

Emergence of a Novel Recombinant of CV-A5 in HFMD Epidemics in Xiangyang, China

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Abstract

Hand, foot and mouth disease (HFMD) is caused by a variety of enterovirus serotypes and the etiological spectrum worldwide has been changing since a large scale of outbreaks occurred in 1997. A large number of clinical specimens of HFMD patients were collected in Xiangyang and genotyping was performed by qRT-PCR, conventional PCR amplification and sequencing. Among the 146 CV-A5 cases detected, the complete genome sequences of representative strains were determined for genotyping and for recombination analysis. It was found that CV-A5 was one of the six major serotypes that caused the epidemic from October 2016 to December 2017. Phylogenetic analyses based on the VP1 sequences showed that these CV-A5 belong to the genotype D which dominantly circulates in China. Recombination occurred between the CV-A5 and CV-A2 strains with a breakpoint at the nt position 3,791 in the 2A region. The result may explain the emergence of CV-A5 as one of the major pathogens of HFMD.

Introduction

Hand, foot and mouth disease is caused by a variety of enteroviruses. At least more than 40 enterovirus serotypes were identified in epidemic and etiological surveillances from clinical samples of HFMD patients in mainland China and other countries [1-6]. The major serotypes currently prevailing in China are CV-A6, CV-A10, CV-A16 and EV-A71 and other serotypes which either cause sporadic cases or small outbreaks [7]. HFMD is characterized by fever, sore throat, general malaise and vesicular eruptions on the hands, feet, oral mucosa and tongue. Severe cases include complications such as encephalitis, meningitis, acute flaccid paralysis, cardiorespiratory failure and even death. Severe and fatal cases are mainly caused by EV-A71 but other serotypes are also increasingly involved in large proportions [6]. Etiological spectrum of HFMD has been changing characteristically driven by the emergence of new predominant enterovirus serotypes such as CV-A6, CV-A10 and other serotypes. The driving forces of evolution are point mutations, intertypic and intratypic recombination [8]. CV-A5 previously cause sporadically HFMD, or herpangina, acute gastroenteritis and onychomadesis at low frequency or small enterovirus outbreaks worldwide [9-21]. CV-A5, however, was recently detected in a relatively-large proportion of HFMD cases in a largescale etiological investigation in Xiangyang performed in our laboratory. We collected a total of 3703 case HFMD samples, of which 3201 were typed. Among them, 1906 samples were typed as CV-A6(51.47%), 490 samples were typed as CV-A16(13.23%), 370 samples were typed as CV-A10(9.99%), 146 samples were typed as CV-A5(3.94) and 121 samples were typed as CV-A2(2.62%). It seemed that these six serotypes were popular at the same time in Xiangyang[22].

CV-A5 is a member of enterovirus species A in the genus enterovirus within the family Picornaviridae in the order Picornavirales [23]. It has been further divided into clades A, B, C and D based on the divergence of the VP1 nucleotide sequence [24]. The single-stranded, polyadenylated, positive-sense RNA genome is proximately 7,400 nucleotides in length. The genome contains a single open reading frame (ORF) flanked by the untranslated regions (UTR) at both 5'- and 3'-ends with a virus encoded peptide (3BVPg) covalently linked to the 5'-end. The polyprotein is co-translationally cleaved by viral proteases 2APro,

3Cpro (or 3CDpro) into precursors such as P1, P2, P3, VP0, 3CD and mature structural proteins VP1-VP4 (1A-1D) and nonstructural 2A-2C, 3A-3D.

In this study, the complete genome sequences of three RD cell isolates of CV-A5 were determined. This represented 146 CV-A5 clinical specimens in an epidemic of HFMD outbreak in Xiangyang in 2017. Phylogenetic analysis revealed that a novel recombinant emerged carrying the 5'-terminal sequence of CV-A5 and the 3'-terminal sequence of CV-A2 with the recombination junction located at 2A region. The recombinant may be responsible for the high proportion of CV-A5 HFMD cases among all enteroviruses detected. This novel recombinant may explain the outbreak caused by CV-A5 and emphasizes the role played by recombination in the emerging, prevailing and changing of etiological spectrum of HFMD-associated enteroviruses.

Materials And Methods

Enterovirus isolation and RNA extraction

Viruses were isolated from rectal swabs by inoculation on RD and Vero cells, respectively, by conventional methods [25]. Samples were identified by molecular typing method. The viral RNAs were extracted directly from cell lysates.

