Severe atypical hand-foot-and-mouth disease in adults due to coxsackievirus A6: Clinical presentation and phylogenesis of CV-A6 strains

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ABSTRACT

Background: Typically, hand-foot-and-mouth disease (HFMD) is a mild childhood illness associated with cox-sackievirus (CV)-A16, CV-A6, enterovirus (EV)-A71.
Objectives: To identify the viral agents associated with severe cases of atypical HFMD in Italy.
Study design: Epidemiologically unrelated cases of severe atypical HFMD admitted to the Emergency Room (ER) of IRCCS San Martino IST (Genoa, Italy) in 2014–2016 were investigated. Serologic screening for viral positivity was performed against exanthem-inducing agents. Ten cases with serology indicative of recent EV infection were selected. Molecular assays were used to detect viral genomes in blood [EVs, Parvovirus B19 (PVB19), herpes-viruses (CMV; EBV, HHV-6, -7, -8)].
Results: CV-A6 was detected in 10 cases of severe atypical HFMD. Two cases were also infected with PVB19. Herpesviruses were not detected. Phylogenetic analysis mapped the CV-A6 strains into a single cluster related to two recent isolates from a German and an Asian child. Fever, systemic symptoms, severe vasculitis-like rash, and enanthem were predominant at presentation. Spontaneous recovery occurred in 1–3 weeks.
Conclusions: CV-A6 is emerging as a frequent cause of severe atypical HFMD in Italian adults. This viral agent is disseminating worldwide. Dermatologists must identify the manifold alterations caused by EVs and understand the diagnostic power of current virology methods.

1. Background

Infections by human enteroviruses (EVs; over 100 different serotypes belonging to 4 species, A–D) are associated with a wide spectrum of clinical presentations ranging from benign self-limiting disease to severe conditions that include myocarditis, meningoencephalitis, aseptic meningitis, flaccid paralysis [1].

Hand foot and mouth disease (HFMD) is a manifestation of EV infection that occurs predominantly in children [2–20] rather than in adults [21–24]. Coxsackievirus A16 (CV-A16), CV-A6, and EV-A71 are the predominant etiologic agents in pediatric cases [2]. Since 2008, CV-A6 has emerged as a cause of HFMD epidemics in the young [2,20]: Finland [3,4], China [2,5–18], Thailand [9], Japan [10–12], USA [13], France [14], Spain [15,16], the UK [17], Vietnam [18], Singapore and Malaysia [19,20].

Classic HFMD manifests with fever and a distinctive vesicular eruption usually limited to the hands, feet, and oral mucosa. Presentation in adults may be atypical and severe: systemic involvement, high fever, morphologically different lesions in variable areas of the body (face, legs, arms, trunk) [13,17,22–24]. CV-A6-associated HFMD manifests as a vesiculobullous disease localized to areas of atopic dermatitis and perioral surfaces [13,17,22–24]; complications such as onychomadesis and orchiepididimitis are not uncommon [25,26].

Unlike typical HFMD (diagnosis made primarily on physical examination and history), atypical cases may require laboratory tests. Indirect EV diagnostics is based on serology including immunoenzyme assays with antigen mixtures of various virus types and neutralization assays. Gene amplification tests (and VP1 sequencing for EV typing) are

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the preferred tool for direct diagnosis [27; 1].

Epidemiologically unrelated cases of severe atypical HMFD in adults have been examined regarding clinical presentation, viral etiology, and short-term outcome.

1.1. Objective

The study aimed at identifying the viral agents associated with cases of severe atypical HFMD in Italy.

