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Analysis of equine herpesvirus 2 strain variation using monoclonal antibodies to glycoprotein B

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Summary. The antigenic relationships of four genomically divergent strains of equine herpesvirus 2 (EHV2.86/67, EHV2.5FN, EHV2.141 and EHV2.T-2) and equine herpesvirus 5 (EHV5) were examined in ELISA using a panel of EHV2.86/67 gB-specific MAbs. EHV2.86/67 and EHV2.5FN were shown to be more similar to each other than to EHV2.T-2, EHV2.141 or EHV5. Seven of nine EHV2.86/67 gB specific MAbs tested in serum neutralisation assays were shown to neutralise EHV2.86/67 and EHV2.5FN but not EHV2.141, EHV2.T-2 or EHV5. The complete nucleotide and deduced amino acid sequences of EHV2.86/67, EHV2.5FN, EHV2.141 and EHV2.T-2 gB were compared and contrasted with each other and with EHV5 gB. The four EHV2 strains were 94–96% similar at the amino acid level and variability in amino acid sequence mapped to three mains sites designated I, II and III. By contrast, the four EHV2 strains were 77–79% similar to EHV5 gB at the amino acid level. The epitope of these seven gB specific neutralising MAbs has been previously mapped to amino acids 29–74 of EHV2 gB and examination of the deduced amino acid sequence of the four sequenced strains localised the epitope of the seven MAbs to amino acids 30 to 49 located within Site I. Six other divergent strains of EHV2 were examined for variability at Site I using DNA sequencing. Examination of the deduced amino acid sequences of all ten EHV2 strains tested indicated, that based on the epitope of the neutralising MAbs the EHV2 strains formed two distinct antigenic groups, EHV2.86/67-like and EHV2.141-like. EHV5 gB showed divergence from all of the EHV2 gB sequences between amino acids 29–74.

Introduction

EHV2 is a slowly growing, highly cell-associated virus that has been linked with respiratory disease, conjunctivitis and general malaise in the horse [2,15, 20, 25, 26]. However, the frequent isolation of EHV2 from the nasopharynx of normal horses has made it difficult to consistently correlate virus isolation with clinical signs of disease [28]. EHV2 is endemic in the adult horse population and has been isolated from the peripheral blood leukocytes of 89% of horses in Australia [16]. These findings have brought into question the role of EHV2 in equine respiratory tract disease. The determination of the complete nucleotide sequence of EHV2.86/67 and the reclassification of EHV2 as a member of the subfamily *Gammaherpesvirinae* has caused renewed interest in the pathogenesis of disease caused by EHV2 [27].

Marked genomic variability based on restriction endonuclease digested DNA profiles has been documented for different strains of EHV2 [5] and examination of the restriction endonuclease fingerprints of different EHV2 isolates has identified genomically variable strains of EHV2 that were isolated from the same horse [4]. Furthermore, a tentative relationship between the results of DNA restriction endonuclease profiles and the previous results of serum neutralisation data of different isolates of EHV2 has been suggested [4, 22]. These findings suggest that multiple different antigenic types of EHV2 may exist.

gB is the most highly conserved envelope glycoprotein of the all members of the herpesvirus family and is essential for virus replication in vivo [21]. Additionally, antibodies directed against gB are capable of neutralising herpesvirus infection. We have previously characterised EHV2.86/67 gB using a panel of EHV2.86/67 specific MAbs [14]. The results of this study demonstrated that EHV2 gB is a disulphide-linked heterodimer that is a component of the virion envelope. Additionally, using MAbs a neutralisation epitope was identified between amino acids 29–74. We have characterised gB from the closely related gammaherpesvirus, EHV5 and shown that EHV5 gB is also a component of the virion envelope [13]. The aim of the present study was to analyse strain variation of EHV2 gB using a panel of gB specific MAbs, to sequence gB from three genomically diverse strains of EHV2 and to compare these with the gB gene of EHV2.86/67 [27] and with EHV5 [1].

Materials and methods

Computer analysis of EHV2 strain variability

To examine strain variability among different EHV2 isolates four genomically diverse strains of EHV2 were selected for further study based upon examination of a phylogenetic tree constructed using previously published restriction endonuclease data [7, 8]. Data for *Bam*HI, *Eco*RI and *Hin*dIII digests of genomic DNA from 15 EHV2 isolates was used to construct an unrooted evolutionary tree using the GCG computer program RESTML via the Australian National Genomic Information System (ANGIS) computer interface. The program RESTML estimates phylogenies by implementing a maximum likelihood method for restriction site data [11]. RESTML optimises branch lengths and computes likelihoods for branches of each particular tree. The final tree presented in this paper represents the optimal tree of