VP1 region sequencing and typing of CV-A5 strain

The VP1 regions were amplified by Enterovirus VP1 universal primers (292 and 222) [26] listed in Table 1. The reaction was performed by adding 15.2µl reaction buffer, 0.4µl template cDNA, 3.6µl enzyme mixture, 0.8µl forward and reverse primers (1.0 ng/µl each), respectively, and nuclease free water to be added to a total volume of 20µl. The PCR amplification was run under the following conditions: 94°C 5 min, 35 cycles of 94°C 30s, 55°C 30s, and 72°C 30s. VP1 sequences were compared with sequences available in GenBank by Blast. Virus strains showing more than 75 % nucleotide similarity with known enterovirus serotype were considered to be the same serotype.

Full-length genome sequencing

The complete genome of CV-A5 strains were sequenced for further genetic characterization. PCR conditions: 94°C, 5min; 94°C 30s, 52°C, 30s, 72°C kb/30s, 35 cycles. Primers used were listed in Table 2.

Phylogenetic analysis and recombination analysis

The nucleotide homology among 3 CV-A5 Xiang Yang strains was 99.6%, so we selected the CV-A5 R3487 strain to compare its nucleotide and amino acid sequences with those of other Enterovirus by Megalign software. The phylogenetic tree was generated by the MEGA-X software, and the evolutionary distance was calculated with the Neighbor-Joining method using 500 bootstraps for evaluation. The recombination was analyzed by using the MEGA-X and SimPolt software, the Kimura-2-parameter model mapping, and 500 bootstrap for evaluation. The Recombination Detection Program (RDP) was used for

the detection of potential recombinant sequences, and localization of possible recombination breakpoints.

Nucleotide sequence accession number

The entire VP1 nucleotide sequences (888 nucleotides) of CV-A5 strains and the full-length genome sequences of CV-A5 3487 strain was determined in this study had been deposited in GeneBank under the accession no., MN663160.

Results

Detection and cell isolation of CV-A5

In an etiological spectrum study from Oct 2016 to Dec 2017 at Xiangyang, 146 cases of HFMD were caused by CV-A5. The virus identification was performed using rectal swabs from HFMD patients by PCR amplification and sequencing of the 5'-UTR fragments, RD and Vero cell isolation. The result indicated that CV-A5 emerged as one of the six main serotypes associated with HFMD. Four strains were isolated in RD cells and the isolation rate of CV-A5 in RD cells was low (4/146 of CV-A5 positive swabs). Three of four isolates grew at titers higher than 1×10^7 CCID₅₀/ml and were also adapted to grow in Vero cells.

Determination of the complete genome sequence

The complete genome sequences of the three isolates were determined and identities of nucleotide sequence between each pair were higher than 99.5% by blasting analysis (Fig. 1). The genome of the representative strain CVA5-R3487/XY/CHA/2017 (CV-A5-R3487 in short) is 7,407 nucleotides in length. The single ORF is 6,576 bases long encoding a polyprotein of 2,191 amino acid residues and a stop codon. The ORF is flanked by the 5'- and 3'-untranslated regions (5'- and 3'-UTR), 747 and 81 nucleotides long, respectively. The genome is polyadenylated at the terminus of the 3'-UTR and the poly-A tail is at least 24 bases long. The sequence (18 nucleotides) of the 5'-extreme end is the prime sequence based on the sequence of prototype Swartz.

Serotyping and subgenotyping of Xiangyang isolates

Blasting analysis based on the VP1 gene showed that homologies of the Xiangyang strains with the prototype Swartz of CV-A5 were 81.6%-82.0% for nucleotides and 94.9%-95.9% for amino acids, respectively. In contrast, homologies with other serotypes were below 75% for nucleotide sequences and 85% for amino acid sequence. Based on the criterion of the molecular typing recommended by Oberste [9], Xiangyang isolates are CV-A5. Subgenotyping of the three Xiangyang strains within the CV-A5 serotypes was performed by sequence comparison of the VP1 including 16 strains representing subgenotypes A-D (Fig. 1). They clustered with genotype D strains in the phylogenetic tree and shared identities of 95.9-96.3% and 98.3%-99.3% in nucleotide and amino acid sequences, respectively. The Xiangyang isolates were most closely related to the CV-A5 isolated at Wenzhou in 2013 [27]. In contrast,

they were genetically distant with other CV-A5 subgenotypes (or clades) isolated in other areas and different countries previously.

