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Detection of coxsackievirus A6 in formalin-fixed, paraffin-embedded skin biopsy specimens using immunohistochemistry and real-time reverse-transcriptase PCR

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Abstract

Background: Hand, foot, and mouth disease (HFMD), classically a childhood viral infection, has an atypical and severe clinical presentation in adults. Coxsackievirus A6 is a leading cause of atypical HFMD, but current diagnostic methods utilizing formalin-fixed, paraffin-embedded skin biopsy specimens often lack sensitivity and specificity.

Methods: Formalin-fixed, paraffin-embedded skin biopsies from seven case patients with clinical and histopathological suspicion of atypical HFMD were evaluated by coxsackievirus A6 (CVA6) immunohistochemistry, enterovirus-specific conventional reverse transcriptase-PCR with subsequent Sanger sequencing targeting the 5'UTR, and CVA6-specific real-time PCR targeting the VP1 gene.

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Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

Ethics statement

This study was reviewed by the CDC Human Research Protections Office and received a non-research determination, as it does not meet the definition of research under 45 CFR 46.102(l). Therefore, IRB review was not required.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Amy M. Denison: Methodology, Validation, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Julu Bhatnagar:** Writing – review & editing, Supervision. **Richard R. Jahan-Tigh:** Resources, Writing – review & editing. **Pamela Fair:** Validation, Investigation, Writing – review & editing. **Gillian L. Hale:** Conceptualization, Methodology, Investigation, Writing – review & editing, Visualization, Supervision, Project administration.

Results: The CVA6-specific antibody demonstrated appropriate antigen distribution and staining intensity in keratinocytes in all cases. Conventional RT-PCR and sequencing also detected the presence of enterovirus, and CVA6-specific real-time RT-PCR analysis identified CVA6.

Conclusion: Applying these immunohistochemistry and molecular techniques to formalin-fixed, paraffin-embedded tissues, CVA6 was determined to be the causative infectious agent in seven cases of atypical hand, foot, and mouth disease.

Keywords

Hand, foot, and mouth disease (HFMD); Atypical HFMD; Formalin-fixed; Paraffin-embedded (FFPE) tissue; Coxsackievirus A6 (CVA6) real-time; reverse-transcriptase polymerase chain; reaction (rRT-PCR); Enterovirus; Immunohistochemistry (IHC)

1. Introduction

Hand, foot, and mouth disease (HFMD), caused by a subset of viruses in the *Picornaviridae* family, has historically been considered a mild self-limiting viral disease of infancy and young children [1, 2]. In recent years, there is increasing recognition of an atypical disease presentation caused by coxsackievirus (CV) A6 strain that broadly affects adults [3]. Viruses that cause HFMD belong to the enterovirus (EV) genus, and usually consist of EV-71, CVA16, or CVA10, but there is growing awareness for the predilection of CVA6 [4] infections to cause atypical HFMD infections in adults [3, 5, 6], with a prolonged clinical course, higher fever and protracted duration of characteristic vesiculobullous rash involving hands, feet and mouth [7]. Infections in adults are often transmitted within a household, from children to parents [8, 9]. HFMD outbreaks caused by CVA6 continue to increase worldwide [3], and CVA6 is one of the most common circulating strains in the World Health Organization's Western Pacific region, which includes China, where HFMD is a notifiable disease [4, 10, 11]. Other affected countries in this region include Singapore [12], Japan [13], Vietnam [14], and Korea [15], among others.

Characteristic histopathologic features of cutaneous coxsackievirus infection may include epidermal necrosis, intraepidermal vesiculation with associated neutrophilic inflammation, papillary edema and lymphocytic dermatitis. The histological differential diagnosis may include herpes simplex virus (HSV) infections, spongiotic dermatitis, erythema multiforme, and fixed drug eruption [5].

A wide range of specimens can be utilized for diagnosis of EV infections, including stool, blood, tissue biopsies, and oropharyngeal swabs [16]. Culture is the gold standard method for EV surveillance recommended by the World Health Organization [17]. However, in atypical cases when the diagnosis is not initially suspected in adults, a fresh tissue specimen may not be sent for culture studies; rather tissue biopsy may be obtained and fixed in formalin for microscopic examination, precluding culture for confirmatory testing. Molecular assays that target the 5' untranslated region can be used on samples to detect the presence of EV [18], while amplification of the VP1 capsid gene is utilized on viral isolates in order to specifically determine the viral subtype [19]. However, PCR insensitivity and complexity due to the effects of formalin-fixation, and a lack of commercially-available

antibodies for immunohistochemistry (IHC) detection [7, 20] have traditionally limited the specific detection of CVA6 in skin biopsy specimens.

