Pathologic and molecular studies of enterovirus 71 infection in a fatal case from a recent epidemic in China
A case report
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Abstract
Rationale: Enterovirus 71 (EV71) is identified as the primary cause of hand, foot, and mouth disease (HFMD) and mainly infects the young infants. Though some fatal cases have been reported, the underlying mechanisms of EV71 infection remain elusive and more further pathologic and molecular studies of EV71 infection are needed.

Patient concerns: A 26-month-old girl with a history of fever and lethargy for 3 days and intermittent seizures for 2 hours associated with rash on 4 limbs was brought to a hospital.

Diagnoses: The autopsy was performed to identify the cause of death for a medical dispute. The results of histologic examination, immunohistochemistry (IHC), nested reverse transcription polymerase chain reaction (RT-PCR), and viral isolation confirmed that this patient died of EV71 infection.

Interventions: The patient was transferred to neonatal intensive care unit and was intubated and mechanically ventilated. The other treatment included cardiopulmonary resuscitation and intravenous injection of adrenaline.

Outcomes: The patient presented persistent coma and intermittent seizures and suddenly developed respiratory arrest and died 16 hours after admission.

Lessons: Our results suggest that EV71 might invade into the central nervous system (CNS) through peripheral nerves which control the digestive tract in the early stage of infection. In addition, we successfully isolated one EV71 strain. Phylogenetic analysis showed that the isolated strain clustered in the C4a of C4 subgenotype. This case also highlights that rapid deterioration in HFMD cases is still a challenge to physicians and they must pay special attention to the infants with HFMD symptoms, particularly in EV71 epidemic areas for early diagnosis and treatment.

Abbreviations: CNS = central nervous system, EV71 = enterovirus 71, HFMD = hand, foot, and mouth disease, IHC = immunohistochemistry, RT-PCR = reverse transcription polymerase chain reaction.

Keywords: encephalomyelitis, enterovirus 71, immunohistochemistry, pulmonary edema, RT-PCR

1. Introduction
Hand, foot, and mouth disease (HFMD) is a common self-limiting childhood disease and is characterized by fever, oral ulcers, and vesicular lesions on the hands and feet. Enterovirus 71 (EV71), a member of the human enterovirus species A within the family Picornaviridae, is considered as the major pathogen of HFMD.[1] Though EV71 mainly infected the children under 5 years and caused mild HFMD, few cases aged 3 years and younger developed severe neurological complications and even death.[1,2] The neurological complications range from aseptic meningitis to acute flaccid paralysis and brainstem encephalitis, which is associated with systemic features, such as severe pulmonary edema and shock.[1,2] Since EV71 was initially isolated in California in 1969,[3] it has caused many large HFMD outbreaks throughout the world, especially in the Asia-Pacific region.[4-6]

Until now, the underlying mechanisms of EV71 infection, especially the model of viral spread into the central nervous system (CNS) and the mechanism of pulmonary edema have not yet been clarified completely, which was probably due to the scarce cases of autopsy and the lack of appropriate animal...
models. Here we report a fatal case of EV71 infection during the past outbreak of HFMD in China.

2. Case presentation

A 26-month-old girl was brought to a hospital after suddenly becoming unresponsive at home. She had a history of fever and lethargy for 3 days and intermittent seizures for 2 hours prior to admission. On admission, physical examination revealed that the temperature was 38.3°C, heart rate was 106 beats/min, and blood pressure was 118/66 mm Hg. Several vesicular rashes and small papules were found on her hands and feet. No obvious oral ulcers were observed. She was transferred to neonatal intensive care unit. The treatment included tracheal intubation, cardiopulmonary resuscitation and intravenous injection of adrenaline. She presented persistent coma and intermittent seizures and suddenly developed respiratory arrest and died 16 hours after admission. Prior written and informed consent for publication of the case were obtained from the patient’s family and the study was approved by the Human Ethical Commission at Medical School of Ningbo University.

