

## Letter to the Editor



## An imported Genotype D Coxsackievirus B4 Strain Identified in Tianjin, China\*

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Hand, foot, and mouth disease (HFMD) is an infectious disease caused by various enterovirus (EV) serotypes and occurs in infants and children across the world. The mild symptoms of HFMD include fever and a maculopapular rash on the hands, feet, mouth, and buttocks. However, in some patients, this infection causes severe neurological syndromes and even death. EVs belong to the *Enterovirus* genus (family Picornaviridae, order Picornvirales), of which there are 15 species: *Enterovirus A (EV-A)*, *EV-B*, *EV-C*, *EV-D*, *EV-E*, *EV-F*, *EV-G*, *EV-H*, *EV-I*, *EV-J*, *EV-K*, *EV-L*, *Rhinovirus A*, *Rhinovirus B*, and *Rhinovirus C*. Since the 2008 outbreaks, enterovirus 71 (EV-A71) and coxsackievirus A16 (CVA16) have been considered to be the two major HFMD pathogens in China<sup>[1]</sup>. Nevertheless, other prevalent EV serotypes have caused large-scale outbreaks (e.g., CVA4, CVA6, CVA10, and Echovirus 11). Therefore, increasing attention has recently shifted to other *EV-A* and *EV-B* pathogens to determine the spectrum of HFMD-causing pathogens besides the major causative ones.

Coxsackievirus B4 (CVB4) is an *EV-B* type virus. Other than its association with HFMD, CVB4 is associated with a wide range of conditions and diseases such as angina, myocarditis, pancreatitis, viral encephalitis, acute flaccid paralysis, and type 1 diabetes<sup>[2]</sup>. Six CVB4 strains were isolated in Inner Mongolia in 2010<sup>[3]</sup> during the HFMD outbreaks. They were also reported to be isolated from HFMD patients in Shandong (2010–2011) and Guangxi (2010)<sup>[4,5]</sup> and from aseptic encephalitis patients in Yunnan, 2009<sup>[6]</sup>. Otherwise, there have been few reports of CVB4 outbreaks in China.

CVB4 comprises five genotypes: genotypes A–E<sup>[4]</sup>. Most European and American strains are genotypes C and D, but all the strains isolated so far from China are genotype E<sup>[3,4]</sup>. CVB4 might have undergone intertypic recombination with other *EV-B* strains<sup>[3,6]</sup>. In the present study, next-generation sequencing (NGS) by Illumina MiniSeq was used to

identify an imported recombinant CVB4 strain from Tianjin, China. Based on its genetic and recombination characteristics, the strain was assigned to genotype D within a phylogenetic cluster mainly containing strains from Europe and America.

A 0316/TJ/CHN/2019 stool sample was collected from a five-year-old local male patient in Tianjin (2019), whose clinical signs and symptoms included fever (39 °C), abdominal pain, rash, and herpes in the oral mucosa but no respiratory or nervous syndrome. The patient indicated no overseas travel history within the previous six days. The virus was isolated using rhabdomyosarcoma (RD) cells. Thereafter, two pairs of overlapping *EV-B* primers were used to amplify the complete genome sequence from CVB4 by RT-PCR, as described by Joffret et al.<sup>[7]</sup>. The amplification products were subjected to Illumina MiniSeq NGS amplicon sequencing.

A 7,503-nt genome sequence was assembled by *de novo* assembly (CLC Genomics Workbench 20.0.3, QIAGEN), with an average coverage of 14,386.94. After deleting the 5'- and 3'-terminal low-quality nucleotides, a 7,396-nt sequence was assembled as the ultimate complete 0316/TJ/CHN/2019 genome sequence, which has been deposited in GenBank (Accession No. MZ161144).

All the complete genome or coding sequences from the CVB4 isolates and 31 genomes from *EV-B* serotype prototypes were collected from NCBI GenBank, and all the low-quality sequences were removed. The nucleotide and aa genome similarity levels between 0316/TJ/CHN/2019 and CVB4 prototype J.V.B. Benschoten (JVB: GenBank Accession No. X05690) were 77.3% and 96.5%, respectively (Supplementary Table S1 available in [www.besjournal.com](http://www.besjournal.com)). The nucleotide and aa genome similarity levels between 0316/TJ/CHN/2019 and the strains from the mainland of China were 76.7%–78.9% and 96.0%–97.4%, respectively

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(with the highest similarity level shared with the Guangxi JX308222 strain isolated from a fatal disease case in 2010). In addition, the nucleotide and aa genome similarity levels between 0316/TJ/CHN/2019 and the strains outside the mainland of China were 75.7%–89.6% and 96.4%–98.5%, respectively (with the highest similarity level shared with the French 2019/FR/3996 MN590273 strain isolated in 2019).