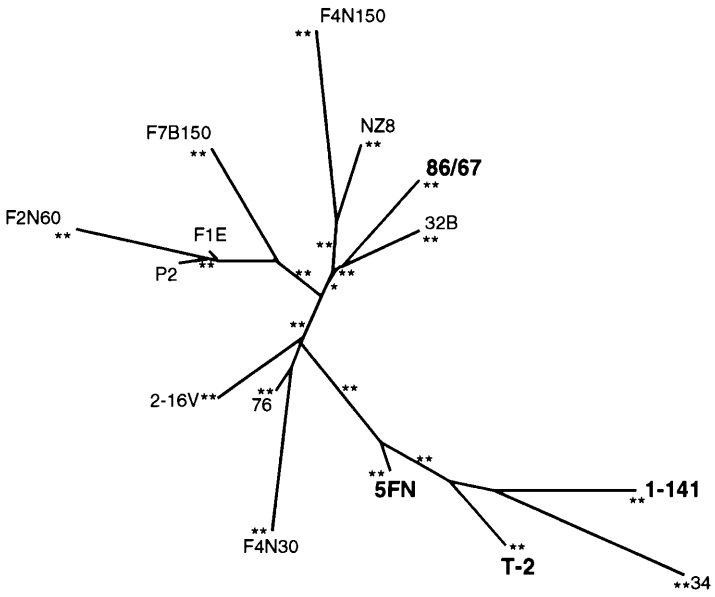


Fig. 1. Unrooted phylogenetic tree of 15 isolates of EHV2 constructed using the computer program RESTML of Felsenstein [11] which estimates phylogenies by maximum likelihood using restriction site data. The tree was constructed using restriction endonuclease enzymes (*Bam*HI, *Eco*RI, *Hin*dIII) and data from 76 restriction sites. The branch lengths are drawn to scale. The significance of approximate confidence limits are marked with an asterisk (*) significantly positive at $P < 0.05$; (**) significantly positive at $P < 0.01$

1755 trees examined. Three strains of EHV2: EHV2.5FN, EHV2.141 and EHV2.5FN were chosen following examination of this unrooted evolutionary tree, as being representative of strains of EHV2 that were most distantly related to the prototype strain EHV2.86/67 (Fig. 1).

Viruses and cell culture

EHV2 strains (EHV2.86/67, EHV2.141, EHV2.T-2, EHV2.5FN) and EHV5 (EHV5.141) were propagated in equine foetal kidney (EFK) monolayer cell cultures. Infected cells were maintained in minimal essential medium (MEM, Gibco BRL) containing Earle's salts, L-glutamine and non-essential amino acids, and supplemented with 1% foetal bovine serum (FBS), 50 μg/ml ampicillin, 0.13M $NaHCO_3$ and 0.015 M-HEPES (Sigma) at pH 7.5.

Purification of virus

Purification of virus from infected EFK cell lysates was performed as described [10]. Briefly, the medium was clarified at 1,500 **g** for 15 min followed by a second clarification of the lysate at 2,500 **g** for 15 min. Extracellular virus was ultracentrifuged at 50,000 **g** for 90 min, resuspended in 1 ml of 0.01M Tris-HCl, pH 7.4, 0.1M NaCl and 0.001M EDTA (TNE) buffer and layered onto performed 5–15% w/v Ficoll-400 (Pharmacia) gradients in TNE and centrifuged at 20,000 **g** for 2 h. The virus band was visualised and collected by side puncture of the centrifuge tube. Peak fractions were diluted 5-fold in TNE and centrifuged at 20,000 **g** for 2 h and resuspended in 1 ml of TNE. Virus protein content was determined using a colourimetric assay (BCA protein kit, Pierce).

Antibodies

The production and characterisation of ten MAbs, recognising EHV2.86/67 gB were previously described [14]. Of these ten MAbs, one (15C1) was not available for use in the present study. Eight gB-specific MAbs (15C7, 4F10, 3D10, 17D8, 19F10, 19B1, 17A7 and 3B1) recognised linear epitopes located in the 89 kDa subunit of gB and one MAb (2E5) recognised a conformational epitope present in gB.

Virus neutralisation

Virus titration and neutralisation assays were performed in sterile 96 well flat-bottom polyvinyl chloride plates (Nunc). EHV2.86/67 was obtained from cell culture supernatants and half log serial dilutions made in MEM supplemented with 2.5% FBS, 50 μg/ml ampicillin and 0.013 M $NaHCO_3$ (incubation medium). In each well 50 μl of diluted virus was added to 50 μl of incubation medium, then approximately 2.4×10^4 EFK cells in 50 μl was added to each well. Plates were incubated in an atmosphere of 5% CO_2 at 37 °C for 7 days, examined for cytopathic effect and the 50% tissue culture infectious dose per ml ($TCID_{50}$/ml) calculated.

