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Highlights

- New recombinant breakpoints are described for EHV-1 and EHV-8 strains
- Insterspecific events of recombination distributes mostly at the repeat regions
- ICP4 is a hotspot for recombination in equid alphaherpesvirus
- Immediately early genes seems to be a primary target for recombination
- EHV1 zebra borne genotypes recombine at different locations with EHV-9

**Comprehensive analysis of equid herpesvirus recombination: an insight into the repeat regions**

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**Abstract**

High-throughput sequencing of genomes has expanded our knowledge of the *Alphaherpesvirinae*, a widely extended subfamily of DNA viruses that recombine to increase their genetic diversity. It has been acknowledged that Equid herpesvirus 1 (EHV-1) and equid herpesvirus 4 (EHV-4), two alphaherpesviruses with an economic impact on the horse industry, can recombine. This work aimed to analyze interspecific recombination between all equid alphaherpesvirus species, using genomes of EHV-1, EHV-3, EHV-4, EHV-6, EHV-8, and EHV-9 available in GenBank. 14 events of recombination by RDP4 and Simplot between EHV-1 x EHV-4, EHV-1 x EHV-9, EHV-8 x EHV-1, and EHV-8 x EHV-9 were identified. 10 out of 14 events involved ORF64, a double-copy gene located at the repeat regions that codifies for the infected cell protein 4 (ICP4). Among the ICP4, recombination can be found between EHV-1 X EHV-9, EHV-8 X EHV-9, and EHV-1 X EHV-4, the former affects zebra-borne genotypes, a type of EHV-1 that infect wild equids, and the latter match with previous breakpoints reported in fields isolates. Consequently, these findings strongly suggest that ICP4 is a hotspot for recombination. This work describes novel recombination events and is the first genome-wide recombination analysis using all available equid alphaherpesvirus species genomes.

**Keywords** Alphaherpesvirus; equid herpesvirus; homologous recombination; ICP4

## 1. Introduction

Equid herpesviruses (EHV) are double-stranded DNA viruses capable of infecting equids that belong to the *Herpesviridae* family. Currently, there are nine EHV that have been described under the subfamilies

*Alphaherpesvirinae* and *Gammaherpersvirinae* which have been numbered from 1 to 9 [1]. Equid alphaherpesvirus includes EHV-1, EHV-3, EHV-4, EHV-6, EHV-8, and EHV-9 [2]. Equid alphaherpesvirus genomes are approximately 150 kb long with a structure consisting of two unique regions, Unique Long (UL) and Unique Short (US), flanked by inverted repeat regions known as Internal Repeat (IRs) and Terminal Repeat (TRs) [3]. Due to the efficient proofreading activity of polymerase and the low synonymous substitution rate, recombination has been mentioned as one of the driving forces to increase the variability of alphaherpesvirus [4]. Anteriorly, recombination was detected in both intraspecific and interspecific in EHV genomes, the latter between EHV-1 x EHV-4, and EHV-1 x EHV-8 [5] [6] [7]. The aim of this study was to determinate interspecific recombination among EHV genomes.

## 2. Materials and Methods

Sixty complete genomes deposited in GenBank until February 2022 were analyzed. Sequences belong to EHV-1 (n=33), EHV-3 (n=2), EHV-4 (n=14), EHV-6 (n=1), EHV-8 (n=8), and EHV-9 (n=2) (Table 1). Partial genomes were excluded. Alignments were prepared using MAFFT version 7 [8] and further inspected with Bioedit [9]. In order to detect recombination, the alignments were analyzed using RDP4 software [10] assuming linear genomes and applying 7 detection methods: RDP, BOOTSCAN, MAXCHI, CHIMAERA, 3SEQ, and GENECONV, SISCAN as secondary scan. A true recombination event was considered only when detected in five or more methods with a p-value <0.05 after Bonferroni correction. To further validate these recombination events and approximate the breakpoints, positive events were analyzed by SimPlot using the allegedly recombinant as the query with a window size of 200 bp and a step size of 20 bp [11]. All recombination breakpoints were checked manually and the identity of the recombination was confirmed by identity matrix and BLAST [12].

