Infectious Medicine 3 (2024) 100115

Contents lists available at ScienceDirect

Infectious Medicine

journal homepage: www.elsevier.com/locate/imj

Review

A review of the recombination events, mechanisms and consequences of Coxsackievirus A6

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ARTICLE INFO

Keywords:
Genetic recombination
CV-A6
Virus evolution
Enterovirus
HFMD

ABSTRACT

Hand, foot, and mouth disease (HFMD) is one of the most common class C infectious diseases, posing a serious threat to public health worldwide. Enterovirus A71 (EV-A71) and coxsackievirus A16 (CV-A16) have been regarded as the major pathogenic agents of HFMD; however, since an outbreak caused by coxsackievirus A6 (CV-A6) in France in 2008, CV-A6 has gradually become the predominant pathogen in many regions. CV-A6 infects not only children but also adults, and causes atypical clinical symptoms such as a more generalized rash, eczema herpeticum, high fever, and onychomadesis, which are different from the symptoms associated with EV-A71 and CV-A16. Importantly, the rate of genetic recombination of CV-A6 is high, which can lead to changes in virulence and the rapid evolution of other characteristics, thus posing a serious threat to public health. To date, no specific vaccines or therapeutics have been approved for CV-A6 prevention or treatment, hence it is essential to fully understand the relationship between recombination and evolution of this virus. Here, we systematically review the genetic recombination events of CV-A6 that have occurred worldwide and explore how these events have promoted virus evolution, thus providing important information regarding future HFMD surveillance and prevention.

1. Introduction

Hand, foot, and mouth disease (HFMD) is a common infectious disease, with epidemic outbreaks of this disease occurring worldwide. Enteroviruses (EVs) are the causative agents of HFMD, and enterovirus A71 (EV-A71) and coxsackievirus A16 (CV-A16) have long been considered the predominant pathogenic agents [1]. However, in recent years coxsackievirus A6 (CV-A6) has supplanted EV-A71 and CV-A16 as the predominant causative agent, being responsible for repeated large-scale HFMD outbreaks [2,3] since its first outbreak in Finland [4]. Similar to other EVs, CV-A6 is a small, non-enveloped virus with a single-stranded, positive-sense RNA genome of approximately 7.4 kb comprising a long open reading frame (ORF) flanked by 5′- and 3′-untranslated regions (UTRs). This ORF is translated into a polyprotein of 2201 amino acids, which can be cleaved into the three polyprotein precursors P1, P2, and P3. P1 encodes the capsid proteins VP1–VP4, whereas P2 and P3 encode the noncapsid proteins 2A–2C and 3A–3D, respectively [5]. VP1, which is highly conserved and serotype-specific, is used in internationally accepted methodology for EV typing [6], whereas 3D is often used for recombinant lineage classification [7]. The clinical symptoms associated with CV-A6 differ from those of EV-A71 and CV-A16, with CV-A6 infection leading to herpangina and atypical HFMD manifesting as a higher and longer-lasting fever, a more widespread rash, and onychomadesis [8]. CV-A6 can in-

Abbreviations: HFMD, Hand, foot, and mouth disease; CV-A6, Coxsackievirus A6; CV-A16, Coxsackievirus A16; EV-A71, Enterovirus A71; CV-A4, Coxsackievirus A4; PV, Poliovirus; EV, Enterovirus; RF, Recombination form; RdRp, RNA-dependent RNA polymerases; UTR, Untranslated region.

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https://doi.org/10.1016/j.imj.2024.100115
Received 11 January 2024; Received in revised form 25 February 2024; Accepted 22 April 2024

fect not only children but also adults, sometimes leading to severe HFMD and fatalities. Since no effective vaccines or drugs are commercially available, CV-A6 is a significant public health burden.

Genetic recombination of RNA viruses, which was initially detected by Hirst [9], is regarded as a driving force of EV evolution. Researchers have demonstrated that the $3D^{pol}$ error-prone RNA-dependent RNA polymerases (RdRps) of EVs lack proofreading function, leading to misincorporations of 10^{-5}–10^{-3} per nucleotide site [10] during genome replication, while recombination can prevent deleterious mutation accumulation, which may account for the high-frequency recombination. Compared with other EVs, CV-A6 strains show a higher diversity of genetic sequences. It was reported that most EV-A71 strains belonging to the same sub-genotype also clustered together in the phylogenetic tree of other regions, such as 2C and 3D, while CV-A6 showed the opposite trend [11]. The evolutionary phenomenon of the noncapsid coding region in the same sub-genotype of CV-A6 might explain why this virus has shown rapid, worldwide dissemination. Based on the VP1 region, CV-A6 strains can be divided into A, B1, B2, C1, C2, and D1–D3 genotypes; D3 can be further divided into D3a and D3b sub-genotypes, with D3a [11] and D3 [12,13] being the predominant genotypes in China and worldwide, respectively, in recent years. Based on the 3D coding region, previous studies categorized global CV-A6 variants into 25 recombination forms (RFs), alphabetically termed RF-A to RF-Y [14-16], among which RF-R, -S, -T, -U, and -V were newly detected in France [17] and RF-Y was newly detected in Thailand during 2019–2022 [18]. It is worth noting that the diversity in the noncapsid coding region within a single sub-genotype observed in CV-A6 is uncommon among the other leading pathogenic agents of HFMD. Researchers have demonstrated that conservation of the CV-A6 capsid gene has led to its high transmissibility, while the noncapsid gene, which is lineage-specific, might affect pathogenicity [11].