For neutralisation assays, MAbs to EHV2.86/67 were tested by incubating 50 μl of incubation medium containing 100 $TCID_{50}$ of EHV2.86/67 with serial two-fold dilutions of antibody in a volume of 100 μl for 1 h at 37 °C. 2.4×10^4 EFK cells in 50 μl incubation media were then added to the wells and the plates incubated as described above. The neutralisation titre of each MAb was defined as the reciprocal of the highest dilution of MAb giving a 90% reduction in CPE.

ELISA

Enzyme linked immunosorbent assays (ELISA) were performed as described previously [9] except that 96 well plates were coated with EHV2 or EHV5 virions at a concentration of 5 μg/ml and added to plates in a total volume of 100 μl and horseradish peroxidase-conjugated affinity purified rabbit anti-mouse antibody (Dako) diluted 1:1000 was used. Plates were developed using a soluble tetramethylbenzidine substrate (Sigma) at 0.1 mg/ml and 0.005% H_2O_2 and read spectrophotometrically at 450 nm using a Titertek Multiskan (Flow Laboratories). Using an ELISA the antibody titre against EHV2.86/67 of each MAb was determined by measuring absorbance at 450 nm of serial half-log dilutions of each MAb ascites fluid. Titre was defined as the negative log of the dilution giving an absorbance of 0.5 at 450 nm.

EHV2 PCR and DNA sequencing strategy

Primers used for PCR amplification of EHV2 DNA were designed from DNA sequences located in the 5′ non-coding region and 3′ non-coding region of EHV2 gB. Primer sequences were designed from the EHV2.86/67 DNA sequence previously determined and were used to produce a set of four PCR products (Table 1). For PCR approximately 0.1μg of EHV2 DNA was added to the PCR mixture consisting of 50 mM KCl, 10 mM Tris-hydrochloride (pH 8.3), 2.0 mM $MgCl_2$, 200 mM of each dNTP, 25 pmol of each primer and 1 unit of *Taq* polymerase (Promega). Prior to amplification DNA was denatured for 2 min at 95 °C. Samples were amplified for 35 cycles at 56 °C with 30 secs denaturation at 95 °C, 30 secs annealing at 60 °C and extension at 72 °C for times of 1 min per kb of predicted DNA product. The EHV2 gB PCR products were electrophoresed on 1.5% agarose gels, excised and purified using a silica based gel extraction kit (Qiagen) prior to cloning into the plasmid pGEM-T (Promega). The recombinant plasmids were selected by blue/white screening and sequenced from the left and right ends. Double stranded DNA sequencing was carried out using the dideoxy

Table 1. PCR primers used for sequencing of the EHV2 gB and EHV2 vIL-10 genes

PCR primer	Sequence
EHV2 5′ F seq	GCTGCGCTTCTCTGCCAGG
EHV2 5′ R seq	GGTCCTTGTCTTCCGTGTTG
EHV2 3′ R	TTGCTCAGCTCGTACCACAATTGA
EHV2 3′ F	ACAGAGAAAAGGCTGTCCAACG
EHV2 69	AAGTTGTGGCTGAAACGACCA
EHV2 70	CCTGCGTCTTCTCCTGGGA
EHV2- 2.7R	TTGCTCAGCTCGTACCACATGAGCG
EHV2 2.5 F	TGATAACGCTACCAGATTCTTTGC
EHV2 gB 2900.r	AAAGAGCAGATACACTTTATG
EHV2 gB 2.4r	CATAATCCCCCTCATCGTCG
EHV2 gB F.f	GGTGCTGGTCTTTGACTCTAAGG
EHV2 2.6 F	AAACTCAACACAATAGCAGTTCTG
EHV2 IL10 F	GCAGATCAGCCATGTTCAGG
EHV2 IL10 R	CCTCAGTTTTTCATCTTTGTGG

chain-termination method [24] using modified *Taq* polymerase (*fmol* cycle sequence kit, Promega) and ^{35}S-dATP (ICN). The products of sequencing reactions were electrophoresed in 6% polyacrylamide gels containing 4.2% urea. DNA sequencing of recombinant pGEM-T vectors was performed using T-7 forward and SP-6 reverse primers (Promega). Following initial sequencing of the three different strains of EHV2, new primers were designed based on conserved sequences obtained for each strain. These primers were used to sequence further upstream (forward) or downstream (reverse) of the gB gene. The EHV2 interleukin-10 homologue (vIL-10) was amplified by PCR using primers designed from the EHV2.86/67 sequence and DNA sequencing of the PCR products performed directly without cloning. The EHV2 gB gene of an additional six EHV2 isolates was analysed by PCR amplification of the DNA coding for amino acids 17 to 84 using the primers EHV2 5′ Fseq and EHV2 5′ Rseq (Table 1), followed by direct DNA sequencing of the products.