## 3. Results and Discussion

The analysis with RDP4 detected 105 recombination events, 14 of which were considered true recombinants in 13 strains. To clarify, a single recombination event can be traceable to multiple sequences, therefore the number of recombinant genomes could be higher than the number of recombinant events. This study relied on a limited number of genomes with limitations in the geographic distribution of the isolates (Table 1). No recombination event was detected involving EHV-3 or EHV-6, which may be caused by the low identity with the other genomes. In summary, recombination was detected between EHV-1 x EHV-4, EHV-1 x EHV-9, EHV-8 x EHV-1 and, EHV-8 x EHV-9. (Table 2)

(Figure 1A). Several events were located at ORF64 that codifies for infected cell proteins 4 (ICP4), among which recombination between zebra-borne EHV-1 and EHV-9 strains was detected for the first time.

7 EHV-1 genomes that recombine with EHV-4 were identified (Figure 1A). All EHV-1 x EHV-4 recombination events involve ICP4 and have breakpoints that match previous reports of recombination (figure 1B). Anteriorly, recombinant events were described in the EHV-1 strain, 97c7 [6]. In this study, the same recombination was found in two non-described genomes, NY03 and 5586 strains, (GenBank accession number KF644569 and AP012321.1) from 3996 bp to the end, at both copies of ICP4, and 272 bp of the intergenic region. Likewise 97c7, NY03 is an abortogenic isolate. Alignments of the 3 strains display a 0.995 of identity at the recombinant region demonstrating that the recombination event is the same in the three isolates. The isolates T953_P15, T953, T953_P135, T953_P210 and OH03 recombine with EHV-4 too (GenBank accession number KP975078.1, KM593996.1, KR021354.1, KR047045.1 and KF644571.1) (Figure 1A). OH03 and T953, also known as Findlay, were isolated from a fatal neurological outbreak [13] and described as recombinants at ICP4 but only at the first copy [5]. The same breakpoints were found in both copies of ICP4, from 3690 to 4038 bp, and in other strains anteriorly not reported as T953_P15, T953_P135 and T953_P210 (Figure 1B).

Both in this work and previous ones, EHV-4 was described as the minor parent but not the recombinant. This could be because of unidirectional recombination, as described in Herpesvirus Simplex Virus (HSV) between HSV-1 and HSV-2, where only HSV-2 recombines [14] [15]. Other reasons could be the lack of geographical representation and the low number of sequences for EHV-4 in comparison with EHV-1.

Recombination was also found among EHV-8, with two recombinant events at two different strains (Figure 1A). A previous report revealed that EHV-8 has a lower identity at ORF24, ORF64 and ORF71 [16]. The recombination events found in this analysis are located at ORF64 and ORF71. Wh isolate recombine at ORF71, which that encodes Glycoprotein J from 433 bp to the end, generating a truncated protein of 544 amino acids. This truncation was previously described and attributed to a frameshift [16]. The recombination also involves a 915 bp of the intergenic region. EHV-8/IR/2010/47 (GenBank accession number MF431612.1) recombines at ICP4 from 177-427 bp (Figure 1A). According to RDP4, this recombination is between EHV-8 and EHV-9. Unlike other events of recombination found in this analysis, the identity between the recombinant and the minor parent, EHV-9, is low at the recombinant

region (0.949). This result can be explained by the fact that EHV-9 is not the real minor parent but it was the only one available for the analysis.

