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Abstract: Abstract:
Recombination in alphaherpesviruses was first described more than sixty years ago. Since then, different techniques have been used to detect recombination in natural (field) and experimental settings. Over the last ten years, next-generation sequencing (NGS) technologies and bioinformatic analyses have greatly increased the accuracy of recombination detection, particularly in field settings, thus contributing greatly to the study of natural alphaherpesvirus recombination in both human and veterinary medicine. Such studies have highlighted the important role that natural recombination plays in the evolution of many alphaherpesviruses. These studies have also shown that recombination can be a safety concern for attenuated alphaherpesvirus vaccines, particularly in veterinary medicine where such vaccines are used extensively, but also potentially in human medicine where attenuated varicella zoster virus vaccines are in use. This review focuses on the contributions that NGS and sequence analysis have made over the last ten years to our understanding of recombination in mammalian and avian alphaherpesviruses, with particular focus on attenuated live vaccine use.

Highlights:

- Natural (field) recombination in alphaherpesviruses is linked with live attenuated vaccine usage.

- Natural recombination drives evolution and pathogenicity of alphaherpesviruses.

- Advances in next-generation sequencing techniques have helped to provide accurate data in regards to natural (field) recombination.

Natural recombination in alphaherpesviruses: insights into viral evolution through full genome sequencing and sequence analysis

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Abstract:

Recombination in alphaherpesviruses was first described more than sixty years ago. Since then, different techniques have been used to detect recombination in natural (field) and experimental settings. Over the last ten years, next-generation sequencing (NGS) technologies and bioinformatic analyses have greatly increased the accuracy of recombination detection, particularly in field settings, thus contributing greatly to the study of natural alphaherpesvirus recombination in both human and veterinary medicine. Such studies have highlighted the important role that natural recombination plays in the evolution of many alphaherpesviruses. These studies have also shown that recombination can be a safety concern for attenuated alphaherpesvirus vaccines, particularly in veterinary medicine where such vaccines are used extensively, but also potentially in human medicine where attenuated varicella zoster virus vaccines are in use. This review focuses on the contributions that NGS and sequence analysis have made over the last ten years to our understanding of recombination in mammalian and avian alphaherpesviruses, with particular focus on attenuated live vaccine use.

Keywords: Alphaherpesvirus, natural recombination, next-generation sequencing, attenuated live vaccine.

33 **Contents:**

1. Introduction

Viruses belonging to the order *Herpesvirales* have a double stranded DNA genome and have been isolated from a wide variety of host including mammals, birds, reptiles, fish and invertebrates (Davison, 2010). Three large subfamilies arose within the family *Herpesviridae* over 80 million years ago. One of these, the subfamily *Alphaherpesvirinae*, includes avian and mammalian alphaherpesvirus lineages (Davison, 2010; McGeoch *et al*., 1995). The evolution of many viruses in this subfamily has been attributed, in part, to the process of recombination. The contribution that recombination makes to the evolution and diversity of alphaherpesviruses is of particular importance as these viruses have a DNA polymerase with a highly efficient proof-reading activity and exonuclease activity (Javier *et al*., 1986; Lee *et al*., 2012; Thiry *et al*., 2005), resulting in low point genetic mutation rates. In herpes simplex virus-1 (HSV-1) the mutation rate is as low as 0.026 to 0.0027 (Drake and Hwang, 2005).

Recombination is the process in which new genetic material (offspring) is generated by shuffling two different DNA sequences from viruses infecting the same host cell at the same time. High rates of *in vivo* intra-species homologous recombination have been demonstrated after experimental co-inoculation of different strains of HSV-1 into mice (Kintner *et al*., 1995), bovine herpesvirus 1 (BoHV-1) into calves (Schynts *et al*., 2003), and pseudorabies virus (PRV) into sheep and pigs (Christensen and Lomniczi, 1993; Henderson *et al*., 1990). *In vitro* intra-species co-inoculation experiments have demonstrated recombination in different alphaherpesviruses strains into cell cultures, including HSV-1 (Kintner *et al*., 1995), BoHV-1 (Muylkens *et al*., 2009), varicella zoster virus (VZV) (Dohner *et al*., 1988), feline herpesvirus 1 (FeHV-1) (Fujita *et al*., 1998) and PRV (Henderson *et al*., 1990). *In vitro* recombination has been detected at a lower rate than in *in vivo* experiments, possibly due to the reduced number of viral replication cycles possible in cell cultures. Therefore, factors that influences the number of viral replication cycles, such as latency/reactivation and use of vaccines that replicate after vaccination, should be considered as factors that may increase the likelihood of recombination. Recombination occurs most commonly between different

strains of the same virus species (intra-species recombination) but inter-species recombination is also possible and has been detected in experimental studies between caprine herpesvirus-1 and -2, and also between BoHV-1 and bovine herpesvirus-5 (BoHV-5) (Meurens *et al*., 2004). In field samples inter-species recombination has been detected between equine herpesviruses 1 and 4 (EHV-1 and EHV-4, respectively) (Pagamjav *et al*., 2005).

Many biological features of alphaherpesviruses, including their infection of epithelial surfaces, rapid infectious cycle, establishment of latent infection with periodic reactivation and high prevalence of infection in many host populations, create a favourable environment for co-infection of host cells, and hence for recombination. The viral, host and cell conditions that influence the likelihood of recombination *in vivo* and *in vitro* under laboratory conditions have been reviewed previously (Thiry *et al*., 2005). The molecular basis of alphaherpesvirus recombination has also been recently reviewed and is hypothesized to be similar to that described for lambda bacteriophages (Lo Piano *et al*., 2011; Weller and Sawitzke, 2014).

Early studies of alphaherpesvirus recombination used strain virulence as a marker to detect recombinants (Wildy, 1955). Analysis of partial genome sequences were then used extensively to study recombination in several alphaherpesviruses, using tools such as PCR followed by restriction endonuclease cleavage fragment analysis of PCR products (PCR plus restriction fragment length polymorphism [PCR-RFLP]), gene deletion mutants, PCR hydrolysis probe assays and bioinformatic comparisons of partial genome sequences to detect recombination (Bowden *et al*., 2004; Christensen and Lomniczi, 1993; Dangler *et al*., 1993; Dohner *et al*., 1988; Glazenburg *et al*., 1994; Henderson *et al*., 1990; Javier *et al*., 1986; Kintner *et al*., 1995; Muylkens *et al*., 2009; Norberg *et al*., 2004; Sakaoka *et al*., 1995; Sakaoka *et al*., 1994; Schynts *et al*., 2003; Umene and Sakaoka, 1997). More recently, lower costs, improved technologies and greater access to next generation sequencing (NGS) techniques (Capobianchi *et al*., 2013; Pareek *et al*., 2011), statistical analysis (Bruen *et al*., 2006; Posada, 2002) and software to detect and estimate the likelihood of recombination (Huson and Bryant, 2006; Kosakovsky Pond *et al*., 2006; Kuhner, 2006; Lole *et al*.,

1999; Martin *et al*., 2010; Martin *et al*., 2011; Martin *et al*., 2015; Pond and Frost, 2005; Wilson and McVean, 2006) have helped us to better understand recombination, and thereby provide insights into the role of recombination in the natural evolution of alphaherpesviruses (Burrel *et al*., 2015; Hughes and Rivailler, 2007; Kolb *et al*., 2013; Kolb *et al*., 2015; Lamers *et al*., 2015; Lee *et al*., 2013; Lee *et al*., 2012; Newman *et al*., 2015; Norberg *et al*., 2015; Norberg *et al*., 2007; Norberg *et al*., 2006; Norberg *et al*., 2011; Peters *et al*., 2006; Szpara *et al*., 2014; Vaz *et al*., 2016a; Vaz *et al*., 2016b; Ye *et al*., 2016; Zhao *et al*., 2015).

