Neonatal hand, foot, and mouth disease due to Coxsackievirus A6 in Shanghai

CURRENT STATUS: UNDER REVIEW

BMC Pediatrics

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SUBJECT AREAS
Pediatrics

KEYWORDS
Hand-foot-and-mouth disease, Neonate, Coxsackievirus A6, Clinical symptom, Transmission route, Immunologic function
Abstract

Background: Evidence of hand, foot, and mouth disease (HFMD) in neonates is limited. The aim of this study was to evaluate the clinical symptoms, possible transmission routes, and prognosis of neonatal HFMD in Shanghai.

Methods: This was a case-control study based on the HFMD registry surveillance system. All neonates and infected family members were enrolled between 2016 and 2017 in Shanghai. Neonates with HFMD were followed for at least half a year. Detailed questionnaires, medical history, and physical examination were recorded. Routine blood examination, liver and renal function, immunophenotypes of peripheral blood lymphocytes (CD3, CD4, and CD8 T-cells; NK cells), immunoglobulin (Ig) M, IgG, and IgA, and cytokine interleukin (IL-1β, IL-2R, IL-6, IL-8, IL-10, and TNF-α) levels were measured. All rectal swab specimens were collected and genotyped for enterovirus. T-test or nonparametric test was used to evaluate the differences. Logistic analysis was applied to calculate the risk of clinical symptoms in the group of HFMD neonates and their paired siblings.

Results: There were 16 neonates among the 12608 diagnosed patients with HFMD, accounting for 0.1%. All neonatal infections were transmitted by other members of the family, mainly the elder siblings, and involved different types of coxsackievirus A6. Coxsackievirus A6 is also the emerging and predominant causative agent of HFMD in Shanghai. None of the neonates with HFMD suffered fever, onychomadesis, or severe complications. However, two elder sibling patients showed lethargy, and one developed hypoperfusion. In the elder siblings with HFMD, the proportion of white blood cells was generally higher than in neonates with HFMD. The immunologic function of the neonates with HFMD was basically normal. The levels of inflammatory markers were higher in both neonates and elder siblings with HFMD compared to their age-matched controls. The clinical symptoms receded after about one week of onset. None of the neonates had sequelae.

Conclusions: All neonates with coxsackievirus A6 HFMD had mild disease with no complications or sequelae. Notably, due to the two-child policy in China, elder siblings may be the main route of HFMD transmission.

Introduction
Hand, foot, and mouth disease (HFMD) is one of the most recognizable viral exanthems in children and adults [1]. In March 2008, a sudden outbreak of HFMD occurred in Anhui Province, China. In May, HFMD was defined as a C-class notifiable disease. The incidence of HFMD ranks first among communicable diseases since 2009, and has become an important public issue [2, 3]. Although HFMD is generally a mild clinical syndrome [4, 5], serious complications may occur [6]. Although HFMD age of onset is widely variable, ranging from neonatal age to 70 years, children aged 5 years and younger are the most susceptible subjects and may develop severe clinical symptoms [7, 8]. It was reported that subjects younger than 3 years have an increased risk of severe HFMD [2, 8]. However, to date, the age-specific risk of severe HFMD in young children has not been established.

Evidence of HFMD in neonates is limited. Thus far, three case reports have been published on 7 cases of neonatal HFMD [9-11]. One study reported on a neonatal enterovirus (EV) 71 infection, while another described a coxsackievirus (CV) B3 infection in 2014. Both two cases progressed to severe HFMD. Another report in 2017 mentioned five benign neonatal cases without associated pathogens. In this prospective cohort study, the neonates with HFMD and their families were recruited in Shanghai in 2016-2017, and the epidemiological features, clinical presentation, pathogens, and immune function in neonates with HFMD were explored in comparison with diseased siblings.