The remaining recombination events involve EHV-1 x EHV-9. Anteriorly, recombination between EHV-1 and EHV-9 in the polymerase gene (ORF30) was described in a polar bear with encephalitis from a zoo in Germany [17]. This case has been argued not as a recombinant but as zebra-borne EHV-1, an independent phylogenetic group capable of infecting wild equids [18]. In this study, the UL30 from the virus that infected the bear using RDP4 was analyzed. (GenBank Accession number JQ692312.1). No evidence of recombination was detected, supporting the results that this is not a recombinant virus [18]. However, recombination and the classification of zebra-borne genotype are not necessarily two independent events: In this work, 4 zebra-borne EHV-1 genomes available in GenBank were included. All of which recombine with EHV-9 (Figure 1A). EHV-9 and Zebra-borne EHV-1 can cross species barrier and co-occurrence of these viruses has been demonstrated in zebras [19].

The zebra-borne EHV-1 genomes, T616, T616 delta 71, 94-137 and, T-529 10/84 (GenBank accession number KF644574, KF644573, KF644575 and KF644580) share a recombination at ICP4 from 2340 to 4077 bp. Another recombination at ICP4 is found but affects only 94-137 and T529 10/84 from 256 to 1917 bp (Figure 1B). The proximity between both ICP4 recombination with only one event affecting 2 strains could be a result of independent recombination.

EHV-1 strains, T-616 and T-616, were sequenced from the same virus isolation [18]. In addition to the ICP4 recombination, both recombine at ICP22 from 1 to 57 bp involving 1610 bp from the non-coding zone and at the Unique Long region (UL) from 4864 to 9706 bp extending from ORF5 to ORF7, homologues of HSV-1 UL54, UL53 and UL52 that codifies for the transcriptional activator ICP27, the glycoprotein k and the DNA helicase/primase complex protein. Interestingly, three immediately early genes (IE) recombine in these isolates: ICP4, ICP22 and ICP27. Additionally, recombination generates a chimeric EHV-1 x EHV-9 UL52, which is one out of seven essential replication proteins, and a complete EHV-9 identity glycoprotein K, a protein associated with the cell-to-cell spread and virus egression [20].

The several breakpoints, with diverse species involved as recombinant and parents, together with previous reports points at ICP4 as a hotspot for EHV recombination (Figure 1B). ICP4 is an immediately early gene (IE) that binds DNA with several functions attributed to such as regulation of the transcriptional cascade, coating the viral genome, and helping circularize the herpes genome [21] [22], [23]. All these

functions are associated with the replication of the genome which is highly linked to recombination. Replicating DNA adopts structures known as concatamers in which recombination occurs. Reports concerning recombination are associated with UL/US segment inversion that generates four isomers for class D genomes like HSV1 and two isomers for Class E genomes like EHV [24].

Whole and partial genomes analysis carried out for bovine herpesvirus 1 and 5 uncovered that recombination events occur in UL and US. It should be noted that for partial analysis, search for breakpoints is biased by the primer design [25] [26]. Recombination leaning towards the repeat region could be a feature for EHV genomes but should be confirmed with analysis incorporating a significant number of genomes from each species

ICP4 and ICP22 are double-copy genes at the repeat regions. Interestingly, recombinant events involving these genes are found in both copies. This could be due to the assembly process after sequencing; despite the advent of high throughput sequencing (HTS), some features like G+C high regions and highly repetitive areas are still a challenge [27]. Some recombinant analyses excluded the TRs to avoid double counting of recombination events. Recombination at the repeat regions was described for HSV1 lab-generated strains, here the same feature was found in naturally circulating variants of EHV [28].

## 4. Conclusions

These findings provide insight into EHV recombination and emphasize the importance of sequencing and surveillance, especially in EHV-1, a highly prevalent virus that causes of outbreaks with different symptomatology that can recombine with EHV-4 and EHV-9.