While previous studies have demonstrated the detection of enteroviruses in formalin-fixed, paraffin-embedded (FFPE) tissues [21, 22], there are no reports to our knowledge of specific assays for detection of CVA6 in FFPE tissues by IHC or RT-PCR. In this study, we describe a series of seven cases in which HFMD was included in the differential diagnosis and were submitted to the Infectious Diseases Pathology Branch of the Centers for Disease Control and Prevention (CDC) for diagnostic evaluation. FFPE skin biopsy specimens were submitted, and histochemical, immunohistochemical and molecular techniques were performed to confirm infection with CVA6.

2. Materials and methods

Skin shave or punch biopsy specimens from seven case patients were obtained between the years 2013 and 2017 from three states (Georgia, Massachusetts, and Tennessee). Six of seven cases were adult male patients ranging from 23 to 73-years-old, while one case patient was a 14-year-old female.

Histopathologic features were evaluated by hematoxylin and eosin stains. Immunohistochemical assays were performed on 4-μm tissue sections of suspected enterovirus cases using indirect immunoalkaline phosphatase detection methodology as previously described [23]. For panenteroviral detection, monoclonal antibody (Millipore Sigma, Burlington, MA, USA; catalog # 3362) diluted at 1:30 was used for detection, and tissues were pretreated with 0.1 mg/mL proteinase K (Millipore Sigma, Burlington, MA, USA; catalog # 03115879001) in 0.6M Tris/0.1% $CaCl_2$ for 15 min. Polyclonal CVA6 antibody (GeneTex, Irvine, CA, USA; catalog GTX132346), which was targeted to an epitope of the VP1 protein, was diluted at 1:1000 for specific detection. Optimal pretreatment included heat-induced epitope retrieval with citrate (Reveal Decloaker, Biocare Medical, Pacheco, CA, USA). Antibodies were diluted in either LabVision Ultra Clean Diluent (ThermoFisher Scientific, Waltham, MA, USA; catalog # TA125UC, discontinued) or DaVinci Green Diluent (Biocare Medical, Pacheco, CA, USA). Appropriate positive and negative controls (normal rabbit serum, 1:1000, CDC) were run in parallel for each case.

RNA was extracted from a 16-μm FFPE tissue section from each case as previously described [24]. To ensure RNA was of sufficient quality, an internal control to the 18S rRNA gene was used (QuantumRNA Classic II 18S Internal Standard, ThermoFisher Scientific, Waltham, MA, USA). Extracts were first assessed for the presence of enterovirus using a 231-bp 5’ untranslated region (5’UTR) enterovirus RT-PCR assay [21]. Sanger sequencing of the amplicon could confirm the presence of enterovirus. RNA from cases with histological and molecular evidence suggestive of atypical HFMD after analysis at CDC were then subjected to rRT-PCR specific to the CVA6 VP1 gene. Using previously published concentrations and primers/probe sequences [25], the Invitrogen SuperScript III Platinum One-Step qRT-PCR kit (ThermoFisher Scientific, Waltham, MA, USA; catalog # 11732020) was used with the following conditions: 1 cycle of 42°C for 30 min, 1 cycle of 95°C for 2 min, and 45 cycles of 95°C for 15 sec and 57°C for 30 sec. Cases of confirmed EV71 (n =

4), and EV groups A (n = 1) and B (n = 5), as well as HSV types 1, 2 and 3 (varicella zoster virus) were included as negative controls in the RT-PCR assay to ensure specificity to CVA6.

3. Results

Clinical data for seven case patients with associated histopathologic features is described in Table 1.

By immunohistochemistry, the CVA6-specific antibody highlighted the skin lesions from all seven case patients with an appropriate distribution and staining intensity (Fig. 1). The six adult cases were also immunoreactive with a nonspecific panenterovirus antibody. Specificity testing was performed by testing the antibody against other viral infections in the clinical differential diagnosis, and with other enteroviruses. The CVA6-specific antibody cross-reacted with one confirmed CVA16-positive FFPE case though the CVA6-specific rRT-PCR was negative on this specimen. There was no cross-reactivity of the CVA6 antibody to cases of EV group B (n = 4), orf virus, and HSV types 1, 2, and 3 (varicella zoster virus).