Materials And Methods

Participants and specimens

This was a case-control study based on the National Registry of HFMD. The Chinese government established a network-based national surveillance system for HFMD since 2009. In Shanghai, local health providers and physicians are required to report clinically diagnosed HFMD cases to the Shanghai Municipal Centre for Disease Control and Prevention (CDC) within 24 h via the surveillance system. Basic epidemiologic and clinical information is recorded for each HFMD patient [12]. Sixteen local CDC representing as many districts are responsible for sample collection and transport. The specimens of patients were sampled for pathogen testing at local sentinel hospitals in each district. At least ten outpatients were diagnosed with HFMD each month. The clinicians can also test the specimens as the condition requires. Throat and/or faecal swabs were then sent directly to
microbiology laboratories at the local CDCs, where the presence of EV71, CV-A16, CV-A6, CV-A10, and other EVs was confirmed by real-time PCR [13]. The vast majority of children with HFMD are treated in two designated hospitals, the Children’s Hospital of Fudan University and the Xinhua Hospital affiliated to Shanghai Jiao Tong University School of Medicine.

All cases were diagnosed according to the criteria specified by the HFMD Prevention and Treatment Guidelines [14]. Patients who had a rash, with or without fever, and no other organ damage, were classified as having common HFMD. Those with any complication (i.e., aseptic meningitis, brainstem encephalitis, encephalitis, encephalomyelitis, acute flaccid paralysis or autonomic nervous system dysregulation, pulmonary oedema, pulmonary haemorrhage, or cardiopulmonary failure), or those who died, were classified as having severe HFMD. From January 2016 to December 2017, 12608 patients were diagnosed with HFMD at Xinhua Hospital affiliated to Shanghai Jiao Tong University School of Medicine. The distribution of patients covered all 16 municipal districts in Shanghai.

Patients who met the following criteria were recruited in our study: 1. neonates diagnosed less than 28 days after birth; 2. skin lesions manifested as small vesicles, papulovesicular lesions or macular rashes on the palms, soles, buttocks, and oral mucosa, or were present on the limbs, trunks or facial areas. All family members were included in the screening. Because the prognosis of neonatal HFMD is unknown, they were all admitted to the hospital for observation, including routine clinical blood examination, evaluation of biochemical and immune function, and virus detection. The clinical specimens (e.g., rectal swabs and plasma) were collected from each patient within one day of diagnosis. To evaluate alterations in parameters, such as immune function, we also recruited age- and birth weight-matched non-infected neonates (e.g., infants with breast milk jaundice) as neonatal controls, and age-matched preoperative patients without infection (e.g., subjects with hypospadias) as elder sibling controls. Finally, 16 neonates with HFMD and their infected families were included in the study and followed up for at least six months for sequelae.

This study was approved by the Ethics Committee of Xinhua Hospital, affiliated to Shanghai Jiao Tong University School of Medicine (XHEC-C-2018-082), and the procedures were carried out in accordance with the Helsinki Declaration. Parents or guardians of each case or control were required to sign a
written informed consent form. The relevant tests were paid by the research group.

**Data collection**

Demographic data, clinical manifestations, and laboratory findings of each participant were recorded. Fever, timing, and distribution of skin lesions were evaluated. The skin lesions were classified into 8 groups based on the site: perinasal, perioral, scalp, palms/soles, lower limbs, upper limbs, abdomen, and intraoral lesions.

Complete blood cell count, liver and kidney function, and the levels of myocardial enzymes, immunoglobulins, lymphocyte subsets, and cytokines were assessed in cases and controls. The immunophenotypes of peripheral blood lymphocytes (CD3, CD4, and CD8 T-cells, NK cells) were determined by flow cytometry (Becton Dickinson Immunocytometry Systems) and analysed by Cell Quest software (Becton Dickinson). The serum levels of immunoglobulin (Ig) M, IgG, and IgA were detected by turbidimetric immunoassay. ELISA (Quantikine; R&D Systems) was used for quantitative determination of the cytokines IL-1β, IL-2R, IL-6, IL-8, IL-10, and TNF-α. The assays were performed according to the manufacturer’s instructions.

The EVs were genotyped from rectal swab specimens. Viral RNA was extracted directly from clinical specimens using a QIAamp Viral RNA Mini Kit (Qiagen, Santa Clara, CA) and stored at -80 °C. EV identification and serotyping of EV71 and CV-A16 from samples were performed by real-time reverse transcription-polymerase chain reaction (RT-PCR), as previously described [15, 16]. To further identify enteroviral serotypes other than EV71 and CV-A16, semi-nested RT-PCR and sequencing were conducted as previously described [17]. The serotype was determined by comparison of the viral sequences with the corresponding sequences of the EV prototype strains using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

