to CV-A16 (n=21).

Thirty-six of the 42 (85.7 %) strains amplified in the 5’NCR also showed amplification of the VP1/2A junction region or VP1 region. Sequence analysis of the PCR products revealed the presence of CV-A16 in West Bengal (n=9, 2009), Orissa (n=5, 2009), Kerala (n=2, 2010) and Tamil Nadu (n=7, 2010) and that of CV-A6 (n=10, 2009), EV-71 (n=1, 2009), CV-A10 (n=1, 2009) and E-9 (n=1, 2009) in Kerala. Genogroupwise distribution showed predominance of HEV-A (97.2 %, CV-A16, CV-A6, CV-A10 and EV-71) followed by HEV-B (2.8 %, E-9).

Phylogenetic analysis

A phylogenetic tree constructed based on partial VP1 gene sequences of CV-A16 strains (n=23) of the present study grouped separately from those available in GenBank (Fig. 1). However, three distinct subclusters indicating 2.9–4.7 % nucleotide divergence were identified for the strains from West Bengal/Orissa, Kerala and Tamil Nadu. The nucleotide identities exhibited by these strains were highest (94.9–97.1 %) with a Malaysian CV-A16 strain (AM292476) and lowest (59.9–61.3 %) with an EV-71 (BrCr, U22521) strain.

Table 1. Distribution of positivity to EV RNA in HFMD patients by state and specimen type

<table>
<thead>
<tr>
<th>State (no. of cases)</th>
<th>Vesicular fluid</th>
<th>Serum</th>
<th>Throat swabs</th>
<th>Stool</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kerala (n=37)</td>
<td>7/10 (70%)</td>
<td>1/22 (4.5%)</td>
<td>NA</td>
<td>13/16 (81.25%)</td>
<td>21/48 (43.75%)</td>
</tr>
<tr>
<td>Orissa (n=7)</td>
<td>NA</td>
<td>5/7 (71.4 %)</td>
<td>NA</td>
<td>NA</td>
<td>5/7 (71.4 %)</td>
</tr>
<tr>
<td>West Bengal (n=8)</td>
<td>5/7 (71.4 %)</td>
<td>1/3 (33.3 %)</td>
<td>2/6 (33.3 %)</td>
<td>1/2 (50 %)</td>
<td>9/18 (50 %)</td>
</tr>
<tr>
<td>Tamil Nadu (n=9)</td>
<td>6/7 (85.7 %)</td>
<td>1/9 (11.1 %)</td>
<td>NA</td>
<td>NA</td>
<td>7/16 (43.7 %)</td>
</tr>
<tr>
<td>Total (n=61)</td>
<td>18/24 (75 %)</td>
<td>8/41 (19.5 %)</td>
<td>2/6 (33.3 %)</td>
<td>14/18 (77.7 %)</td>
<td>42/89 (47.1 %)</td>
</tr>
</tbody>
</table>

NA, Specimens not available.
Phylogenetic analysis of the VP1/2A junction region (420 bp) of 10 CV-A6 strains showed clustering with two Japanese CV-A6 strains (AB162726 and AB114111) with 94.8–95.7% nucleotide identity. With CV-A16 (G-10, U05876) and EV-71 (BrCr, U22521) strains, the nucleotide identities were found to be 60.7–61.2% and 59.3–60.2% respectively (Fig. 2).
Comparative analysis of the VP1 gene sequence of the EV-71 strain detected in only one of the samples showed higher (94.0–94.4%) nucleotide identity with Australian strains (AF135946, AY722909) than with the BrCr strain isolated from an encephalitis case in California and strain R13223-IND-01 recovered from a case of acute flaccid paralysis in India (82.4–83.2%). With G-10 of CV-A16 type and Gdula of CV-A6 type strains, the nucleotide identities were 57.2% and 51% only, respectively. An E-9 strain identified in the study showed 94.4% and 75.6% nucleotide identity respectively with the E-9 strains (AM711104 and AM711072) isolated from meningitis cases in France and E-9 strain-Hill (X84981) from the USA in the VP1/2A junction region. A CV-A10 strain identified in the study showed 95.1% nucleotide identity with strain AY956577 from Germany.
DISCUSSION

Aetiology of multiple enterovirus serotypes has been reported for HFMD worldwide (Cherry, 1992; Chang et al., 2010). Serotypic identification and classification of EV rely on virus isolation in cell culture. In India, the role of enteroviruses in causing HFMD is not very clear on account of lack of accurate diagnosis, as most of the cases reported earlier were diagnosed on the basis of clinical signs compatible with HFMD (Sarma et al., 2009). A single study conducted to investigate an outbreak of HFMD in Calicut, Kerala, identified association of EV-71 by an immunological approach (Sasidharan et al., 2005). In the present study, use of sensitive molecular methods, RT-PCR and sequencing targeted against conserved and variable regions of the EV genome revealed the presence of significant EV RNA positivity as well as multiple types in the clinical specimens.

To date, EV-71 and/or CV-A16 have been described as predominant causative serotypes of HFMD all over the world (Ang et al., 2009; Hosoya et al., 2007; Solomon et al., 2010) while infections with CV-A6 and CV-A10 have been mainly detected in Finland (Blomqvist et al., 2010). China and Singapore have reported circulation of multiple EV serotypes along with coinfection in HFMD cases (Shah et al., 2003; De et al., 2011). Interestingly, the present study demonstrated association of CV-A16 and CV-A6 as major and CV-A10, EV-71 and E-9 as rare pathogens of HFMD in India. The virus strains detected in different specimens from the same patient represented the same type, with no co-infections. Of the 16 strains from Kerala identified as CV-A6 on the basis of the 5'NCR, only 10 could be typed using primers specific to the VP1 region. The failure of amplification of typing regions in six specimens may have been due to a low viral load. It may be noted that the analysis of data obtained in this study was limited due to the small size of samples available from both years, 2009 and 2010. Despite these constraints, the yearwise distribution of EV serotypes in Kerala may suggest cyclic predominance of CV-A6 and CV-A16. However, in order to ascertain this pattern continued surveillance is necessary.

The majority of the HFMD patients of the present study were school children, aged ≤10 years. This could be due to the lack of immune response of this population resulting from the absence of previous exposure to the circulating EV types or recent introduction of causative agents in the environment from a distant source. EV infections have been described to be associated with climatic conditions, especially temperature (Urashima et al., 2003). The high humidity (>60%) and high average temperature (>28 °C) in the southern and eastern states during the study period might have contributed to the occurrence of HFMD infections. Recently, nail shedding has been reported in HFMD patients within 1–2 months after the onset of disease (Osterback et al., 2009; Davia et al., 2011). However, in the present study this observation could not be made as the patients were from remote rural areas, and no complaints were received from the parents.

In summary, the present investigation highlights the co-circulation of CV-A16, CV-A6, CV-A10, EV-71 and E-9 serotypes causing HFMD in India. These results emphasize the need for continuous monitoring of HFMD in India and facilitation of the diagnosis of the associated EV infections using molecular and/or serological approaches.

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