Molecular testing performed on RNA extracted from FFPE skin biopsies using broad-range 5'UTR enterovirus RT-PCR, followed by Sanger sequencing of the amplicons and analysis using the National Center for Biotechnology Information Basic Local Alignment Search Tool, detected the presence of group A enterovirus in all cases [21]. The prototypic Gdula strain [26] was initially chosen as a positive control for the CVA6 rRT-PCR assay, but no amplification was observed. Therefore, to fully ensure validity of the rRT-PCR results, two of the seven cases identified in this study were also verified as CVA6 using whole genome sequencing by the CDC's Polio and Picornavirus Laboratory Branch (GenBank accession numbers SAMN18206580 and SAMN18206581; SRA database BioProject PRJNA707479). Insufficient tissue specimen quantity prohibited analysis of additional specimens by whole genome sequencing. Therefore, RNA from case patient three was used as a positive control in the rRT-PCR assay in subsequent runs. The rRT-PCR assay confirmed the presence of CVA6 in all seven cases. All 10 EV71 and EV group A and B positive cases included as controls, as well as HSV types 1, 2 and 3 were negative by the rRT-PCR, as expected. Further comparison of the primer/probe annealing regions of the two cases verified by whole genome sequencing to the Gdula strain show several mismatches, especially in the nucleotides that correspond to the probe's sequence (Fig. 2).

4. Discussion

Atypical HFMD caused by CVA6 is increasing in prevalence among adults [3], but specific detection of CVA6 in FFPE tissue specimens has been met with diagnostic challenges [20]. The CVA6-specific immunohistochemical and rRT-PCR assays developed herein meet the important need for improved diagnostic modalities in atypical HFMD infections in FFPE tissues.

The CVA6 antibody used in IHC demonstrates appropriate immunostaining intensity and distribution in all seven PCR-confirmed CVA6 cases tested (Fig. 1), and did not cross-react with other common viral exanthems The antibody was noted to cross-react to one PCR-

confirmed case of CVA16, and therefore could potentially cross-react with this viral strain, but limited internal human control tissue precluded more extensive testing of the antibody against CVA16.

There are inherent limitations in utilizing FFPE biopsy specimens for immunohistochemical and molecular analysis. For example, prolonged tissue fixation can limit nucleic acid quality in PCR though skin biopsies are small specimens that tend to fix well. Temporal association of tissue sampling in relation to the stage of illness and viral burden may also affect the sensitivity of both assays, but all case patients in this study were found to have abundant viral antigens when the vesiculobullous lesion was sampled. Our study would further be strengthened by correlating viral detection in tissues to detection in other specimens such as stool; however, the atypical presentation of enterovirus in an adult and the retrospective design of the study precluded that comparison. Future studies in which a variety of sample types are taken simultaneously would be of value in further elucidating the pathogenesis and natural progression of the disease when it presents in an atypical adult patient population.

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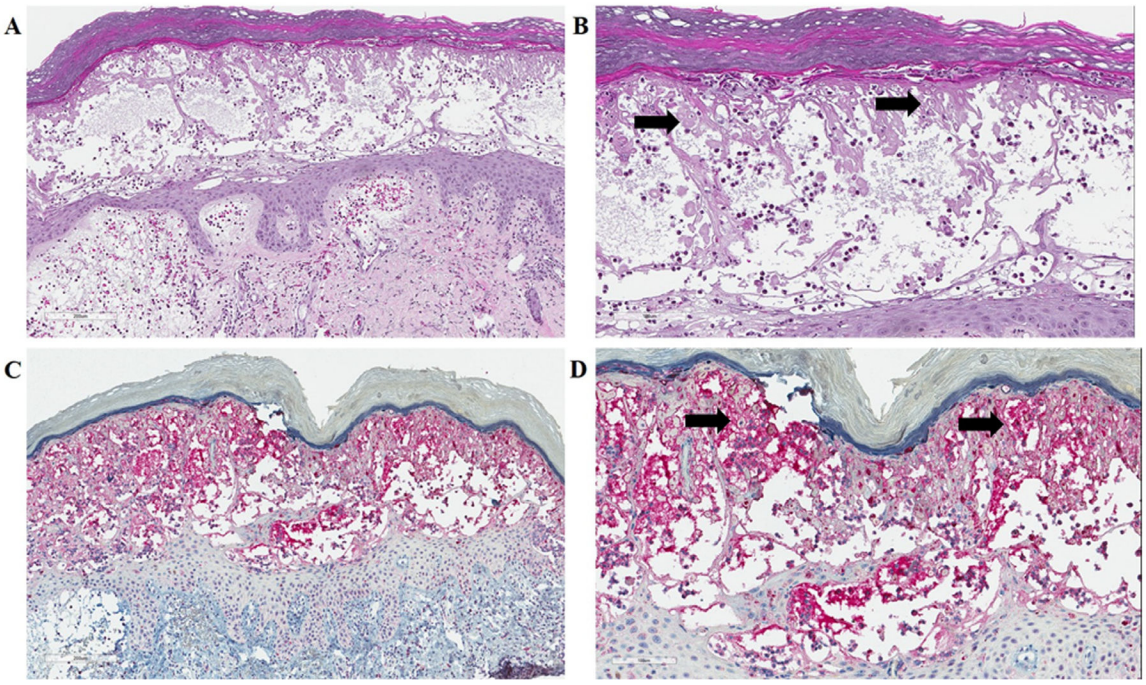