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**Figure Caption**

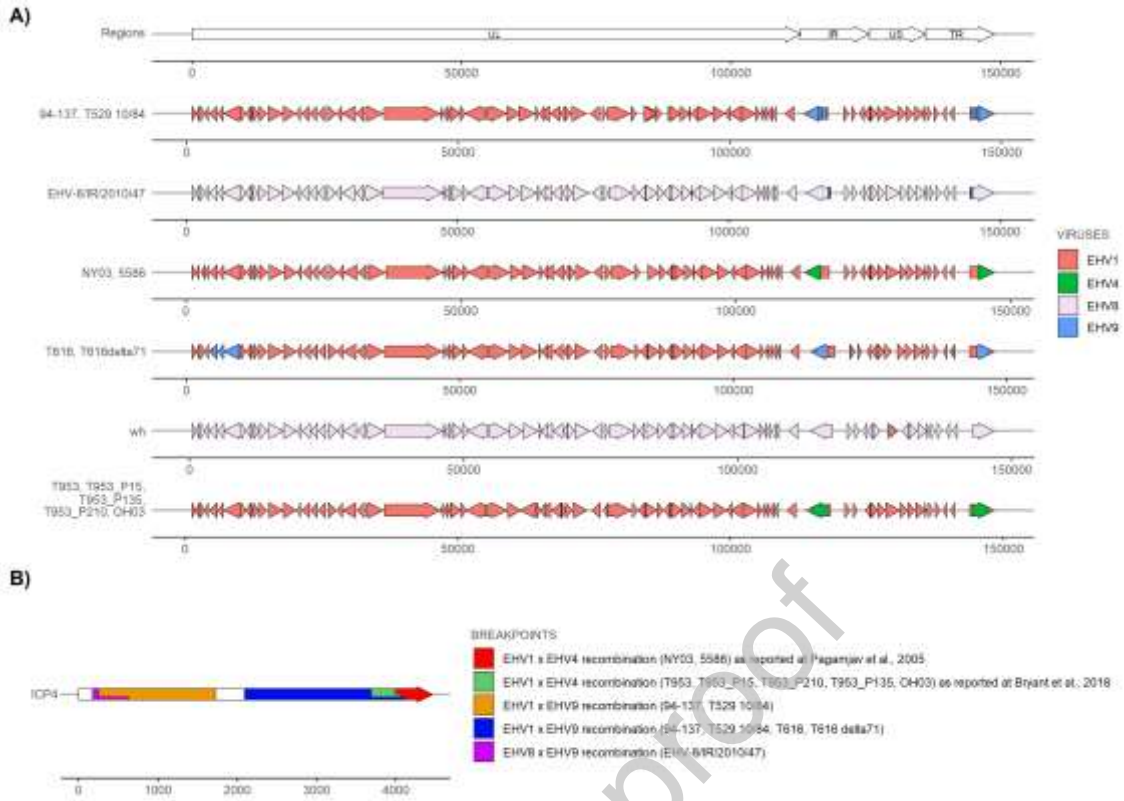


**Figure 1.**

**Schematic representation of the recombinant events. Figures were generated in R 4.2.0 with the packages genes https://wilkox.org/gggenes/. The abscissa represents nucleotide position (A) Genomic organization of the recombinant viruses B) Distribution of recombinant breakpoints at ICP4 gene.**

**Table 1.**

| Table 1. Sequences used in this study | | | | | | |
|---|---|---|---|---|---|---|
| GenBank | Strain | Specie | Host | Country | Year | Source |
| NC_001491.2<br>AY665713.1 | Ab4 | EHV1 | Equus caballus | UK | 1980 | Neuropathogenic<br>(quadriplegic gelding ) |