Compared with the JVB CVB4 prototype, 19 aa substitutions were identified in the capsid protein (Supplementary Table S2 available in [www.besjournal.com](http://www.besjournal.com)), and 57 such substitutions in nonstructural proteins (23 in *P2* and 34 in *P3*) were detected in 0316/TJ/CHN/2019. Of the 19 aa substitutions found in the capsid protein from 0316/TJ/CHN/2019, only 9 were also found in other strains isolated within the mainland of China, whereas the remaining 10 substitutions were unique to the Chinese strains (Supplementary Table S2).

The point aa site mutation in the capsid protein from CVB4 has been reported in a few studies. One study suggested that the 16–24 aa region in the *VP4* capsid protein is the target of infection-enhancing antibodies in CVB4<sup>[8]</sup>. Ser-16 in *VP4* and Met-129 in *VP1* have been identified as virulence-related determinants<sup>[9,10]</sup>. The 0316/TJ/CHN/2019 strain possesses both Ser-16 in *VP4* and Met-129 in *VP1* of the capsid protein, indicating that it is a virulent strain.

In EVs, *VP1* is considered useful for standard molecular genotyping because it is serotype-specific. However, because intertypic recombination frequently occurs among EVs serotypes, a new genotyping method based on whole-genome sequences has been developed to enable the accurate determination of genetic relationships in these viruses. Phylogenetic analyses based on the 852-nt *VP1* and full-length genome sequences were performed, and neighbor-joining trees were constructed using the Kimura 2-parameter model in MEGA 5.05. The results were tested by the bootstrap method with a bootstrap value of 1,000.

In this study, the *VP1* and whole-genome sequence-based phylogenetic analyses supported the division of the CVB4 strains into five genotypes, although with a minimal difference in genotypes B and C typing (Figure 1). The genotyping difference between *VP1* and the whole-genome sequences showed that substantial recombination had occurred in the CVB4 strains, highlighting the need for phylogenetic analyses to be based on the complete genome sequences.

In the *VP1* tree, all the CVB4 strains clustered into five genotypes (genotypes A–E). The prototype JVB and a type 1 diabetes-causing strain from Italy clustered as genotype A with a similarity value of 99.8%. A Russian strain and two type 1 diabetes-causing strains clustered in genotype B (similarity values, 88.5%–98.6%). Genotype C contained 15 Denmark strains (with similarity values of 86.1%–100.0%). The 0316/TJ/CHN/2019 strain clustered into genotype D (with similarity values of 83.9%–98.1%) with strains isolated from the USA, Uganda, Australia, Japan, and European countries. Other strains from the mainland of China clustered into genotype E (similarity values, 87.6%–100.0%), with strains from Taiwan, Australia, and Japan. The mean similarity values among the different genotypes ranged from 85.6% (genotypes B and C) to 76.9% (genotypes D and E).