This review aims to summarise and update our understanding of natural recombination in alphaherpesviruses and the influence of natural recombination on viral evolution, focusing on the contributions that full genome sequencing and sequence analysis have made to this field over the last 10 years. This review covers natural recombination in human alphaherpesviruses, other mammalian alphaherpesviruses and avian alphaherpesviruses.

2. Natural recombination in human alphaherpesviruses

Herpes simplex virus-1, -2 and VZV are important causes of human disease worldwide. Infection with HSV-1 is commonly associated with ulcerated oral lesions and HSV-2 with genital lesions, although both viruses can cause lesions at both anatomical sites (Lowhagen *et al*., 2002). Additionally, HSV-1 can cause keratitis and subsequent blindness, along with sporadic encephalitis (Liesegang, 2001). Infection with VZV causes varicella (chickenpox) and herpes zoster (shingles) (Zerboni *et al*., 2014). Evidence of natural recombination has been available since 2004 for HSV-1, 2003 for VZV, and 2007 for HSV-2. These studies have demonstrated that these three human alphaherpesviruses show differences in recombination (Table 1).

2.1 Herpes simplex virus-1

Early HSV-1 studies in mice demonstrated that a high proportion of recombinant viruses were generated following co-inoculation, and many of the recombinants had higher levels of virulence

than the parental strains (Brandt and Grau, 1990; Kintner *et al*., 1995). This potential for recombination to result in increased virulence highlighted the importance of studying HSV-1 recombination in clinical settings. In 2004, Bowden *et al*., sequenced approximately 4% of the HSV-1 genome in 14 clinical samples from the UK and Korea, performed phylogenetic network analyses on the data to estimate recombination, and concluded that recombination plays a major role in generating diversity within HSV-1 (Bowden *et al*., 2004). In the same year, Norberg *et al*., sequenced approximately 2.3% of the HSV-1 genome in 28 clinical samples from Sweden, also detecting recombination (Norberg *et al*., 2004).

From 2011 onwards, techniques shifted to detection of natural HSV-1 recombination by bioinformatic analysis of whole genome sequences obtained by NGS, providing a higher level of accuracy in identifying and characterising these phenomena (Kolb *et al*., 2011; Kolb *et al*., 2013; Norberg *et al*., 2011; Szpara *et al*., 2014). In 2011 the genomes of ten clinical and two laboratory HSV-1 strains isolated in 1972, were sequenced and analysed. Significant recombination was detected, including recombination events between the HSV-1 field strains F and 17. Additionally, the full genome sequences of these 12 HSV-1s facilitated classification of HSV-1 into 3 clades: A, B and C (Norberg *et al*., 2011). Kolb *et al* reached similar conclusions about the number of HSV-1 clades (Kolb *et al*., 2011). In regard to recombination, Kolb *et al*., (2011) showed that some field strains (TFT401 and CJ970) were unstable in their phylogenetic classification using the whole genome sequence and identified cross over points using similarity plots in the UL1, UL11, UL43, UL49A, US4 and US7 genes. Additionally, they found that some nucleotide sequences that code for selected proteins resulted in variable phylogenetic groupings depending on the parameters used to build the phylogenetic trees. They concluded that recombination was likely and that each viral genome is a unique mosaic (Kolb *et al*., 2011).

The most recent and comprehensive studies of HSV-1 genetic diversity, recombination and genome evolution are those that have examined full genome sequences of HSV-1 from four continents. Kolb

154 *et al*., (2013) examined 31 full genome sequences as well as partial genome sequences obtained
155 from the NCBI reference database. Szpara *et al*., (2014) examined whole genome sequences
156 obtained by NGS of 20 field strains of HSV-1 obtained from China, Japan, Kenya and South Korea
157 and compared them with the genome sequences available for HSV-1 strains from the United States
158 (US), Europe and Japan. Both these studies confirmed that recombination in HSV-1 is widespread,
159 frequent, historical and ongoing (Kolb *et al*., 2013; Szpara *et al*., 2014). In addition, these two
160 studies found that the HSV-1 strains clustered into six groups, rather than the three groups
161 described previously. The clustering correlated with the geographic origin of the isolates,
162 highlighting the need to include isolates from different regions in order to comprehensively
163 examine phylogeny and recombination (Kolb *et al*., 2013; Szpara *et al*., 2014).
164 The high levels of HSV-1 recombination detected in these studies have been hypothesised to be due
165 to the co-existence of many different HSV-1 strains within the same geographical region (Norberg
166 *et al*., 2004; Schmidt-Chanasit *et al*., 2009) and to the high number of HSV-1 replication cycles that
167 which may arise from more frequent HSV-1 reactivation from latency, compared to HSV-2 or VZV
168 (Kaufman *et al*., 2005; Wang *et al*., 2010). This suggests that the latency and reactivation
169 characteristics of alphaherpesviruses are also factors that can influence recombination, with viruses
170 that establish and re-activate from latency more frequently having a higher likelihood of
171 recombination.
172
173 *2.2* Herpes simplex virus-2

174 The first study to detect recombination in HSV-2 using bioinformatics analyses of partial DNA
175 sequences from clinical isolates was published by Norberg *et al*., (2007). Approximately 3.5% of
176 the genome of 47 clinical isolates of HSV-2 from Norway, Sweden, and Tanzania was examined
177 and it was found that HSV-2 was clustered into 2 genogroups, rather than the 3 groups seen in
178 HSV-1 isolates using similar techniques. Isolates from Norway and Sweden were clustered into
179 only one genogroup. It was hypothesised that the lower level of genetic diversity seen in HSV-2,

compared to HSV-1, was directly related to recombination (Norberg *et al*., 2007). Importantly, however, these conclusions were based on the analysis of only a limited number of samples, and of partial genome sequences. Only three genes within the unique short (US) region of the HSV-2 genome were examined (US4, US7 and US8) (Norberg *et al*., 2007). Studies on HSV-1 had detected variation in diversity across the whole genome, which can influence recombination analysis (Szpara *et al*., 2014). Thus, in order to comprehensively examine recombination in HSV-2, and also compare recombination between HSV-1 and HSV-2, an analysis of HSV-2 recombination analyses at the whole genome level was required.

