PHYLOGENETIC ANALYSIS OF EQUINE HERPES VIRUS 4 GLYCOPROTEINS IN COMPARISON TO EQUINE ABORTION VIRUS

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ABSTRACT

Equine Herpes Virus-1(EHV-1) and 4 (EHV-4), both viruses are found in horse populations all over the world and leading to a feverish respiratory disease per annum among horses in areas with intense horse populations. Abortion in mares infection with EHV-4 was rarely, from another side mare may abort several weeks to months after clinical or subclinical infection with EHV-1. Nucleotide sequences of EHV-4 genes encoding each glycoprotein showed that the amino acid sequences (gB, gE, gG, gH, gK, gL, gM and gN of TH20p) resemble that of the reference genome (NS80567) except (gC, gD, gI and gP2) at certain positions. By constructing phylogenetic tree using glycoproteins sequences of EHV-4, EHV-4 reference genome and EHV-1 (strain 89c25p) revealed that there are two main clusters within each clusters.

Key words: Equine, Abortion, EHV-1, EHV-4, Phylogenetic analysis

INTRODUCTION

Equine herpesvirus 1 and 4 are a members of genus *Varicellovirus, Alphaherpesvirinae* subfamily (Roizman, 1996; Davison *et al.,* 2009). Equine herpesvirus-1 (EHV-1) is an important, ubiquitous equine viral pathogen that exerts its major impact by inducing abortion in pregnant mares, early neonatal death, respiratory disease in foals and myeloencephalopathy (Reed and Toribio, 2004; Patel and Heldens, 2005). The complete genome sequences of EHV-4 was reported and that the virus has a linear double stranded DNA (Cullinane *et al.,* 1988; Roizman *et al.,* 1992; Telford *et al.,* 1998). Sequence analysis showed that there is (55-84%) DNA homology depending on the gene and that the homology at the amino acid level ranged from 55% to 96% (Telford *et al.,* 1998). The EHV-4 mainly causes respiratory disease in horse and rarely causes abortion, while the EHV-1 primarily causes abortion and myeolo-encephalopthy in addition to respiratory disease (Slater *et al.,* 1994). Both EHV-4 and EHV-1 cause economic losses and negative impact on equine as a result of morbidity or mortality from virus-induced abortion and respiratory disease. However, EHV-4-specific vaccine has not been available, EHV-1 and EHV-4 have resistant reactions and build latent infection, there are contrasts concerning infection section and their capacity to meddle with the intrinsic insusceptible reaction (Ma,G. *et al.,* 2013).

MATERIALS AND METHODS

1 - Cells
FHK-Tcl3.1 (Maeda *et al.,* 2007; Andoh *et al.,* 2009) and 293T cells were maintained in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% heat-inactivated fetal calf serum (FCS; JR Scientific, Woodland, CA, U.S.A.), 100 units/ml penicillin and 100 µg/ml streptomycin (GIBCO) at 37°C with 5% CO_2.

2 - Viruses
EHV-4 TH20 strain was isolated from a colt suffering from respiratory disease at1952 (Kawakami *et al.,* 1962) was plaque-purified three times in primary fetal horse kidney (FHK) cells and termed as TH20p.

3 - Construction of expression plasmids
For expression of glycoproteins of EHV-4 each gene was amplified from the genome of EHV-4 TH20p by

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polymerase chain reaction (PCR) using primers (Table 1). PCR was performed by using Takara LA PCR Kit Ver. 2.1 (Takara), and the amplified fragments were digested with restriction enzymes, and were then cloned into the expression plasmid pCAGGS, which was kindly provided by Dr. Miyazaki (Osaka University). The nucleotide sequences of at least two constructed plasmids were determined using a Big Dye Terminator v3.1 kit (Applied Biosystems). All plasmids were purified by QIAprep Spin Miniprep Kit (QIAGEN) or QIAfilter plasmid Midi Kit (QIAGEN) (Mahmoud, 2013).

4 - Bioinformatics analysis

Sequence alignments, translations, and comparisons were carried out using BIOEDIT (Version 7.0.9.0, Hall, T.A, 1999). The BLAST algorithm was used to search the NCBI Gen Bank (http://www.ncbi. nlm.hih.gov/) databases for homologous sequences. Neighbor-joining trees (Saitou and Nei, 1987) were constructed on the basis of genetic distances, estimated by Kimura's (1980) two-parameter method, using MEGA 5 (Kumar et al., 2001; http://www.megasoftware.net). The reliability of the trees was estimated by bootstrap confidence values (Felsenstein, 1985) and 500 bootstrap replications were used.

RESULTS

1 - Sequence analysis of each glycoprotein of EHV-4 strain TH20p

Nucleotide sequences of genes encoding each glycoprotein showed that the amino acid sequences of gB, gE, gG, gH, gK, gL, gM and gN of TH20p were identical to those of NS80567, but those of gC, gD, gI and gp2 were different at positions 309 (Aspartic acid to Glutamic acid), 27 and 382 (leucine to valine and glycine to glutamic acid, respectively), 134 and 298 (arginine to tryptophan and aspartic acid to alanine, respectively) and 376, 463 and 509 (leucine to serine, lysine to arginine and Glutamine to Arginine, respectively), respectively.