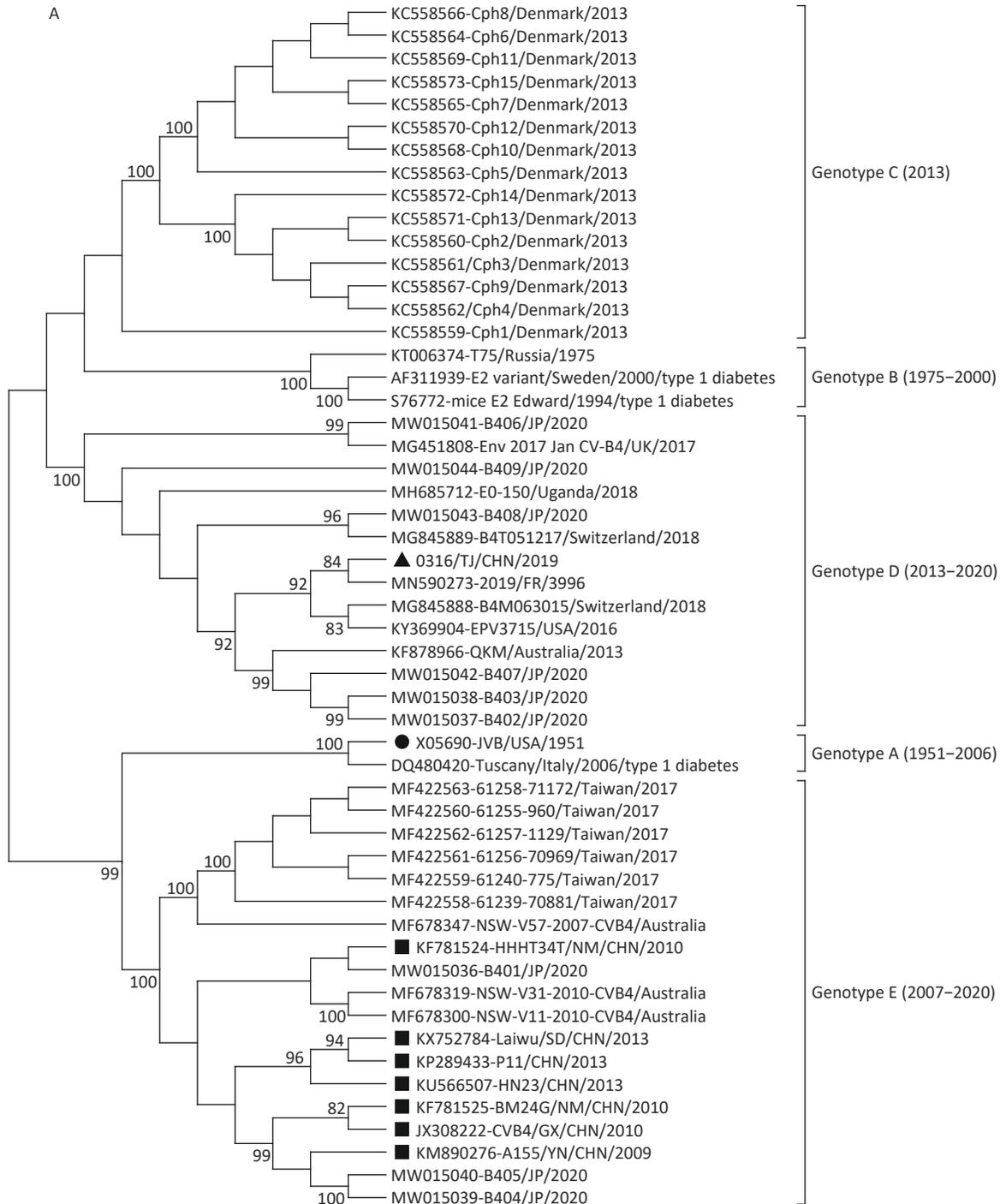
Both *VP1* and the whole-genome sequence trees revealed that, unlike the genotype E strains from the mainland of China, the Tianjin 0316/TJ/CHN/2019 strain clustered into genotype D with the strains mainly isolated from the USA, Japan, Australia, and European countries (Figure 1). This conclusion was further supported by homology analysis, which showed that, based on the complete genome sequences, 0316/TJ/CHN/2019 shares the highest similarity (89.6%) with a French strain and, based on the complete *VP1* sequence, shares the highest similarity (92.7%) with an American strain. Because the local Chinese patient did not have any overseas contact, it seems that the CVB4 genotype D strain has been imported into Tianjin.