Prior to 2014 there were only two full genome sequences for HSV-2, one published in 1998 (Dolan *et al*., 1998), and another one in 2014 (Colgrove *et al*., 2014). Kolb *et al*., (2015) determined the complete genome sequences of an additional six HSV-2 clinical isolates. Bootscan analysis of the eight complete genome sequences demonstrated that the HSV-2 genomes were mosaics, suggesting frequent recombination randomly along the genome (Kolb *et al*., 2015). In a separate study, 34 near complete genome sequences were determined for HSV-2 isolates from Africa, USA and Japan. Bootscan and phylogenetic analyses of these sequences suggested that HSV-2 had five major crossover points and that recombination in HSV-2 did not occur as frequently as in HSV-1 (Newman *et al*., 2015). Another recent study has reported that a new HSV-2 variant (HSV-2v) from west and central Africa (mostly from immunocompromised patients infected with human immunodeficiency virus) differed significantly from the classical HSV-2 prototype and contained a UL30 gene (encoding DNA polymerase) that clustered closely with the chimpanzee herpesvirus (ChHV), providing evidence of an inter-species recombination event (Burrel *et al*., 2015). Herpes simplex virus 2 and ChHV have genetically similar genomes with 88.3% pairwise identity and 88.3% identical sites between ChHV (Genbank accession number JQ360576) and HSV-2, (Genbank accession number Z86099) as determined using Multiple Alignment with Fast Fourier Transformation (MAFFT) version 7 within Geneious V8.0.4 (Katoh and Standley, 2013). A high level of identity between viruses has been shown to play a role in promoting recombination. This

level of identity is similar to what has been described for EHV-1 and -4, another pair of alphaherpesviruses in which natural inter-species recombination has been detected (Pagamjav *et al*., 2005).

2.3 Varicella zoster virus

Varicella zoster virus is the only human alphaherpesvirus for which live attenuated vaccines are in widespread use. These live attenuated vaccines (Takahashi *et al*., 1974) contain a heterogeneous mixture of related VZV haplotypes (Depledge *et al*., 2014) and are used in several countries including Japan, Korea, the US, Canada, Australia, Germany, Costa Rica, Uruguay, and Qatar (Norberg *et al*., 2015). As VZV recombination has been observed in cell culture (Dohner *et al*., 1988) there is potential for natural recombination, including between vaccine and wild type viruses, to occur (Quinlivan *et al*., 2009). This has been the focus of a number of studies examining VZV recombination. VZV recombination has been investigated using partial and full genome sequence analyses to identify viral groups (clades), and to detect recombination events (Norberg *et al*., 2015; Norberg *et al*., 2006; Norberg *et al*., 2011; Peters *et al*., 2006).

Early studies used restriction endonuclease digestion profiles to examine differences between VZV genomes (Takada *et al*., 1995). Later, DNA sequencing and bioinformatic analyses were used to classify, determine similarities and detect recombination events between VZV isolates. Single nucleotide polymorphism (SNP) analysis (Wagenaar *et al*., 2003), heteroduplex mobility assays to locate informative SNPs along the genome (Barrett-Muir *et al*., 2003) and targeted sequencing of different regions of the VZV were used to detect and compare SNP patterns (Loparev *et al*., 2004). These studies showed that recombination occurs among VZV isolates and also enabled the classification of VZV isolates into different phylogenetic groups. Loparev *et al*., (2004) were able to classify 326 VZV isolates from the six continents into the European (E), Japanese (J) and the Mosaic (M) groups by sequencing and analysing approximately 0.3% of the full VZV genome sequence. This study also sequenced and analysed approximately 6.9% of the full VZV genome

sequence of 16 isolates. Specifically, the M group was identified as carrying SNP patterns of both the E and J group, and it was hypothesised that M strains resulted from recombination after mixed infection (Loparev *et al*., 2004). A separate study classified isolates into four major clades, with clade A containing European/North American (Dumas) isolates, clade B Japanese (vaccine-Oka) isolates, clade C Asian-like isolates sharing some European/North American features, and clade D containing European/North American-like isolates sharing some features of the Asian strains (Wagenaar *et al*., 2003). These classifications provided the first insight into natural recombination in VZV, but more detailed understanding was later achieved by full genome sequence analyses. Complete genome sequence analyses were first used to detect natural VZV recombination in 2006 (Norberg *et al*., 2006; Peters *et al*., 2006). The two studies reporting these findings had different foundations but both reached similar conclusions about recombination. One investigated recombination within the clades C and D (Asian-like and European/North American-like, respectively) at the full genome level (Peters *et al*., 2006). This study determined the full genome sequences of 11 VZV isolates that were considered representative for Canada and the USA, and compared them to seven other sequences that were publicly available (Peters *et al*., 2006). They detected evidence of recombination within a Canadian isolate (VZV-8) within clade C that contained mixtures of genetic characteristics from clade A (Dumas strain from Europe/North America) and clade B (pOka strain from Japan). It was hypothesised that this isolate arose as a result of a recombination event between the vaccine strain vOka from Japan and VZV field strains from Canada, as the vaccine strain is used in Canada (Peters *et al*., 2006). However, as the vaccines contains mixtures of distinct genetic subtypes (Quinlivan *et al*., 2005; Vassilev, 2005) and the sequences available at the time in 2006 did not include all the strains within the vaccine, it was not possible to reach this conclusion definitely (Peters *et al*., 2006).

The second study (Norberg *et al*., 2006) determined the full genome sequences of two VZV strains (DR and 123) that were classified previously into group M (mosaic) by Loparev *et al*., (2004) and thus contained genetic features from groups E (European) and J (Japanese). At the whole genome

level, the DR and 123 strains were shown to be well separated from each other and from groups E and J. Thus, group M was further divided into M1, represented by the 123 strain, and M2, represented by the DR strain. Bootscan analysis suggested that that the DR and 123 strains contain putative recombination dependent sites. Specifically, some genomic regions clustered with pOka, while other regions of the VZV genome clustered with the European strains (Norberg *et al*., 2006). Subsequent phylogenetic network analysis revealed that the SVETA strain, which is a Russian isolate and was thought to belong to clade 1 (European clade), had been involved in an intra-clade recombination event (Norberg *et al*., 2011), and significant intra-clade recombination events were found among other VZV isolates (Zell *et al*., 2012).

After intra-clade recombination was observed, it was hypothesised that an increase in the number of full genome sequences available for analysis would reveal a greater number of recombinants. Additionally, it was hypothesised that human migration, along with widespread distribution of the attenuated VZV vaccine strains (Takahashi *et al*., 1974), may contribute to an apparent disappearance of VZV clades as recombination events would result in isolates becoming genomic mosaics similar to HSV-1 (Norberg *et al*., 2004; Sauerbrei and Wutzler, 2007; Sauerbrei *et al*., 2008). However, recent studies that have included several isolates from different continents have shown that the only clade to consistently display a relatively high level of recombination is the clade that contains the pOka and vOka vaccine strains (Norberg *et al*., 2015). Detection of recombination in the other clades appears to be dependent on the analytical method used to detect evidence of recombination (Norberg *et al*., 2015).

The relatively low rate of recombination in VZV compared to HSV-1 may be due to the distinct biology and epidemiology of VZV (Kaufman *et al*., 2005; Schmidt-Chanasit *et al*., 2009; Wang *et al*., 2010) as well as geographical separations of strains (Norberg *et al*., 2004; Schmidt-Chanasit *et al*., 2009). However, ongoing monitoring of recombination in field isolates of VZV is needed, as recombination between attenuated viruses such as vaccine strains has been detected, to create recombinant virulent progeny in other alphaherpesviruses (Lee *et al*., 2012), as well as in several

other virus families (Becher *et al*., 2001; Camus-Bouclainville *et al*., 2011; Chong *et al*., 2010; Cuervo *et al*., 2001; Dahourou *et al*., 2002; Holmes *et al*., 1999; Liu *et al*., 2003; Norberg *et al*., 2013; Seligman and Gould, 2004; Wenhui *et al*., 2012).

