Abstract

Introduction: Hand, foot, and mouth disease (HFMD), an entroviral disease has emerged as a major emerging infection in India. This is caused most commonly by enterovirus 71 (EV71) and coxsackievirus A16 (CVA16) but can also be due to CVA4-10, CVA24, CVB2-5, and echovirus 18 (Echo18). Virological analysis of the cases of HFMD has been infrequently done in India. West Bengal is one of the worst affected states in India. Objective: To document the clinical and etiological aspect, the changing patterns and clinic-virological correlation. Method: a total of 62 samples of throat swab were collected from affected children over 3 successive years in Kolkata, West Bengal, India. Result: Five cases had a previous history of HFMD during the last 1–5 years. Fever was usually of mild degree (highest 102°C). There was no apparent correlation between fever of >100°C and a positive test. There was no correlation of viral strain and clinical severity. A test positive for the Viral RNA was noted among 64.51% (40/62) cases. Multiple strains were characteristically present in each year. CVA6, EV71 were found in 2013, CVA6, EV71 in 2014, and CVA6, CVA16 in 2015. Conclusion: Presence of multiple strains explained the frequent occurrence of relapses. We expect this small study will serve as an important document for all future studies on HFMD.

KEY WORDS: Coxsackievirus, enterovirus 71, epidemiological trend, hand, foot, and mouth disease, West Bengal

Introduction

Hand, foot, and mouth disease (HFMD) has emerged as a major emerging infection in India.[1] Progressively larger scale of involvement, mostly of pediatric population, in West Bengal, is found in India since its first reported detection in 2007.[2,3]

HFMD is an entroviral disease, caused most commonly by enterovirus 71 (EV71) and coxsackievirus A16 (CVA16). Other strains such as CVA4-10, CVA24, CVA2-5, and echovirus 18 (Echo18) can also rarely cause HFMD.[4] Entroviruses (picornaviridae family) can be classified into coxsackie Group A (Types 1–22, 24), coxsackie Group B (Types 1–6), echoviruses (Types 1–7, 9, 11–27, 29–34), and enteroviruses (Types 68–71).

EV71 is a human enterovirus A species.[5,6] EV71 is a nonenveloped, positive, single-stranded RNA virus. It has four structural capsid proteins, including VP1, VP2, VP3, and VP4.[7] VP1 capsid antigen contains highly variable genetic sequences and is possibly most pathogenic. In comparison to other viruses, EV71-induced HFMD may lead to more serious complications such as brainstem encephalitis, cardiopulmonary disorders, and may even lead to fatalities.[4]

HFMD is transmitted through direct contact with the infected mucus, saliva, or feces or indirectly through contact with contaminated surfaces.[8]

Pathogenesis of HFMD is yet elusive. The presence of proinflammatory mediators such as interleukin (IL)-4, IL-5, IL-22, IL-23, IL-2, tumor necrosis factor-a, IL-1b,
IL-6 and high-mobility group box 1 have been suggested to mediate a key role.\textsuperscript{[4,5,9]}

So far, there are no effective antiviral drugs or vaccines against this infection. However, many vaccines including inactivated virus vaccines, attenuated live virus vaccines, subunit vaccines, DNA vaccines, and virus-like particle vaccines have been tried.\textsuperscript{[10,11]} Inactivated virus vaccines are considered advantageous and promising.\textsuperscript{[12,13]}

Virological analysis of the cases of HFMD has been infrequently done. This study has tried to identify the causative viral strain of HFMD for 3 successive years to assess the trends in molecular epidemiology.

**Materials and Methods**

Patients attending the dermatology outpatient department with typical clinical features of HFMD were included for the study after taking consent or assent. Cases were clinically diagnosed independently by two senior dermatologists. All atypical cases were excluded. Cases were then selected for virological analysis. Study was approved by Institutional Ethical Committee.

Cases of HFMD with classical presentation and having active oral lesions were selected during monsoon and postmonsoon session between July and November, in 3 successive years (2013, 2014, and 2015).

A total of 62 samples of throat swab were collected from children who cooperated and in whom parents gave consent. Throat swab samples were assessed for viral detection within 2–3 days on the onset of manifestation. All samples were transported maintaining proper cold chain to the laboratory.