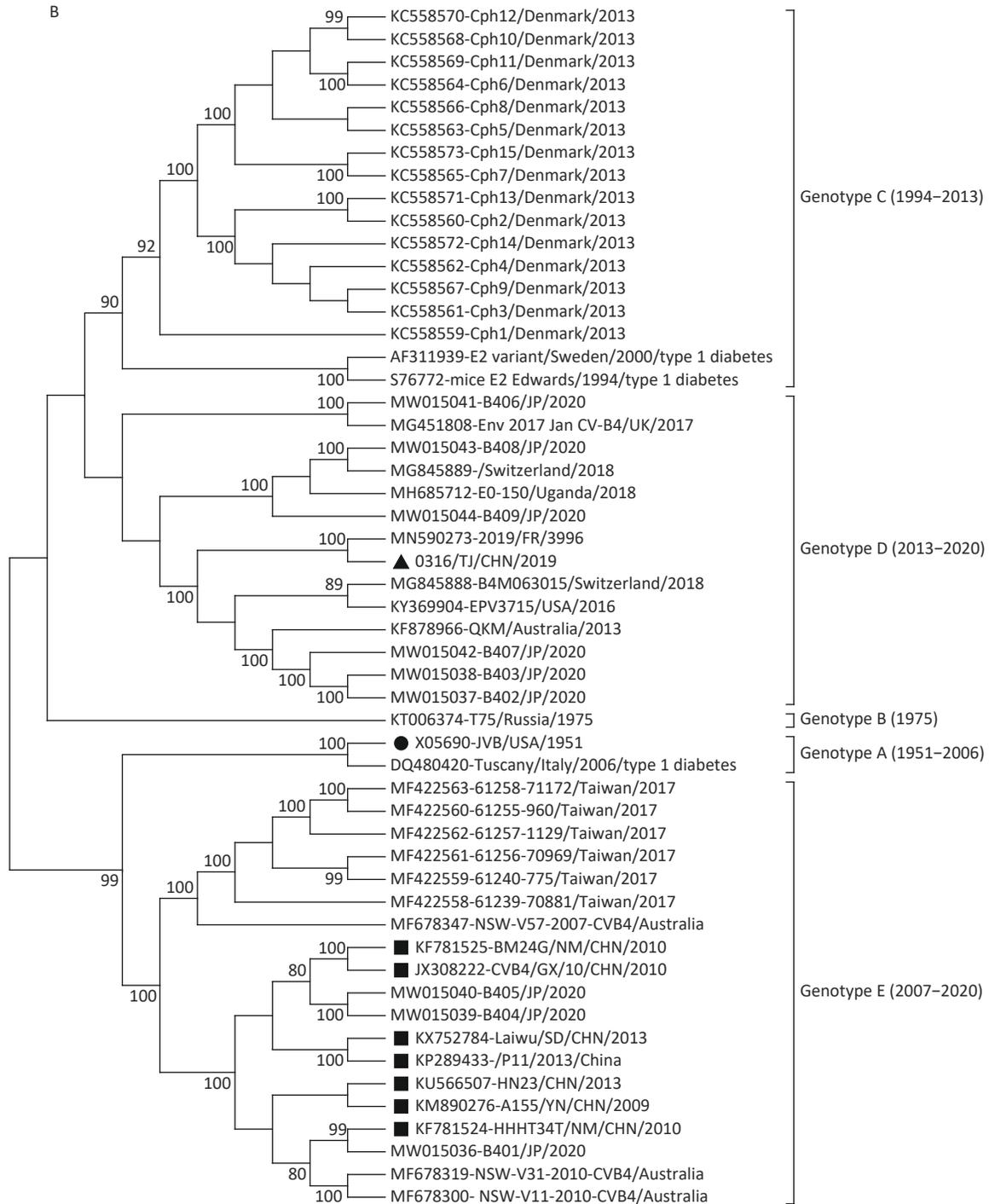
Previous studies<sup>[3,4]</sup> indicate that genotype E strains are the only strains derived from those circulating in the mainland of China and Australia since 2007. However, in this study, we added strains from Taiwan (2017) and Japan (2020) to the genotype E cluster (Figure 1). The similarities based on the *VP1* sequences among genotype E strains ranged from 87.6% to 100.0%, conforming to the criterion normally used for genotyping EVs (i.e., no greater than 15% nucleotide sequence differences in the *VP1* sequences). Our study has illustrated that the genotype E strains did not only occur in the mainland of China and Australia but also occurred in a vast region around the western Pacific Ocean, including Taiwan, China and Japan.