3. Natural recombination in other mammalian herpesviruses

Over the past ten years natural recombination has been assessed in alphaherpesviruses from five non-human mammalian hosts; EHV-1, EHV-4, EHV-9, FeHV-1 and PRV (Greenwood *et al*., 2012; Pagamjav *et al*., 2005; Vaz *et al*., 2016a; Vaz *et al*., 2016b; Ye *et al*., 2016) (Table 2). This represents only a small proportion of the alphaherpesviruses of importance in veterinary medicine. There have been, however, other efforts to better understand recombination in a wider range of mammalian alphaherpesviruses using experimental *in vitro* and *in vivo* studies, including BoHV-1, BoHV-5 (Meurens *et al*., 2004; Muylkens *et al*., 2009; Schynts *et al*., 2003), PRV (Christensen and Lomniczi, 1993; Henderson *et al*., 1990) and FeHV-1 (Fujita *et al*., 1998). Live attenuated vaccines are used extensively in veterinary medicine and there is clear evidence that attenuated live vaccines can naturally recombine to generate more virulent and dominant progeny (Lee *et al*., 2012). Therefore, the study of natural recombination in vaccinated and unvaccinated animals is of importance in improving animal health and disease control in the field of veterinary medicine.

3.1 Equine alphaherpesviruses

Equine herpesviruses 1 and 4 cause significant losses in horse industries worldwide (Allen *et al*., 2004). Equine herpesvirus-1 and 4 are genetically similar (Telford *et al*., 1998), but they differ significantly in terms of their pathogenesis and epidemiology (Allen *et al*., 2004; Patel and Heldens, 2005). Infection with EHV-1 causes respiratory disease in young horses, myeloencephalitis in older horses, abortion in mares and systemic perinatal disease (Allen *et al*., 2004; Patel and Heldens, 2005). Infection with EHV-4 also causes upper respiratory tract infection, but EHV-4 infection rarely induces systemic disease or abortions in mares, probably because of its limited capacity to

infect mononuclear cells, and therefore produce viraemia (Patel and Heldens, 2005; Vandekerckhove *et al*., 2011). The differences in the epidemiology of EHV-1 and EHV-4 have been well described (Allen *et al*., 2004; Patel and Heldens, 2005). One key epidemiological difference between these viruses is the substantially lower prevalence of infection with EHV-1 compared to that seen for EHV-4 (Gilkerson *et al*., 1999).

Natural recombination in EHV was first reported by Pagamjav *et al*., (2005). The field isolate EHV-1 B was shown to have arisen as a result of an inter-species recombination event between EHV-1 and EHV-4, and then spread among horse populations to become a dominant strain (Pagamjav *et al*., 2005). The recombination event described by Pagamjav et al., (2012) was the first evidence of natural inter-species recombination involving EHV-1. In 2012 another natural recombination event involving EHV-1 and equid herpesvirus 9 (EHV-9) was detected in a zoo in Germany by Greenwood *et al*., (2012) following analysis of the sequence of six virus genes using a distance based method within the Recombination Analysis Tool 1.0 (RAT v1.0). Interestingly, the recombinant was isolated from a polar bear with fatal encephalitis, even though neither EHV-1 nor EHV-9 naturally infect polar bears (Greenwood *et al*., 2012). More recent work has suggested that the recombination event involving these two viruses most likely occurred in zebras and was then transmitted to the polar bear (Abdelgawad *et al*., 2016). This recombination event has some similarities to the one detected between the HSV-2 and ChHV (Burrel *et al*., 2015), as both reports shown recombination within the UL30 gene (Burrel *et al*., 2015; Greenwood *et al*., 2012). The significance of the UL30 gene as a site of recombination, and potentially as an influence on the host range of the resultant recombinant viruses, warrants investigation in future studies.

The high level of genetic similarity between EHV-1 and EHV-9, and also between EHV-1 and EHV-4 (Telford *et al*., 1992; Telford *et al*., 1998) are likely to have facilitated these inter-species recombination events (Pagamjav *et al*., 2005). Alignment of EHV-1 genome sequences (Genbank accession numbers: AY464052, KF644566, KF644567, KF644568, KF644570, KF644572, KF644576, KF644577, KF644578, KF644579, KT324724, KT324725, KT324726, KT324727,

KT324728, KT324729, KT324729, KT324730, KT324731, KT324732, KT324733, KT324734, NC_001491) and EHV-4 partial and full genome sequences (Genbank accession numbers: KT324735, KT324736, KT324737, KT324738, KT324739, KT324740, KT324741, KT324742, KT324743, KT324744, KT324745, KT324746, KT324747, KT324748, NC_001844) showed there was 86.6% pairwise identity and 70.6% identical sites between EHV-1 and EHV-4. Alignments between whole genome sequences of EHV-1 (Genbank accession numbers listed above) and EHV-9 (Genbank accession number: NC_011644) showed 98% pairwaise identity and 87.9% identical sites. Both alignments were done by using MAFFT version 7 within Geneious V8.0.4 (Katoh and Standley, 2013).

Recently, our laboratory has determined the complete genomic sequences of 11 EHV-1 and 14 EHV-4 isolates from Australia and New Zealand (Vaz *et al*., 2016a). Phylogenetic analysis of EHV-4 isolates revealed evidence of widespread recombination. In contrast, analyses of the 11 EHV-1 isolates from Australia and New Zealand, along with another 13 international EHV-1 isolates, detected limited or no evidence of recombination, depending on the method of analysis used. Pathogenesis and epidemiology can influence recombination as both these factors have an impact on the ability of viral infections to overlap in space (i.e. the same cell) and time (Thiry *et al*., 2005). Other factors that promote alphaherpesviruses recombination include similar high loads of each co-infecting virus, similar levels of virulence and invasiveness, and similar tissue distributions of the co-infecting viruses (Thiry *et al*., 2005). EHV-1 and EHV-4 infections of the respiratory tract of horses result in similar viral titres, replication kinetics and durations of virus infection (Allen *et al*., 2004). A key difference between the pathogenesis of the two viruses lies in the ability of EHV-1 to efficiently penetrate the basement membrane of the respiratory mucosa (Vandekerckhove *et al*., 2011) and disseminate to other sites, including the vascular endothelium of the placenta and central nervous system, via a leukocyte-associated viraemia (Gryspeerdt *et al*., 2010). This involves additional episodes of viral amplification compared to EHV-4, which would seemingly increase the opportunities for recombination in EHV-1 if all other factors were constant. However, the much

361 higher frequency of natural recombination in EHV-4 compared to EHV-1 suggests that other
362 factors, such as a lower prevalence of infection with EHV-1, may reduce opportunities for co-
363 infection and may have a greater impact on natural recombination (Vaz *et al*., 2016a). It is
364 interesting to note that, similar to the situation with HSV-1 and HSV-2, less recombination is
365 evident in the less genetically diverse EHV-1, compared to the more genetically diverse EHV-4, but
366 the relationship between the level of recombination and the level of genetic diversity requires
367 further investigation.
368
369 *3.2* Pseudorabies virus

370 Pseudorabies virus is the aetiological agent of Aujeszky's disease in pigs and causes economic loss
371 in the pig industry. Although PRV has been the target of eradication programs in some countries, it
372 remains endemic in some regions including Asia, west/east Europe and South America. Pigs
373 infected with PRV display a range of clinical signs, depending of the age of the affected animal,
374 including neurological, respiratory and reproductive disease. Pseudorabies virus can also infect and
375 cause disease in a wide variety of other hosts (Mettenleiter, 2008), including dogs, cats, cattle and
376 small ruminants.