**Statistical analysis**

We calculated the means and standard deviations of normally distributed variables, and the medians (interval of quartiles) of variables with skewed distributed. For pairwise comparisons, T-test was used
in case of normal distribution, while nonparametric tests were applied in case of non-normal distribution. All cytokine levels showed non-normal distributions. Thus, they were log-transformed to obtain normal distributions. Frequency and percent values were calculated for categorical variables, and the chi-square test was used to determine the differences in these variables between neonatal and paired siblings with HFMD. Logistic analysis was applied to calculate the risk of clinical symptoms in these two groups. All statistical analyses were conducted using SPSS 17.0 software. A $p$-value <0.01 was regarded as statistically significant.

Results

Clinical presentation of neonates and elder siblings with HFMD

There were 16 neonates among the 12608 diagnosed HFMD cases at Xinhua Hospital affiliated to Shanghai Jiao Tong University in Shanghai 2016-2017, accounting for 0.1%. Of the 12608 cases, 14 had severe HFMD, most of which due to EV71 infection. A total of 259 patients were sampled from this sentinel hospital for routine EV surveillance. Of these, 206 were positive cases, with a positive rate of 79.5%. CV-A6 was the predominant causative agent of HFMD (Figure 1). All neonatal cases were not severe, while two elder siblings developed severe HFMD. Half of the neonates with HFMD were diagnosed during summer.

The median age of the neonates with HFMD was 25 days, ranging from 21 to 27 days. All the neonates were full-term with a normal birth weight. The median age of their elder siblings was 4.1 years, ranging from 2.4 to 6.3 years. The percentage of males was 37.5% among neonates and 62.5% among elder siblings. As shown in Table 1, the HFMD symptoms were milder in neonates than in their elder siblings. None of the neonates developed complications such as hypoperfusion, lethargy, onychomadesis, or others. Most of the elder siblings, but none of the neonates, had fever. Ten elder sibling patients had vomiting symptoms, two had lethargy, and one developed hypoperfusion. The prevalence of vomiting was 5 times higher in the elder siblings than in the neonates. Cutaneous lesions, especially intraoral erosions, were more common in elder siblings than in neonatal cases. In neonatal cases, the site of the rash was not typical, and was mainly in the perioral area and the upper limbs (Figure 2). After about one week of symptomatic treatment, the clinical symptoms receded, and
no sequelae occurred within half a year. Interestingly, all infected neonates had an elder sibling affected, and two parents in the 16 families had oral infections by herpes viruses other than herpes simplex.

**Laboratory findings**

We found that the white blood cell (WBC) count was higher in elder siblings with HFMD compared to age-matched controls (Table 2). However, such differences were not detected between neonatal cases and controls. No statistically significant differences were found in liver function, kidney function, and cardiac enzymes between cases and controls, either in neonates or their elder siblings. Regarding immune function, as shown in Table 3, the levels of the inflammatory markers IL-1β, IL-2R, IL-6, and TNF-α were higher in cases compared to controls, among both neonates and their elder siblings (P <0.01). The levels of IgA and IgM were higher in the elder sibling patients than in the neonates, which may be due to age-related immunological development. In the neonates with HFMD, the Ig levels were normal, but the level of CD8 T-cells was lower compared to age-matched controls. In particular, the neonate cases exhibited a median CD8 T-cell count of 534.0 (314.2, 824.6)/μL, while their controls exhibited a median CD8 T-cell count of 970.0 (904.5, 1150.5)/μL (P <0.01). There were no significant differences in other T cell types in any of the groups.

Aetiological evaluation revealed that all neonatal and elder sibling cases were infected with CV-A6 (CV-A6/P619/2013/China capsid protein, P2 protein, CV-A6/P477/2013/China capsid protein, P2 protein, CV-A6/P471/2013/China capsid protein, P2 protein, CV-A6/P424/2013/China capsid protein, P2 protein). In each family, same viral genotype was found, suggesting the homogeneity of the infection in the family.