Our phylogenetic analyses based on the *P1*, *P2*, and *P3* genome regions among the CVB4 strains and other *EV-B* strains suggested that although all the CVB4 strains shared the same cluster with the CVB4 JVB prototype in the *P1* region (Figure 2A), different

genetic relationships exist among the CVB4 strains in the *P2* and *P3* regions. In the *P2* genome region, all the CVB4 genotypes B–E strains, including 0316/TJ/CHN/2019, clustered together with Echovirus 27 prototype Bacon (AY302551) and Echovirus 30 prototype Bastianni (AF162711, [Figure 2B](#)). In the *P3* genome region, JVB and two Chinese CVB4 strains both clustered with Echoviruses 13, 25,

26, and 27 and CVB6. CVB4 genotype B (a Russian T75 KT006374 isolate) and two genotype D strains both clustered with Echovirus 12. Furthermore, other CV-B4 strains, including five Chinese strains and 0316/TJ/CHN/2019, shared the closest relationship with Echovirus 30 ([Figure 2C](#)). The similarity values for 0316/TJ/CHN/2019 and Echoviruses 27 and 30 in the *P2* region were 76.1%





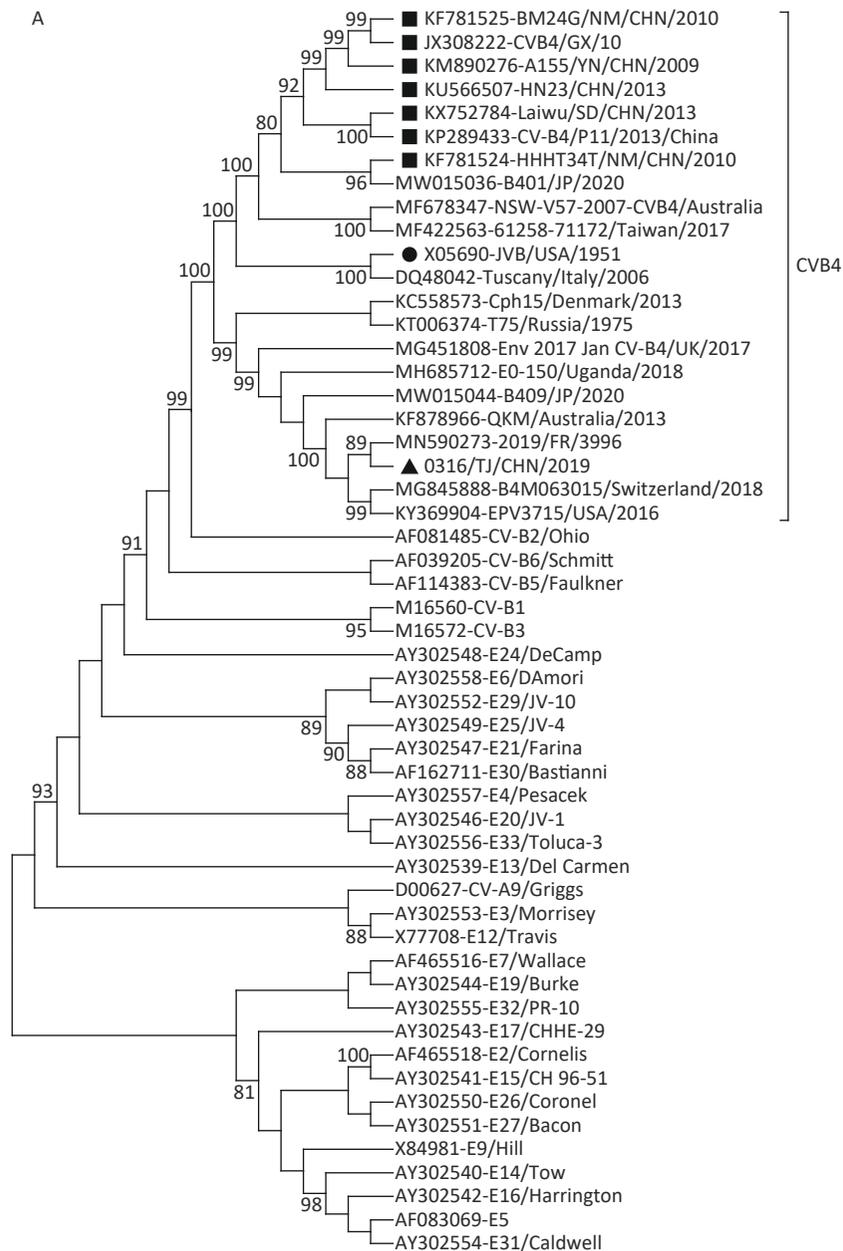
**Figure 1.** Neighbor-joining trees for CVB4 strains based on complete *VP1* sequences (A) and full-length genome sequences (B). The strain indicated by the circle is the prototype JVB. The strain indicated by the triangle is 0316/TJ/CHN/2019. The strains indicated by squares are the isolates from the mainland of China.

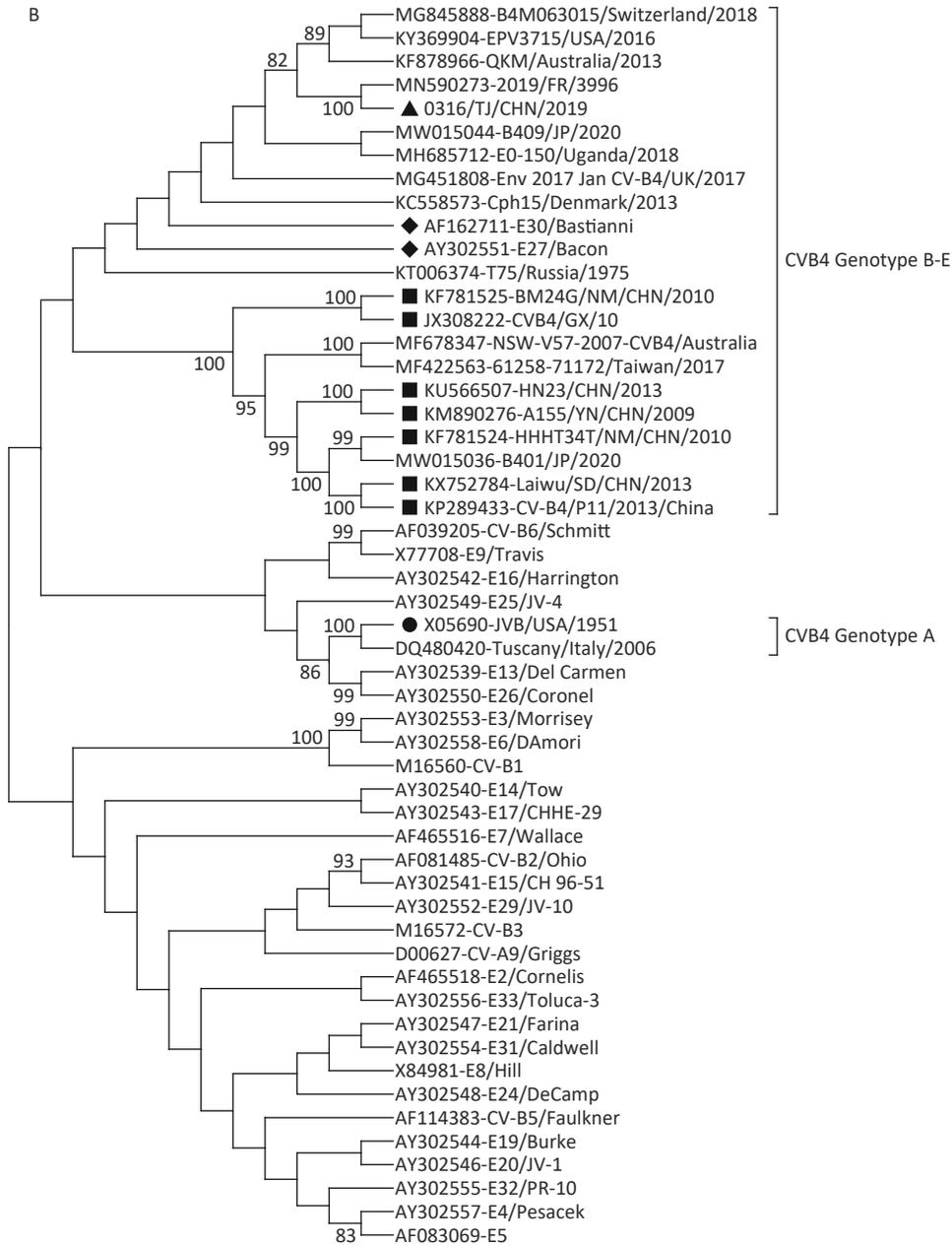
and 76.9%, respectively. These values are somewhat lower than the 80.2% for the P3 region and Echovirus 30. However, 0316/TJ/CHN/2019 shared only 74.5% and 75.2% similarity with CVB4 prototype JVB in the P2 and P3 region, respectively.