377 A live attenuated vaccine, Bartha-K61, has been used to control disease due to PRV infection in
378 many countries, including China. Despite vaccination programs, disease outbreaks caused by new
379 PRV variants have been reported in China since 2011 (Luo *et al*., 2014). The first study using
380 whole genome sequence analysis to detect natural recombination in PRV has been published
381 recently (Ye *et al*., 2016). This study has shown that a historical Chinese PRV strain (SC) isolated
382 during the 1980s is a recombinant derived from an endemic Chinese PRV strain and a Bartha-like
383 strain (Ye *et al*., 2016). Experimental studies have shown that Bartha-K61 can induce protection
384 against the SC strain but not against the new variants (Luo *et al*., 2014). The SC strain has genomic
385 regions similar to the Bartha strain, so it has been hypothesised that these similar regions may
386 account for the capacity of the Bartha strain vaccine to protect against the SC strain, but not the new

387 variants, and that this difference in protective immunity may have permitted the new variants to
388 circulate in pig herds and cause disease (Ye *et al*., 2016)

389

390 *3.3* Feline herpesvirus-1

391 Recombination between FeHV-1 isolates has been demonstrated *in vitro* (Fujita *et al*., 1998), but
392 only one study has investigated natural recombination in FeHV-1 using NGS (Vaz *et al*., 2016b).
393 Feline herpesvirus-1 is the aetiological agent of feline viral rhinotracheitis and also a common cause
394 of ocular lesions in cats (Maes, 2012). Inactivated and attenuated vaccines are used widely, but do
395 not prevent infection (Jas *et al*., 2009). Previous studies using techniques such as restriction
396 endonuclease digestion of the genome, and analysis of partial genomic sequences suggested low
397 levels of diversity among several isolates (Maeda *et al*., 1995). Comparison of the whole genomes
398 of 24 historical and contemporary FHV-1 clinical isolates and 2 US-origin commercial vaccine
399 viruses in use worldwide confirmed that FeHV-1 isolates are highly homogeneous and has revealed
400 no evidence of recombination (Vaz *et al*., 2016b). This is the first alphaherpesvirus in which
401 recombination has been shown to occur under experimental (*in vitro*) conditions, but not under
402 natural *in vivo* conditions. Low rates of FeHV-1 recombination *in vivo* have been hypothesised
403 previously, as FeHV-1 is more homogeneous than other alphaherpesviruses (Fujita *et al*., 1998), but
404 analyses of a larger number of FeHV-1 clinical isolates, from more diverse geographical regions are
405 required in order to fully assess recombination during natural FeHV-1 infection.

406

407 **4. Natural recombination in avian alphaherpesviruses**

408 Natural recombination has been described in infectious laryngotracheitis virus (ILTV) and Marek's
409 disease virus type 1 (MDV-1, also called *Gallid herpesvirus-2, GaHV-2*) (Hughes and Rivailler,
410 2007; Lee *et al*., 2013; Lee *et al*., 2012) (Table 3). Marek's disease virus induces T cell lymphomas
411 in susceptible birds (Morrow and Fehler, 2004), while ILTV causes upper respiratory tract disease

412 in chickens (Garcia *et al*., 2013). Both diseases are highly contagious and cause economic losses in
413 poultry industries worldwide. Live attenuated vaccines are widely used to help control both these
414 diseases.
415
416 *4.1* Marek's disease virus

417 Four complete GaHV-2 genome sequences (CVI988, GA, Md5 and Md11) were compared in 2007
418 and genes with unusually high degrees of synonymous divergence were identified, suggesting the
419 past homologous recombination events (Hughes and Rivailler, 2007). This study identified three
420 clusters of orthologous genes based on their patterns of synonymous substitutions in order to use
421 them for further recombination analysis (Hughes and Rivailler, 2007). Eight loci within the four
422 GaHV-2 genomic sequences were highly homogenous, suggesting homologous recombination
423 between the vaccine strain CVI988, the highly virulent field strain (Md5) and the virulent Md11
424 strain. Additionally, phylogenetic analyses of the GA, Md5 and Md11 strains found that the virulent
425 GA strain generally clustered separately from the highly virulent Md5 and Md11 strains, but further
426 analyses of the UL 49.5 and RL ORF12 genes detected a high level of homogeneity between the
427 GA, Md5 and Md11 strains, suggesting that recombination had resulted in the transfer of virulence
428 factors between these strains. The approach used in this study to detect recombination differed from
429 those used for other alphaherpesviruses, such as HSV-1, HSV2, VZV, EHV-1, EHV-4, FeHV-1 and
430 ILTV (Bowden *et al*., 2004; Hughes and Rivailler, 2007; Kolb *et al*., 2013; Kolb *et al*., 2015; Lee *et*
431 *al*., 2013; Lee *et al*., 2012; Norberg *et al*., 2015; Norberg *et al*., 2007; Norberg *et al*., 2006; Norberg
432 *et al*., 2011; Peters *et al*., 2006; Szpara *et al*., 2014; Vaz *et al*., 2016a; Vaz *et al*., 2016b). Instead,
433 the method used was extrapolated from those used to detect sites of homologous recombination
434 within bacterial genomes. This approach examines the synonymous substitution distribution
435 patterns among orthologous protein coding genes, with higher synonymous substitutions per site
436 providing evidence of recombination (Hughes and Langley, 2007).

Analyses of GaHV-2 genome sequences for evidence of recombination, using methods similar to those used for other alphaherpesviruses, would help to further explore these findings. To this end, we examined the 15 GaHV-2 genome sequences publicly available at NCBI database (Table 4) for recombination using the SplitsTree 4 software and RDP4 software packages, as detailed previously (Vaz *et al*., 2016a; Vaz *et al*., 2016b). These analyses revealed evidence of recombination within the unique short region of the GaHV-2 genome using the SplitsTree 4 software (Figure 1C) (Huson and Bryant, 2006) and in all regions of the GaHV-2 genome using RDP4 software (Table 5). Together these results provide evidence of recombination in GaHV-2 but further study into the importance of recombination for GaHV-2 evolution and genome diversification is warranted, including examination of a larger number of GaHV-2 field isolates.