Immunofluorescence analysis

For immunofluorescent antibody studies, EFK cells were grown and infected with EHV2.86/67 on microscope slides (LabTek) and fixed in 90% methanol containing 0.6% hydrogen peroxide. The EFK cells were then blocked with phosphate buffered saline containing 10 mg/ml bovine serum albumin (BSA_{10}PBS) for 2 h at room temperature prior to addition of MAb 19F10 or 2E5 at dilutions ranging from 1:50 to 1:4000 in PBS containing 5 mg/ml bovine serum albumin (BSA_5PBS) for 1 h. Slides were then washed four times in PBS containing 0.05% Tween 20 (Sigma) and probed with fluorescein isothiocyanate-conjugated rabbit anti-mouse antibodies (Dako) at a dilution of 1:40. Optimal visualisation of fluorescence was determined to occur at a MAb dilution of 1:100. For analysis of EHV2 gB strain variation using indirect immunofluorescence (IFA), cell monolayers were infected with two dilutions of virus, corresponding to approximately 1000 and 100 $TCID_{50}$ of each EHV2 strain, EHV5, EHV1 or were mock-infected. At approximately 48 h post infection EFK cell monolayers were fixed and the IFA was performed using a 1:100 dilution of MAbs 2E5 or 19F10.

Results

ELISA

ELISA was performed to assess the ability of MAbs raised to EHV2.86/67 to bind different strains of EHV2 and EHV5. The titre of the MAbs was determined for each of four EHV2 isolates and EHV5 (Table 2a).

The results of the ELISA demonstrated that seven of nine gB-specific MAbs (17A7, 19B1, 19F10, 3B1, 4F10, 17D8, 3D10) had a similar titre when tested

Table 2a. ELISA titre[a] using EHV2.86/67 gB MAbs against four EHV2 strains and EHV5

	Virus				
MAb	EHV2.86/67	EHV2.5FN	EHV2.141	EHV2.T-2	EHV5
4F10	5.7	5.2	2.0	2.0	2.0
17A7	4.2	3.5	1.1	1.1	1.5
19B1	5.1	4.9	1.1	1.1	1.0
19F10	5.8	5.7	1.0	1.0	1.0
17D8	5.8	5.8	1.4	1.4	2.0
3D10	4.9	4.4	1.6	1.6	2.2
3B1	5.2	5.2	2.0	2.0	2.0
2E5	6.4	5.9	5.5	4.2	4.2
15C7	4.2	2.2	2.0	2.0	2.0

[a]Antibody titre against four strains of EHV2 and EHV5 was determined by measuring absorbance at 450 nm of serial half-log dilutions of each MAb ascites fluid. Titre was defined as the negative log of the dilution giving an absorbance of 0.5 at 450 nm

Table 2b. Serum neutralisation assays using EHV2.86/67 MAbs against four EHV2 strains and EHV5

	Virus				
MAb	EHV2.86/67	EHV2.5FN	EHV2.141	EHV2.T-2	EHV5
4F10	3.5[a]	3.0	< 1	< 1	< 1
17A7	3.5	3.5	< 1	< 1	< 1
19B1	3.0	3.0	< 1	< 1	< 1
19F10	3.5	3.5	< 1	< 1	< 1
17D8	4.5	3.5	< 1	< 1	< 1
3D10	3.0	3.0	< 1	< 1	< 1
3B1	3.5	3.5	< 1	< 1	< 1
2E5	< 1[b]	< 1	< 1	< 1	< 1
15C7	< 1	< 1	< 1	< 1	< 1

[a]Titres are expressed as the log of the reciprocal of the highest dilution of MAb giving a 90% reduction in cytopathic effect

[b]Indicates that cytopathic effect was present at the lowest dilution of antibody tested

with EHV2.86/67 or EHV2.5FN but showed no significant binding to EHV2.141, EHV2.T-2 or EHV5 (Table 2a). MAb 15C7 had a higher titre against EHV2.86/67 than for each of the three other EHV2 strains or EHV5. By comparison, MAb 2E5 had a similar titre for all four strains of EHV2 and EHV5 although the titre was 10–100 fold lower against EHV2.141, EHV2.T-2 and EHV5. These findings suggest that the epitope recognised by MAb 2E5 was conserved among the four different strains of EHV2 and EHV5.

