LETTER

An outbreak of a novel recombinant Coxsackievirus A4 in a kindergarten, Shandong province, China, 2021

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ABSTRACT

In 2021, twenty children exhibiting influenza-like illnesses were reported from a kindergarten in Shandong Province, China. Eleven genomes of Coxsackievirus A4 (CV-A4) were obtained from the pediatric cases, sharing <93% genome sequence identities with known CV-A4 strains. Further analyses suggested potential genetic recombination in the P3 region of the novel strains.

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Coxsackievirus A4 (CV-A4), family Picornaviridae, genus Enterovirus, possesses a single-stranded, ~7.4 kb RNA genome encoding a single polyprotein [1, 2]. CV-A4 was first described in 1948 [3, 4], and is epidemiologically linked with a variety of diseases [5], including febrile illness [6], acute polyradiculoneuritis, herpangina [7], mucocutaneous lymph node syndrome, bilateral idiopathic retinal vasculitis, acute flaccid paralysis (AFP) [8], myocarditis [9], and hand-foot-mouth disease (HFMD) [7, 10, 11] which present in pediatric populations worldwide [5, 12]. These diverse clinical entities demonstrate that CV-A4 exerts a significant global public health disease burden. Herein, we report an outbreak caused by a novel CV-A4 variant in young children with influenza-like illness in China, 2021.

From May 17-23, 2021, twenty children aged six (one on May 17, four on May 19, eight on May 20, six on May 21, and one on May 23) in a kindergarten in Taian city, Shandong Province, China presented with influenza-like symptoms, including high fever (>38°C), sore throat, and swollen tonsils and three cases with nausea and vomiting. There were twelve classes in the kindergarten, each with its own play room, dormitory, cloakroom, and toilet. The twenty sick children were from the same class (n = 32), and no children with similar diseases were identified in other classes. Following the doctor’s advice, three children were hospitalized and 17 were quarantined and treated at home, until defervescence and accompanying clinical symptoms abated.

To identify the causative pathogen, nasopharyngeal (n = 11) and anal swabs (n = 4) from eleven symptomatic children were collected on May 20, 2021, as well as six environmental samples from desktops in the classroom, doorknobs in the cloakroom and bathroom, and sinks, urinals and mops in the bathroom. All swabs and environmental samples tested negative for influenza virus A, influenza virus B, SARS-CoV-2 (BioPerfectus technologies, Jiangsu, China), rotavirus, and norovirus (BioPerfectus technologies, Jiangsu, China) by qRT-PCR. The nasopharyngeal swabs from patients 01 and 02 (Supplementary Table 1) tested positive for rhinovirus and/or enterovirus by the QIAstat-Dx assay (Qiagen, Germany). Sixteen RNA sequencing libraries were constructed (MGIEasy mRNA Library Prep Kit, BGI, China) according to the BGI mRNA Library Preparation protocol, including 15 libraries for each swab from the eleven sick children and one library for an environmental sample from
doorknobs in the cloakroom (Supplementary Table 1). Paired-end reads of 100 bp in length were sequenced on the MGISEQ-2000 platform (BGI, China). CV-A4 was identified in all the sequencing libraries [13] and eleven full-length CV-A4 genome sequences were obtained, with reads annotated as CV-A4 also notably identified from the environmental sample (Supplementary Table 1). This provided convincing evidence of CV-A4 being the causative agent responsible for this outbreak. The full-length CV-A4 genomes were further confirmed by Sanger sequencing (Supplementary Table 2) GenBank database: ON730807-ON730817 (Supplementary Table 1).

The eleven complete CV-A4 genomes shared >99.99% nucleotide identities (100% amino acid identities) with each other, with a single nucleotide difference, indicative of a point source of infection. A Blastn search of the novel CV-A4 genomes revealed that the most closely related strain available from GenBank was MN964078|CV-A4/2018/herpangina/8 [7] (92.59% identity) collected from a neighbouring province - Jiangsu, while VP1 sequences shared the highest nucleotide identities with MK388501|CV-A4/17-96-HeN-2017 (96.06%) from another neighbouring province - Henan. Therefore, we considered the possibility that the CV-A4 strain causing the present outbreak might represent a novel variant.

