ORIGINAL ARTICLE

Rare occurrence of Coxsackievirus B4 along with Coxsackieviruses A6 and A10 from hand, foot and mouth disease

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Abstract

Background: Hand, Foot and Mouth Disease (HFMD) has been widely spread in Asia and resulted in a high disease burden among children in many countries. It remains a common problem in India, yet its etiology is largely unknown as diagnosis is based on clinical characteristics. *Aim and Objectives:* To investigate the circulation of strains, clinical, molecular and virological aspects of the disease. *Material and Methods:* Vesicular lesion and throat swab specimens were referred from eight children for laboratory diagnosis of HFMD. The detection of enteroviruses was performed by nested PCR, virus isolation using HeLa cell line and sequencing of 5′UTR. *Results:* Coxsackieviruses A6, A10 and B4 were identified by nested PCR, isolated in HeLa cell line and reconfirmed. The phylogenetic analysis of Coxsackieviruses A6, A10 and B4 showed similarity with Beijing-China, Pune-India, and France respectively. *Conclusion:* The findings of this study indicate the co-circulation of Coxsackieviruses A6, A10 and B4. Coxsackievirus B4 was isolated and detected for the first time from Pune, India. Additionally, results emphasize the importance of virus isolation using cell lines for laboratory diagnosis. Point-of-Care testing and vaccine development would be beneficial towards control and prevention of HFMD.

Keywords: Coxsackievirus B4, Enteroviruses, Hand, foot and mouth disease

Introduction

Hand, Foot and Mouth Disease (HFMD) is a prevalent viral illness affecting infants, children and occasionally adults that can result in complications associated with the central nervous system or death [1]. HFMD causing viruses are particularly widespread in Asia, and are responsible for millions of infections each year [2]. Several fatal outbreaks have been reported in Malaysia between April and June 1997 with 29 deaths [3], Vietnam in 2011 with 170 deaths [4] and Cambodia in 2012 with 98 deaths [5]. These fatal outbreaks have influenced the severity of HFMD and led to the demand for control measures. In India, the first report of HFMD outbreak was from Calicut in 2004 followed by a massive outbreak in 2007 in and around Kolkata, West Bengal [6].

Coxsackieviruses (CV) belong to the genus Enterovirus within the Picornaviridae family. The etiological agents that majorly cause HFMD are human enteroviruses species A (HEV-A), specifically enterovirus 71 (EV71) and Coxsackievirus A16 (CA16). Coxsackievirus A6 and A10 are also associated with HFMD among other HEV-A serotypes. Coxsackievirus group B (CV-B) is associated with a plethora of diseases but has a low

isolation rate in HFMD. These viruses can cause severe central nervous system and heart related diseases. CV-B has caused many disease outbreaks worldwide. Coxsackievirus B3 (CV-B3) and Coxsackievirus B5 (CV-B5) are considered the main serotypes causing HFMD and aseptic meningitis. It has been reported in the literature that CV-B4 is closely associated with HFMD, acute pancreatitis, and type I diabetes [7].

HFMD was classified as a class C infectious disease due to its high incidence rate in the pediatric population nearly affecting 2 million children in China [8]. It is usually mild and self-limiting with symptoms including fever >38°C, vesicular rash on the palms, soles, tongue or buttocks, sore throat, and ulcers in front portion of the mouth [9], but neurological, cardio-respiratory complications and even fatalities may occur when the causative virus is EV-71 [10].

Virus isolation and molecular identification in culture or from throat swabs, stool or vesicular fluid samples aid in laboratory diagnosis of HFMD. It can transmit via oral-fecal route, contact with contaminated material surfaces, vesicular fluid, food, or water from infected person [6]. The incubation period is usually 3 to 5 days but can be up to 2 weeks. It can be asymptomatic but contagious as the infectious period starts a few days to about a week before onset of illness [9].