The main objective of this study was to document clinicoepidemiological profile of HFMD and molecular detection of circulating enteroviruses causing the disease.

**Viral RNA isolation and detection**

Swab samples were suspended in 1 ml phosphate-buffered saline in 15 ml sterile centrifuge tube. Supernatant was collected after centrifugation at 5000 rpm for 5 min at 4°C, and viral RNA was isolated using QIAamp viral RNA mini kit (Qiagen, Germany) as per manufacturer protocol from 140 μl supernatant.

Viral RNA was eluted in 50 μl elution buffer and stored at −80°C for future use. Enterovirus RNA detection was done based on 5’ untranslated region of the viral genome by nested real time-polymerase chain reaction (RT-PCR) according to a previous Indian publication.\textsuperscript{[14]}

Complementary DNA (cDNA) from viral RNA was made using random primer using SuperScript III (Life Technologies, USA) according to manufacturer protocol followed by nested RT-PCR. A 400 bp PCR amplified product was observed in ethidium bromide-stained agarose gel under Gel Documentation system (BioRad, USA) for positive cases.

VP1-2A junction region was identified to distinguish EV71 and various strain of coxsackievirus isolated in our study participants. For that, cDNA was synthesized from Enterovirus RNA-positive sample with reverse primer 011 (5’-GCICGGAYTGTTGICCRRAA-3’). The resulting cDNA was further amplified to the VP1-2A junction region using forward primer 040 (5’ATGTAYRTCICICIGCIGGC-3’) and reverse primer 011 (5’-GCICGGAYTGTTGICCRRAA-3’).

PCR condition involved initial denaturation at 95°C for 15 min, followed by 94°C for 1 min, annealing at 50°C at 1 min, extension at 72°C for 1 min, and finally 5 min at 72°C. A 458 bp PCR product was visualized under Gel Documentation system (BioRad, USA) for positive cases after ethidium bromide staining. Amplified PCR products were gel purified using the QIAquick Gel Extraction kit (Qiagen, Germany) and directly analyzed for sequencing. The sequencing reaction was carried out from both directions using the ABI PRISM BigDye Terminator Cycle Sequencing ready reaction Kit (Applied Biosystem) according to manufacturer’s protocol. The sequences were determined in an automated DNA sequencer.

**Results**

Patients presented with round to oval papulovesicular eruption usually with an erythematous border on hands, feet, knees, buttocks, perioral, lips, and intraoral lesions over gum, tongue, and buccal mucosa [Figures 1 and 2]. Extensive eruption involving larger parts of the trunk was also seen [Figure 3]. Dribbling of saliva and difficulty in taking food were commonly complained in children who had prominent oral lesions. Vesicles healed without any complication in all cases in <7 days (5–6 days). History of involvement of other siblings was uncommon (only 5 cases, 8%).

**Figure 1:** Erythematous papulovesicular eruptions on the palms in a child with hand, foot, and mouth disease
Males slightly outnumbered females each year. Average ages of the patients also did not show much variation. Severity of the disease as assessed clinically was almost similar in these years. Fever usually of mild degree (highest 102°C) was present during the initial 2–3 days and was much less common in 2015. There was no apparent correlation between fever of >100°C and a positive test. Clinicodemographic details of the cases have been mentioned in Table 1.

Five cases had a previous history of HFMD during the last 1–5 years.

Of the total HFMD cases selected, viral RNA-positive cases were 64.51% (40/62) from clinically suspected cases [Figure 4]. Six positive samples, 2 from each year (2013–2015), were sequenced using the protocol mentioned above [Figure 5]. Among these, 3 samples (50%) were found to be CVA6 (accession No. KX499459, KX523849, and KX523851) and 2 (33.3%) were EV71 (accession No. KX499460 and KX5238450), and one (16.7%) was CVA16 (accession no KX523865). Multiple strains were characteristically present in each year: CVA6, EV71 were found in 2013, CVA6, EV71 in 2014, and CVA6, CVA16 in 2015. There was no correlation of viral strain and clinical severity.

