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LETTER

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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

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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 eleven 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.

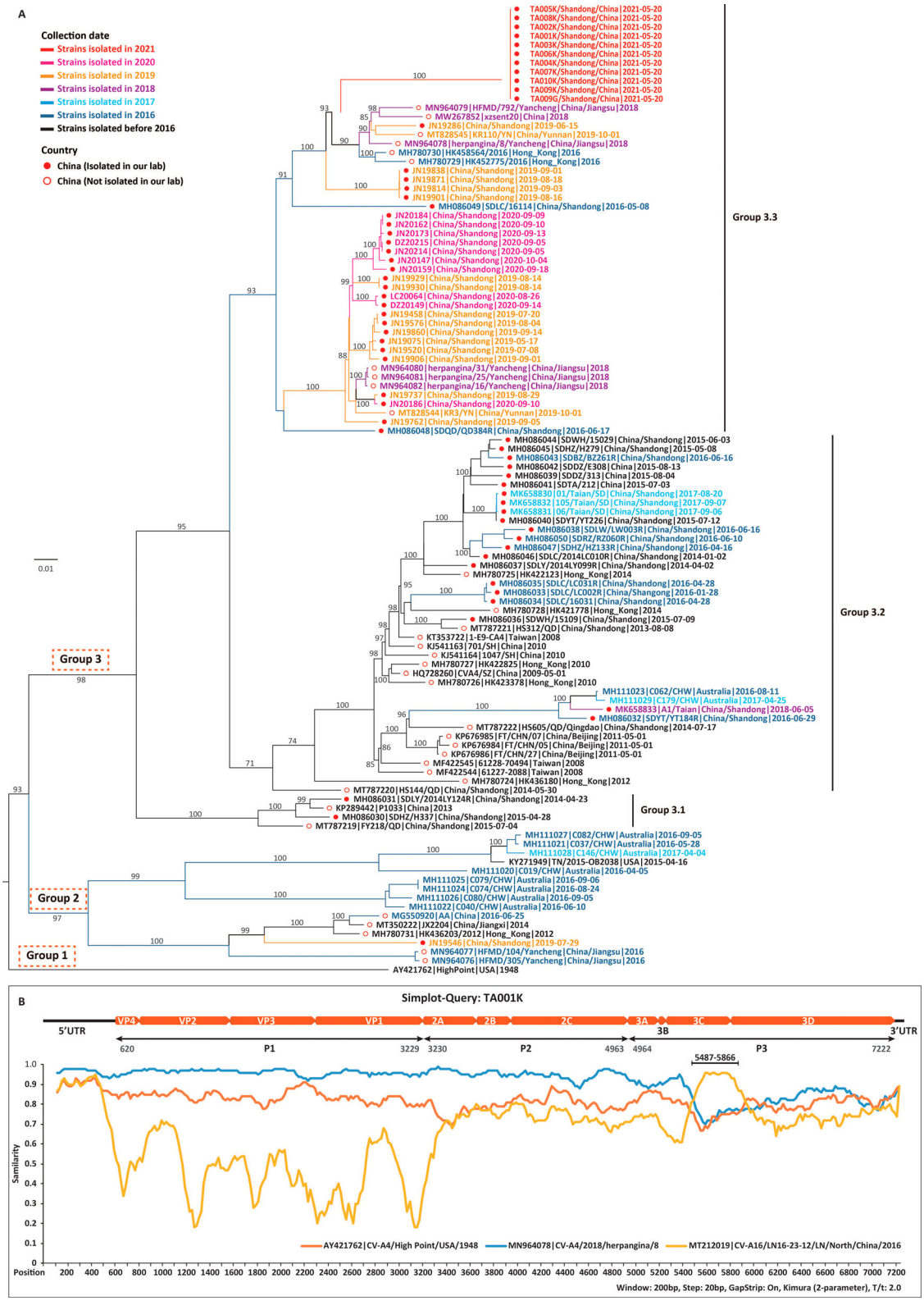

Figure 1. (A) Phylogenetic analysis of 11 full-length CV-A4 genomes from the kindergarten outbreak and reference strains available from GenBank. Font colour in the phylogenetic tree represents the collection date of the CV-A4 strains. Maximum likelihood trees in this study were estimated using RAxML (version 8.1.6) [14] under the GTRGAMMA nucleotide substitution model with 1000 bootstrap replicates. The solid red circle represents the Chinese CV-A4 genomes identified by our laboratory, and the open red circle represents the Chinese CV-A4 genomes described by other laboratories. (B) Sequence identity between CV-A4 genomes from the kindergarten outbreak (exemplified by TA001K) and its most closely related strains. Similarity comparison of the potential recombination event in the TA001K genome was performed using Simplot [15], with a window size of 200 bp and a step size of 20 bp. The breakpoints were defined by RDP4 [16].

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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References

[1] Dalldorf G. The coxsackie virus group. Ann N Y Acad Sci. 1953;56(3):583–586.
[2] Knowles NJ, Hovi T, Hyypiä T, et al. Picornaviridae. In: virus taxonomy: classification and nomenclature of viruses: ninth report of the international committee on taxonomy of viruses. San Diego: Elsevier; 2012.
[3] Melnick JL. Studies on the coxsackie viruses; properties, immunological aspects and distribution in nature. Bull N Y Acad Med. 1950;26(5):342–356.
[4] Oberste MS, Penaranda S, Maher K, et al. Complete genome sequences of all members of the species human enterovirus A. J Gen Virol. 2004;85(Pt 6):1597–1607.