| | | | | | | |
|---|---|---|---|---|---|---|
| MT063054.1 | YM2019 | EHV1 | Equus caballus | China | 2019 | Lung, Aborted fetus |
| MF975656.1 | RacL11 | EHV1 | Equus caballus | USA | Unknown | Aborted fetus |
| KF644567.1 | FL06 | EHV1 | Unknown | USA | Unknown | Unknown |
| AB992258.1 | HH1 | EHV1 | Equus caballus | Japan | Unknown | Aborted fetus |
| KP975078.1 | T953_P15 | EHV1 | Equus caballus | USA | 2009 | Neuropathogenic |
| KF644571.1 | OH03 | EHV1 | Equus caballus | USA | 2003 | Fatal myeloencephalopathy outbreak |
| KF644569.1 | NY03 | EHV1 | Equus caballus | USA | 2003 | Abortion outbreak |
| KF644572.1 | VA02 | EHV1 | Unknown | USA | unknown | Unkwnon |
| KF644570.1 | NY05 | EHV1 | Unknown | USA | Unknown | Unknown |
| KF644568.1 | NMKT04 | EHV1 | Unknown | UK | Unknown | Unknown |
| KF644579.1 | 89c25 | EHV1 | Unknown | Japan | Unknown | Unknown |
| KF644577.1 | 89c105 | EHV1 | Unknown | Japan | Unknown | Unknown |
| KF644566.1 | 90c16 | EHV1 | Unknown | Japan | Unknown | Unknown |
| KF644578.1 | 01c1 | EHV1 | Unknown | Japan | Unknown | Unknown |
| KF644576.1 | 00c19 | EHV1 | Unknown | Japan | Unknown | Unknown |
| MW855962.1 | FR/Valencia2/2021 | EHV1 | Equus caballus | France | 2021 | Neurological Outbreak |
| MW855961.1 | FR/Valencia1/2021 | EHV1 | Equus caballus | France | 2021 | Neurological Outbreak |
| MW855959.1 | BE/21P41/2021 | EHV1 | Equus caballus | Belgium | 2021 | Neurological Outbreak |
| MW855960.1 | BE/21P43_BD5/2021 | EHV1 | Equus caballus | Belgium | 2021 | Neurological Outbreak |
| MW855958.1 | BE/21P40/2021 | EHV1 | Equus caballus | Belgium | 2021 | Neurological outbreak |
| MF975655.1 | KyA | EHV1 | Culture passages | USA | Unknown | Several culture passages |
| LC193725.1 | Ab4p_attB_delta_VP22 | EHV1 | Equus caballus | Constructed virus | Constructed virus | Unknown |
| KF644580.1 | T-529 10/84 | EHV1 | Equus hemionus onager | USA | 1984 | aborted fetus |
| KF644574.1 | T-616 | EHV1 | Equus grevyi | USA | 1984 | Aborted fetus |
| KF644573.1 | T-616 delta71 | EHV1 | Equus grevyi | USA | 1984 | Aborted fetus |
| KF644575.1 | 94-137 | EHV1 | Eudorcas thomsonii | USA | 1994 | Fatal encephalitis |
| KM593996.1 | T953 (Findlay) | EHV1 | Equus caballus | USA | 2003 | Neurologic |
| KR021354.1 | T953_p135 | EHV1 | Equus caballus | USA | 2010 | Unknown |
| KR047045.1 | T953_P210 | EHV1 | Equus caballus | USA | 2010 | Unknown |