In this review, taking information from studies on other EVs into account, we summarize the global recombination events for CV-A6 and explore the mechanisms responsible for this recombination and its role in virus evolution. We therefore provide information relevant to the effective surveillance and prevention of HFMD.

2. Global genetic recombination events for CV-A6

It is well-established that CV-A6 has undergone highly frequent recombination events that correlate with pathogenicity during the processes of transmission and evolution [19,20]. Here, we detail the genetic diversity of CV-A6 strains by summarizing the recombination events that have been reported for CV-A6 worldwide.

2.1. CV-A6 recombination in China

2.1.1. CV-A6 recombination events not leading to the division of RFs

During an outbreak of HFMD in Shanghai in 2013, a recombinant CV-A6 strain, with high similarity in the 2C and 3′-UTR regions to CV-A4 circulating in Shanghai, was isolated [21]. This recombinant strain caused a more generalized rash, implying that recombination may have led to a change in pathogenicity associated with more severe lesions [22]. Another study from Wenzhou, China, reported a more widespread rash, larger blisters, subsequent desquamation, and onychomadesis caused by CV-A6, with the recombination breakpoints in this strain located in the 2A, 3A, and 5′-UTR regions; however, the relationship between recombination and clinical symptoms was not confirmed further in their study [23]. Among the 39 strains isolated in northeast China in 2013, four strains with full length were probably recombinant products of the CV-A6 prototype strain Gdula and CV-A4, and the recombination breakpoints were mainly located in the P2 region. Recombinant strains show differences in reproducibility, their ability to release from cells, and pathogenicity in mice. An interesting phenomenon was that the nonlethal strain Changchun098 resided in a separate cluster from the three lethal strains Changchun046, Changchun097, and Changchun099 according to phylogenetic analysis based on the P2 coding region. However, a similar phenomenon was not observed for other fragments such as the 5′-UTR, P1, and P3. Further genome analysis showed that Changchun098 shared higher similarity with Gdula in the P2 region than the other three strains, implying that P2 may contribute to the virulence of CV-A6-Changchun strains, and that genetic recombination in this region can affect the virulence of the recombinant strains. Further experiments demonstrated that 2C played an important role in the pathogenicity of CV-A6 by causing autophagy and inducing cell death, which was consistent with the epidemiological results [24].

2.1.2. CV-A6 of different RFs reported in China

Qiao et al. reported two recombination events for CV-A6 in Nanjing, China, with CV-A6 strains obtaining partial 2B (4001–4045 bp) and 2C (4866–4873 bp) regions from CV-A4 and CV-A8, respectively. There were two types of recombination patterns of CV-A6 co-circulating in Nanjing, namely RF-A and RF-J, but whether the virulence differs between these RFs remains unknown [25]. Among the four recombinant strains reported in Hong Kong, China, HK459455/2013 belonging to RF-J and HK458288/2015 belonging to RF-L caused herpangina, HK446377/2015 belonging to RF-L caused HFMD, and HK463069/2015 belonging to RF-M caused acute encephalitis. Further analysis revealed that HK459455/2013 originated from the re-

combination of CV-A6 and CV-A4 in the P2 region, HK458288/2015 and HK446377/2015 were the recombinant products of CV-A6 and CV-A4 in the 3D region, and the 3D region of HK463069/2015 originated from the C4 genotype of EV-A71 [16]. By performing a large-scale genetic analysis of global CV-A6 variants from 2010 to 2018, researchers revealed that recombination of the non-structural region led to the emergence of RF-J, -K2, and -L, which evolved from RF-A in mainland China with a mutation rate of 5.20×10^{-3} nucleotide substitutions/site/year, which was slightly higher than that of other strains worldwide. RF-J had undergone three obvious recombination events with Shenzhen CV-A4 strain (HQ728260/2009), Shenzhen CV-A8 strain (KM609478/2012), and Guangdong EV-A71 strain (JF799986/2009). RF-K2 was reported to be a recombinant of Jiangsu CV-A14 strain (KP036482/2012) in the P3 region. Moreover, RF-L had undergone two recombination events with Shenzhen strains CV-A4 (HQ728260/2009) and CV-A8 (KM609478/2012). Further analysis revealed that RF-J infection may cause a more generalized rash and RF-L tended to cause severe HFMD compared with RF-A. It is therefore clear that the high conservation of the structural coding regions means that CV-A6 remains highly transmissible, while the lineage specificity of the non-structural coding regions may correlate with the degree of pathogenicity. This study also highlighted that recombination within the same genotype to produce new lineages was not common among other pathogens of HFMD [11]. Yu et al. reported that recombination and the change in sub-genotype may explain why CV-A6 is the predominant pathogen of HFMD in Beijing, with the two RFs, RF-C and -D, predominating in 2010 and 2011, then changing to RF-A, -L, and -J from 2013. Recombination breakpoints were mainly located in the P2 and P3 regions, especially 2C and 3D [26]. CV-A6 strains isolated in Beijing from 2017 to 2019 tended to recombine with EV-A114 in the 2B and 3D regions and RF-A was the predominant recombinant lineage; however, this study did not explore virulence changes after recombination [27].