2. Study design

The study - conducted in accordance with the Declaration of Helsinki and local laws and regulations – was approved by the Ethics Committee of IRCCS San Martino IST (protocol #PR033REG016; Genoa, Italy). Informed consent was obtained for investigated cases. From November 2014 to March 2016, adult subjects (> 18 yrs) admitted to the Emergency Room (ER) of IRCCS San Martino IST (Genoa, Italy) with suspected severe HFMD (systemic symptoms, vesiculo-bullous and/or maculopapular petechial lesions, acro-facial and oral mucosa involvement, body surface area involvement > 25%) were taken into consideration. Definitive diagnosis was made at the Dermatology Department of the above Medical Center. At the time of clinical onset, HFMD patients were immunocompetent, not affected by chronic conditions, not receiving drug treatments. Serology to ex-anthem-associated viruses was used as preliminary screening [IgG and IgM levels to EVs, Parvovirus B19 (PVB19); cytomegalovirus (CMV), Epstein-Barr virus (EBV), herpesvirus-6 (HHV-6), -7 (HHV7)]. Commercial tests were used to this end: EV: Virion Serion, Alifax, Polverara, Italy; PVB19, CMV, EBV: DiaSorin, Saluggia, Italy; HHV-6 and -7: PanBio, Italy. Blood samples were taken within 1 week from eruption and 2 weeks later to assess seroconversion. Serum and plasma were stored at -70 °C for serology and molecular assays.

Molecular assays were run for all members of the EV genus using in-house tests [28] and to exanthem-related viruses (PVB19; CMV; EBV; HHV-6, -7, -8) using previously reported methods [29].

2.1. Additional serology

Anti-nuclear antibodies (ANA), anti -neutrophil cytoplasmic antibodies (ANCA), rheumatoid factor (RF), anti-citrulline antibody (anti-CCP) were measured for differential diagnosis and to exclude vasculitis. Rapid Plasma Reagin (RPR) and *Treponema pallidum* passive particle agglutination (TPPA) tests were run to exclude secondary syphilis.

2.2. Virus detection

Nucleic acids were extracted from 0.6 ml of plasma (m2000sp instrument; Abbott Molecular) [29]. Multiple RT-PCR assays targeting conserved genome regions of all EV types (5′UTR, 2C, 3Dpol) were performed [28]. Assays for CMV, EBV, and PB19 V DNA were done with commercial real time PCR kits (Abbott Molecular, Rome, Italy), whereas in-house assays were used for HHV-6, -7, and -8 [29].

2.3. Nucleotide sequencing of EV amplicons

Electrophoretic bands of interest were cut from 3 percent agarose gels, re-amplified by PCR with relevant primers, sequenced by the Sanger method (ABI Prims 3100; Thermo-Fisher, Monza, Italy). The Sequencher v5.0 program (Gencodes, Anne Arbor MI, USA) was used to align sequences with equivalent regions of CV-A6 reference genotypes. The Clustal X program was used to prepare neighbor joining trees after bootstrapping data 1000 times. The resulting phylogenetic tree was visualized with the Unrooted and NJplot programs.

2.4. Deep sequencing by Illumina technology

Sequences of viral RNA from three patients were determined using Illumina deep sequencing approach. Total RNA extracted from patient EDTA blood samples was fragmented using a focused ultrasonicator (Covaris) to generate fragments of 200–250 nt. The NEBNext mRNA library prep master mix set for Illumina (New England Biolabs) was used. Fragmented RNA was reverse transcribed obtaining cDNA. DNA fragments were end-repaired and ligated to Illumina paired-end adaptors. Fragments were subjected to size-selection using Agencourt AMPure XP beads (Beckman Coulter) to obtain homogeneous fragments of 370 bp and PCR amplified (12 cycles) with NEBNext multiplex oligonucleotide primers. After purification on magnetic beads, libraries were evaluated for size and quality (BioAnalyzer, Agilent Technologies; Qubit 2.0 Fluorometer, Invitrogen Life Technologies). Deep sequencing was performed using MiSeq (Illumina) producing 250-nt paired-end reads, as well as HiSeq2500 (Illumina) producing 101-nucleotide paired-end reads.

Sequence reads were analyzed using the HIVE software [30]. Reads were aligned to a curated database of 500 full-length enterovirus sequences, separated into discrete subpopulations and assembled into consensus sequences [31]. The number of reads and the depth of coverage in one sample (case #1) produced a near-complete genome of 7420 nt (excepting the 5′- and 3′-ends where sequencing coverage was low). Consensus sequences from other samples (cases #5, #9) contained internal gaps due to the insufficient number of reads and produced 1600 nt and 2556 nt sequences. Consensus sequences were aligned to reference sequences of CV-A6 and used for phylogenetic analysis using in-house SWARM and HIVE software [30]. The GenBank accession No. MH371303 has been given to CV-A6 sequences of Italian cases.