Recombination analysis

Investigation on genetic basis of the emerging CV-A5 in this epidemic was performed by recombination analysis. The complete sequences of 8 CV-A5 strains available at GenBank were used for alignment with CV-A5-R3487/XY/CHN by using MEGA6.0 software. The multi-sequences were scanned by SimPlot software (Version 3.5.1) to generate the similarity map as shown in Fig. 2. It showed that homologies with 8 CV-A5 strains decreased dramatically from the downstream 2B region to the 3'-UTR. This implied that there might be a recombination with other serotypes with a breakpoint at the 2A/2B junction. CV-A5-R3487 strain was aligned with other EV-A serotypes by Blasting. The results showed that from 2B to the 3'-UTR, it shared the highest similarity to the CV-A2/SZ/CHN/2015 strain (KX595284). As mentioned above, from the 5'-UTR to 2A region it shared the highest similarity to the CV-A5/WZ/CHN/2013 strain (KP289362.1). Taken together, the results suggested that there might be a recombinant event between CV-A5-R3487 and an imported CV-A2 strain or a local CV-A2 strain.

Previously, we reported that a Xiangyang CV-A2 strain was a recombinant which shared the high sequence identity with CV-A5 Swartz strain in P2 region [28]. Therefore, the Xiangyang CVA2-R1580/XY/CHN/2017, Shenzhen CV-A2/SZ/CHN/2015 (KX595284), CV-A5 Swartz and Xiangyang CV-A5-R3487 were used for detailed recombination analysis. As shown in Fig. 3a, the 3'-half of CV-A5-R3487 genome was derived from Shenzhen CV-A2 strain but not from Xiangyang CV-A2 strain. Further analysis (Fig. 3b) demonstrated that CV-A5-R3487 was indeed a recombinant carrying the 3'-half of genome of a CV-A2 strain with a breakpoint at the nt position 3,791 within the 2A region. Finally, up- and down-stream regions before and after the breakpoint 3,791 were aligned for sequence comparison. The result showed that the 5'-half (nt 1-3791) and 3'-half (nt 3,792-7,404) were clustered with CV-A5 and CV-A2 strains tested, respectively (Fig. 4a and Fig. 4b). The novel CV-A5 R3487 recombinant obtained the 5'-UTR, P1 and partial 2A regions and downstream partial 2A, 2B, 2C, P3 and 3'UTR regions of CV-A2 KX595284 strain. A novel CV-A5/CV-A4 recombinant emerged and caused an outbreak of HFMD in Xiangyang in 2017.

Discussion

RNA viruses exist as quasispecies when they evolve in nature and are passaged in cells or experiment animals [29-31]. The lack of fidelity and proofreading of the RNA dependent RNA polymerase (RdRp) of these viruses results in high error rates through nucleotide mis-incorporation during genome replication. Thus explaining how these viruses rapidly mutate and evolve [32-33]. The emergence, evolution and virulence/epidemics of RNA viruses are also driven by recombination and this includes enteroviruses. The mechanisms involve template switching [34-35] by the RdRp via an intermediate step of duplicated segments [36] or the replication-independent joining of RNA molecules [37]. It is believed that

recombination is the correction of deleterious mutations providing advantage for emerging new viruses [38].

Numerous intertypic and intratypic recombination events have been reported mostly in the same enterovirus species within and between serotypes of species A to D. Co-circulation and co-infection of several enteroviruses facilitate the emergence of recombinants. Recombination may act against Muller's Ratchet [39-40] and as a counterbalancing force against high mutation rates [38] by eradicating deleterious mutations. It may also lead to the combination of advantageous properties from various genomes into a new one such as emergence of drug resistance or even evasion from the immune system.

Emergence of new recombinants is often responsible for the infection, transmission, and pathogenesis of these viruses and the consequence may be deadly sometimes. For example, a recombinant CV-A2 caused the deaths of several (this sounds smoother but I dont know if its factually correct) children in Hong Kong in 2012 [41] and a recombinant of CV-B4 resulted in a fatal case of HFMD in Guangxi in 2012 [42]. Emergence and spread of novel recombinants are often associated with an outbreak of a disease in human population [43]. It is believed that a recombinant of EV-A71 with CV-A16 was one of the reasons behind the outbreak of HFMD epidemic of in Fuyang, China in 2008 [44].