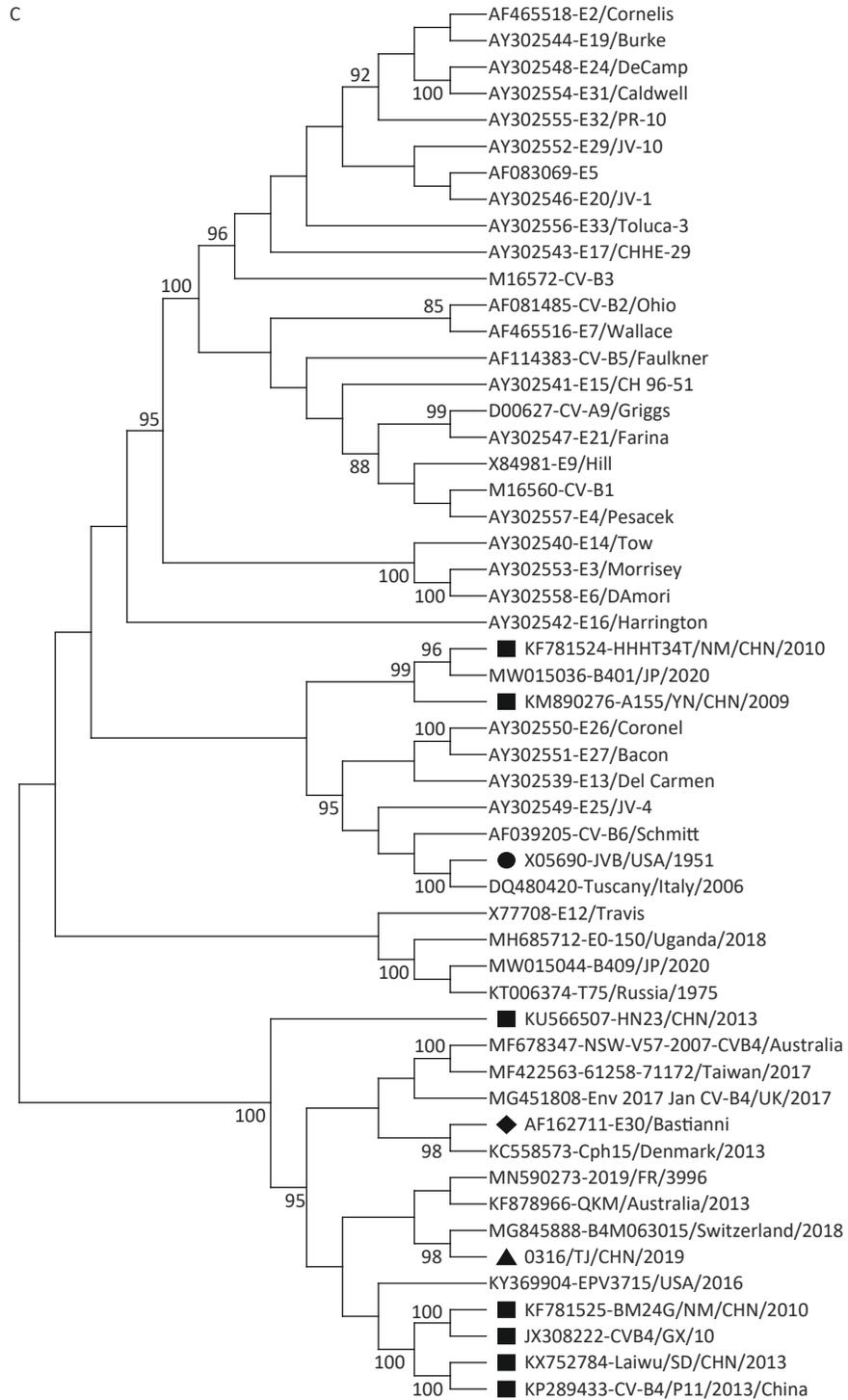
The Simplot and bootscanning analyses were constructed using Simplot 3.5.1 (Stuart Ray, Johns Hopkins University, Baltimore, MD, USA), with a sliding window of 200-bp and steps of 20-bp. The Simplot (Figure 3A) and bootscanning (Figure 3B) analyses revealed that 0316/TJ/CHN/2019 was most similar to JVB in the P1 region (average bootscanning value of 80.5%), whereas 0316/TJ/CHN/2019 and