4.2 Infectious laryngotracheitis virus

Natural recombination in ILTV was first described by our laboratory in the context of attenuated vaccine use in Australia (Lee *et al*., 2012). This study provided clear evidence of natural recombination in ILTV, and also demonstrated safety concerns associated with the use of live attenuated alphaherpesvirus vaccines, a risk that had previously only been hypothesised. Prior to the detection of ILTV recombinants, two new genotypes of ILTV were shown to be dominant in Australia. These new genotypes, named as class 8 and 9 ILTV, had similar PCR-RFLP patterns, and clustered close to the Class 7 genotype (which includes the Serva vaccine strain) (Blacker *et al*., 2011). These studies, together with similar findings around the world led to the hypothesis that live attenuated ILTV vaccines could displace wild type strains and cause outbreaks of disease (Garcia and Riblet, 2001; Graham *et al*., 2000). However, the subsequent whole genome sequencing studies showed that natural recombination between the vaccine strains in use in Australia was responsible for the rise of the virulent Class 8 and 9 ILTV strains (Lee *et al*., 2012). These conclusions were supported by the use of the BootScan algorithm within the SimPlot program, which revealed the locations of breakpoints for intra-species recombination events involving the Serva and Australian

origin SA2 and A20 vaccine strains (Lee *et al*., 2012). These findings echoed earlier studies that showed that recombination between two attenuated HSV-1 strains could generate more virulent strains in a mouse model of infection (Javier *et al*., 1986).

In order to further investigate natural recombination in ILTV, full genome sequence data of other Australian ILTV isolates, along with full genome sequences of isolates from the US, were compared. The analyses revealed extensive recombination networks between ILTV isolates from both Australia and the US, and also uncovered new phylogenetic relationships between isolates (Lee *et al*., 2013). The importance of recombination in the biology and epidemiology of ILTV was then demonstrated further in 2016 in a study showing that a new virulent genotype of ILTV (Class 10 ILTV) had emerged in Australian poultry flocks as a result of recombination and had become dominant in some geographical areas (Agnew-Crumpton *et al*., 2016). These studies that have detected and characterised natural recombination in ILTV have provided new insights into the epidemiology of the disease caused by this virus and have explained the continuing occurrence of disease outbreaks associated with novel viral genotypes in Australia.

5 Conclusions

Alphaherpesviruses have been shown to display a high rate of recombination *in vitro* and *in vivo* under experimental conditions. However, under natural conditions, detection of recombination varies from limited or absent, in FeHV-1 and EHV-1 (Vaz *et al*., 2016a; Vaz *et al*., 2016b) to widespread, in HSV-1, EHV-4 and ILTV (Kolb *et al*., 2015; Lee *et al*., 2013; Lee *et al*., 2012; Szpara *et al*., 2014; Vaz *et al*., 2016a). These findings suggest that the contribution that recombination makes to genomic diversification and evolution in alphaherpesviruses varies across the different virus species. This highlights the importance of complementing studies that examine recombination in experimental settings with studies that look into recombination in field isolates from naturally infected hosts. The most comprehensive studies in this area have included analyses

of a large number of historical and contemporary whole genome sequences of field isolates from diverse geographical regions.

Most studies of natural alphaherpesvirus recombination have focused on human alphaherpesviruses. However, attenuated herpesvirus vaccines are used in both human medicine (against VZV) and veterinary medicine. Their use is particularly widespread in livestock species, poultry and pets. Recombination involving vaccine strains has been described in VZV, MDV (GaHV-2), ILTV (GaHV-1) and PRV, but the recombination events in these virus species have differed in their nature and consequences. In ILTV, two vaccine strains recombined to produce a virulent recombinant that became a dominant field strain (Lee *et al*., 2012). In PRV, recombination occurred between a vaccine strain and a field strain, potentially contributing to vaccine-induced selection and protection of another, genetically less similar, field strain (Ye *et al*., 2016). These studies provide examples of how vaccines and recombination can have an impact on viral evolution and alter the selection pressure on a viral population to result in greater dissemination of more pathogenic viruses. As live attenuated vaccines are favoured in veterinary medicine, their impact on viral ecology and evolution should be evaluated and monitored at a population level. Targeted monitoring of recombination after the introduction of new vaccine strains would be helpful for detecting the rise of new, potentially more virulent strains. Consideration of recombination risks in the assessment of vaccine safety during the process of registration could also be advantageous. Currently, natural recombination has not been assessed in BoHV-1 or herpesvirus of turkeys (HVT) but live vaccines using these viruses are in widespread use in the cattle and poultry industries, respectively, with the latter being used as vaccine vector to express exogenous proteins from other avian pathogens (Kapczynski *et al*., 2015; Li *et al*., 2011; Roh *et al*., 2016). Examining natural recombination in these two viruses should be included in future research in order to determine potential risks to animal health.

Over the last 10 years the development of new more sophisticated and accessible NGS techniques, along with advances in computational and statistical analysis, has dramatically enhanced the study

514 of natural recombination in alphaherpesviruses. Detection of recombination using more than one
515 approach is recommended and can now be achieved using a number of different bioinformatic
516 analysis software. Commonly used programs include the Recombination Detection Program (RDP),
517 SplitsTree software, the Genetic Algorithm Recombination Detection (GARD) program and
518 SimPlot software. The latest version of RDP (RDP4) was made available in 2015 (Martin *et al*.,
519 2015) and includes nine non-parametric recombination detection methods (Martin *et al*., 2010).
520 SplitTree software uses phylogenetic and reticulate networks, in addition to the pairwise homology
521 test (PHI test), to search for the presence of recombination in a given set of aligned sequences
522 (Huson and Bryant, 2006). The GARD program searches for evidence of segment-specific
523 phylogenies. GARD is a likelihood-based model selection procedure that searches multiple
524 sequence alignments for evidence of recombination breakpoints and identifies putative recombinant
525 sequences (Kosakovsky Pond *et al*., 2006). SimPlot allows the analysis of sequence alignments, and
526 searches for breakpoint locations. This program ignores sites containing gaps, and produces a
527 similarity plot identifying the sequence position and the similarity value at each point in the
528 sequence (Lole *et al*., 1999). The application of more than one method is desirable to increase the
529 robustness and consistency of the results, since a true recombination event should be independent of
530 the method of analysis.

531 Sequence quality is also crucial to the accurate detection of recombination events in full genome
532 sequences. Consideration of a measurement of quality such as Phred score and avoiding low
533 sequence quality and parental sequence uncertainty is strongly recommended, since detection of
534 breakpoint locations, and thus recombination, may be unreliable in low quality sequences (Posada,
535 2002; Posada and Crandall, 2001). Inclusion of detailed information describing the methods used in
536 recombination analyses is also desirable, specifically in regard to the preparation of the sequences
537 that are used for recombination analysis such as the trimming of short sequence repeats (SSR),
538 tandem repeat regions (TRR), and terminal repeated regions (TR). It is recommended to remove
539 these sequences from analyses as they can bias recombination analysis (Dutch *et al*., 1995; Lee *et*

al., 2015; Newman *et al*., 2015; Vaz *et al*., 2016a). Finally, studies focused on natural herpesvirus recombination should aim to include high quality information about the samples and virus isolates so that the results from recombination analyses can be considered in the context of relevant clinical and epidemiological information. Pairing recombination analyses with an understanding of the epidemiology and pathogenesis of the viruses offers the greatest potential to understand the importance of recombination and the role that it may be playing in virus evolution. Indeed, some of the most important findings relating to recombination and the involvement of attenuated vaccines have come from studies that have integrated recombination, epidemiological and viral pathogenesis. Maintaining such a focus in future studies will be important for achieving a comprehensive understanding of alphaherpesvirus recombination.