### Discussion

HFMD has been reported from many Indian states such as West Bengal, Kerala, hills of northern India, Odisha, Maharashtra, and Karnataka. There are reports even from Andaman Islands.

Molecular detection of the prevalent viral strain is crucial. This infection is a new entrant in India despite its presence for many decades in many other neighboring Southeast Asian countries where it has started manifesting with great virulence. Unfortunately, only few Indian studies have attempted for virological analysis.

Various strains have been detected from the HFMD cases in India during the last few years. Another recent study that collected samples from different regions on India reported the presence of CVA16 (61.7%), CVA6 (34.04%), CVA4, and Echo12 (4.3%) among the 94 positive samples. One past Indian study reported the presence of CVA16 from Karnataka among 50% of the positive cases (2 out of 4 cases). CVA16 was also reported previously from cases of HFMD in Andaman island.

One multicentric study done few years back and collected sample from many states including West Bengal, the state where this present study is being done, reportedly found CVA16 in all those cases. Our study however found the presence of multiple strains in this region.

### Conclusion

This study showed that multiple strains of HFMD virus are presently circulating in this area. We understand that this is a small study and only limited number of samples has been sequenced for species identification. However, in-depth analysis of entrovirus is technically difficult and is expensive. There is significant lacking of studies performing virological analysis among the HFMD cases in India.

| Total cases | Positive (%) | Negative (%) | Male (%) | Female (%) | Average age | Fever >100°C (%) |
|-------------|--------------|--------------|----------|------------|-------------|------------------|
| 2013        | 14           | 9 (64.3)     | 5 (35.7) | 8 (57)     | 4.7±2.17    | 1 (7), positive test |
| 2014        | 18           | 10 (55.6)    | 8 (44.4) | 10 (55.6)  | 5±3986      | 5 (2.8), 3 (60) had positive test |
| 2015        | 30           | 21 (70)      | 9 (30)   | 18 (60)    | 4.9±3.418   | 5 (0.2), 4 (80) had positive test |

**Figure 2:** Extensive papulovesicular eruptions of hand, foot, and mouth disease on the buttocks

**Figure 3:** Wide spread vesicular eruptions of hand, foot, and mouth disease involving back, neck, and proximal arm

| Table 1: Clinicodemographic details of hand, foot, and mouth disease during the year 2013-2015 |
|--------------------------------------------------|
| Total cases | Positive (%) | Negative (%) | Male (%) | Female (%) | Average age | Fever >100°C (%) |
|------------|--------------|--------------|----------|------------|-------------|------------------|
| 2013       | 14           | 9 (64.3)     | 5 (35.7) | 8 (57)     | 4.7±2.17    | 1 (7), positive test |
| 2014       | 18           | 10 (55.6)    | 8 (44.4) | 10 (55.6)  | 5±3986      | 5 (2.8), 3 (60) had positive test |
| 2015       | 30           | 21 (70)      | 9 (30)   | 18 (60)    | 4.9±3.418   | 5 (0.2), 4 (80) had positive test |
As there is complete dearth of information on the changing epidemiological trend of the viral strains over the years from a specific geographical location in India, we expect that this study will serve the vital purpose of documentation of the etiological agents of HFMD in India and will be of great help for all future studies on this disease.

**Financial support and sponsorship**

This study has been done with an academic grant from the Indian Association of Dermatologists, Venereologists, and Leprologists Academy (IADVL Academy).

**Conflicts of interest**

There are no conflicts of interest.

---

### What is new?

Multiple strains of HFMD virus are presently circulating in West Bengal. There is no apparent correlation between viral strains and clinical severity but frequent relapse might be due to the circulation of multiple strains.