2 - Phylogenetic analysis of EHV4 glycoproteins in relation with strain NS80567 and EHV-1

The EHV4 glycoproteins are grouped in 3 different clusters; the first cluster (gC4, gP24, gH4 and gK4), the second cluster (gE4, gL4, gI4 and gM4) and the third one (gG4, gB4, gD4 &gN4) (Fig. 1).By constructing a tree containing the glycoproteins of EHV4, EHV4 reference strain (NS80567) and EHV-1 revealed that there are two main clusters within each clusters, each glycoprotein of the EHV-4 are grouped together except glycoprotein D (gD_EHV-1) (Fig. 1). The evolutionary history (Fig. 2) was inferred using the Minimum Evolution Method (Rzhetsky and Nei, 1992). The optimal tree with the sum of branch length = 19.96041633 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965). The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm at a search level of 1 (Nei and Kumar, 2000). The Neighbor-joining algorithm was used to generate the initial tree (Saitou and Nei, 1987). The analysis involved 36 amino acid sequences. All positions containing gaps and missing data were eliminated; there were a total of 102 positions in the final dataset evolutionary analyses were conducted in MEGA6 (Tamura *et al.,* 2013). Evolutionary pressures on proteins are frequently measured by the ratio of substitution rates at non-synonymous and synonymous sites. The dN/dSratio was first established for application to distantly diverged sequences, the alterations among which represent substitutions that have fixed along independent lineages, the dN/dS ration is greater than 1, so the sites are under positive or diversifying selection (Tabel 2). Diagrammatic representation of EHV4 glycoproteins in comparison with the reference strain (accession number AF030027) through using an online program (https://server.gview.ca/job/ 350CA7A8BCAE7E9A5F5CB572551A7348/results - Fig. 3).

Table 1: Primers based on the complete genome of EHV-4 NS80567

Genes	Primers	
	Name	Sequences[a]
gB	F	5´-ACGAATTCatgtccacttgttgccgtgc-3`
	R	5´-ACCTCGAGttaaaccatttttcgctttcca-3`
gC	F	5´-ACGAATTCatgggtttggtaaatataatgcg-3`
	R	5´-ACCTCGAGttagaagtctgctttcttgtac-3`
gD	F	5´-ACAGATCTatgtctaccttcaagcctatg-3`
	R	5´-ACCTCGAGttacggaagctgagtatatttg-3`
gE	F	5´-ACAGATCTatggagctgttagactcccgc-3`
	R	5´-ACCTCGAGtcattttaggatagacctgagc-3`
gG	F	5´-ACAGATCTatgttggctgtgggagcaact-3`
	R	5´-ACCTCGAGttatgcaacgtactcaagtcg-3`
gH	F	5´-ACGAATTCatgtcacaaccgtatctaaaaat-3`
	R	5´- ACCTCGAGttactcagagtttaataacaaact-3`
gI	F	5´-ACGAATTCatggccaaacatactgtattgt-3`
	R	5´-ACCTCGAGttagtcttcctcttttatggcc-3`
gK	F	5´-ACAGATCTatgttattcggggggagaacg-3`
	R	5´-ACGCATGCttattgtgacaaggactcgccc-3`
gL	F	5´-ACAGATCTatgtatcactcccaattgggtg-3`
	R	5´-ACCTCGAGtcattctgaagtttcttgaatt-3`
gM	F	5´-ACAGATCTatggcacgacgtggcgctgct-3`
	R	5´-ACCTCGAGTtaacggtaatctctgcgcgag-3`
gN	F	5´-ACAGATCTatgttgtcagcgagattagtg-3`
	R	5´-ACCTCGAGtcagtgtaggtgtcgcaacat-3`
gp2	F	5´-ACAGATCTatggggttcatctatgttagc-3`
	R	5´-ACCTCGAGttaatatacggacgcccggggg-3`

position of genome of Equine herpesvirus 4 strain NS80567, complete genome (Accession No. AF030027.1)
a: Capital letters indicate the restriction endonuclease digestion site and additional two nucleotides.

Table 2: Estimates of Average Codon-based Evolutionary Divergence over all Sequence Pairs

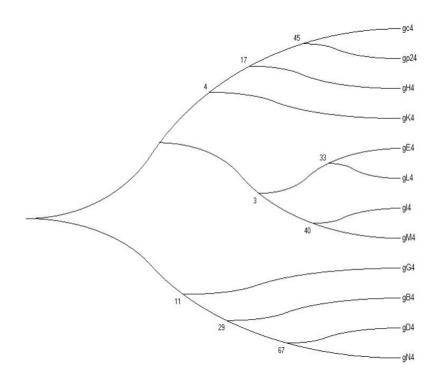| Non-Synonymous sub.		Synonymous sub.		dN-dS		dS-dN		dN/dS
dN	SE	dS	SE	value	SE	value	S.E.	1.001
2.269	0.084	2.266	0.078	0.191	0.176	-0.191	0.176	