2.2. CV-A6 recombination events worldwide

To determine whether virus-specific factors were involved in the changes in clinical symptoms observed with CV-A6, Gaunt et al. found that all Finnish CV-A6 strains related to atypical HFMD in 2008 belonged to RF-A, while all strains in Edinburgh related to herpetic eczema in early 2014 belonged to RF-H. This indicated a strong correlation between genetic recombination and altered clinical symptoms. Further analysis demonstrated that both RF-A and RF-G had a strong correlation with atypical HFMD, while RF-B and RF-E mainly caused herpangina, and RF-H mainly caused herpetic eczema. This provided strong evidence that non-structural coding regions may play an essential role in the diversity of clinical phenotypes of CV-A6 infection. However, corresponding experimental data and statistical analyses to support these findings are needed in future research [14]. In another study, recombination breakpoints were detected in the 2A–2C and 5′-UTR regions among 151 CV-A6 strains collected from Germany, Spain, Sweden, Denmark, and Thailand from 2013 to 2014, and most strains belonged to RF-A (105/151) and RF-F (37/151). Regretfully, there was a lack of information about the association between recombination and clinical changes in this study [15]. Joanna et al. reported that CV-A6 in Australia had undergone recombination events in the 2C region with CV-A4, CV-A2, and EV-A71 and called for greater surveillance of EVs to improve strategies for outbreak preparedness and vaccine development [28]. During 2010–2018 in France, most strains of CV-A6 reportedly belonged to RF-A, -H, -G, and -F, among which RF-A was predominant, and the newly emerged RF-V was detected. The researchers also claimed that 1–6 RFs are circulating each year, reflecting the frequency of recombination. It was stated that the large-scale spread of CV-A6 worldwide began from 2005 to 2007, which was consistent with the appearance of D3/RF-A [17]. In recent research, CV-A6 was demonstrated to be the predominant pathogen of HFMD in Thailand, with four RFs reported, namely RF-A, -N, -H, and -Y, with RF-A being the most common. RF-Y was a newly discovered recombination lineage that shared high similarity with CV-A10 in the 3D region. More detailed analysis showed that the genetic recombination event generating RF-Y occurred 4.8 years ago. This study also reported that the recombination frequency of CV-A6 peaks every five years; however, analysis on full-length genomes was lacking [18].

2.3. Summary of global CV-A6 recombination events

Genetic recombination of CV-A6 is frequent both in China and in other countries (Table 1). Human EVs have a high mutation rate due to evolutionary pressure and frequent recombination, with the rate for CV-A6 reported to be 4.20×10^{-3}–4.73×10^{-3} substitutions/site/year [11,14,18]. However, it is interesting that CV-A6 in mainland China evolved faster according to the substitution rate of P1 (5.20×10^{-3} substitutions/site/year), although the reason for this phenomenon remains unknown.

The recombination breakpoints of EV-A, -B, and -C are distributed across the 5′-UTR, P1, P2, and P3 regions [29–31], among which P1 is not often reported. The recombination breakpoints of EV-B and -C are mostly located at the junction of the 5′-UTR and P1 regions and the start of P2, while recombination of EV-A occurs relatively uniformly in both the P2 and P3 regions [32]. This was consistent with the CV-A6 recombination events reported globally. Researchers found that recombining the capsid

Table 1
Genetic recombination events for CV-A6 worldwide.