The prevailing EV-A71 is a replication domains donor to other species A enteroviruses as recently reported research on recombination [45]. CV-A5 also dominated its replication part of the genome to EV-A17 and the recombinant circulated in Eastern Asia [46]. There is evidence that suggests recombination contribute to the switch of dominant serotypes and changes in of clinical manifestation [47]. In the outbreak of HFMD in Shanghai in 2012, a recombinant of CV-A6 emerged, which resulted in more generalized skin lesions than normal symptoms induced with non-recombinant CVA6 [47]. Between 2012 and 2013 in many cities and areas, CV-A6 and CV-A10 have emerged and even became dominant serotypes [4]. Changes of etiological spectrum may result from mutations and recombination. Following the largescale vaccination of EV-A71 vaccine in China, it is important to closely monitor the newly-emerged pathogens for disease control measures and for strategy of strategic development of multivalent vaccines against HFMD[48].

In Xiangyang, China in 2017, the HFMD outbreak was mainly caused by six main serotypes including CV-A6, A16, A10, A2, A5 and EV-A71. and Meanwhile other 12 serotypes were also detected in clinical samples of HFMD patients at very low proportions. The co-circulation of multiple serotypes increased the chances of co-infection and intertypic recombination [45]. In a period of nine months in 2017, CV-A2 and CV-A5 were co-circulating with case numbers of 117 and 146, respectively. Complete sequence analysis of 3 representative CV-A5 isolates showed that they were recombinants between CV-A5 with a breakpoint at 2A region.

The limitation of this study is that only 8 and 4 cell isolates were obtained for CV-A2 and CV-A5 and that the complete genome sequences of all CV-A5 samples were difficult to complete due to degradation of viral RNA. The ratio of parental strains to recombinants is not known. It is not clear whether recombination occurred in or spread to Xiangyang as the parental donor strain providing P2 and P3

region was not detected. Nonetheless, further detailed study should be performed to investigate the origin, evolution of CV-A5 parental strain and recombinant, and its pathogenicity in mouse model and human. Following the largescale vaccination of EV-A71 vaccine in China, it is worthwhile to closely monitor the newly-emerged pathogens for disease control measures and for strategic development of multivalent vaccines against HFMD.

Abbreviations

HFMD: hand, foot and mouse disease; ORF: open reading frame; RDP: recombination detection program; RdRp: RNA dependent RNA polymerase

Declarations

Compliance with Ethical Standards

Conflict of interest

The authors declare that they have no conflict of interest.

Research involving Human Participants and/or Animals

This work was approved by Medical Ethics Committee of Xiangyang Disease Control Center.

Informed consent

Informed consents were obtained from legal guardians of all patients prior to the collection of throat swab samples.

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Author contributions

Y.T.Y. and S.S. wrote the main manuscript text, analysed the data and prepared figures and tables.

Y.T.Y. and S.S. designed the work. All authors reviewed the manuscript.

Data availability statement

The data used to support the findings of this study are available from the corresponding author upon request.

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Tables

Table 1

Universal primers for Enterovirus VP1

Name	Primer sequence ^a (5'-3')	Position ^b (nt)
292	5'-MIGCIGYIGARACNGG-3'	2612-2627
222	5'-CICCIGGIGGIAYRWACAT-3'	2969-2951

a: IUB fuzzy code; I, deoxyinosine

b: The location relative to the genome of the Poliovirus type I Mahoney strain

Table 2

Primers used for PCR amplification and sequencing of CV-A5 genome

Name	Primer sequence (5'-3')	Position (nt)
A5YYT 19F	TTAAAACAGCCTGTGGGTTGTACC	1-24
A5YYT 6F	GCAATCCACTATGGTAGGACAAC	1998-2021
A5YYT 9R	ACCTACTGCAAGCATGAGATG	3630-3610
A5YYT 9F	GCACCTTTTCAGTGAGATTCGTT	3128-3250
A5YYT 13R	AACCCAGCAAAGAGCTTGTAGATA	4825-4806
A5YYT 20F	AAGAGAGTCTACGCCCTGGAG	4366-4386
A5YYT 23R	TTGACCCCTTGTTTCATCCACTAAT	5588-5565
A5YYT 24F	TCACTGCTGAGAAGGAATATCAGG	5407-5430
A5YYT 24R	CCAAAATGTATCTGGATTGCACCC	6585-6562
A5YYT PolyA	AGTCAAGTTACATAGTAGGCTACAGTAACTGCTTTTTTTTTTTTTTTTTTTT	7370-7419