Figure 1: Neighbor joining tree showing the relationship between different glycoprotein of EHV4.
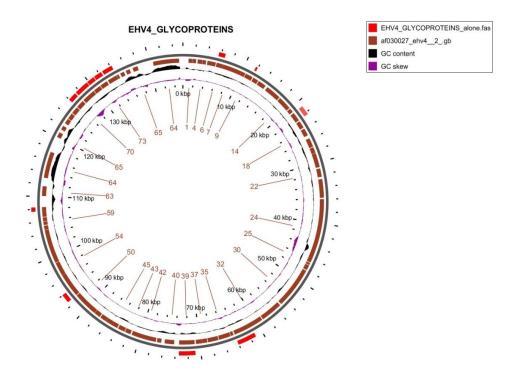

Figure 2: The BLAST Atlas analysis type displays regions of the uploaded glycoproteins sequences where there are BLAST hits to the reference genome.
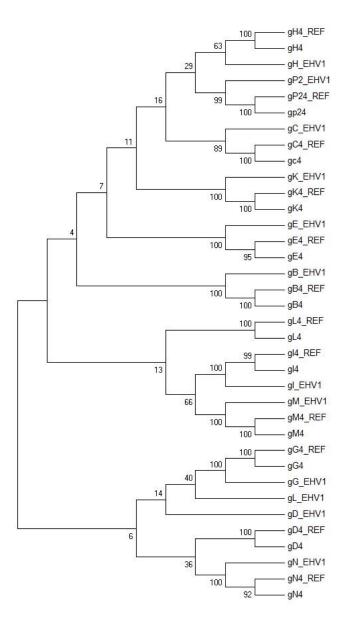
Figure 3: Neighbor joining tree showing the relationship between different glycoprotein of EHV-4 and EHV-1.

DISCUSSION

Viral glycoproteins play important roles in the biology of herpesviruses, envelope glycoproteins of EHV-4 encodes 12 different glycoproteins, which are involved in virus attachment, penetration and egress, cell to-cell spread as well as pathogenicity and virulence. The result of sequence analysis of amino acid EHV-4 strain TH20p indicated that there is no difference in amino acid for gH, gk, gM and gN comparing to reference strain NS80567 of EHV-4. The rapid accumulation of DNA sequences provides increased opportunities to study molecular evolution and phylogenetic relationships many methods were used to constructphylogenetic trees for organisms, based on the introduction of alignment of DNA or proteinhomologous sequences, followed by tree construction based on various statistical criteria such as maximum likelihood, parsimony, distance matrices, paralinear distances and invariants.The pattern of nucleotide substitution within the different glycoproteins was examined, the overall average value for the number of synonymous nucleotide substitution (ds) per site and the number of non-synonymous nucleotide substitution (dn) per site were calculated separately for the EHV-4 glycoproteins. The result of nonsynonymous substitutions was slightly higher than that of synonymous substitutions (Table 2), this result indicates slightly *diversifying selection or positive selection working on these glycoproteins* (Nei and Gojobori, 1986; Tamura *et al.,* 2013). The phylogenetic Neighbor-joining trees (Saitou and Nei, 1987) were constructed on the basis of genetic distances (Kimura, 1980) two-parameter

method by using MEGA 6 (Tamura *et al.,* 2013) for the 12 glycoproteins of EHV4 (Fig. 1-2) and 36 glycoproteins of EHV4, EHV4 reference strain (NS80567) and EHV-1 (Fig. 3). These trees revealed that these similarities between glycoproteins may be due to the pathogen which could provide selection pressure to keep specific MHC alleles which is necessary to face a specific immune response to a common pathogen (Zhou and Hickford, 2004), or from a common ancestor. These alleles predate speciation according to the trans-species hypothesis (Klein *et al.,* 1998) who argued that over evolutionary time a group of ancestral alleles were passed on to a species' descendants, while mutations accumulate further through time.

CONCLUSION

The non-synonymous substitutions were slightly higher than that of synonymous substitutions; this result indicates slightly diversifying selection or positive selection working on these glycoproteins.

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تحليل التطور لبروتينات فيروس هربس الخيول ٤ بالمقارنة بفيروس هربس الخيول المجهض

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فيروسات هربس الخيول ١ و ٤، من الفيروسات التى تصيب الخيول فى جميع أنحاء العالم، وتؤدى إلى أمراض فى الجهاز التنفسى بالاضافة إلى الإجهاض للخيول. هذه الدراسة أظهرت تسلسل النوكليوتيدات من فيروس هربس الخيول ٤ أن تسلسل الأحماض الأمينية لكل من البروتينات السكرية (gB, gE, gE, gG, gH, gK, gL, gM and gN) فى العتره المعزولة (TH20p) متشابه مع العتره المرجعية (NS80567) ما عدا البروتينات السكرية الاتية (gC, gD, gI and gP2) وعن طريق بناء شجرة النشوء والتطور باستخدام بروتينات التسلسل للعترة المعزولة والعترة المرجعية ومقارنتهم بفيروس هربس الخيول ١ أن هناك مجموعتين رئيسيتين داخل كل المجموعات فى شجرة النشوء والتطور.

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