To better understand the origins and evolutionary characteristics of the 11 CV-A4 genomes in the outbreak, phylogenetic analyses were performed using the maximum likelihood method [14]. 51 CV-A4 genomes obtained by our laboratory from HFMD cases in Shandong province characterized from 2014–2020 were also included, including 25 published CV-A4 genomes from 2014–2017 [5, 11] and 26 previously-undescribed CV-A4 genomes determined between 2018 and 2020 (GenBank accession numbers: ON730850-ON730875). In addition, 46 reference genomes available in the GenBank database were also included. Among the 108 CV-A4 genomes, a total of 96 genomes were annotated from China, including 66 identified from 11 cities in Shandong province between 2014-2020, including Taian, suggesting continuous circulation of CV-A4 in this region.

Consistent with previous reports [11], the 108 CV-A4 genomes formed three well-supported groups in the phylogeny: Groups 1, 2 and 3 (Figure 1). The prototype strain High Point/USA/1948 formed an independent branch designated Group 1. 13.9% of the CV-A4 genomes (n = 15) formed Group 2, including eight strains from Australia (2016-2017) and one strain from the USA (2015) forming a sub-group, and six strains from China (2012-2019) forming another sub-group. Notably, 85.2% (n = 92) of the strains fell within Group 3, including 90 strains from China (2008-2021) and two strains from Australia (2016-2017). Group 3 could be further divided into three separable sub-groups: Groups 3.1–3.3 (Figure 1), and the mean genetic distance between these groups was >5.0%. Strains of Group 3.1 (n = 4) were mainly identified between the years 2013-2015; those of Group 3.2 (n = 40) mainly from 2008-2018, while the majority of Group 3.3 strains (n = 48) were annotated from 2018-2021, including the eleven novel CV-A4 from infected children described here. It should be noted that strains from both Groups 2 and 3 circulate in Shandong province, with Group 3 representing the predominant lineage in this region at present.

We further analyzed the phylogenetic relationships of the VP1 gene of the CV-A4 strains available from GenBank (Supplementary Figure 1). As previously reported, four genotypes could be classified for the VP1 gene and genotype D was dominant in China [5]. The eleven genomes described in the present study fell within genotype D, sub-genotype D2 [5]. The novel strains from Shandong clustered with the CV-A4 VP1 gene sequences circulating in China between 2016-2019. Indeed, there were 34 nucleotide mutations in the VP1 genes of the eleven novel genomes compared with the closest relatives within sub-genotype D2, while they possessed 100% amino acid identities, indicative of numerous synonymous mutations in the novel CV-A4 variants causing the outbreak.

Strikingly, a long branch length was evident which separated the eleven novel genomes and all the other CV-A4 strains both in the phylogenetic trees constructed using the complete genome and VP1 gene sequences. Indeed, the eleven CV-A4 genomes possessed ≥540 nucleotide substitutions (93.5%–95.6%) compared with other Chinese strains in Group 3.3 across their entire genomes, with 90.9%–96.1% nucleotide identities in region P1, 87.9%–95.2% identities in region P2, and 81.2%–83.3% identities in region P3. This suggested a recombination event in the P3 region of the novel genomes, which was confirmed by analysis employing Simplot [15] and RDP4 (3SEQ) [16], with breakpoints encompassing genomic positions 5487–5866 in the 3C and 3D genes (Figure 1 and Supplementary Figure 2). MT212019|CV-A16/LN16-23-12/LN/North/China/2016, shared the highest nucleotide identities with a representative strain of the novel variants (denoted TA001K) in the recombination region. The universal primers to detect all known enteroviruses are designed based on the most conserved 5’UTR region and the recombination event was found in the 3C-3D region in the novel CV-A4 virus. Therefore, we infer that routine qRT-PCR EV molecular diagnostic assays should be capable of detecting the novel recombinant as well. Taken together, these findings underscore the genetic diversity of CV-A4 and the possible emergence of novel variants, which enhances our understanding of the evolutionary dynamics of this pathogen during the COVID-19 pandemic.
In conclusion, we describe a novel recombinant CV-A4 variant as the causative agent of an outbreak cluster of infected children with influenza-like illness in a kindergarten in Shandong Province, China in 2021. Notably, the infected children did not develop herpangina. This once again highlights the wide spectrum of...
clinical symptoms associated with CV-A4 infection, although the reasons behind this remain imperfectly understood. Eleven CV-A4 full-length genomes of Group 3.3 were obtained from this outbreak. We also report an additional 26 previously undescribed genomes identified between 2018 and 2020 during our routine surveillance, which greatly enriches the genomic resources available for analysis of the understudied CV-A4 pathogen. Although the incidence of human enteroviruses has decreased during the COVID-19 pandemic, our findings highlight the ongoing circulation and genetic variation within CV-A4. Increased genome surveillance of human enteroviruses is warranted in China and elsewhere.

Disclosure statement
No potential conflict of interest was reported by the author(s).

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