**Discussion**

Neonatal HFMD is rarely reported in the literature. According to this study, the proportion of neonatal HFMD cases, among all cases, was only 0.1% in Shanghai, 2016-2017. All 16 neonates became infected from other family members, mainly their elder siblings. They were diagnosed with different subtypes of CV-A6 infection aetiologically. Neonatal HFMD cases showed normal immune function.
Almost all cytokines exhibited higher plasma levels in cases than in controls. HFMD is a common acute EV infection, characterized by short-lasting fever, mouth ulcers, and vesicles on the hands, feet, or hips [18]. It can be transmitted both horizontally (faecal-oral/respiratory route) and vertically (prenatal infection). Most new-borns presenting with serious EV disease acquire the infection from a symptomatic mother in the perinatal period; up to 60% of the mothers of infected infants report a febrile illness during the last week of pregnancy [19]. Additionally, serious EV disease may be acquired via nosocomial transmission, spreading throughout nurseries via caregivers engaged in mouth care, gavage feeding, and other activities requiring direct contact. Close contact with infected family members or caregivers is also an important route of transmission. In our study, the age of neonatal onset ranged between 19 and 28 days, and the mothers had no prenatal infection symptoms; therefore, vertical transmission was not considered. In China, mothers usually rest indoors for one full month after giving birth, avoiding contact with people outside of the family. Therefore, the chances of infection are relatively low for mothers. With the adoption of the two-child policy, the risk of infection is very high for elder siblings, who are generally pre-schoolers in kindergartens [20]. In addition, according to epidemiological investigation, elder siblings were infected earlier than the neonates, by the same pathogen strain. This further supported within-family transmission. However, establishing the transmission pathway is still a difficult challenge.

The genus enterovirus includes four species (A-D) known to infect humans. CV-A16 and EV71 are the serotypes most frequently associated with HFMD, responsible for most of the large outbreaks [21]. Among healthy individuals in Shanghai, 50.5% and 54.2% are positive for neutralising antibodies against EV71 and CV-A16, respectively [13]. Beginning in 2008, CV-A6 has been increasingly reported as a cause of HFMD outbreaks worldwide, and may be associated with more severe diseases than typical HFMD [4, 22-28]. Sporadic cases and epidemics of CV-A6 have been reported, principally affecting elder children, adolescents, and adults rather than young infants. CV-A6 has replaced EV71 and CV-A16 as the most common pathogen causing HFMD in Shanghai [12]. Thus, we reasoned that most pregnant women could have been infected asymptotically, but the proportion of neutralizing
antibodies in maternal blood was too low to protect the children.

In the literature, significant clinical differences were reported in HFMD manifestations depending on the pathogen. Genetic typing to establish the exact virus strain is usually not necessary to confirm the HFMD diagnosis. However, in some cases of HFMD, identification of the virus type is crucial for appropriate disease management and to reliably assess the risk of potential complications. The sole published case of neonatal EV71 infection was quite severe. Another reported case of CV-B3 infection, which was not fatal and self-limited in children, also caused severe disease in a neonatal case. However, none of the five neonates clinically diagnosed with HFMD in southeast China developed brainstem encephalitis or pulmonary oedema, and all recovered well. CV-A6 had a broad spectrum of manifestations [29]. In our study, the symptoms of neonatal HFMD were mild. Moreover, the risk of neonatal cases with fever, vomiting, and onychomadesis was lower than in elder children. Some HFMD cases exhibited an atypical skin presentation with facial involvement and vesiculobullous lesions on the body.

Immunological reactions may be critical for HFMD. Almost all fatal HFMD cases had symptoms of autonomic nervous system dysregulation and increased sympathetic discharge, indicating the involvement of reticular formation [30]. Systemic inflammatory response also played an important role. Many studies showed that virus infection activated the host’s immune system, released cytokines, and caused tissue and cell damage [31]. In our study, cytokine and WBC levels were increased in both neonatal and elder sibling patients. However, neonatal HFMD cases showed significantly lower CD8 T-cell counts compared to diseased elder siblings, which is not uncommon in acute viral infection. The T-cell subset assay is an accurate method to evaluate cellular immunity, and abnormal results may indicate occurrence or aggravation of viral diseases. Regarding the T-cell phenotype in children with HFMD, Wang and colleagues found that CD4 T-cells, CD8 T-cells, and NK cells were depleted in patients with pulmonary oedema, possibly resulting in impaired EV71 clearance [32]. Another study had found that CD4 T-cells decreased while CD8 T-cells showed no change [33]. There are few reports on neonatal HFMD. We speculate that the low level of CD8 T-cells may be related to the sampling time. Although the samples were taken upon admission, there may have been
a delay between the onset at home and the appearance of a rash. Since only the symptomatic population was considered, this study may contain a selection bias. In addition, some results, such as the low level of CD8-T cells, need to be confirmed by further studies.