As a result of its self-limiting nature, HFMD has received less attention from the medical community, researchers, public health department and policy makers. The non-availability of effective antiviral drugs, vaccines or stringent preventive policy is major evidence of this. It is becoming a reason of concern in Southeast Asian countries with increasing reports of fatal outbreaks [11]. The present study reports the investigation of Enterovirus (EV) etiology in cases of HFMD that occurred during September 2022 in and around Pune, Maharashtra.

Material and Methods

Clinical specimens and their collection

Patients were identified on the basis of clinical presentation. Throat and vesicular swab samples were collected in Viral Transport Medium (VTM) vials from eight suspected pediatric cases and received by our laboratory situated at Dr. D. Y. Patil Medical College, Hospital and Research Centre, Pimpri Pune, with clinical evidence of HFMD. Ethical clearance was obtained from the Institutional Ethics Committee with reference number I.E.S.C./02/ 2023.

Viral nucleic acid extraction and nested Reverse Transcriptase – Polymerase Chain Reaction (RT-PCR)

TRUPCR viral RNA extraction kit was used for nucleic acid extraction from the clinical samples and cultured viruses. Briefly, 200 µl of sample was added in 560 µl lysis buffer and incubated for 10 min at room temperature (15-25°C). About 560 µl ethanol (90-100%) was then added and incubated for 5 min at room temperature. About 630 µl lysate was centrifuged at 10000 rpm for 1 min (repeated twice). This was followed by addition of 500 µl wash buffer (BAW 1) and centrifugation at 10000 rpm for 1 min; 500 µl wash buffer 2 (BAW2) centrifuged at 14000 rpm for 3 min. Lastly, 40 µl elution buffer was added and centrifuged at 10000 rpm for 1 min. The extracted RNA was further used for PCR. Nested RT-PCR assay was performed as per the protocol [12] with modifications by adding a cDNA synthesis step with the reaction conditions:

25˚C 10 min, 37˚C 120 min, 85˚C 5 min. For first PCR, 25 µL reaction mixture containing 12.5 µL of 2X master mix, 1.5 µL of 10 Mm PanEV primers for 5'UTR outer region (F1 5'-CYTTGTGCGCCT GTTTT-3') (R1 5'-ATTGTCACCATAAGCAG CC-3') and 5 µL viral RNA were prepared and amplified as follows: 50˚C for 30 min, 94˚C for 2 min, 40 cycles of 94˚C for 15 sec, 55˚C for 30 sec and 68˚C for 40 sec and final extension at 68˚C for 5 min. Nested PCR was performed using 2 µL first-round product as template, with 5'UTR inner region primers (F2 5'-CAAGYACTTCTGTMWCCCC-3') and (R2 5'-CCCAAAGTAGTCGGTTCC-3') with similar reaction system and conditions except there was no reverse transcription step. Six µL nested PCR products were subjected to 2.0% agarose gel electrophoresis. For differential diagnosis nucleic acid from the 08 samples were subjected to Herpes Simplex Virus (HSV) detection using TRUPCR HSV 1/2 kit with the following reaction conditions: 94°C for 10 min, 40 cycles of 94°C 15 sec, 58°C 45 sec, 72°C 15 sec.

Virus isolation in cell culture

Isolation of viruses from HFMD suspected samples was performed following the standard protocol by World Health Organization [13]. HeLa cells (P-122) were cultured in minimum essential medium supplemented with 10% fetal bovine serum, 100 IU penicillin/mL and 100 mg streptomycin/mL at 37°C, 5% CO_2. The cells were trypsinized and 2×10^5 cells seeded in a 24 well plate. After 48 hours of incubation, supernatant from vesicular swabs were inoculated and incubated at 37°C. Cells were examined daily, harvested upon appearance of cytopathic effect and detected by nested RT-PCR.

Sequencing

Forward and reverse sequencing of purified PCR products were performed with PAN EV-F2 and PAN EV-R2 primers using ABI BigDye Terminator kit version 3.1 and an ABI 3130 automated genetic analyzer (Applied Biosystems, USA) by the Sanger sequencing method. Bioedit software was used to assemble the obtained sequences. Clustal W program was applied for multiple sequence alignment. A phylogenetic tree was produced using maximum likelihood method by Kimura 2 parameter model (Mega11.0).