Ability of MAbs to neutralise different strains of EHV2 and EHV5

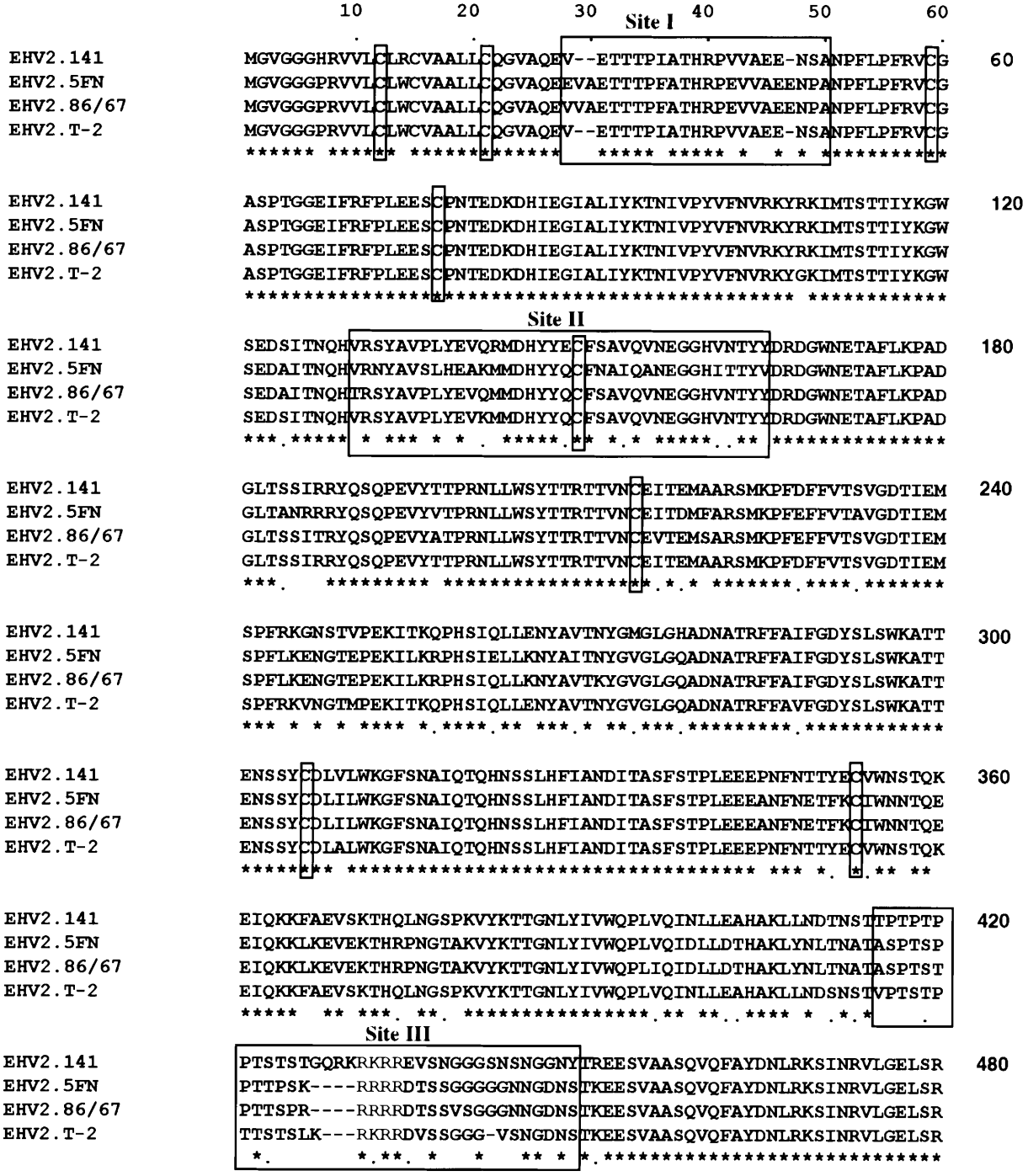
The ability of the MAbs to neutralise EHV2.86/67, EHV2.5FN, EHV2.T-2, EHV2.141 and EHV5 was determined. Seven MAbs (4F10, 3D10, 17D8, 19F10, 19B1, 17A7 and 3B1) neutralised EHV2.86/67 and EHV2.5FN but not EHV2.141, EHV2.T-2 and EHV5 (Table 2b) reflecting their ability to bind these strains in ELISA. MAb 15C7 did not neutralise any of the EHV2 strains or EHV5. The conformation dependent MAb 2E5 did not neutralise any of the EHV2 strains tested or EHV5.

Analysis of multiple strains of EHV2 gB and EHV5 gB by IFA

Monoclonal antibody 2E5 and 19F10 were used in IFA with EHV2.86/67, EHV2.5FN, EHV2.141, EHV2.T-2 and EHV5 infected cells as well as 6 additional strains of EHV2 to determine their antigenic relatedness. Using MAb 2E5, positive fluorescence was seen for all 10 strains of EHV2 tested and for EHV5. Some variability in the intensity of fluorescence in IFA was seen using MAb 2E5, although positive fluorescence was clearly visible for all EHV2 strains tested. By contrast MAb 19F10, capable of binding EHV2.86/67 and EHV2.5FN in ELISA, bound to only six of the 10 strains of EHV2 tested (Table 3). Neither MAb showed fluorescence in IFA analysis of EHV1 or mock-infected EFK monolayers.