Figures

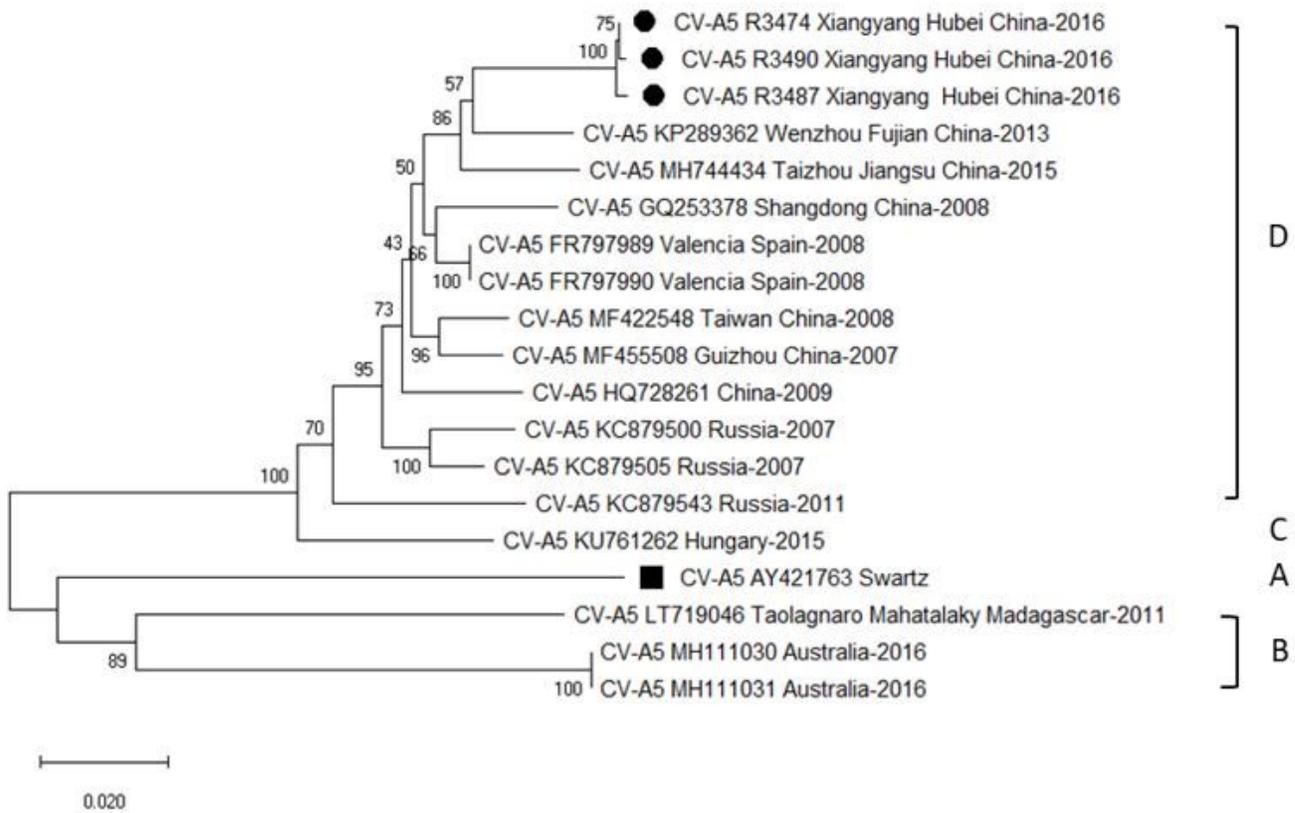


Figure 1

Phylogenetic analysis based on the CV-A5 VP1 sequences. ● represents the strain isolated at Xiangyang isolares, and ■ indicates the CV-A5 prototype Swartz. The GenBank accession No, isolation year and place of each reference strain are indicated. The phylogenetic tree was reconstructed using neighbor-joining method with the Kimura (2-parameter) model, 500 bootstraps for evaluation. The genotype demarcation was shown.

