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Case Report

Simultaneous detection of enterovirus-D68 and vaccine-related poliovirus 3 in the stool samples of a 5-month hospitalized child with acute respiratory disease: A case report

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ABSTRACT

Human enterovirus (EV) infections can lead to various manifestations, with variable correlations between genotypes and symptoms. Human enterovirus D68 (EV-D68) was considered to be associated with acute respiratory disease and acute flaccid myelitis. In this short report, both EV-D68 and poliovirus 3 were detected in the stool of a hospitalized 5-month child who presented with acute respiratory symptoms and who was recently vaccinated with oral polio vaccine (OPV), using a metatranscriptomic high-throughput sequencing method. The nearly full-length genome sequences with complete open reading frames of EV-D68 and poliovirus 3 were assembled. One previously-reported neurovirulence-related amino acid substitution (T860N) in the EV-D68 VP1 region was observed, but the patient showed no neurological symptoms. More attention should be paid to EV-D68, and continuous multiple syndrome-based surveillance on non-polio enterovirus is called for.

1. Introduction

Enteroviruses (EVs) belong to the subfamily *Ensavirinae* in the family *Picornaviridae*. Originally, EVs were classified into over one hundred serotypes according to their serological characteristics [1]. However, according to the genome organization, sequence similarity, and biological properties, human EV is now classified into four species: human enterovirus A to D (EV-A to EV-D).

The EV genome is a single-stranded positive-sense RNA of about 7.5 kb. One single open reading frame (ORF) encodes four structural proteins (VP1 to VP4), and the VP1 nucleotide sequence correlates well with EV serotypes in most molecular studies [2]. The clinical manifestations of EV infection include hand, foot, and mouth disease, herpangina, encephalitis, acute and chronic cardiac disease, respiratory disease, acute flaccid myelitis (AFM), and acute flaccid paralysis (AFP) [3,4].

Poliovirus (PV) was known to most people worldwide as the cause of polio, which caused many paralytic poliomyelitis epidemics in the past century [5]. With the extensive use of the Sabin oral polio vaccine (OPV), wild poliovirus has been eliminated in most countries. However, as a live virus, OPV can revert to being the neurovirulent and cause neurological symptoms. Enterovirus-D68 (EV-D68), a member of the species human EV-D, first isolated in California in 1962, was considered to be associated with acute respiratory disease and AFM [6,7]. No antivirals or vaccines are available for EV-D68 [8]. As a unique genotype of human EV, EV-D68 shares more physiochemical properties with human rhinoviruses than enteroviruses and has mainly been isolated from respiratory samples [9]. Before the large-scale outbreak in 2014, a few cases of EV-D68 infection were reported [7,10]. In South China, EV-D68 is the most common EV found in hospitalized children with respiratory symptoms [11,12].

In this study, we report an unusual case with acute respiratory disease and simultaneous detection of EV-D68 and vaccine-related PV3 in the stool sample.

2. Case presentation

A 5-month girl with a high spiking fever (39.9 ℃) was admitted into a district-level maternal and child healthcare hospital's emergency department in Guangzhou, China, on June 8, 2022. Blood routine anal-

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ysis showed hyperleukocytosis (white blood cell count, WBC = 20.36*10^9/L) and increased C-reactive protein (CRP, 40.2 mg/L). Nasopharyngeal swab test results were negative for influenza A/B or severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) ribonucleic acid (RNA) using reverse transcriptase-polymerase chain reaction (RT-PCR) detection method. The patient was immediately transferred to the pediatric inpatient department and treated with ceftriaxone sodium. Twenty-four h after hospitalization, WBC and CRP increased to 26.48*10^9/L and 43.1 mg/L. Forty-eight hours after antibiotic therapy, the patient showed signs of recovery with the temperature back to normal, and the chest X-ray showed bronchopneumonia and blood cultures were performed with negative results. On the fourth day of hospitalization, the level of WBC and CRP decreased to normal levels. After six days of hospitalization, the patient fully recovered and was discharged on June 13, 2022.