Table 3. Analysis of EHV2 strain variability using IFA and DNA sequence analysis

Virus	Year isolated	IFA using MAb 19F10	IFA using MAb 2E5	Site I DNA sequence similarity
EHV2.86/67	1967	+	+	86/67
EHV2.5FN	1969	+	+	86/67
EHV2.-16V	1967	+	+	86/67
EHV2.FN60	1972	+	+	86/67
EHV2.32B	1967	+	+	86/67
EHV2.691	1969	+	+	86/67
EHV2.1039	1994	−	+	141
EHV2.1-57	1967	−	+	141
EHV2.T-2	1983	−	+	141
EHV2.141	1967	−	+	141
EHV5	1967	−	+	Neither similarity


10
20
30
40
50
60
Site I
EHV2.141 MGVGGGHRVVLCLRCVAALLCQGVAQEV--ETTTPIATHRPVVAEE-NSANPFLPFRVCG 60
EHV2.5FN MGVGGGPRVVLCLWCVAALLCQGVAQEEVAETTTPFATHRPEVVAEENPANPFLPFRVCG
EHV2.86/67 MGVGGGPRVVLCLWCVAALLCQGVAQEVVAETTTPFATHRPEVVAEENPANPFLPFRVCG
EHV2.T-2 MGVGGGPRVVLCLWCVAALLCQGVAQEV--ETTTPIATHRPVVAEE-NSANPFLPFRVCG
EHV2.141 ASPTGGEIFRFPLEESCPNTEDKDHIEGIALIYKTNIVPYVFNVRKYRKIMTSTTIYKGW 120
EHV2.5FN ASPTGGEIFRFPLEESCPNTEDKDHIEGIALIYKTNIVPYVFNVRKYRKIMTSTTIYKGW
EHV2.86/67 ASPTGGEIFRFPLEESCPNTEDKDHIEGIALIYKTNIVPYVFNVRKYRKIMTSTTIYKGW
EHV2.T-2 ASPTGGEIFRFPLEESCPNTEDKDHIEGIALIYKTNIVPYVFNVRKYGKIMTSTTIYKGW
Site II
EHV2.141 SEDSITNQHVRSYAVPLYEVQRMDHYYECFSAVQVNEGGHVNTYYDRDGWNETAFLKPAD 180
EHV2.5FN SEDAITNQHVRNYAVSLHEAKMMDHYYQCFNAIQANEGGHITTYYDRDGWNETAFLKPAD
EHV2.86/67 SEDAITNQHIRSYAVPLYEVQMMDHYYQCFSAVQVNEGGHVNTYYDRDGWNETAFLKPAD
EHV2.T-2 SEDSITNQHVRSYAVPLYEVKMMDHYYQCFSAVQVNEGGHVNTYYDRDGWNETAFLKPAD
EHV2.141 GLTSSIRRYQSQPEVYTTPRNLLWSYTTRTTVNCEITEMAARSMKPFDFFVTSVGDTIEM 240
EHV2.5FN GLTANRRRYQSQPEVYVTPRNLLWSYTTRTTVNCEITDMFARSMKPFEFFVTAVGDTIEM
EHV2.86/67 GLTSSITRYQSQPEVYATPRNLLWSYTTRTTVNCEVTEMSARSMKPFEFFVTSVGDTIEM
EHV2.T-2 GLTSSIRRYQSQPEVYTTPRNLLWSYTTRTTVNCEITEMAARSMKPFDFFVTSVGDTIEM
EHV2.141 SPFRKGNSTVPEKITKQPHSIQLLENYAVTNYGMGLGHADNATRFFAIFGDYSLSWKATT 300
EHV2.5FN SPFLKENGTEPEKILKRPHSIELLKNYAITNYGVGLGQADNATRFFAIFGDYSLSWKATT
EHV2.86/67 SPFLKENGTEPEKILKRPHSIQLLKNYAVTKYGVGLGQADNATRFFAIFGDYSLSWKATT
EHV2.T-2 SPFRKVNGTMPEKITKQPHSIQLLENYAVTNYGVGLGQADNATRFFAVFGDYSLSWKATT
EHV2.141 ENSSYCDLVLWKGFSNAIQTQHNSSLHFIANDITASFSTPLEEEPNFNTTYECVWNSTQK 360
EHV2.5FN ENSSYCDLILWKGFSNAIQTQHNSSLHFIANDITASFSTPLEEEANFNETFKCIWNNTQE
EHV2.86/67 ENSSYCDLILWKGFSNAIQTQHNSSLHFIANDITASFSTPLEEEANFNETFKCIWNNTQE
EHV2.T-2 ENSSYCDLALWKGFSNAIQTQHNSSLHFIANDITASFSTPLEEEPNFNTTYECVWNSTQK
EHV2.141 EIQKKFAEVSKTHQLNGSPKVYKTTGNLYIVWQPLVQINLLEAHAKLLNDTNSTTPTPTP 420
EHV2.5FN EIQKKLKEVEKTHRPNGTAKVYKTTGNLYIVWQPLVQIDLLDTHAKLYNLTNATASPTSP
EHV2.86/67 EIQKKLKEVEKTHRPNGTAKVYKTTGNLYIVWQPLIQIDLLDTHAKLYNLTNATASPTST
EHV2.T-2 EIQKKFAEVSKTHQLNGSPKVYKTTGNLYIVWQPLVQINLLEAHAKLLNDSNSTVPTSTP
Site III
EHV2.141 PTSTSTGQRKRKRREVSNGGGSNSNGGNYTREESVAASQVQFAYDNLRKSINRVLGELSR 480
EHV2.5FN PTTPSK----RRRRDTSSGGGGGNNGDNSTKEESVAASQVQFAYDNLRKSINRVLGELSR
EHV2.86/67 PTTSPR----RRRRDTSSVSGGGNNGDNSTKEESVAASQVQFAYDNLRKSINRVLGELSR
EHV2.T-2 TTSTSLK---RKRRDVSSGGG-VSNGDNSTKEESVAASQVQFAYDNLRKSINRVLGELSR