Results

Clinical description

Patients presented with fever, severe headache and bodyache, ear pain followed by ulcers in the throat and mouth. The rashes appeared on the face, hands, involving palms, buttocks and soles as shown in (Figure 1) and the detailed clinical description is presented in (Table 1).

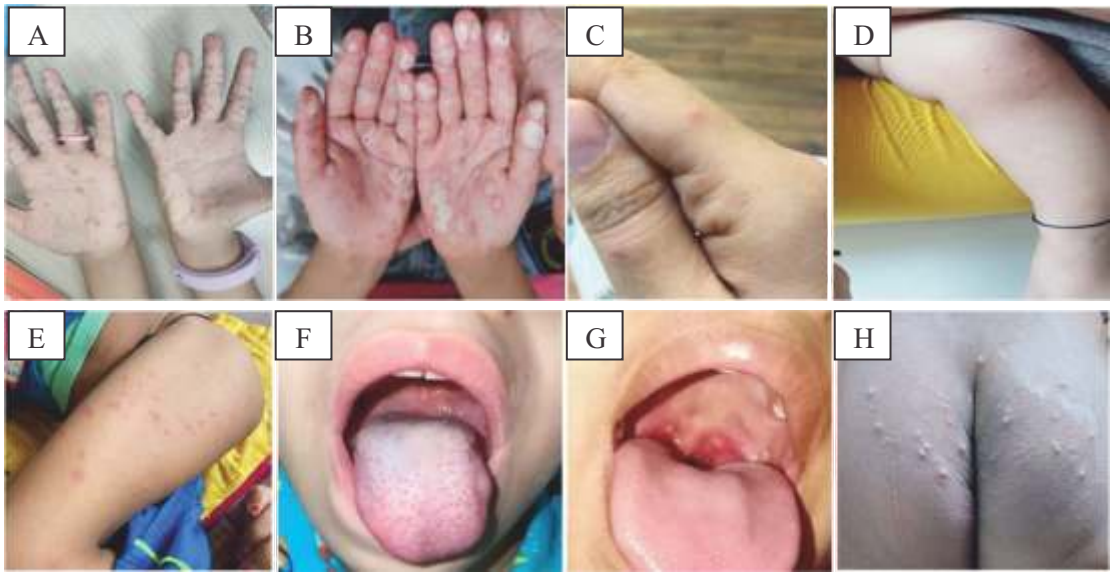

Figure 1: Numerous small macules, papules and vesicles in and around (a, b) palms; (c) finger; (d) knees (e); thighs; (f, g) oral cavity;(h) buttocks

Table 1: Clinical characteristics of HFMD patients

Symptom/ Characteristics	Description
Fever duration	2 to 4 days
Fever severity	Moderate to high grade
Headache	Severe
Bodyache	Severe
Ear pain	Present in some cases
Throat and mouth ulcers	Painful, associated with difficulty in swallowing, leading to decreased food intake.
Rashes	70 to 80 % patients affected; appeared on the face, hands, palms, soles and buttocks; macular, papular and sometimes vesicular; itchy especially when healing and exfoliation of the nails
Rashes healing	Resulted in peeling and scaling of the skin after 2 to 3 weeks
Generalized abdominal pain	Present during acute phase
Prostration	Extreme fatigue and weakness
Recovery duration	Generally within 7 to 10 days
Complications	No complication reported

Laboratory investigations and virus isolation

To determine the prevalence of HFMD, 08 vesicular and throat swab specimens from children with typical symptoms of fever and vesicular eruption on the palms and soles were obtained and examined with nested RT-PCR approach. Of the 8 children, 6 were boys and 2 girls, with a male to female ratio of 3:1. The ages ranged from 2 to 10 years, with a mean age being 4.7 years. Seven were positive and one negative for PanEV by nested PCR (Figure 2A). Positive samples showed a band size of 389 bp. Cytopathic effects were seen on the 3-day post inoculation (dpi). Morphological changes such as rounding of cells and fusion with adjacent cells were observed. The nested RT-PCR results of all the virus isolates turned to be positive for PanEV-5'UTR along with the sample that tested negative before virus isolation (Figure 2B). Samples were also tested for Herpes Simplex Virus (HSV) to rule out differential diagnosis but yielded non-detectable results.