[5] Wang M, Li J, Yao MX, et al. Genome analysis of coxsackievirus A4 isolates from hand, foot, and mouth disease cases in shandong, China. Front Microbiol. 2019;10:1001.
[6] Li JS, Dong XG, Qin M, et al. Outbreak of febrile illness caused by coxsackievirus A4 in a nursery school in Beijing, China. Virol J. 2015;12:92.
[7] Guo WP, Chen GQ, Xie GC, et al. Mosaic genome of human coxsackievirus A4 associated with herpangina and HFMD in yancheng, China, 2016 and 2018. Int J Infecti Dis. 2020;96:538–540.
[8] Chen P, Wang H, Tao Z, et al. Multiple transmission chains of coxsackievirus A4 co-circulating in China and neighboring countries in recent years: phylogenetic and spatiotemporal analyses based on virological surveillance. Mol Phylogenet Evol. 2018;118:23–31.
[9] Akuzawa N, Harada N, Hatori T, et al. Myocarditis, hepatitis, and pancreatitis in a patient with coxsackievirus A4 infection: a case report. Virol J. 2014;11:3.
[10] Ji TJ, Guo Y, Lv LK, et al. Emerging recombination of the C2 sub-genotype of HFMD-associated CV-A4 is persistently and extensively circulating in China. Sci Rep. 2019;9(1):13668.
[11] Wei Q, Liu J, Li J, et al. Complete genome characteristics of coxsackievirus A4 isolated from taian, 2017-2018. Chinese J Exp and Clin Virol. 2019;33 (004):347–353.
[12] Chu PY, Lu PL, Tsai YL, et al. Spatiotemporal phylogenetic analysis and molecular characterization of coxsackievirus A4. Infect Genet Evol. 2011;11 (6):1426–1435.
[13] Hu T, Li J, Zhou H, et al. Bioinformatics resources for SARS-CoV-2 discovery and surveillance. Brief Bioinformatics. 2021;22(2):631–641.
[14] Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics. 2014;30(9):1312–1313.
[15] Lole KS, Bollinger RC, Paranjape RS, et al. Fulllength human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. J Virol. 1999;73(1):152–160.
[16] Martin DP, Murrell B, Golden M, et al. RDP4: detection and analysis of recombination patterns in virus genomes. Virus Evol. 2015;1(1):vev003.