This review has highlighted the significance of natural recombination in many viruses of importance to human and veterinary medicine and has demonstrated the power of using full genome sequencing and sequence analysis to examine natural herpesvirus recombination. It is likely that continued advances in technology and methodologies, further reductions in the cost of NGS techniques and improved bioinformatics tools to assess recombination will enable further developments in the field of recombination, in particular by facilitating the affordable examination of increasing numbers of clinical isolates from diverse widespread geographical regions.

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Conflict of interest

The authors declare there is no conflict of interest in submission of this paper.

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943

944 **Figure captions:**

945 **Figure 1.** Recombination network trees generated using SplitsTree4 from alignment of 15
946 publically available GaHV-2 genome sequences on Genbank. **A)** Alignment of complete genomes
947 excluding the terminal sequence repeats. **B)** Internal repeat region. **C)** Unique short region. The
948 multiple reticulate networks indicate recombination events between the isolates. The bar indicates
949 the rate of evolution in sequence substitution per site. *P* values for the PHI test for detecting
950 recombination, as implemented in SplitsTree4, are shown and were highly significant for the
951 complete genome and for the unique short region. **D)** Unique long region. Details about the
952 nucleotide sequences used in these analyses are shown in Table 4.

953

Table(s)

Table 1. Timeline and summary of key studies examining natural recombination in human alphaherpesviruses.

Year	Summary of study findings	Reference
Herpes simplex virus 1 (HSV-1)		
2004	Evidence of recombination in partial genome sequences (4% of the genome) from 14 clinical samples from UK compared with samples from Seoul and South Korea.	Bowden *et al*., 2004
2004	Evidence of recombination in partial genome sequences (2.3% of the genome) in 28 clinical samples from Sweden.	Norberg *et al*., 2004
2011	Whole genome sequencing and analysis of seven clinical isolates shows one isolate had variable phylogenetic features, potentially due to recombination.	Kolb *et al*., 2011
2013	Widespread recombination was detected following whole genome sequencing of 31 clinical isolates from diverse geographical regions.	Kolb *et al*., 2013
2014	Widespread recombination was found following whole genome sequencing of 20 field strains obtained from China, Japan, Kenya and South Korea and comparison with those available from the US, Europe and Japan..	Szpara *et al*., 2014
Herpes simplex virus-2 (HSV-2)		
2007	Analysis of partial genome sequences (3.5% of the genome) from 47 diverse clinical isolates shows HSV-2 has fewer genogroups than HSV-1.	Norberg *et al*., 2007
2015	Whole genome sequencing and analysis of six clinical isolates, along with analysis of two other available genome reveals evidence of recombination.	Kolb *et al*., 2015
2015	Analysis of 34 near complete genome sequences from clinical isolates from diverse geographical regions shows that recombination is present but is less frequent than in HSV-1	Newman *et al*., 2015
Varicella zoster virus (VZV)*		
2003	Analysis of SNP patterns from geographically diverse isolates classified VZV into four major groups and showed evidence of potential recombination	Wagenaar *et al*., 2003
2004	Analysis of SNP patterns from geographically diverse isolates determined three major groups and showed evidence of potential recombination in one group/	Loparev *et al*., 2004
2006	Whole genome sequencing of 11 representative isolates from North America identified recombination potentially involving the VZV vaccine strain.	Peters *et al*., 2006
2006	Whole genome sequencing of isolates previously identified by Loparev *et al*., (2004) detected recombination, potentially involving the VZV vaccine.	Norberg *et al*, 2006
2011	Whole genome sequencing of a Russian clinical isolate showed significant intra-clade recombination events	Norberg *et al*., 2011
2012	Whole genome sequencing of 21 isolates revealed two novel genotypes and evidence of recombination	Zell, *et al*.,*2012*
2015	Whole genome sequences of 37 isolates from diverse geographical regions confirmed that viruses in the Japanese vaccine-like group consistently display higher levels of recombination.	Norberg *et al*., 2015

*** Live attenuated vaccine is in use**

Table 2. Timeline and summary of key studies examining natural recombination in other (non-human) mammalian alphaherpesviruses.

Year	Summary of study findings	Reference
Equine herpesvirus-1*, -4* and -9 (EHV-1, -4 and -9)		
2005	Analysis of partial genome sequence detected the first natural inter-species recombination reported between EHV-1 and EHV-4.	Pagamjav *et al*., 2005
2012	Analyses of partial genome sequences of an isolate from a polar bear in a zoo revealed inter-species recombination between EHV-1 and EHV-9	Greenwood *et al*., 2012
2016	Whole genome sequencing of 11 EHV-1 and 14 EHV-4 isolates from Australia and New Zealand, and comparison with other available genomes, showed widespread recombination in EHV-4 but not in EHV-1.	Vaz *et al*., 2016a
Pseudorabies virus (PRV)*		
2016	Whole genome sequencing of isolates from China demonstrated recombination involving a vaccine-like strain, potentially altering selection pressures in vaccinated pig populations	Ye *et al*., 2016
Feline herpesvirus-1 (FeHV-1)*		
2016	Analyses of 24 whole genome sequences from clinical samples and 2 genome sequences of US origin vaccines found no evidence of recombination.	Vaz *et al*.,2016b

*** Live attenuated vaccine is in use**

Table 3. Timeline and summary of key studies examining natural recombination in avian alphaherpesviruses.

Year	Summary of study findings	Reference
Marek's disease virus (MDV)*		
2007	Four whole genome sequences were analyzed and recombination was identified between a vaccine strain and a highly virulent field strain	Hughes and Rivallier *et al*., 2007
Infectious laryngotracheitis virus (ILTV)*		
2012	Whole genome sequence analyses of newly emerged field isolates found that two attenuated vaccine strains had recombined to generate virulent viruses.	Lee *et al*., 2012
2013	Whole genome sequence analyses of current and historical isolates in Australia and the US revealed extensive recombination networks	Lee *et al*., 2013
2016	A newly emerged virulent field strain in Australia was sequenced and shown to be a recombinant virus.	Agnew-Crumpton *et al*., 2016

*** Live attenuated vaccine is in use**

Table 4. Publically available full genome sequences of GaHV-2 used in recombination analyses.

Isolate (GenBank accession number)	Year of isolation (Reference)	Country	Comments	Genome sequence reference
GA (AF147806)	1964 (Eidson and Schmittle, 1968)	USA	Isolated from ovarian tumour	(Lee *et al*., 2000)
Md5 (AF243438)	1980 (Witter *et al*., 1980)	USA	Very virulent isolate from spleen of commercial broilers	(Tulman *et al*., 2000)
Md11(AY510475)	1980 (Witter *et al*., 1980)	USA	Isolated and maintained in duck embryo fibroblast	(Niikura *et al*., 2006)
CVI988 (DQ530348)	1972 (Rispens *et al*., 1972)	Worldwide use	Vaccine used since 1990	(Spatz *et al*., 2007a)
RB-1B (EF523390)	1982 (Schat *et al*., 1982)	USA	Highly oncogenic	(Spatz *et al*., 2007b)
CU-2 (EU499381)	1973 (Smith and Calnek, 1973)	USA	Mildly virulent	(Spatz and Rue, 2008)
814 (JF742597)	1980	China	Isolated from healthy chickens	(Zhang *et al*., 2012)
LMS (JQ314003)	2007	China	Isolated from broilers with severe disease	(Cheng *et al*., 2012)
648A (JQ806361, JQ806362, JQ809691, JQ809692, JQ820250 and JQ836662)	1994 (Witter, 1997)	USA	Serial passages of isolate 648A (passages p11, p31, p41, p61, p81, p101)	(Spatz *et al*., 2012)
GX0101 (JX844666)	2001 (Zhang *et al*., 2012)	China	Isolated from layers with severe tumours	(Su *et al*., 2012)