Time	Location	Number of patients	Number of CV-A6 strains	Genotype	Recombination breakouts	Parents	Recombination forms	Association between recombination and clinical symptom changes
2013.1–2013.12	Nanjing, China [25]	16551 HFMD patients	Selected 28 strains for further study of VP1, 2C and 3D, among which full-length sequences of 8 strains were sequenced	N/A	4001–4045bp(2B)4866–4873bp(2C)	CV-A2, CV-A4, CV-A8, EV-A71	co-existence of RF-A and RF-J	N/A
2013	Shanghai, China [21]	N/A	N/A	N/A	2C, 3′ -UTR	CV-A4	N/A	N/A
2012.1–2013.9	Shanghai, China [22]	626 HFMD patients	Recombinant CV-A6 accounted for 21.9% (64/292) of all CV-A6 strains	D6, D7	2C	CV-A4	N/A	Recombinant strains caused wider rash than did the non-recombinant CV-A6.
2017–2019	Beijing, China [27]	1721 HFMD patients	VP1 of 120 strains were used for genotyping, 14 were full-length sequenced	D3a more than D3b	2B and 3D	EV-A114	RF-A	N/A
2013	Northeast China [24]	N/A	39 circulating CV-A6 strains	N/A	P2/P3, especially in P2	CV-A4, CV-A6	N/A	Changchun098 strain which had a higher similarity with Gdula showed less virulence.
2010.1–2017.12	Hong Kong, China [16]	36 CV-A6 positive patients	28 strains	D5	3D, P2/P3	EV-A71 CV-A4, CV-A6	RF-J, -L, -M	RF-J,-L caused herpangina, while RF-M caused acute encephalitis.
2010–2016	Beijing, China [26]	N/A	64 CV-A6 positive samples	D2→D3, (2013)	noncapsid regions, especially P2 and P3	CV-A4, CV-A6	RF-C, -D(2010,2011), RF-A, -J, -L(2013)	Recombination of CV-A6 may be a cause of it being the predominant pathogen for HFMD.
2013	Wenzhou, China [23]	955 HFMD patients	CV-A6 was the predominant (77.8%)	N/A	3′ end of 5′ -UTR and 2A, 3A	CV-A2, CV-A8	N/A	CV-A6 caused severer skin lesions than EV-A71 and CV-A16; nail loss was significantly associated with desquamation ($p = 0.002$). However, comparation of clinical symptoms between non-recombinants and recombinants were not mentioned.
2010–2018	Mainland China [11]	N/A	336 strains, among which 158 (2 were reported as fatal cases, 17 were severe cases, and 139 were mild cases) were isloated in this study, 178 were downloaded from GeneBank	D2, D3	Noncapsid region	EV-A71, CV-A4, CV-A8, CV-A14	7 RFs including -A, -C, -D, -K1, -K2, -J, and -L, with most of the Chinese CV-A6 strains belonging to lineages -A, -J, and -L	Lineage-L may be more likely to cause severe HFMD than lineage-A.
2014.1–2014.2	Edinburgh [14]	Children and young adults with CVA6-associated eczema herpeticum	N/A	N/A	2A-2C,VP1, VP3, 5′ -UTR	N/A	Except for 8 RFs circulating worldwide over the past 10 years, all CV-A6 associated with eczema herpeticum cases in Edinburgh in 2014 belonged to RF-H .	RF-A and RF-G caused atypical HFMD, RF-B and RF-E mainly caused herpangina, RF-H mainly caused herpetic eczema.
2013 and 2014	Germany, Spain, Sweden, Denmark, Thailand [15]	N/A	151 variants for VP1 and 3D analysis; 39 variants for nearly full-length analysis	N/A	2A-2C and 5′ -UTRs	N/A	RF-A, -F, -G, -H	N/A
2016.2–2017.7	western Sydney, Australia [28]	N/A	24 strains for whole genome sequencing	N/A	2C	CV-A4, CV-A2, EV-A71	N/A	N/A
2010–2018	France [17]	Throat specimens of 245 children	213 complete CVA6 genomes	D1, D3	N/A	N/A	RF-A(58%),-H (19%),-G(6.1%), -F(5.2%), -B, -N, -V	N/A
2019.1–2022.10	Thailand [18]	N/A	CV-A6 (23.7%) was the predominant genotype	The majority were D3.1, others belonged to D3.2	3D	CV-A10	RF-A(147,84.5%), RF-N (11, 6.3%), RF-H (1, 0.6%), and newly RF-Y (15, 8.6%)	N/A

Note: N/A, not applicable.

region with different serotypes of EVs resulted in poor replication ability or even the loss of replication of poliovirus (PV), while the opposite was true for recombination in the noncapsid region. This demonstrated that recombination in the capsid region negatively effects the survival of EVs and illustrates why recombination in the P1 region is rare [33]. Other studies revealed that different EVs recognize different cell receptors and that recombined capsid protein may not produce the correct recognition structure thus preventing cell adhesion and/or entry, which may also explain why the recombination sites of EVs are mostly located in the P2 and P3 regions [34,35].

As for recombination in non-structural regions, the 2A–2C, 3D, and 5′-UTR regions of CV-A6 are predominantly reported [14-16,18,21-28]. CV-A6 can recombine with other CV-A6 strains as well as other EVs, among which CV-A6 prototype Gdula, CV-A4, CV-A8, and EV-A71 are most frequently reported. The length of recombination breakpoints varies for different recombination events. To date, 25 RFs (RF-A–RF-Y) have been reported worldwide, among which RF-A is the most common and is the ancestor of the other RFs. Many studies have also reported an association between different RFs of CV-A6 and changes in clinical symptoms, although most of these studies lack experimental data to support the epidemiological findings. Further research is therefore needed to provide stronger evidence that genetic recombination can impact on disease manifestations and severity.

3. Potential mechanism of genetic recombination

Currently there are two possible mechanisms underlying the genetic recombination of EVs, namely replicative recombination and non-replicative recombination [36-38]. According to the genetic characteristics of recombinant products, recombination can also be divided into homologous recombination and non-homologous recombination [39,40], which are also known as precise recombination and non-precise recombination. Homologous recombination always occurs at the same site in two parental chains, whose products have the same structure as the parental chains with no base insertions or deletions, whereas for non-homologous recombination the recombination breakpoints of the two parental chains are located at different sites [41] (Fig. 1). In other words, homologous recombination occurs in regions with highly similar sequences and is always reported in RNA viruses, while non-homologous recombination usually occurs in regions with different sequences and may produce harmful genotypes, making it uncommon [42-44]. Non-homologous recombination can be observed under experimental conditions and is not stable [41] and the repetitive and abnormal sequences generated during non-homologous recombination may be deleted through unknown pathways or replaced by homologous recombination strains with stronger adaptability [45].

Genetic recombination of EVs also includes interspecies recombination and intra-species recombination [43], the former being relatively rare [38]. However, both EV-D111 and EV-D120 can infect animals, and EV-D111 of humans and animals belongs to the same branch in phylogenetic trees based on VP1, indicating the possibility of cross-species transmission of EVs [46]. Although there have been no reports of cross-species transmission of CV-A6, surveillance should be strengthened based on this possibility. Intra-species recombination was first discovered in PV by Hirst [9], which was also the first case of RNA virus genetic recombination.

3.1. Replicative recombination

Since it is widely recognized that PV homologous recombination is mediated by template switching during replication (also known as copy-choice recombination or template-switching replication) [47], it can be deduced that other EVs such as CV-A6 might also follow the same mechanism. This is supported by the fact that CV-A6 has a single-stranded, positive-sense RNA genome, similar to PV.