3. Results

3.1. Clinical presentation and virologic features of atypical HFMD

Subjects presenting to the ER with systemic symptoms, fever ≥ 38 °C, cutaneous and mucosal evidence of atypical HFMD were investigated. Patients had no previous history of skin disease and recalled prodromal symptoms for 3–6 days (fever, asthenia, arthralgia, myalgia), were not related geographically, did not share common sources of infection. Among 20 cases, 14 had positive or borderline EV serology. Ten out of these 14 cases carried EV genome in blood and were investigated in detail. In 4/14 cases no virus could be detected; these cases were not further investigated. The investigated cases had typical HFMD [1 case (petechial maculo-papules and vesicles in the extensor surfaces of hands, feet, oral mucosa)], atypical HFMD [5 cases (absence of involvement of one typical site, or involvement of adjacent sites as face, scalp, ankles)], atypical exanthems [4 cases (maculopapular eruption all over the body)]. The clinical presentation, viral serology, and viral genome determinations are summarized in Table 1.

Notably, vesicular lesions were frequently spread on the trunk mimicking chickenpox. However, chickenpox gives more severe prodromal symptoms and a centripetal distribution of lesions that usually appear extremely numerous and in crops. Besides, chickenpox lesions quickly progress from papules to vesicles, then to pustules, with the different forms appearing at different stages. Laboratory results excluded vasculitis and secondary syphilis. All patients recovered within 3 weeks with no short-term complications, except for patient #3 and #6 who developed bilateral orchiepididymitis and onychomadesis, respectively.

Five patients had erythematous vesicular lesions (mainly on hands and feet; mostly bilateral). Maculopapular purpuric lesions were found in 4 cases. Lesions were on extensor surfaces and palm and soles. Three patients had lesions on the trunk. Some patients had lesions in the perioral area, scalp, elbows, legs. Buttocks were involved in only two

Table 1
Clinical presentation and virological features of atypical HFMD.

Case No. (Age years /Sex)	Presentation to Emergency Department	Systemic symptoms	Clinical findings (body sites involved)	Resolution time, days post-diagnosis	Enterovirus		Parvovirus B19	
					Serology[a]	RNA (EV type)	Serology[a]	DNA
1 (50/M)	Yes	Malaise, Fever, Arthralgia	EV (feet, hands and scalp); V (hard palate)	21	IgG+ /IgM -	+ (CV-A6)[b]	IgG+ /IgM-	+
2 (35/F)	Yes	Malaise, Fever, Arthralgia	MP (trunk, hands and feet); PE (hard palate)	14	IgG+/IgM -	+ (CV-A6)	IgG-/IgM-	–
3 (18/M)	Yes, requiring hospitalization	Malaise, Fever, Anorexia	EV (face, hands, scalp and legs, forearms); MP (trunk); V (hard palate)	21	IgG+ /IgM -	+ (CV-A6)	IgG+ /IgM-	–
4 (21/F)	Yes	Malaise, Fever, Arthralgia	MP (trunk, hands and feet); M (hard palate); EP (elbows)	14	IgG+ /IgM -	+ (CV-A6)	IgG-/IgM-	–
5 (51/M)	Yes, requiring hospitalization	Malaise, high Fever, Arthralgia, Myalgia	EV (face, hands, scalp, legs, hard palate)	14	IgG+ /IgM-	+ (CV-A6)	IgG+ /IgM-	–
6 (28/M)	Yes	Malaise, Fever, Anorexia	EV (face, scalp, hands; hard palate)	14	IgG+ /IgM-	+ (CV-A6)	IgG+ /IgM-	–
7 (43/M)	Yes	Malaise, Fever, Anorexia	EV (face, neck, hands and feet, hard palate)	14	IgG+ /IgM-	+ (CV-A6)	IgG-/IgM-	–
8 (56/F)	Yes	Malaise, Low-fever, Arthralgia, Anorexia	MP (hands, feet and legs)	14	IgG ± /IgM-	+ (CV-A6)	IgG+ /IgM-	–
9 (52/M)	Yes	Malaise, Low-fever, Arthralgia	V (feet, hands, hard palate)	7	IgG ± /IgM-	+ (CV-A6)	IgG-/IgM-	–
10 (33/F)	Yes	Malaise, Fever, Arthralgia	MP (trunk, hands and feet); EP (elbows); M (hard palate)	7	IgG-/IgM+	+ (CV-A6)	IgG+ /IgM+	+