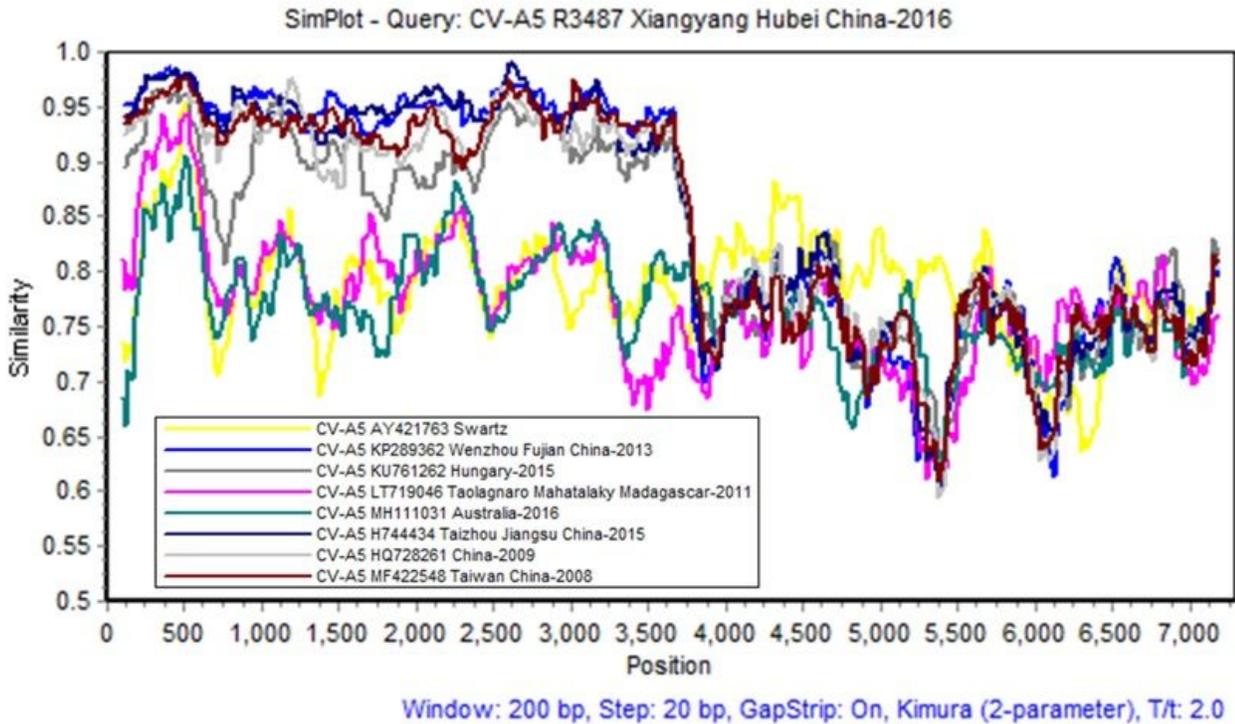
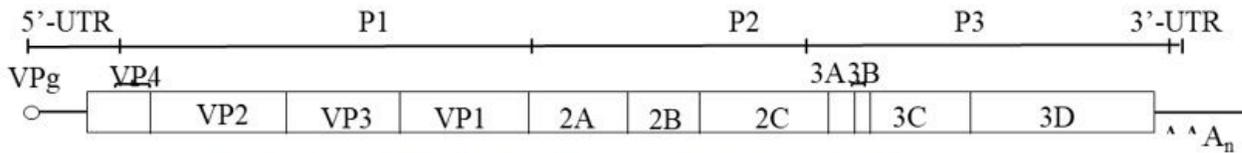


Figure 2

The similarity of CV-A5 R 3487 strain with other 8 CV-A5 serotypes strains. Window=200 bp, Step: 20 bp, GapStrip: On, Kimura (2-parameter), T/t: 2.0