Sequence analysis

Sequence analysis of the eight PanEV-positive amplicons was performed using BLAST in NCBI that identified the causative agent of six cases as CVA6, one CVA10 and one as CVB4. Accession numbers of the sequences submitted to Genbank were S1-OQ186454, S2-OQ186606, S3-OQ184962, S4-OQ186455, S5-OQ186607, S6-OQ200608, S7-OQ200609 and S8-OQ184963. To determine the genetic characteristics and strains circulating in the geographic location, phylogenetic analysis of these strains was based on the alignment of partial 5' UTR gene sequences. A total of 20 CVA6 strains were used for phylogenetic analysis including 06 CVA6 strains identified in this study showed maximum similarity of 99.71% with China. 2 CVA6 strains from India and 12 international representative strains from China (n=3), France (n=3), Australia (n=2), Japan (n=2), Thailand (n=1) and Malaysia (n=1) were downloaded from the Genbank database. A similar analysis of the CVA10 and CVB4 strains was performed. Ten CVA10 and CVB4 strains were used for phylogenetic analysis, including 01 CVA10 that showed highest similarity with Pune (n=5), and others with China (n=1), France (n=1), Japan (n=1) and Australia (n=1) whereas 01 CVB4 strain identified in this study and 09 other CVB4 strains from USA (n=1), France (n=1), Switzerland (n=1), Japan (n=4), China (n=1), Africa (n=1) showing maximum similarity with France (Figure 3).

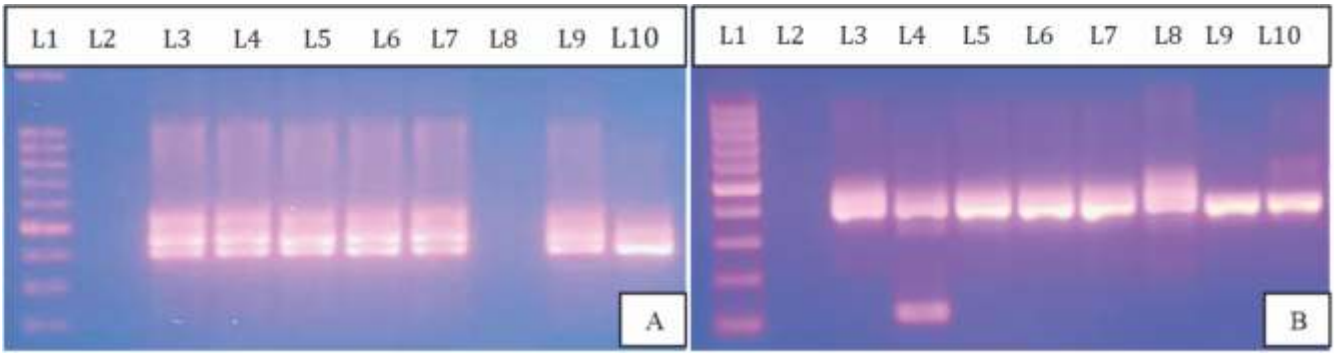

Figure 2: Gel electrophoresis results of nested RT-PCR. Figure 2A- before virus isolation; Figure 2B- after virus isolation. Left to right: Lane 1 -100 bp DNA ladder (Promega); Lane 2 – negative control; Lane 3 to10 - HFMD Pan- EV samples (amplicon size 389 bp)