3.1.1. The core content of replicative recombination

The core content of replicative recombination is based on the replication selection hypothesis proposed by Copper et al. [48], which is a widely accepted concept. During virus replication, copy choice recombination occurs when the RdRp dissociates from the virus genome to prevent the synthesis of new negative-stranded RNA molecules and then binds to a second genome from another virus to continue the replicative process, generating a new mosaic-like genome with regions originating from different parental strains [49-51]. Several researchers reported similar recombination mechanisms for RNA viruses, suggesting that RNA polymerase regulates RNA replication and switches from one RNA molecule (donor template) to another (receptor template) during the synthesis process [52,53]. Correspondingly, the RNA polymerase in most EVs is RdRp, and in retroviruses is RT [50]. Consistent with this hypothesis, Lowry et al. illustrated that recombination of EVs was a “copy-choice” process with the polymerase switching template during negative-strand synthesis [54] and at the same time maintaining binding with newly-formed nucleic acid chains, resulting in RNA molecules with mixed ancestors.

Recombination of EVs is a biphasic replicative process. In the first stage, greater than genome length “imprecise” intermediates with duplicate fragments (up to hundreds of bases) from the parental gene are generated. In the second stage, the virus undergoes an “elimination” program during the passage process and variants with

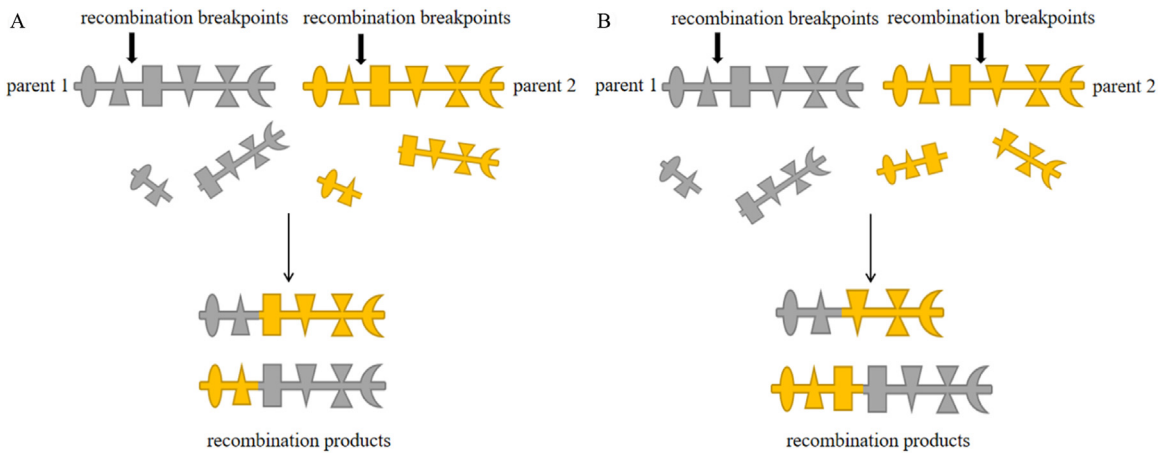

Fig. 1. Mechanisms of homologous recombination and non-homologous recombination. (A) Homologous recombination occurs when two parental genomes (gray and yellow) have the same recombination breakpoints, and the recombination products have the same length and structure as their parental chains. (B) Non-homologous recombination will lead to base insertion or deletion because parental chains have different recombination breakpoints.

stronger adaptability are selected to continue replicating. Repeated fragments produced in the first stage are deleted during this process, ultimately generating a genome of the appropriate length [55]. This study introduced the concepts of precise and imprecise recombination, but further research is needed on the detailed mechanism of the "elimination" process. Hence, replicative recombination is currently the most widely recognized concept.

3.1.2. Conditions for the occurrence of replicative recombination

A prerequisite for replicative recombination is that two parental strains must share homologous regions. The presence of a specific base composition, such as high U-A or G-C, at the crossover site is also essential. Similar secondary structure between the two parental genomes is also considered as an important prerequisite. Evidence suggests that co-infections of multiple viruses within cells of the same host, as well as the simultaneous replication of all RNA viruses, are also of great importance for recombination mediated by replicative mechanisms [41,49].

3.1.3. Factors that influence replicative recombination

Multiple factors such as RdRp, gene structure, base composition, sequence consistency of the RNA template, and transcription dynamics have been identified to be involved in replicative recombination. For example, during RNA replication, the insertion of incorrect bases can stop the process, thus leading to the occurrence of recombination [56].