Abbreviations: V, vesicular; VP, vesicular-pustular; PE, petechial; M, macular; P, papular; MP, maculopapular; EV, erythematovesicular; EP, erythematous plaques; High fever: ≥ 38 °C (≥ 100.4 °F); Low-fever: 37–38 °C (98.6–100.4 °F).

[a] IgG positivity denotes seroconversion at 2 weeks post-diagnosis.

[b] CV-A6, Coxsackievirus A6.

Fig. 1. Clinical aspect of atypical hand-foot-and-mouth disease associated with Coxsackievirus A6 infection. A–B, Patient 2: maculopapular lesions on the trunk and petechiae in the palms. C, Patient 3: erythematovesicular lesions on forearms. D, Patient 4: Erythematous plaques on the elbows. E–F, Patient 6: erythematous-vesicles on the face (lesions in the seborrheic and perioral/perinasal regions) and vesicles and erosions of the hard palate.

cases (case #1 and #10 that were co-infected with PVB19). Lesions were reported as painful, burning or itchy. Cutaneous lesions healed within 2–3 weeks with desquamation. Oral lesions were present in 9/10 patients. Clinical aspect of atypical HFMD associated with Coxsackievirus A6 infection are presented in Fig. 1.

3.2. Virus detection in blood and properties of CV-A6 genomes

Results of EV and PVB19 detection are shown in Table 1. Short EV amplicons (150–250 nt) were sequenced by the Sanger method (5′UTR, 2C, 3D regions [28]). Deep sequencing could attribute infection to the CV-A6 type. EV serology showed IgM positivity in only one patient (case #10) and IgG seroconversion at 2 weeks in 7 patients (cases #1 to #7). The PVB19 genome was detected in cases #1 and #10. In case 10, IgM positivity for PVB19 indicates primary infection. EBV, CMV, HHV-6, -7 and -8 were not detected.

3.3. Phylogenetic analysis of CV-A6 strains

To assemble consensus nucleotide sequences from deep sequencing data individual Illumina sequence reads were mapped on a curated representative database of 50 aligned full-length sequences of reference CV-A6 strains present in GenBank. A phylogenetic tree was constructed using UPGMA method using the in-house SWARM and HIVE software [30] (Fig. 2). For some isolates, only partial nucleotide sequences were available. In this case the missing parts of the genome were disregarded. All phylogenetic distances were normalized by the number of nucleotides used for pairwise comparisons. All strains clustered together close to the sequence of strain DE/G4/A/2014 isolated in 2014 from stools of a 15-month-old child with HFMD in Germany (0.67% nucleotide differences, with only 3 amino acid substitutions in capsid proteins), sequences of Hyogo9205 and Hyogo9426 strains isolated from 3-4-year-old children in 2013 in Japan (2.41% and 2.51%, respectively) [14], and the CV-A6/Shenzhen94/CHN/2014 isolate obtained from a Chinese child in 2014 (2.97%). Based on the molecular clock rate established for a different EV (poliovirus) [34], the difference between the Italian and the German isolate corresponds to less than one year of circulation, and 2–3 years of circulation for the closest Asian

isolate. These viruses belong to clade RF-A of CV-A6 according to a recent study [35]. Therefore, it may be speculated that the infecting strain originated in Asia and was brought to Europe in 2013-2014. Partial 5'-UTR sequences (118 to 325 nt) could be obtained for ten CV-A6 strains. Analysis of these strains confirmed that all sub-grouped into the genetic group A.