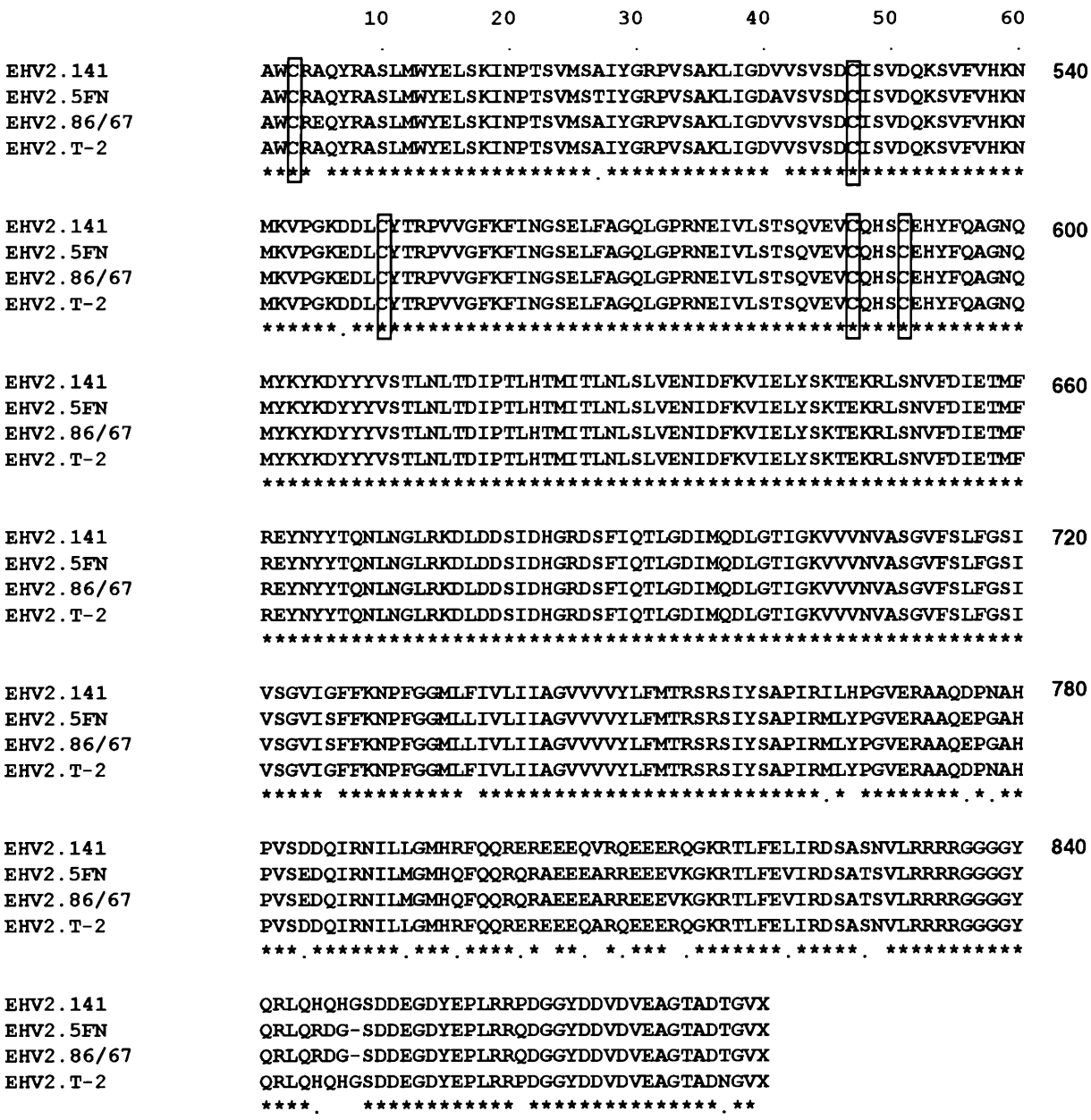


Fig. 2. Multiple sequence alignment of the deduced amino acid sequence of the EHV2.86/67 gB gene with the amino acid sequences of EHV2.5FN, EHV2.141 and EHV2.T-2 using the program ECLUSTALW. Identical amino acids are marked with an asterisk and conserved amino acids are marked with a dot. Dashes represent gaps introduced to optimise alignments. Three sites (Sites I-III) of increased amino acid variability (boxes) are shown. The amino acid residue numbers are indicated to the right of the alignment