(1) The role of viral RdRp in EV recombination
Numerous studies have illustrated that recombination of EVs is related to the accuracy or fidelity of the viral RdRp. The higher the fidelity of RdRp, the less likely it is to undergo genetic recombination [52]. Mutations in RdRp can impact on genetic recombination and the key sites have been identified. Researchers found that the amino acid mutation D79H decreased the recombination rate but did not affect the replication rate and fidelity, whereas mutation H273R affected fidelity thus increasing the mutation rate. It was demonstrated that H273R and G64S mutants correlated with low-fidelity and high-fidelity, respectively. The D79H mutation did not affect virulence, but H273R and G64S mutations were associated with a less virulent phenotype. The specific mechanism was that a single D79H mutation could not lead to a decrease in accumulated favorable mutations and an increase in harmful mutations in the virus strain [57]. However, double mutations affecting both recombination (D79H) and fidelity (H273R or G64S) can decrease the recombination rate whilst also changing the fidelity of replication, thus dramatically reducing the adaptability of the virus in the host and altering the tissue tropism, leading to weaker and less virulent strains. These findings have important implications for the treatment and prevention of viral infections. However, a D79H mutation in the 3D coding region had no observable impact on the frequency of recombination in one study [58]. In another study, a mutation (L420A) in RdRp, which reduced recombination in PV, similarly reduced EV-A71 recombination, suggesting conservation of RdRp-mediated recombination mechanisms [59], which was consistent with the results of Brian et al. [58].

(2) RdRp is not the only factor that can affect EV recombination
The speed and fidelity of the RdRp are not the only determinants of recombination efficiency and mechanism; other biochemical properties of RdRp may also affect the incidence of recombination [60]. The contribution of secondary structure and homologous sequences among the parental genes is controversial. Some studies found that recombination breakpoints were mostly located in regions with RNA secondary

structures and homologous sequences [61], highlighting the importance of sequence identity between the nascent strand and the acceptor RNA, as well as the donor templates. While others illustrated that there was no significant correlation between the frequency of viral recombination and the above two factors, attaching greater importance to genome function and fitness for genetic recombination [54,62]. Additionally, the relationship between recombination and base composition varies among viruses. In plant viruses and retroviruses, a higher U-A base composition could increase the frequency of recombination, and recombination breakpoints were often located in areas with a higher proportion of U-A bases [63]. However, a high proportion of G-C rather than U-A will increase the recombination frequency of PV [61]. Moreover, a recent study reported that GC-rich sequences could increase the recombination frequency of PV and Brome Mosaic Virus (BMV), while recombination sites were often found to be located in AU-rich sequences in many positive-strand RNA viruses such as PV, because the weak annealing of A-U nucleotides may promote the nascent strand to dissociate from the donor strand, thus facilitating the initiation of the template-switching process [41]. Further research is needed to explore the molecular mechanisms that contribute to the above phenomena.

3.2. Non-replicative recombination

Non-replicative recombination (also known as fracture-connection recombination or the breaking-joining model) was first demonstrated for bacteriophage Qβ [64]. Non-replicative recombination has since been confirmed for Hepatitis C Virus (HCV), Bovine Viral Diarrhea Virus (BVDV), and PV [65-67], but to date no evidence has been found in CV-A6. This theory suggests that different RNA chains are cleaved and then their exposed ends are rejoined at the cleavage site by a trans-esterification reaction, which is not related to the activity of replication enzymes. It is evident that neither the 5′ or 3′ components that undergo non-replicative recombination need to be translated, suggesting that viral proteins are not involved in this process [38,66]. Gmyl et al. demonstrated that PV can still undergo homologous and non-homologous recombination in the absence of viral RdRp [68]. In other words, non-replicative recombination can occur between both homologous and non-homologous RNA fragments, and does not require consistent or specific RNA sequences except for RNA secondary structures such as pseudo-knots, bulges, or loops [41,67]. Although viral proteins such as RdRps are not essential during the non-replicative process, researchers suggested that there must be a need for host cell proteins in this process [69]. Several enzymatic mechanisms have been proposed to explain non-replicative recombination, and it is the secondary structure of the RNA rather than the similarity of sequences that is widely considered as the main factor mediating this type of recombination [50].

In summary, both of the above mechanisms are feasible (Fig. 2). However, due to the inability to distinguish the end products of different recombination mechanisms in epidemic strains, the natural recombination mechanism of viruses cannot be determined, and it is not yet possible to determine which theory dominates under natural conditions [38]. Nevertheless, the replicative mechanism is widely recognized. The prerequisite of both mechanisms is that two or more virus strains co-circulating in a limited geographic area over a short period of time simultaneously infect the same host cell, providing spatial support for genetic recombination. Co-localization of parental genomes can also not be ignored, and recombinant gene fragments need to be compatible. The recombinant products can replicate and produce infectious offspring virus particles. The prevalence of recombinant strains also requires them to be competitive enough to survive during the limited transmission process [41,69]. Although there are fewer studies on the recombination mechanism of CV-A6, there has been much research on other EVs, such as PV and EV-A71. In the future, it is important to explore the mechanism of CV-A6 recombination events to better explain the diversity of CV-A6.

4. Recombination promotes the occurrence of new strains with different clinical symptoms and virulence

To date, many scientists have regarded genetic recombination as the driving force of CV-A6 evolution. It is well-established that recombination can lead to a change in virulence, drug-resistance, antigenicity, and transmissibility [38], thus leading to outbreaks of HFMD. Recombination in CV-A6 can also generate strains capable of inducing specific clinical symptoms such as onychomadesis and a generalized rash [14,22]. In summary, recombination and mutation are the core processes driving virus evolution, and are crucial for the transmission and virulence of viruses.