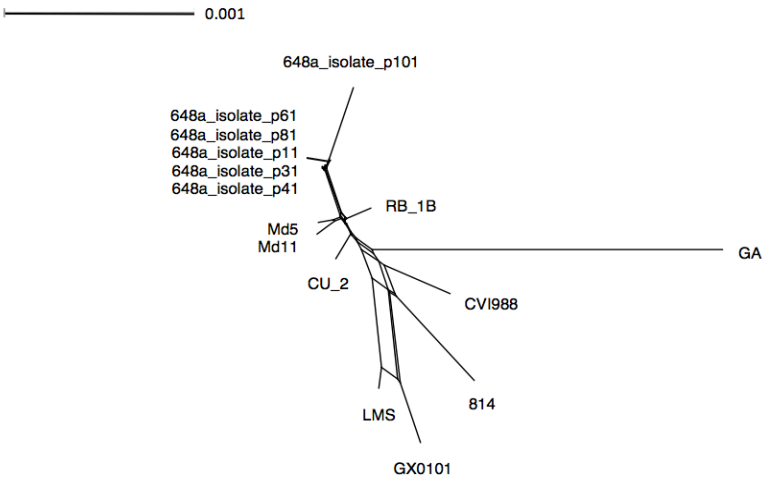
Table 5. Recombination breakpoint analysis of GaHV-2 genome sequences using RDP4

Genome region	Breakpoint (in alignment)		Possible viruses involved in recombination event **R:** Recombinant, **M:** Major parent, **m:** Minor parent	Method of breakpoint detection in RDP4 software
	Breakpoint beginning 99% CI*	**Breakpoint ending 99% CI***		
Internal/terminal repeat	18291 – 19335	19926 - 21087	**R:** GA, **M:** unknown, **m:** GX0101	GENECONV, MaxChi, 3Seq.
Unique long	7627 – 9282	9365 – 9689	**R:** 648a isolate_p101, **M:** 648a isolate_p81, **m:** unknown.	GENECONV, Bootscan, MaxChi, Chimaera, 3Seq
	51568 – 73297	108359 – 3016	**R:** GA, **M:** unknown, **m:** Md5	MaxChi, Chimaera, SiScan, 3Seq.
	91057 – 58003	91057 - 58003	**R:** GX0101, **M:** 814, **m:** unknown	GENECONV, MaxChi, SiScan, 3Seq.
	82371 – 93738	93740 – 101624	**R:** 814, **M:** unknown, **m:** Md5	MaxChi, Chimaera, 3Seq.
Unique short	2289 – 3999	5959 – 7480	**R:** CVI988, **M:** LMS, **m:**GX0101	GENECONV, MaxChi, Chimaera, SiScan, 3Seq.

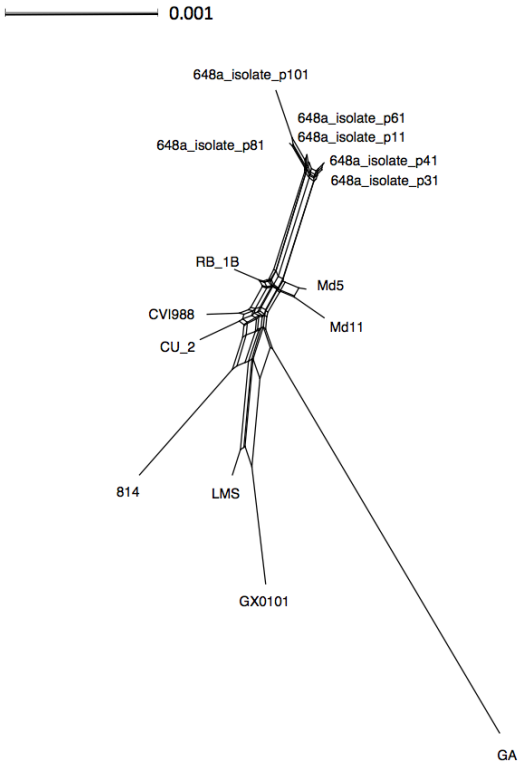
*CI = confidence interval

Figure(s)

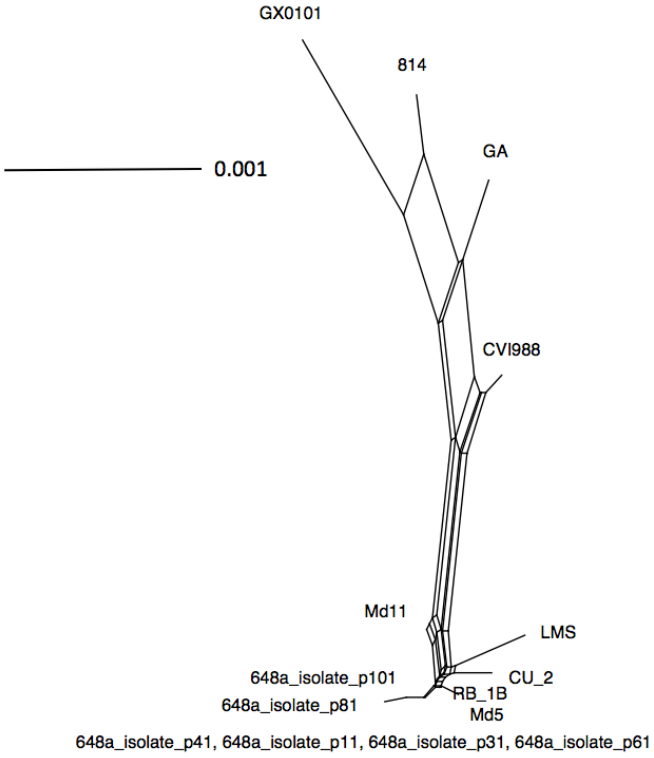
A) Complete genome (p = 9.751E-9)



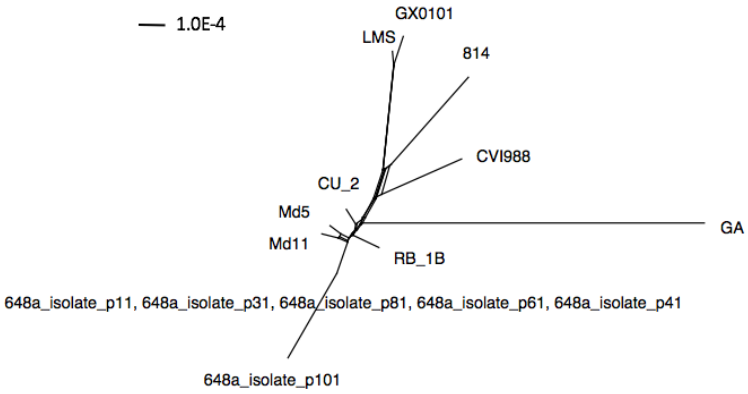
B) Internal repeat (p = 0.05766)



C) Unique short (p = 0.002421)



D) Unique long (p = 0.2554)



Supplementary Material

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