Echovirus 30 prototype Bastianni were most similar in the P3 region, with an average bootscanning value of 77.1% (Figure 3B). Both phylogenetic and bootscanning analyses suggested that recombination events have occurred between 0316/TJ/CHN/2019 and the other EV-B strains.

Frequent recombination within the 5'-UTR, P2, and P3 regions is a common mechanism seen in the evolution of EV-B species<sup>[3,6,11]</sup>. Whereas the 5'-UTR sequences in EV-Bs cluster with EV-A members, the P2 and P3 genome regions have only recombined within the EV-B species, resulting in complex mosaic sequence patterning of the nonstructural protein's







**Figure 2.** Neighbor-joining trees based on the *P1* (A), *P2* (B), and *P3* (C) genome regions among the CVB4 strains and other EV-B strains. The strain indicated by a circle in each panel is prototype JVB. The strain indicated by a triangle in each panel is 0316/TJ/CHN/2019. The strains indicated by squares are isolates from the mainland of China. The strains indicated by rhomboids are Echovirus 27 and Echovirus 30.

coding sequences<sup>[11]</sup>. The *P1* region-based phylogenetic analysis supports the monophyletic evolution of CVB4 (Figure 2A). However, *P2* and *P3* region-based phylogenetic trees and the bootscanning analysis both revealed that 0316/TJ/CHN/2019 is also a recombinant strain (Figure 3). Therefore, considering the high evolution rate and frequent recombination events within EVs, surveillance measures and monitoring, especially including the NGS method, should be enhanced to prevent and control the diseases caused by CVB4.