4.1. Recombination and changes in virulence

4.1.1. Recombination-enhanced virulence

Typically, recombination is considered an important factor that can enhance the virulence of EVs, leading to more severe clinical symptoms and even fatalities [70-72]. Recombination between circulating strains may also lead to viral disease outbreaks. In 2013, an epidemic strain generated from recombination between CV-A6 and CV-A4 caused an outbreak of HFMD in Shanghai. The recombinant strains caused more severe skin lesions than

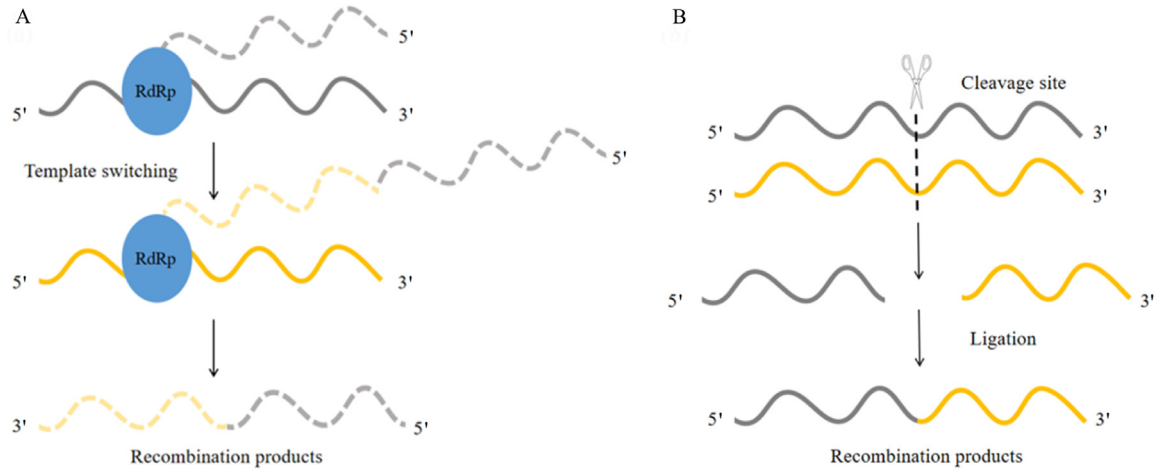

Fig. 2. Comparison of the replicative and non-replicative recombination mechanisms. (A) Replicative recombination is mediated by RdRp (blue), RdRp dissociates from the viral genome (gray) to prevent the synthesis of new negative-stranded RNA molecules and then binds to a second genome (yellow) from another virus to continue the replicative process, generating a new mosaic-like genome with regions originating from different parental chains. (B) During non-replicative recombination, different RNA chains are cleaved and then their exposed ends are rejoined at the cleavage site by a trans-esterification reaction, which is not related to the activity of replication enzymes.

non-recombinant strains, possibly due to the increased pathogenicity caused by recombination [22]. This type of recombination was also found in Beijing at the same time and caused a heavy burden on public health [26]. In the same year, CV-A6 strains underwent recombination with EV-A71 in the connecting zone of the P1 and P2 regions, which may have been related to the outbreak of HFMD in Guangdong [73]. In 2015, a study in Hong Kong reported that CV-A6 recombinant strains that had acquired the 3D region from EV-A71, caused acute encephalitis in children [16]. Another study in Changchun found that recombinant CV-A6 strains that had higher similarity to Gdula in the P2 region, showed weaker virulence in mice than recombinant CV-A6 strains that had higher similarity to CV-A4 in the P2 region, suggesting that the degree of genetic recombination may be highly related to the virulence of the recombinant strains [24]. In other research, patients infected with recombinant CV-A6 had more extensive skin lesions in their upper limbs, lower limbs, and anterior abdomen than non-recombinants, with statistically significant differences, but the mechanism remains unclear [22]. However, a further study by the same group found no significant differences in biological characteristics between recombinant and non-recombinant CV-A6 strains.

Additionally, recombination can also lead to the increased virulence of other EVs. Recombination between PV Sabin 2 and non-polio EV-C in the 5′-UTR resulted in 25%–50% paralysis in mice, which was higher than that of non-recombinants [74]. Moreover, recombination between PV live vaccines with non-poliovirus C could restore the pathogenicity of the vaccine strains, resulting in an outbreak of vaccine-derived poliovirus. Therefore, the recombination of PV vaccine strains cannot be ignored [41]. Based on phylogenetic analysis of the P3 region, enterovirus C could be divided into three branches. Branch I mainly included strains from healthy children and children with acute flaccid paralysis and acute hemorrhagic conjunctivitis, while strains causing respiratory diseases only clustered in Branch III. Distinct from strains in Branch III, strains in both Branch I and Branch II could replicate in the gastrointestinal tract. This suggests an association between the P3 region and disease manifestations [75]. However, due to the limited sample size of the above studies, more clinical data and virus genome sequences are needed to verify this conclusion.

4.1.2. Recombination-decreased virulence

Several researchers maintain that some recombinants display decreased virulence. Compared with recombination in the 5′-UTR, recombination in the P2 or/and the P3/3′-UTR region of Sabin2 and non-polio EV-C generated strains with attenuated virulence lacking neurotoxicity [74]. Considering that virulence may decrease or increase after recombination, it is imperative that changes in virulence are urgently analyzed when new recombination events occur.