### References

1. Sarma N. Hand, foot, and mouth disease: Current scenario and Indian perspective. Indian J Dermatol Venereol Leprol 2013;79:165-75.
2. Sarma N, Sarkar A, Mukherjee A, Ghosh A, Dhar S, Malakar R. Epidemic of hand, foot and mouth disease in West Bengal, India in August, 2007: A multicentric study. Indian J Dermatol 2009;54:26-30.
3. Sarma N. Relapse of hand foot and mouth disease: Are we at more risk? Indian J Dermatol 2013;58:78-9.
4. Ooi MH, Wong SC, Lewthwaite P, Cardosa MJ, Solomon T. Clinical features, diagnosis, and management of enterovirus 71. Lancet Neurol 2010;9:1097-105.
5. Chang PC, Chen SC, Chen KT. The current status of the disease caused by Enterovirus 71 infections: Epidemiology, pathogenesis, molecular epidemiology, and vaccine development. Int J Environ Res Public Health 2016;13. pii: E890.
6. Zheng W, Shi H, Chen Y, Xu Z, Chen J, Jin L. Alteration of serum high-mobility group protein 1 (HMGB1) levels in children with enterovirus 71-induced hand, foot, and mouth disease. Medicine (Baltimore) 2017;96:e6764.
7. Brown BA, Oberste MS, Alexander JP Jr., Kennett ML, Pallansch MA. Molecular epidemiology and evolution of enterovirus 71 strains isolated from 1970 to 1998. J Virol 1999;73:9969-75.
8. Du Z, Zhang W, Zhang D, Hao Y. Estimating the basic reproduction rate of HFMD using the time series SIR model in Guangdong, China. PLoS One 2017;12:e0179623.
9. Lin TY, Hsia SH, Huang YC, Wu CT, Chang LY. Proinflammatory cytokine reactions in enterovirus 71 infections of the central nervous system. Clin Infect Dis 2003;36:269-74.
10. Liang ZL, Mao QY, Wang YP, Zhu H, Li JX, Yao X, et al. Progress on the research and development of inactivated EV71 whole-virus vaccines. Hum Vacc Immunother 2013;9:1701-5.
11. In HJ, Lim E, Lee JA, Kim HJ, Kim JW, Hyeon JY, et al. An inactivated hand-foot-and-mouth disease vaccine using the enterovirus 71 (C4a) strain isolated from a Korean patient induces a strong immunogenic response in mice. PLoS One 2017;12:e0178259.
12. Heinsbroek E, Ruitenbergh EJ. The global introduction of inactivated polio vaccine can circumvent the oral polio vaccine paradox. Vaccine 2010;28:3778-83.
13. Mao Q, Li N, Yu X, Yao X, Li F, Lu F, et al. Antigenicity, animal protective effect and genetic characteristics of candidate vaccine strains of enterovirus 71. Arch Virol 2012;157:37-41.
14. Gopalkrishna V, Patil PR, Patil GP, Chitambar SD. Circulation of multiple enterovirus serotypes causing hand, foot and mouth disease in India. J Med Microbiol 2012;61(Pt 3):420-5.
15. Nanda C, Singh R, Rana SK. An outbreak of hand-foot-mouth disease: A report from the hills of northern India. Natl Med J India 2015;28:126-8.
16. Kar BR, Dwibedi B, Kar SK. An outbreak of hand, foot and mouth disease in Bhubaneswar, Odisha. Indian Pediatr 2013;50:139-42.
17. Hegde R, Kowalli S, Nagaraja K, Dharanesha NK, Seema CM, Khan TA, et al. Serosurveillance of foot and mouth disease in Karnataka state, India: A 3 years study. Virusdisease 2016;27:294-302.
18. Palani S, Nagarajan M, Biswas AK, Maile A, Paluru V. B1c genetic subtype of coxsackievirus A16 associated with hand, foot and mouth disease in Andaman Islands, India. Trans R Soc Trop Med Hyg 2016;110:421-3.
19. Ganorkar NN, Patil PR, Tikute SS, Gopalkrishna V. Genetic characterization of enterovirus strains identified in Hand, Foot and Mouth Disease (HFMD): Emergence of B1c, C1 subgenotypes, E2 sublineage of CVA16, EV71 and CVA6 strains in India. Infect Genet Evol 2017;54:192-9.
20. Sinha DP, Raut CG, Jayaprakash H, Hanumaiah H, Shaikh NJ, Manjunatha MJ. Molecular diagnosis of enteroviruses associated with Hand, Foot and Mouth Disease (HFMD). Indian J Pediatr 2014;81:1242.