Supplementary retrospective investigation was conducted and hospital records showed that the patient was admitted into the emergency department in the same hospital on May 1, 2022, with a clinical diagnosis of acute bronchitis. Additionally, the vaccination record shows that the patient has been vaccinated with the third dose of bivalent OPV (Sabin strain) in May 26, 2022, just about two weeks before the stool sample was collected. Earlier on March 18 and April 21, the patient had the first two polio vaccinations with inactivated polio vaccine (IPV). Clinical manifestation and vaccination records of the patient were listed in Table 1.

Table 1
Summary of medicine and vaccination records of the patient.

Date	Event
Clinical manifestation	
May 1, 2022	The patient was admitted into the emergency department due to acute bronchitis; She was not hospitalized.
Jun. 8, 2022	Admitted to the emergency department;High spiking fever (39.9 ℃); WBC 20.36*10^9/L，CRP 40.2 mg/L;SARS-CoV-2(-), flu-A/B(-); Hospitalized; Treated with ceftriaxone sodium.
Jun. 10, 2022	Body temperature back to normal; Chest X-ray showed bronchopneumonia; Blood cultures resulted in a negative.
Jun. 13, 2022	After complete recovery, the patient was discharged; WBC 5.72*10^9/L，CRP 4.4 mg/L.
Vaccination records	
Mar. 18, 2022	Received first polio vaccination (IPV).
Apr. 21, 2022	Received second polio vaccination (IPV).
May 26, 2022	Received third polio vaccination (OPV).

Abbreviations: WBC, white blood cell; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; flu-A/B, influenza virus A/B; CRP, C-reactive protein; IPV, inactivated poliovirus vaccine; OPV, oral polio vaccine.

The stool sample of this patient was collected on June 9, 2022 (two days after symptoms presented) and sent to Guangzhou Center for Disease Control and Prevention (GZCDC) for regional enterovirus surveillance on July 26, 2022. Viral RNA was extracted directly from the stool with QIAamp MinElute Virus Kits (Qiagen, 57704) and subjected to an EV-universal real-time RT-PCR. The result revealed EV-positive with a Ct-value of 28.05. The RNA samples were then subjected to a nested RT-PCR to amplify the partial VP1 region, using paired primers 222/224 (1st round) and AN88/89 (2nd round) as previously described [2]. The amplicon was sequenced by the Sanger method. The 350 bp consensus was compared with those available at GenBank, returned due to poliovirus 3 (PV3). A metatranscriptomic PE150 sequencing of this stool sample was performed on the Illumina Novaseq platform for further investigation. After data filtering and quality control, 119,365,412 reads of downstream data were used for analysis. A total of 500 contigs (length > 500 bp) were extracted in the primary de novo assembling by CLC workbench, which indicated that the simultaneous presence of PV3 (2 contigs, contig length = 3,606 bp and 1,301 bp) and EV-D68 (3 contigs, contig length = 2,040 bp, 1,496 bp, and 1,284 bp). The original reads were then mapped to the PV3 (NCBI accession No. MG212494) and EV-D68 references (MW697453), and the consensuses were generated at > 10× level of coverage depth. The sequence length of PV3 consensus was 7,410 bp, with a genome coverage of 99.70% and average coverage depth of 421.25 ×, and the sequence length of EV-D68 consensus was 7,310 bp, with a genome coverage of 99.23% and average coverage depth of 210.98 ×. Additionally, overlapped regions of the PV3 consensus obtained by Sanger sequencing and metatranscriptomic sequencing were highly matched, with a consistency level of 100.00%. To confirm the co-infection of both PV3 and EV-D68 and rule out the potential contamination of libraries, PV3 and EV-D68 specific real-time RT-PCR was conducted on the RNA sample of original stool samples, which resulted in both positive with Ct-values of 32.40 (PV3) and 30.11 (EV-D68) [13,14]. Simultaneously, virus isolation was conducted with Vero, RD, and Hep-2 cell lines for three generations. However, neither PV3 nor EV-D68 was isolated. The consensuses were submitted to the NCBI database under the accession numbers OQ451882 (EV-D68) and OQ451883 (PV3). After reference selection and sequence alignment, maximum likelihood phylogenetic analysis of both the EV-D68 and PV3 sequences (Fig. 1 A and B) were performed using the online tool PhyML 3.0 (https://www.atgc-montpellier.fr/phyml/) with an automatic nucleotide substitution selection model within Smart Model Selection (SMS; https://www.atgc-montpellier.fr/sms/) [15,16]. In this case, the ORF of the PV3 sequence showed high similarity with those former reported vaccine-related poliovirus sequences. Compared with the sequence of the Sabin-3 poliovirus vaccine strain (AY184221), one single nucleotide mutation in the VP1 region (C17T, caused an amino acid substitution T6I in the VP1 region) was observed in this case. The EV-D68 sequence grouped into the clade B3 and exhibited a phylogenetic relatedness with the EV-D68 isolate SHFD528 collected in 2018 (MW697453). Besides, one previously-reported neurovirulence-related amino acid substitution (T860N) in the EV-D68 sequence was observed (Table 2).