Fig. 1.

Hematoxylin and eosin (H&E) staining (A&B) and immunohistochemical (IHC) staining (C&D) as Exemplified by Case Patient Number Seven. A. H&E 10X. The shave biopsy of the right dorsal index finger joint shows intraepidermal vesiculation with associated neutrophilic inflammation, papillary edema and a lymphocytic dermatitis. B. H&E 20X. Higher power demonstrates epidermal necrosis in the area of vesiculation (arrows), intralesional neutrophils and edema. C. IHC 10X. CVA6 immunostain using a red chromogen highlights the extensive intracytoplasmic viral antigens throughout the lesion. D. IHC 20X. A higher power image of the CVA6 immunostain shows extensive cytoplasmic staining of the infected cells in the area of vesiculation (arrows).

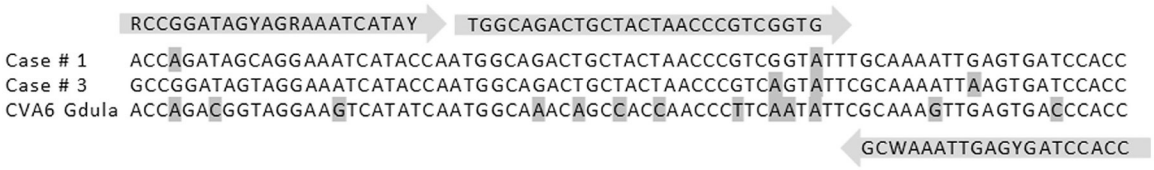

Fig. 2.

5’ to 3’ nucleotide sequence of the portion of the EV VP1 gene in which the forward and reverse primers and probe anneal (primer/probe sequences and orientation in gray arrows) from cases 1 and 3 as well as the Gdula strain. Nucleotide mismatches are also shaded in gray, demonstrating the larger number of divergent nucleotides present in the Gdula strain.

Table 1

Age, gender, biopsy site and type, and histologic impression and features of each of the seven cases.

Case No.	Age	Gender	Biopsy site	Biopsy type	Provided clinical history and submitter's histologic impression	Histopathologic features
1	14	F	Right medial dorsal foot	Punch	HFMD versus erythema multiforme.	Interface, superficial and deep lymphohistiocytic dermatitis with epidermal ballooning and necrosis
2	34	M	Right foot	Shave	Acute eruption on hands, feet and buttocks compatible with HFMD.	Intraepidermal vesicle
3	23	M	Right ventral proximal forearm	Punch	Bullae, erosions with yellow-honey colored crust; HFMD versus bullous erythema multiforme, or HSV.	Intraepidermal vesicle
4	33	M	Right arm	Punch	Recent-onset rash involving face, palms, soles, and genitals; Skin biopsy suspicious for HFMD. Serology positive for CVB5 titer (1:18).	Intraepidermal vesicle with neutrophilic infiltrate, dyskeratosis and acantholysis
5	39	M	Left index finger	Punch	Painful 2-mm red papules on fingers, palms, and plantar feet, sore throat. Histology favors HFMD.	Lymphocytic dermatitis with epidermal reticular degeneration, vesiculation and necrosis
6	38	M	Not specified ("skin rash")	Punch	Papular rash on hands and feet after exposure to child with suspected Coxsackie (A6) infection (atypical HFMD).	Acral skin with mild lymphocytic dermatitis, dermal papillary edema, and focal epidermal necrosis
7	73	M	Right dorsal index finger joint	Shave	History of diabetes and hypertension, status post ICD placement, presented with a rash on extremities. Vesicular lesions on hands, and faint plaques with dusky erythema on the forearms and torso.	Vesicular dermatitis