4. Discussion

Classical HFMD is characterized by a distinctive vesicular eruption affecting the mouth and extremities. The illness occurs worldwide, is self-limiting, affects children under 10 years. HFMD is primarily associated with EV types of the A species [CV-A4, -A5, -A6, -A7, -A10, A-16, -A24; EV-A71, though types of the B species (CV-A9, CV-B2 to -B5; Echovirus-4, -18, -19)] have also been reported [34]. HFMD cases associated with CV-A6- and CV-A10 are emerging worldwide [2,4]. However, most reports are dealing with pediatric cases [2–20]. The severity of EV diseases is related to age-and to the virus type [21].

Dermatologic lesions presented at different stages in each patient, with variation in morphology and extension. Initial signs consisted of erythematous maculopapular lesions (healing with desquamation) and erythematous vesicular lesions that progressed to shallow ulcerations, then crusts without sequelae. Vesicular lesions had a wider distribution than in classical HFMD. Chickenpox and rubella were considered for differential diagnosis, but HFMD lesions were not pustular and umbilicated. Erythematous or purpuric macular lesions on palms and soles may mimic those of secondary syphilis, vasculitis or drug reaction. Differential diagnosis was also based on serologic tests. Involvement of dorsum of hands and feet is more frequent in CV-A6 infection than in classic HFMD [22–24]. Case #8 was missing oral involvement, while three patients exhibited peri-oral erythematous papules (with buccal involvement), a finding unique to CV-A6. Two cases developed complications: orchiepididimitis or onychomadesis (a late lesion of the nail matrix possibly related to infection of proliferating stem cells) [25,26].

Two cases (#1 and #10) were co-infected by CV-A6 and PVB19. This may have contributed to the eruption involving inguinal folds and buttocks in a "baboon-like" fashion [35].

Among different cases, the amount of virus in plasma varied. In case
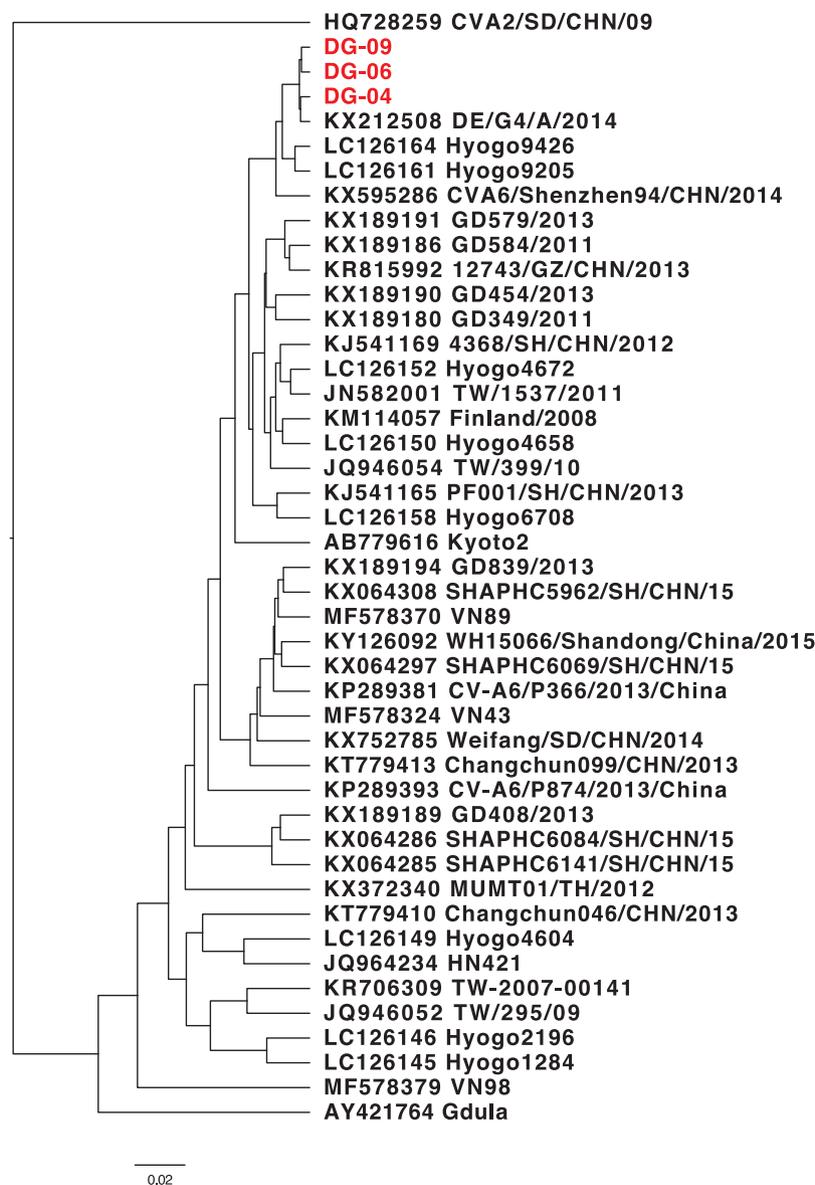
Fig. 2. Phylogenetic analysis of CV-A6 strains showing relatedness of complete and partial nucleotide sequences determined by direct deep sequencing (red) with reference full-length genomes of Coxsackievirus A6 (black). The position of the tree root was determined relative to Coxsackie A2, another representative of the enterovirus A species. The phylogenetic tree was constructed using the UPGMA method. The scale bar indicates branch distance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