Conclusions
Neonatal HFMD caused by CV-A6 shows mild clinical symptoms and basically normal immune function. Neonatal HFMD is not necessarily very serious, and the severity of the disease may depend on the pathogen. In China, with the gradual adoption of the two-child policy, the elder brother or sister is the main source of infection. In case of infection, control measures should be in place.

Abbreviations
CV, coxsackievirus; EV, enterovirus; HFMD, hand foot and mouth disease; WBC, white blood cell

Declarations

Ethics approval and consent to participate:
This study was approved by the Ethics Committee of Xinhua Hospital (XHEC-C-2018-082), and the procedures were carried out in accordance with approved guidelines.

Consent for publication:
Not applicable.

Availability of data and material:
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests:
The authors declare that they have no competing interests.

Funding:
The funding received for this work was from the National Natural Science Foundation of China.

Number: NSFC 81530086.

Authors' contributions:
LH and WZ had the idea for the study, designed and revised the manuscript. SX and HL were major contributors in writing the manuscript. GX, DZ, XL, HY, and YQ collected the data. PQ and WZ detected the pathogens. XZ analysed the data. All authors read and approved the final manuscript.
Acknowledgements:

The authors thank all the other members of the Department of Paediatric Infectious Diseases, Xinhua Hospital, affiliated to Shanghai Jiao Tong University School of Medicine: Heyu Huang, Xinxin Zeng.

Writing assistance was provided by Elsevier Language Editing Services.

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Tables
Table 1. Clinical features of neonates and paired older siblings with hand, foot, and mouth disease
| Clinical features                  | Neonates (N = 16) | Older siblings (N = 16) | Odds ratio | P-ν |
|----------------------------------|-------------------|------------------------|------------|-----|
| Fever (temperature ≥39 °C)       | 0                 | 14 (87.5)              | 0 (0.0, 0.1) *b | < 0 |
| Vomiting                         | 2 (12.5)          | 10 (62.5)              | 0.2 (0.1, 0.8) * | < 0 |
| Seizure                          | 0                 | 0                      | 1.0 (0.1, 7.3) b | 0.50 |
| Symptom of hypoperfusion         | 0                 | 1 (6.3)                | 0 (0.0, 19.0) b | 0.50 |
| Lethargy                         | 0                 | 2 (12.5)               | 0 (0.0, 3.4) b | 0.50 |

Cutaneous areas affected

|                    | Neonate HFMD | Neonate control | P-value |
|--------------------|--------------|-----------------|---------|
| Perinasal          | 4 (25.0)     | 3 (18.8)        | 1.4 (0.2, 9.1) 0.50 |
| Perioral           | 13 (81.3)    | 14 (87.5)       | 0.9 (0.7, 1.3) 0.50 |
| Scalp              | 0            | 0               | 1.0 (0.1, 7.3) b 0.50 |
| Palms/soles        | 6 (37.5)     | 13 (81.3)       | 0.4 (0.2, 0.8) b 0.50 |
| Lower limbs        | 2 (12.5)     | 9 (56.3)        | 0.3 (0.1, 1.0) b 0.50 |
| Upper limbs        | 14 (87.5)    | 13 (81.3)       | 1.3 (0.4, 4.0) b 0.50 |
| Abdomen            | 0            | 1 (6.3)         | 0 (0.0, 19.0) b 0.50 |
| Intraoral erosions | 10 (62.5)    | 16 (100.0)      | 0.4 (0.2, 0.6) * < 0 |
| Onychomadesis      | 0            | 5 (31.3)        | 0 (0.0, 0.7) * 0.50 |
| Complications      | 0            | 1 (6.3)         | 0 (0.0, 19.0) b 0.50 |

^a Two-tailed exact P-value.
^b Conditional maximum likelihood estimate of odds ratio.
*Statistical significance.