Fig. 3a

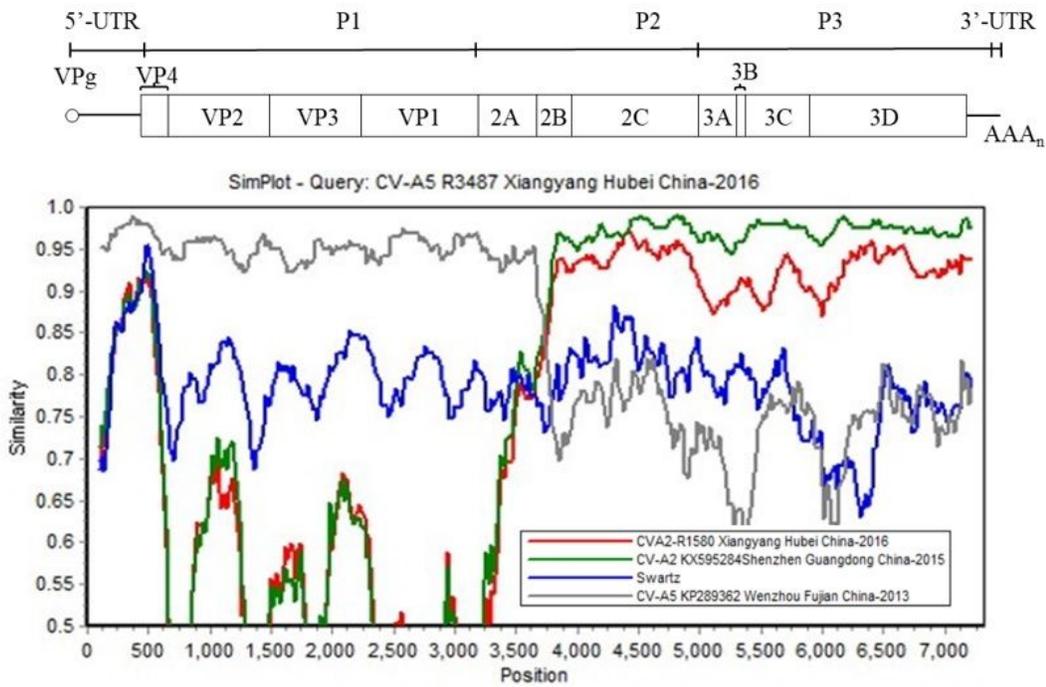


Fig. 3b

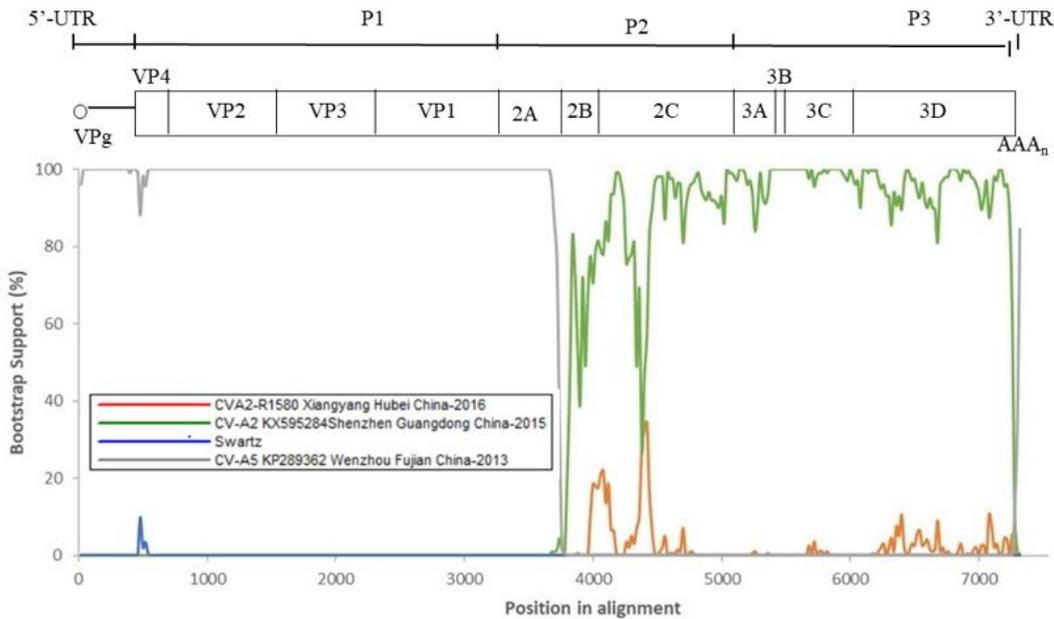


Figure 3

CV-A5-R3487 recombination analysis CV-A2 KX595284 (Green), CV-A2 1580 (Orange), CV-A5 Swartz (Blue) and CV-A5 KP289362 (Grey) were used to perform Similarity plot (Window=200 bp, Step: 20 bp, GapStrip: On, Kimura (2-parameter), T/t: 2.0) (a) and BootScan analysis (b).