#1 it was large enough to enable generation of a near complete genome sequence. In other cases, the number of reads was insufficient for determining the entire genome, and only its fragments could be used to determine the relatedness of CV-A6 strains. This confirms the power of metagenomic analysis that allows virus identification directly in clinical specimens even when present at low levels. Phylogenesis revealed that Italian strains were closely related to a virus (DE/G4/A/2014) obtained from a German child in 2014. Likely, the latter virus had been imported to Europe from Asia [33].

Unlike cases from Finland [3,4], China [2,5–18], Thailand [9], Japan [10–12], USA [13], France [14], Spain [15,16], the UK [17], Vietnam [18], Singapore and Malaysia [19,20] in which the virus selectively hit children, the Italian series identifies CV-A6 as a pathogen for adults [22]. The unusual age distribution and severe clinical presentation could imply strain-specific shifts associated with the worldwide spread of this agent, or – alternatively - immunologic differences between distant populations due to dissimilar age-related exposure to EVs that could be linked to different hygienic conditions [36].

In the young, systemic infections by EV-A71 and CV-A6 are accompanied by a cytokine/chemokine storm [23]. Comparable reactions may certainly occur in adults, could contribute to general symptoms and dermatologic manifestations and represent an interesting topic for research [23].

Attention to typical and atypical HFMD (conditions that easily call for medical assistance) may lead to the identification of EV types circulating in different geographic areas. The identification of virus types is essential from epidemiology and needed for implementing vaccines and antivirals [37]. In fact, the prevention of EV-induced diseases may be of great value due to the possibility of chronic infection and long-term consequences [28,37]. We have previously shown that EVs of the B species can be linked to cases of type 1 diabetes and chronic cardiomyopathy and that polioviruses (EVs of the C species) are associated with the post-polio syndrome [28].

It has long been said that "inside as in the skin, inside and on the skin" (*Intus ut in cute, intus et in cute*; Marcus Tullius Cicero, 106-43 BC). Thus, it is no surprise that dermatologic manifestations are mirroring

the pathophysiology of internal organs [38]. Hence, clinicians must identify the wide range of cutaneous and mucosal alterations caused by the different EV types, need to prescribe appropriate diagnostic tests and to uncover the diagnostic and epidemiologic power of current virologic methods.

Conflicts of interests

None declared.

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Authors' contributions

FB, FD, KC, AT designed the study. GC, AP, FD were responsible for clinical aspects and analyzed the results. AG, AP, HM, ML, KC, AT performed laboratory determinations and analyzed laboratory data. FB, AT, FD and KC drafted and revised the manuscript. All authors participated in data interpretation and revised the manuscript for intellectual content.

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