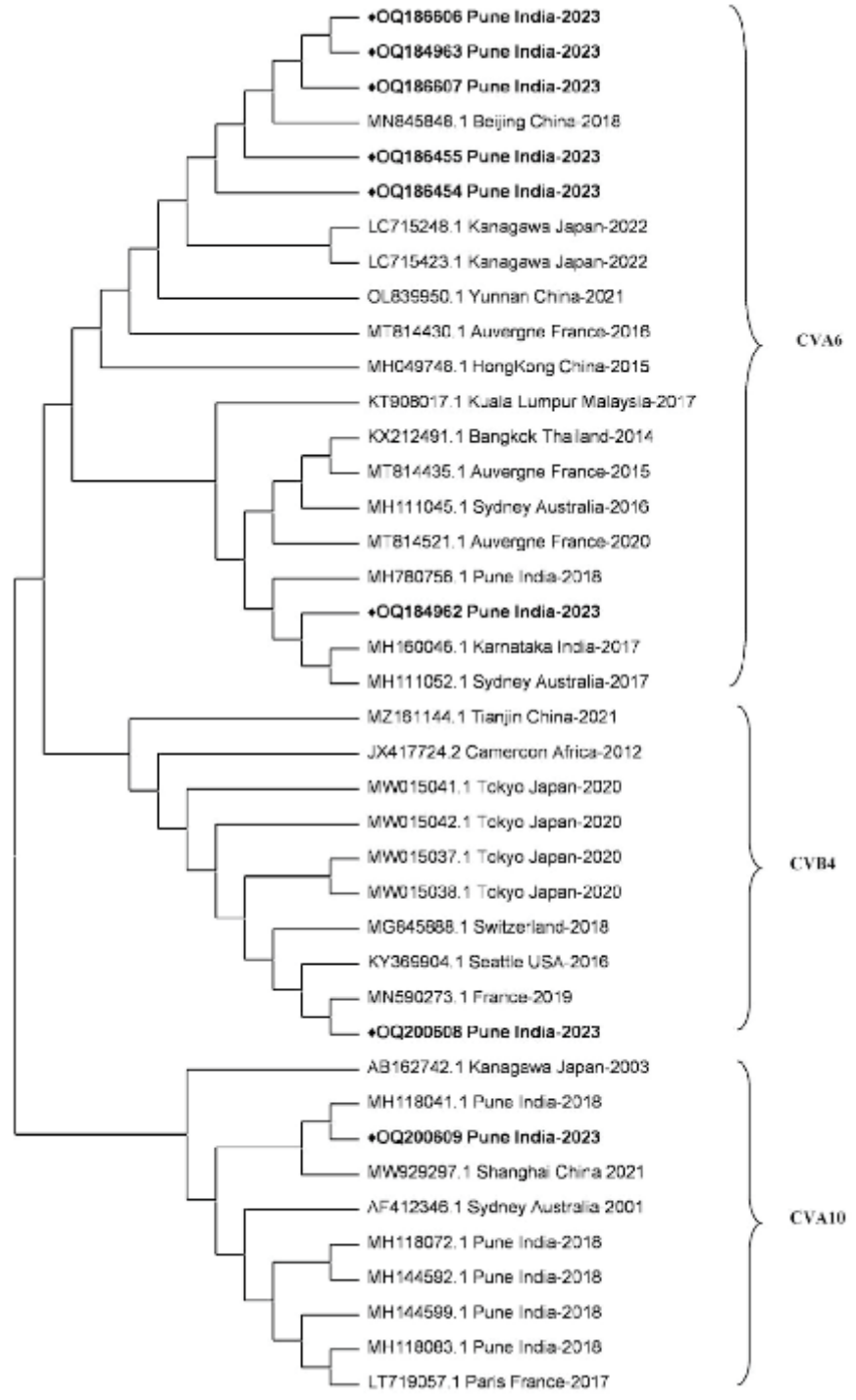

Figure 3: Phylogenetic tree was constructed from the nucleotides sequence of HFMD viral pathogens: CV-A6, CV-A10 and CVB4 with the Maximum likelihood method using Kimura-2 parameter models. The nucleotide sequences determined in this study were deposited in the Genbank database and the strains are indicated using (♦) symbol and boldfont.