Fig. 4a

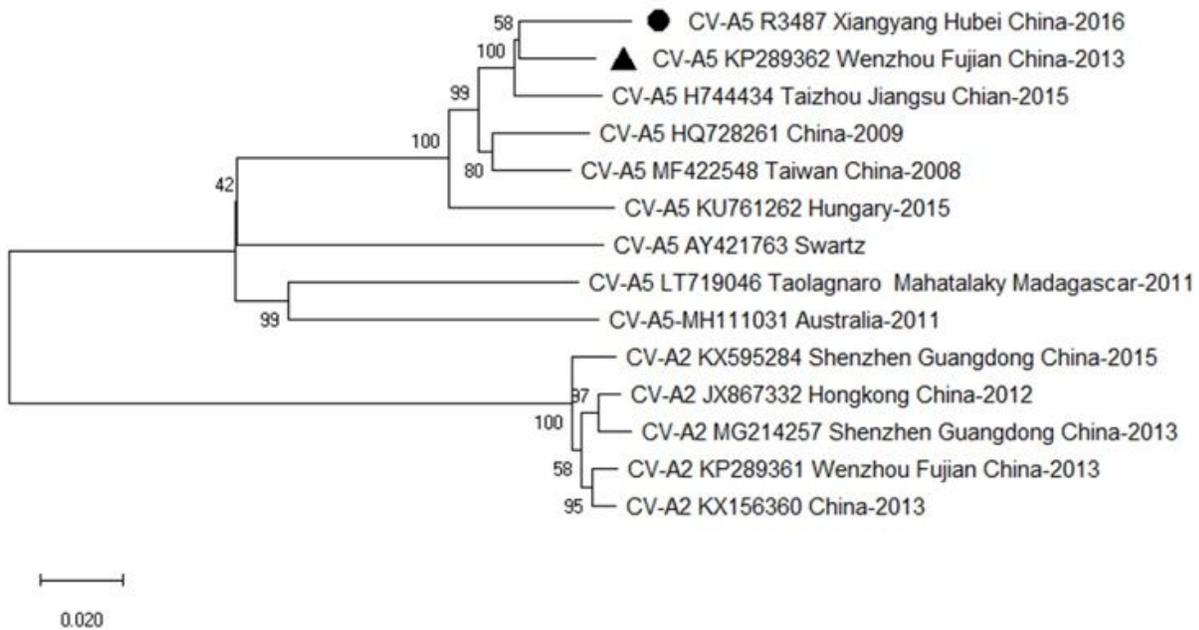


Fig. 4b

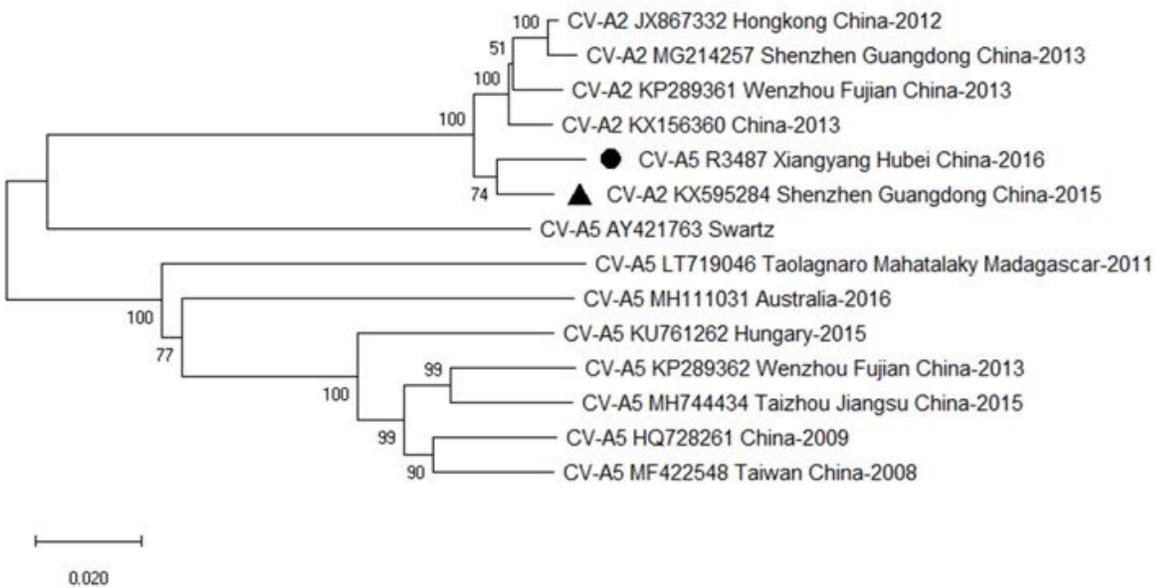


Figure 4

Phylogenetic trees based on 5'UTR-2A (3791) (a), and 2A (3792)- 3'UTR region (b) nucleotide sequences of CV-A5 and CV-A2 strains. Represents the strain isolated from Xiang Yang. Represents the contrast strains. The phylogenetic tree was reconstructed using neighbor-joining method with the Kimura 2-parameter model, 500 bootstrap for evaluation.