3. Discussion

In this report, both EV-D68 and vaccine-related PV3 were detected in the stool sample of a hospitalized 5-month child with acute respiratory disease using a metatranscriptomic high-throughput sequencing method. Additionally, the nearly full-length genome sequences of both EV-D68 and PV3 were successfully assembled. Based on the clinical symptoms, laboratory diagnosis results, the effect of antibiotic treatment, and the vaccination record, we speculated that the main reason for the patient's hospitalization might be the secondary bacterial bronchopneumonia after the respiratory EV-D68 infection. However, the detection of PV3 in the stool sample is only a coincidence caused by OPV vaccination [17].

In a previous study, six neurovirulence-related amino acid substitutions in EV-D68 (M291T, V341A, T860N, D927N, S1108G, and R2005K) were reported, but the functional consequences of these polymorphisms are entirely unknown, and further investigation will be needed to determine their clinical significance [18]. Of the six neurovirulence-related amino acid substitutions, T860N was observed in this case. The EV-D68 from patients with AFM often carried the six substitutions simultaneously, as in patients with other clinical manifestations (encephalitis or respiratory diseases), EV-D68 occasionally carried one substitution [18,19]. Although the clinical significance of these substitutions was still unclear, the absence of neurological

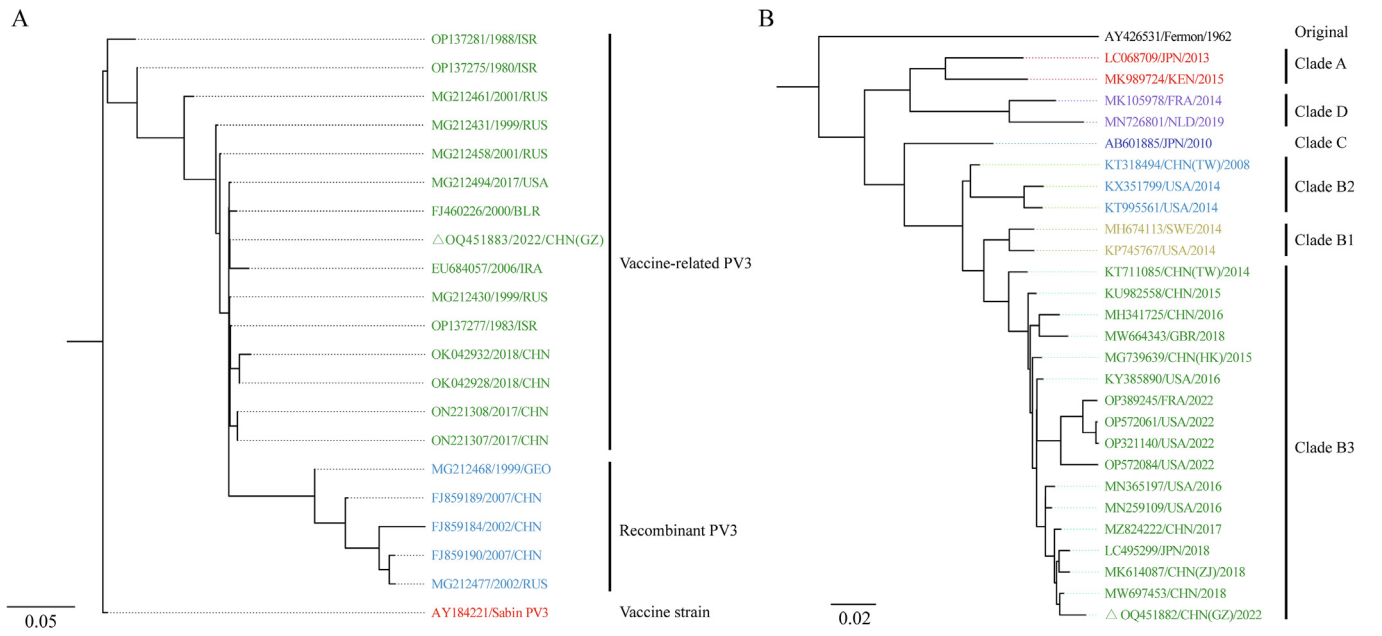

Fig. 1. Phylogenetic analysis based on poliovirus 3 (PV3) (A) and enterovirus D68 (EV-D68) (B) nearly full-length sequences. The sequences of this study are marked with triangles. In addition, the strain names, years of collection, and GenBank accession numbers are presented.

Table 2
Coding polymorphisms of six neurovirulence-related amino acid substitutions in the EV-D68 polyprotein with different clinical manifestations.

NCBI accession No.	AA position [19]						Clinical manifestations
	291	341	860	927	1108	2005	
AY426531 (Fermon)	M	V	T	D	S	R	Respiratory disease
KP100794	T	A	N	N	G	K	AFM
KP126911	T	A	N	N	G	K	AFM
KM892501	M	V	T	D	G	R	Encephalitis
KP240936	M	V	T	D	S	R	Pneumonia
KM851230	M	V	T	D	S	R	Respiratory disease
KM892500	V	V	T	D	S	R	Respiratory disease
OQ451882*	M	V	**N**	D	S	R	Respiratory disease

*The EV-D68 sequence was obtained in this case.
Abbreviations: EV-D68, enterovirus D68; AA, amino acid; NCBI, national center for biotechnology information; AFM, acute flaccid myelitis.

symptoms in the patient could be explained by the combined effects among the six substitutions.