The autopsy was performed to identify the cause of death for a medical dispute. External examination revealed a few vesicles on the instep of the right foot. Subcutaneous hemorrhage was seen in the skin with vesicles after its incision. Marked pulmonary congestion and edema were seen in both lungs grossly. Tissue blocks from the CNS and non-CNS tissues were fixed in 10% buffered formalin for a week and sampled routinely. The hematoxylin–eosin (H&E) staining was processed routinely and showed typical features of acute encephalomyelitis, manifested as perivascular cuffing (Fig. 1A), microglia nodules (Fig. 1A), encephalomalacia (Fig. 1B) and necrosis with neuronophagia. The lesions were mainly located in the brainstem and the anterior spinal cord. The basal ganglia, thalamus, and dentate nucleus were also involved. The lungs revealed marked pulmonary edema with multifocal pulmonary hemorrhage and mild inflammatory infiltration (Fig. 1C). Mild mononuclear lymphocytes infiltration in the myocardial interstitium and epicardium were observed. Reactive lymphoid hyperplasia in the enlarged tonsils and the submucous lymphatic tissues of larynx (Fig. 1D) were seen. Multifocal inflammatory infiltration accompanied by destruction of ducts and acini in the submandibular gland was also observed (Fig. 1E).

A polyclonal mouse anti-EV71 antibody (1:1000 dilution; a gift from the State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences) was used as the primary antibody for immunohistochemistry (IHC) staining. The IHC staining was performed using a standard avidin-biotin immunoperoxidase technique.[7] Control sections were incubated with non-immune medium instead of primary antibody. IHC staining showed that viral antigens were mainly located in the cytoplasm of neurons and neuronal processes in the brainstem.

![Figure 1. Pathologic findings and the distribution of viral antigens. (A) Perivascular cuffing and microglia nodule (circle) in the pons. (B) Encephalomalacia in the medulla. (C) Remarkable pulmonary edema and focal pulmonary hemorrhage. (D) Reactive lymphoid hyperplasia in the submucous lymphatic tissues of the larynx. (E) Focal inflammatory infiltration accompanied by destruction of ducts and acini in the submandibular gland. Viral antigens in the neuronal bodies and processes in the medulla (F), in a microglia nodule in the thalamus (G), in a macrophage in the faecal tonsil (H), and in the glandular epithelial cells of the large intestine (I). (A–E, H&E staining; F–I, Immunohistochemistry staining; A D E G H I × 200; B × 100; C ×40; F ×400).](image-url)
Scattered positive viral antigens were also seen in the macrophages in the faucial tonsil (Fig. 1H), as well as in the epithelial cells of intestines, including the small and large intestines (Fig. 1I). No viral antigens were observed in the other areas of CNS and other non-CNS organs.

At autopsy, fresh tissues were obtained for viral identification and isolation. Nested reverse transcription polymerase chain reaction (RT-PCR) detection of EV71 RNA was performed in fresh tissues. Virus RNA was extracted from the fresh tissues using the RNeasy Mini Kit (QiaGen, Hilden, Germany), and converted to cDNA (One Step RNA PCR Kit, TaKaRa, Kyoto, Japan) according to the manufacturer’s instructions. The virus used as the positive control was previously isolated from a fatal human case of EV71 encephalomyelitis and obtained from the State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences. The procedure of nested RT-PCR consisted of 2 PCR cycles. The 2 round primers have been previously published which were designed from the published whole VP1 sequence of the standard EV71 BrCr strain,[8] and the first round primer was designed to amplify the whole VP1 sequence. Enterovirus-specific RT-PCR products with a predicted size of 261 base pairs were identified in RNA extracts from the brainstem, spinal cord, thalamus, intestines, and the skin with vesicles (Fig. 2). However, the lungs were negative for RT-PCR and no swabs were obtained for test.