4.2. Recombination may change biological characteristics other than virulence

It is also possible that genetic recombination has little effect on virulence and instead leads to changes in other biological characteristics. Researchers found that both recombinant and non-recombinant EVs could lead to either mild or severe clinical symptoms, suggesting that there may be no difference in virulence between recombinant and non-recombinant EVs [76,77]. In addition, by constructing recombinant chimeric viruses of cVDVP (circulating vaccine-derived poliovirus) and non-

PV, researchers found different recombinant strains had different temperature sensitivity and plaque size without changes in replication ability [74]. The above studies provide evidence that genetic recombination may not always lead to disease outbreaks, and such events may remain undetected, making it difficult to determine the initial source of gene fragments in the recombinants. Therefore, this hidden type of recombination may also have public health implications and should be taken into consideration.

4.3. Recombination and viral antigenicity

Although changes in viral antigenicity caused by recombination have barely been reported in CV-A6, studies with EV-A71 confirmed that recombination may increase virus binding ability and reduce its neutralizing effect on patient serum. Huang et al. found that the B4 and B5 genotypes of EV-A71 showed different antigenicity and the recombinants EV-A71 VP1-98K/145Q/164E showed increased virus-binding ability, which may affect the efficacy of vaccines [78]. Based on antigen analysis of serum from infected children, genotype A viruses differed in antigenicity between genotype B5 and C4a viruses [79]. In 2008, during the prevalence of EV-A71 in Taiwan, China, Huang et al. reported a new sub-genotype, named C2-like, which may be generated by the recombination of C2 and B3 sub-genotypes. Compared with other sub-genotypes, the neutralization ability of C2-like was generally poor, with a maximum difference of up to 128 times [80]. Collectively, these data suggested that genetic recombination might play an important role in viral antigenicity and may present a challenge to vaccine development. Regrettably, it remains elusive whether recombination can change the antigenicity of CV-A6, which is worthy of further investigation. Considering that recombination has been shown to affect the efficacy of the EV-A71 and PV vaccines, greater efforts should be devoted to real-time monitoring of recombinant viruses, and recombination should not be ignored when selecting strains for CV-A6 vaccine development.

5. Perspectives and conclusion

HFMD has always been the predominant Class C infectious disease [81], posing a serious threat to public health. Currently, there is no vaccine available to prevent HFMD pathogenic agents, except for EV-A71, and no antiviral drugs for HFMD treatment have been approved [82,83]. Previous studies have shown extensive recombination in the genome of CV-A6, which may be a key factor in its rapid evolution. This likely contributed to it becoming the dominant pathogen of HFMD and to the fact that it induces unique clinical symptoms compared with EV-A71 and CV-A16 [84]. However, little is known regarding the mechanism of viral genetic recombination, and there is a lack of research on the underlying reasons for the frequent recombination of CV-A6.

In this review, we discussed the frequently occurring genetic recombination events of CV-A6 and highlighted the underlying mechanism of how it promotes viral evolution. Global CV-A6 strains have undergone frequent recombination during the evolutionary process, with consequential changes in virulence and clinical outcomes. During this process, CV-A6 has acquired partial genomes from other viruses, especially EVs. The underlying mechanism mediating recombination may be divided into replicative and non-replicative mechanisms, the former is accepted to occur naturally, while the latter has only been proven experimentally [85].

Recently, HFMD caused by EV-A71 has rarely been detected [86], while CV-A6 is becoming the predominant causative agent. However, it remains to be proven whether this phenomenon is caused by the widespread use of EV-A71 vaccines or by specific evolutionary processes. Genetic recombination has been commonly observed among EVs [87-89], and as we mentioned above, many studies have proven its association with changes in virulence and antigenicity, thus leading to altered clinical symptoms and disease outbreaks. Taken together, we may hypothesize that genetic recombination plays an important role in CV-A6 evolution. Unfortunately, studies to date lack sufficient experimental data to support this hypothesis and the detailed mechanisms remain unclear. Further research is therefore needed on virus genetic recombination and its relationship with pathogenesis. Researchers have used reverse genetic systems to explore the mechanisms of recombination [54], and the construction of such a system in CV-A6, along with an animal model [90,91], may aid our understanding of the *in vivo* evolutionary process and the interactions of this virus with the host. More attention should be paid to the impact of CV-A6 recombination on vaccine development and safety. It is also essential to strengthen the monitoring and whole genome sequencing of CV-A6 to detect significant population size fluctuations and new genetic recombination events in a timely manner, thus optimizing public health strategies and implementing effective measures to prevent large-scale outbreaks.

By summarizing the data on genetic recombination of CV-A6 and other EVs, this review will not only enhance our understanding of the mechanism and impact of viral genome recombination but will also potentially lead to the development of anti-HFMD strategies, suggesting the necessity of continuous surveillance of HFMD pathogens to determine whether the predominant lineage will change in the future.

This review also enriches the information on global CV-A6 genetic recombination and its influence on viral evolution and transmission. It highlights two key problems

that urgently need to be resolved. First, much of the previous research has been based on only partial regions of the CV-A6 genome, such as VP1 for genotyping and 3D for recombinant lineage categorizing [92-94], leading to a lack of essential information in other regions. So it is necessary in future studies to use the full-length CV-A6 sequence for systematic and comprehensive analyses. Second, the association between recombination and atypical HFMD has been reported in many studies but experimental data is lacking, indicating the need for more comprehensive research into the mechanisms involved, as well as the pathogenesis of CV-A6.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author contributions

Zequn Wang: Conceptualization, Writing – review & editing. **Hongling Wen**: Conceptualization, Supervision.

Acknowledgments

None.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data available statement

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

Ethics statement

Ethics approval were waived for this study because no patients' data were reported.

Informed consent

Not applicable.

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