Table 2. Routine blood test of neonates and paired older siblings with hand, foot, and mouth disease

| Parameter             | Neonate HFMD | Neonate control | P-value* | Older-sibling |
|-----------------------|--------------|-----------------|----------|---------------|
| WBC count (x10^9/L)   | 8.5 (4.2, 12.8) | 7.1 (5.5, 9.8) | 0.51     | 15.5 (6.4, 20.6) |
| RBC count (x10^12/L)  | 4.3 (3.2, 5.1)  | 4.1 (3.3, 4.6) | 0.20     | 4.3 (3.5, 5.5) |
| Platelet count (x10^9/L) | 378.5 (164.6, 406.9) | 349.0 (248.5, 398.0) | 0.01 | 283.1 (233.7) |
| Leukomonocytes (%)    | 53.4 (24.8, 76.4) | 54.6 (43.7, 61.4) | 0.84 | 47.2 (30.5, 67.0) |
| Monocytes (%)         | 11.2 (9.4, 14.7)  | 9.7 (7.8, 11.7) | 0.12 | 10.3 (7.9, 17.0) |
| Neutrophils (%)       | 26.6 (19.2, 49.2) | 22.0 (13.7, 34.2) | 0.48 | 38.6 (13.2, 78.1) |
| Haemoglobin (g/L)     | 103.2 (89.7, 118.1) | 140.0 (107.0, 154.5) | 0.05 | 118.1 (101.2) |

P-value*: T-test between neonatal HFMD cases and controls.
P-value*: T-test between older siblings with HFMD and age-compared controls.
P-value*: T-test between neonatal older-sibling HFMD cases.
*Statistical significance.
### Table 3 Examination of the immune function in neonates and paired older siblings with hand, foot, and mouth disease

| Parameter                        | Neonate HFMD | Neonate control | P-value\(^1\) | Older-sibling HFMD | P-value\(^2\) | P-value\(^3\) |
|----------------------------------|--------------|----------------|---------------|-------------------|---------------|---------------|
| CD3 T-cell                       | 2837.2 (2243.3, 3982.1) | 3536.3 (3196.6, 4450.2) | 0.01          | 221               |               |               |
| CD4 T-cell                       | 2161.6 (1845.8, 4132.7) | 2488.5 (2165.5, 3379.0) | 0.48          | 122               |               |               |
| CD8 T-cell                       | 534.0 (314.2, 824.6)   | 970.0 (904.5, 1150.5)   | < 0.01*       | 911               |               |               |
| CD16+CD56+ (NK cell)            | 250.3 (123.9, 325.4)   | 361.0 (239.5, 478.5)    | 0.72          | 573               |               |               |
| IgG                              | 3.2 (2.3, 4.1)         | 6.2 (5.2, 7.0)          | 0.68          | 9.7               |               |               |
| IgA                              | < 0.3              | < 0.3                    | 1             | 2.0               |               |               |
| IgM                              | 0.4 (0.2, 0.6)        | 0.2 (0.2, 0.3)           | < 0.01*       | 2.5               |               |               |
| IL-1β                            | 730.1 (384.8, 937.5)   | 61.9 (22.5, 71.5)        | < 0.01*       | 101               |               |               |
| IL-2R                            | 1516.4 (497.3, 2732.3) | 1438.5 (1277.5, 1556.5) | < 0.01*       | 139               |               |               |
| IL-6                             | 32.4 (28.2, 67.3)     | 3.8 (3.1, 48.4)          | < 0.01*       | 16.4              |               |               |
| IL-8                             | 63.6 (14.6, 1283.6)    | 110.8 (19.7, 1866.0)     | < 0.01*       | 58.4              |               |               |
| IL-10                            | 26.4 (14.2, 46.8)     | 17.5 (12.3, 53.1)        | 0.01\(^c\)    | 79.4              |               |               |
| TNF-α                            | 15.3 (9.5, 38.2)      | 13.2 (11.0, 26.7)        | < 0.01*       | 18.6              |               |               |

P-value\(^1\): T-test between neonatal HFMD and controls.

P-value\(^2\): T-test between older siblings with HFMD and age-compared controls.

P-value\(^3\): T-test between neonatal and older-sibling HFMD cases.

*Statistical significance.

\(^c\)T-test after log-transformed.

**Figures**
Figure 1

Enterovirus-positive HFMD cases, 2016-2017.
Figure 2

A 22-day-old boy with CV-A6 infection showing vesicles on the upper limb.