The EV71 RNA-positive fresh brainstem tissue homogenates were sent for viral culture. Human rhabdomyosarcoma (RD) cells were used for EV71 isolation and detailed procedures for the viral isolation were described previously,[9] and we successfully isolated one EV71 strain. The entire VP1 gene of the EV71 isolate from this case was amplified by RT-PCR (QIAGEN, OneStep RT-PCR Kit, Hilden, Germany) with the first round primer as mentioned above.

The reference strains with whole VP1 genome sequence obtained from GenBank (Table 1S, http://links.lww.com/MD/C664) were used for phylogenetic analysis. The phylogenetic tree was constructed using MEGA 5.0 software. Phylogenetic analysis of the isolated strain and reference strains based on the nucleotide sequences of complete VP1 region showed that the isolated strain clustered in the C4a of C4 subgenotype (Fig. 3).

3. Discussion

Several large HFMD outbreaks have occurred in China in the past years.[5] Though the prevalence of severe cases was estimated to be low (<1%),[10] thousands of cases died during these outbreaks since millions of children were infected. Among these fatal patients, only very few cases were sent for autopsy which are

![Figure 2. Enterovirus-specific RT-PCR products with a predicted size of 261 base pairs were identified in RNA extracts from the different fresh autopsy tissues. M, DNA ladder. RT-PCR = reverse transcription polymerase chain reaction.](image)

![Figure 3. Phylogenetic analysis based on EV71 VP1 nucleotide sequences (891 bp). Strain isolated from the patient in this study. The phylogenetic tree was constructed using the neighbor joining method with a bootstrap value of 1000 and the scale reflects the number of nucleotide substitution per site along the branches.](image)
very important for studying the pathogenesis of EV71 infection. In recent years, with the increasing conflict between hospitals and patients, the number of medical dispute increases year by year and have called increasingly public attention in China. In this reported case, the autopsy was conducted to determine the cause of death for a medical dispute. The hospital was accused for its delayed diagnose and mistreatment.

Though it has been postulated that positive viral antigens mainly located in the neurons in different parts of the CNS demonstrated that EV71 is a neuronotropic virus. There are some different findings in our case. Inflammatory infiltration accompanied by destruction of ducts and acini in the submandibular gland and scattered positive viral antigens in the faucial tonsil and in the epithelial cells of intestines indicated a link from the digestive tract to the CNS. Therefore, we speculate that EV71 might invade into the CNS through peripheral nerves which controlled the digestive tract to the CNS. Therefore, we speculate that EV71 might invade into the CNS through peripheral nerves which controlled the digestive tract in the early stage of infection, and more further studies are needed to reveal the exact process of infection. The infection in this stage was likely to be transient and caused the negative viral antigens in the submandibular gland.

Although pulmonary edema is closely associated with CNS involvement, the exact mechanism for pulmonary edema in EV71 encephalitis is still unclear. Now, pulmonary edema is speculated to be neurogenic caused by damage in the brainstem.

However, whether cardiac dysfunction, increased vascular permeability, and cytokine storm contribute remain controversial. In addition, current animal models of EV71 infection do not present the full spectrum of clinical and pathological features observed in fatal human cases, especially the pulmonary edema[12] limits their further utility in understanding the viral pathogenesis of pulmonary edema. Although pulmonary edema was remarkable and was the final cause of death in this case, we did not detect EV71 viral antigens and RNA in the lungs. The results suggested that pulmonary edema is not caused by the direct viral invasion to the lung. In this case, reactive lymphoid hyperplasia in the enlarged tonsils and submucous lymphatic tissues of the larynx, as well as mild mononuclear lymphocytes infiltration in the myocardial interstitium and epicardium were observed. These findings suggested that the immune response and myocardial damage were also involved in the development of the disease, such as the pulmonary edema.

In summary, our results suggested that the differences of clinical outcomes are possibly due to the differences of the virulence[9,16] and the exact molecular virulence determinants of EV71 remain unclear. Therefore, future investigations to finish the whole genome sequencing and use large group of EV71 genomes to identify the virulence determinants and reveal whether the differences in EV71 genomes are responsible for different clinical presentations are required.

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