| | | | | | | |
|---|---|---|---|---|---|---|
| AY464052.1 | V592 | EHV1 | Unknown | UK | 1985 | Abortion outbreak |
| AP012321.1 | 5586 | EHV1 | Equus caballus | Unknown | Unknown | Unknown |
| NC_024771.1<br>KM051845.1 | AR/2007/C3A | EHV3 | Equus caballus | Argentina | 2007 | equine coital exanthema |
| NC_001844.1<br>AF030027.1 | NS80567 | EHV4 | Equus caballus | Ireland | 1942 | upper respiratory disease |
| LC075588 | 12-I-203 | EHV4 | Equus caballus | Japan | 2012 | Aborted fetus |
| LC075587.1 | 11-10 | EHV4 | Equus caballus | Japan | 2011 | Respiratory disease |
| LC075585.1 | 03-VR | EHV4 | Equus caballus | Japan | 2003 | Horse with respiratory disease |
| LC075584.1 | 01-10-1 | EHV4 | Equus caballus | Japan | 2001 | Horse with respiratory disease |
| LC075583.1 | 91c1 | EHV4 | Equus caballus | Japan | 1991 | Aborted horse fetus |
| LC075586.1 | 05-I-202 | EHV4 | Equus caballus | Japan | 2005 | Respiratory disease |
| LC063142.1 | TH20p | EHV4 | Equus caballus | Japan | 1962 | Respiratory disease |
| LC075582.1 | 83-MB | EHV4 | Equus caballus | Japan | 1983 | Respiratory disease |
| MW892438.1 | DE17_4 | EHV4 | Equus caballus | Germany | 2017 | Respiratory outbreak |
| MW892436.1 | DE17_2 | EHV4 | Equus caballus | Germany | 2017 | Respiratory outbreak |
| MW892435.1 | DE17_1 | EHV4 | Equus caballus | Germany | 2017 | Respiratory outbreak |
| MW892437.1 | DE17_3 | EHV4 | Equus caballus | Germany | 2017 | Respiratory outbreak |
| MT012704.1 | AsHV/Bari/2011/740 | EHV6 | Equus asinus | Italy | 2011 | ulcerative stomatitis outbreak |
| NC_017826.1<br>JQ343919.1 | Wh | EHV8 | Equus caballus | China | 2010 | Fever and nasal discharge |
| MF431614.1 | EHV-8/IR/2015/40 | EHV8 | Equus asinus | Ireland | 2015 | Neurological disease |
| MF431613.1 | EHV-8/IR/2010/16 | EHV8 | Equus asinus | Ireland | 2010 | Respiratory disease |
| MF431613 | EHV-8/IR/2003/19 | EHV8 | Equus caballus | Ireland | 2003 | Aborted fetus |
| MF431612.1 | EHV-8/IR/2010/47 | EHV8 | Equus caballus | Ireland | 2010 | Aborted fetus |
| MW822570.1 | SD2020113 | EHV8 | Equus asinus | China | 2019 | Unknown |
| MW816102.1 | SDLC66 | EHV8 | Equus asinus | China | 2019 | Unknown |

| | | | | | | |
|---|---|---|---|---|---|---|
| NC_011644.1<br>AP010838.1 | P19 | EHV9 | Giraffa reticulata | USA | Unknown | Fatal encephalitis |

**Table 2.**

| Table 2. Recombinant events detected in this work | | | | | |
|---|---|---|---|---|---|
| Gene/s | Recombinant EHV | Recombinant genomes | GenBank accession number | Breakpoint* | Minor parental |
| ORF64 | EHV-1 | NY03, 5586 | AP012321.1, KF644569.1 | 112276-113013<br>146548-147285 | EHV-4 |
| | | T953_P15, T593, T593_P135,T953_P210,OH03 | KP975078.1,KM593996.1, KR021354.1, KR047045.1, KF644571.1 | 114265-114613<br>147558-147906 | |
| | | T616 delta71,T616,94-137,T-529 10/84 | KF644573.1, KF644574.1, KF644575.1, KF644580.1 | 116240-114503<br>145581-147318 | EHV-9 |
| | | 94-137,T-529 10/84 | KF644575.1, KF644580.1 | 116132-117793<br>144506-146167 | |
| | EHV-8 | EHV-8/IR/2010/47 | MF431612.1 | 118292-118542<br>144691-144941 | |
| ORF65 | EHV-1 | T616 delta71,T616 | KF644573.1, KF644574.1 | 119720-121386<br>140435-142101 | |
| ORF5, ORF6 and ORF7 | | | | 4864-9706 | |
| ORF71 | EHV-8 | Wh | JQ343919.1 | 127940-130057 | EHV-1 |
| *Breakpoint location are based on the first genome mentionated for each event | | | | | |



Animal welfare/ethical statement: No animal experiment or sample collection were perfomed, therefore ethical approval is not applicable for this article. All genomes analyzed are available in Genbank (https://www.ncbi.nlm.nih.gov/genbank/)