There were occasional reports of EV-D68 and other enteroviruses co-infection, with clinical manifestations of hand, foot, and mouth disease or encephalopathy [20,21]. Detection of EV-D68 in the stool samples of patients with respiratory symptoms is unusual, which might be because patients with respiratory symptoms typically perform throat swab sampling rather than anal swabs or stool collection. It can be informed that it could also be helpful to conduct virus screening in stool samples of patients with respiratory symptoms, which can help the clinical staff to make a precise diagnosis. Traditional molecular diagnostic methods, such as real-time PCR, had some limitations in covering multiple pathogens, and the operational complexity would also increase with the increase of aiming targets [22]. Based on a high-throughput sequencing platform, metagenomic/ metatranscriptomic sequencing methods showed advantages, especially in the precise diagnosis of rare pathogens, which might become a universal, rapid, and effective tool for the syndrome-based surveillance of infectious diseases [23–25].

In this case, we could not determine the bacteria the patient was infected with because the bacterial culture was absent on respiratory tract samples. Fortunately, the antibiotic treatment the doctor gave was timely and practical: the patient's body temperature returned to normal on the third day of hospitalization, and he was discharged on the sixth day. Besides, the association between the patient's primary infection and the detection of EV-D68 was unable to confirm because of the absence of EV-D68 screening on the respiratory tract samples in the early stage and the antibodies detection of PV3 and EV-D68 after the patient recovered. However, it was confirmed that the detection of vaccine-related PV3 had little correlation with the clinical manifestations. Therefore, more attention should be paid to EV-D68 and continuous multiple syndrome-based surveillance on non-polio enterovirus.

Ethics statement

This study was approved by the Ethics Committee of the Center for Disease Control and Prevention (CDC) of Guangzhou (GZCDC-ECHR-2022P0044). All information related to individuals in this study was pseudonymized.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

Author contributions

Wenzhe Su: Investigation, Data curation, Writing – original draft. **Qing Zeng:** Investigation, Data curation. **Jinmei Geng:** Investigation, Data curation. **Jingwen Liu:** Investigation, Data curation. **Huaping Xie:** Investigation, Data curation. **Kuibiao Li:** Investigation, Data curation. **Pengzhe Qin:** Conceptualization, Project administration, Writing – review & editing. **Chaojun Xie:** Resources, Project administration. **Biao Di:** Funding acquisition, Resources, Writing – review & editing.

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