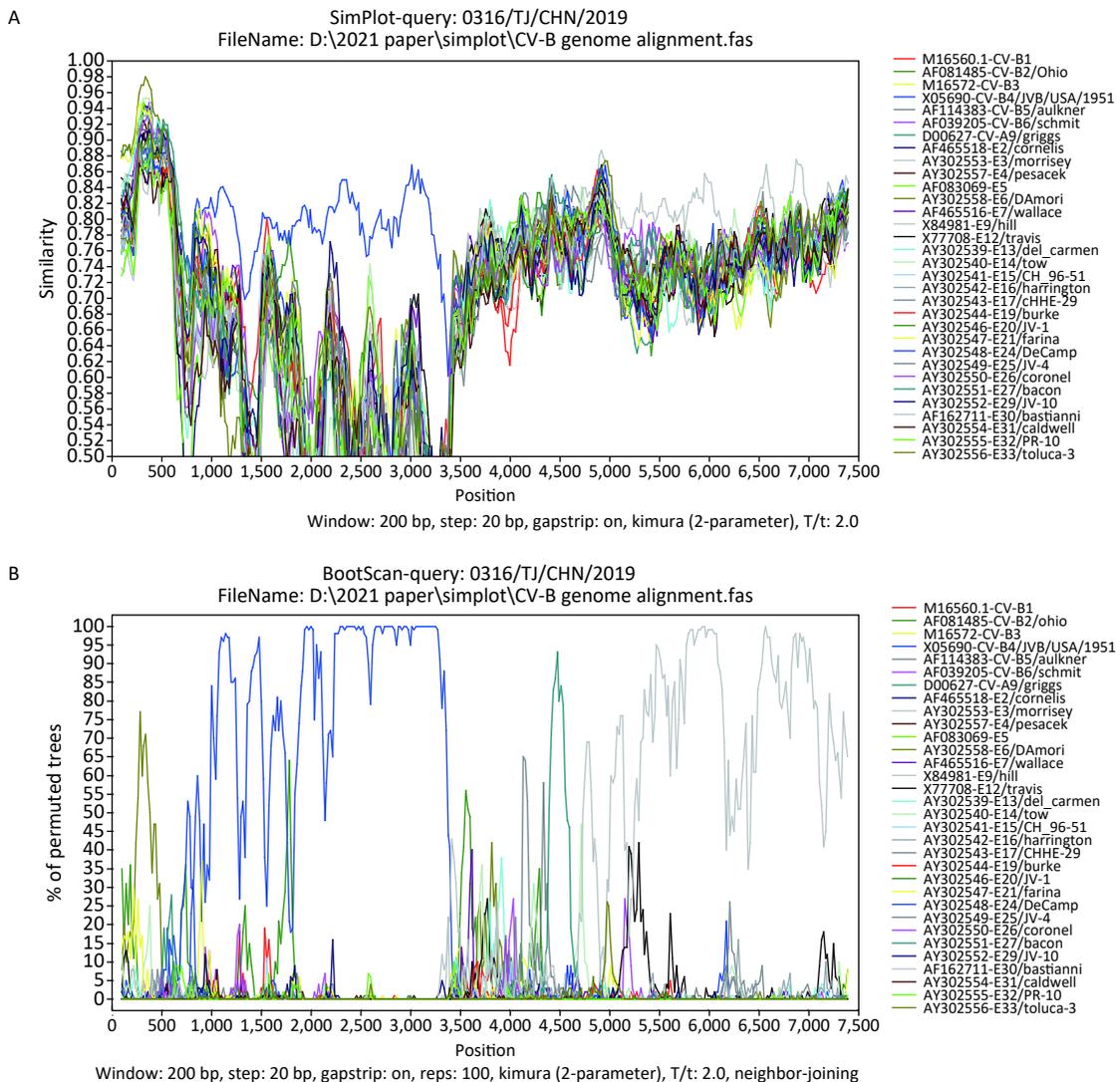
In conclusion, an imported CVB4 strain isolated in Tianjin, China, in 2019 was found to cluster into genotype D. Recombination analysis suggests that the Tianjin strain has recombined with other *EV-B* species, including Echovirus 30, in the *P2* and *P3*

regions. It has also been shown that the CVB4 whole-genome sequence has advantages over the traditional methods, especially for traceability, and NGS is an effective method to trace the source of CVB4, as well as other EVs.

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**Authors' Contributions** Designed the experiments: LI Xiao Yan. Performed the experiments: TAN Zhao Lin, LYU Li Kun. Analyzed the data: Tan Zhao Lin, XIE Tong. Collected the specimens and epidemiological information: LI Li. Wrote the paper: TAN Zhao Lin.

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**Figure 3.** Similarity plot (A) and bootscanning (B) analyses based on the full-length genome sequences from 0316/TJ/CHN/2019 and *EV-B* strains.

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**Supplementary Table S1.** Nucleotide sequences similarities and their deduced amino-acid sequences between 0316/TJ/CHN/2019 and other CVB4 strains

Region	CVB4 prototype JVB		Strains from the mainland of China	
	Nucleotide (%)	Amino acid (%)	Nucleotide (%)	Amino acid (%)
5'-UTR	82.1	–	81.4–82.7	–
VP4	82.3	100.0	73.9–77.5	97.1–98.5
VP2	77.5	97.3	78.0–79.4	96.9–98.1
VP3	79.9	98.3	75.8–77.7	97.9–98.7
VP1	79.5	97.1	76.2–77.7	96.0–97.1
2A	70.4	90.0	68.8–70.2	88.5–89.2
2B	75.2	96.9	70.6–73.8	96.9–98.0
2C	76.1	98.2	77.2–78.2	97.2–99.1
3A	69.9	89.3	74.3–77.1	91.8–97.7
3B	68.0	95.3	70.5–90.1	85.3–95.3
3C	74.9	94.4	71.8–80.4	95.5–98.9
3D	76.7	96.7	76.5–83.5	95.3–98.9
3'-UTR	85.3	–	83.7–91.7	–
CDS	76.7	96.5	76.0–78.5	96.0–97.4

**Supplementary Table S2.** Amino acid substitutions in the 0316/TJ/CHN/2019 capsid protein versus the CVB4 prototype JVB (X05690)

Proteins	Size (aa)	Substitutions
VP4	69	None
VP2	261	A137V, A142T <sup>a</sup> , S154Q <sup>a</sup> , A160T <sup>a</sup> , K163E, Y187F, S249N
VP3	238	I55L, M63K <sup>a</sup> , F213L <sup>a</sup> , T234S <sup>a</sup>
VP1	284	K5D, Y76F <sup>a</sup> , S263N, V271I, A273T, S277N <sup>a</sup> , I279V <sup>a</sup> , Y284C <sup>a</sup>

**Note.** <sup>a</sup>Substitutions first detected in strains from the mainland of China.