Discussion

In the past two decades, several outbreaks of HFMD have been reported from Southeast Asian countries, including Malaysia, Japan, Singapore, Vietnam, Cambodia and China and in recent years from many parts of India [12, 14]. Additionally, co-circulation of various EV serotypes has been observed in HFMD cases from Bhubaneswar, Odisha in 2009 [15], the southern and eastern regions of India in 2009–2010 [16], and north Kerala in 2015–2016 [17], showing that CV-A6 and CV–A16 are the main enteroviruses causing HFMD in India. The present study reports the cases of HFMD that occurred during September through November 2022 in and around Pune. Similarly HFMD cases have been reported in Mumbai, Bangalore and Karnataka in 2013 during September–November [14, 18]. Vesicular lesion and throat swabs were used in the study as sample types whereas previously urine and stool specimens were preferred from children for molecular detections [12, 14, 18].

Similar to other enteroviruses, children below 10 years are the most significant target of HFMD viruses [11]. In this study, HFMD affected children mostly between 2 and 9 years of age with no evidence of infection in adults. Cases were reported with fever, macular, papular, vesicular rashes on palms, buttocks, knees, soles, thighs, and ulcers in the mouth. Nail abnormalities have rarely been documented from previous HFMD outbreaks in India. The association between HFMD and onychomadesis has been described in the United States and Europe but without a link to specific serotype or with a small percentage of CVA6-associated cases. Similar to our present study during 2014, 82.6% cases of onychomadesis were reported from the pediatrics outpatient department of North Bengal Medical College, West Bengal, India and exfoliation of nails was observed [19, 20]. EV-A71 and CV-A16 were the most prevalent HFMD-causing viruses, and EV-A71 was more likely to result in neurological complications and cardiopulmonary failure [21]. No neurological or pulmonary indications were detected, and all cases recovered within 7-10 days after symptomatic treatment.

Molecular detection of the viruses is important to determine the world-wide geographical distribution [22]. CVA10 and CVA6 were co-circulating with EV-71 and CV-A16 in recent three outbreaks [23]. In this study we found three different strains in just 8 samples and that the HFMD infections in Pune were caused by CVA6, CVA10 and CVB4 viruses. The phylogenetic analysis of CVA6 viruses showed their similarity with CVA6 viruses from China, whereas the one CVA10 isolate showed similarity with CVA10 viruses from India and CVB4 showed similarity with France. No EV-71 was detected and isolated from any case during the outbreak.

Virus isolation using various cell lines provides strong evidence for enterovirus coinfection. Additionally, our results emphasize the importance of virus isolation using cell lines for laboratory diagnosis [24]. Differential diagnosis plays a vital role as there is a possibility of missing individual cases and smaller epidemics, due to the rare number of cases, resemblance to common skin diseases like varicella zoster, papular urticaria, impetigo, pompholyx, chickenpox, mosquito bite,

lack of suspicion among clinicians, and most importantly, rapid recovery [25-26]. In China, during 2008 the cases suspected for HFMD, when virus isolated were identified as HSV type 1 [27], whereas in our study all the isolates were negative for HSV. In India, the role of enteroviruses causing HFMD is unexplored in depth, as most of the cases reported earlier have been diagnosed on the basis of clinical signs [18].

Conclusion

The findings of this study indicate the co-circulation of Coxsackieviruses A6, A10 and B4. CVB4 was isolated and detected for the first time from Pune, India. Initially CVB4 was undetectable by PCR possibly due to low viral loads but detected after isolation in cell culture which further suggests the need for diagnostic kits with higher sensitivity and specificity. Point-of-Care testing and vaccine development would be beneficial towards control and prevention of HFMD.

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