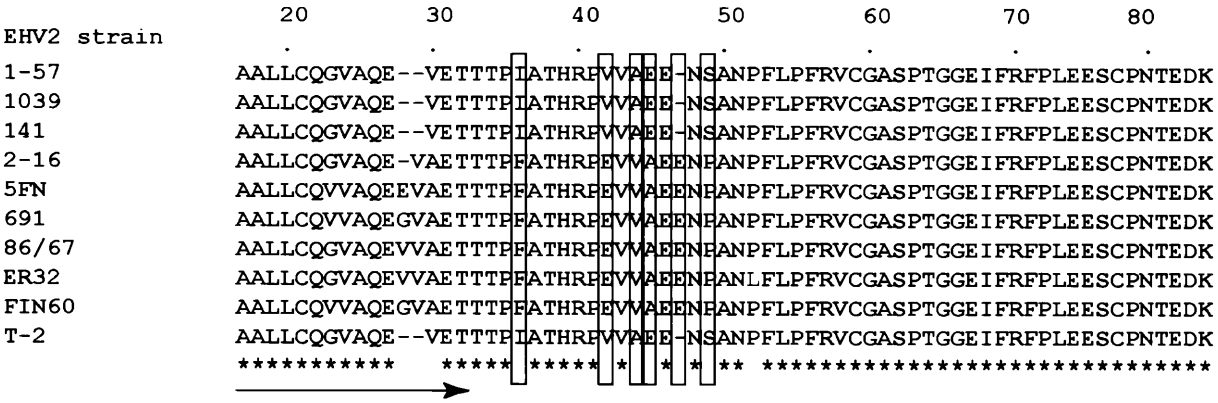


Fig. 3. Multiple alignment of amino acids 17–84 of 10 genomically diverse strains of EHV2 using the computer program ECLUSTALW. The predicted signal cleavage site is shown (arrow) and 5 amino acid substitutions in Site I are shown (boxes)

DNA sequence analysis of gB from four genomically divergent strains

The gB coding sequences of four genomically divergent strains of EHV2 varied from 871 to 877 amino acids because of deletions of nucleotides at one or more sites. The deduced amino acid sequences of the four divergent strains of EHV2 were aligned using the computer program ECLUSTALW of the GCG package (Wisconsin) (Fig. 2). Amino acid variability was mapped to three regions of the EHV2 gB gene. These regions were located between amino acids 30–49 (Site I), 130–165 (Site II) and 415–448 (Site III) (Fig. 2). Site I encompassed the first 19 amino acids of EHV2 gB following removal of the signal sequence and Site III encompassed the region immediately N-terminal to the predicted endoproteolytic cleavage site. Computer analysis using the GCG program BESTFIT showed that the EHV2 gB amino acid sequences ranged between 94–96% identity.

Alignment of the predicted amino acid sequences of the 10 strains of EHV2 showed that the pattern of deletions and point mutations, demonstrated previously between amino acids 30 and 49 of gB (Site I) in the four strains completely sequenced, was present among the seven other strains of EHV2 (Fig. 3). There was some variation in amino acids 28 and 29 with several strains having two amino acids deleted, although these were predicted to be located in the signal sequence and would be assumed not to be found in the mature envelope protein.

DNA sequence analysis of vIL-10 of four EHV2 strains

To determine if the genetic variation observed within gB was also present in other genes of the EHV2 genome the complete nucleotide sequence of the vIL-10 homologue of EHV2.5FN, EHV2.141 and EHV2.T-2 was determined and the deduced amino acid sequence compared with EHV2.86/67. By contrast with the variation observed for gB, there was 99% identity between the vIL-10 of EHV2 strains with conservative amino acid changes occurring at positions 5 and 82 (Fig. 4).

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                          10         20         30         40         50         60
                           .          .          .          .          .          .
EHV-2.141          YDSESGDDCPTLPTSLPHMLHELRAAFSRVKTFFQMKDQLDNMLLDGSLLEDFKGYLGCQ
EHV-2.5FN          YDSEAGDDCPTLPTSLPHMLHELRAAFSRVKTFFQMKDQLDNMLLDGSLLEDFKGYLGCQ
EHV-2.86           YDSESGDDCPTLPTSLPHMLHELRAAFSRVKTFFQMKDQLDNMLLDGSLLEDFKGYLGCQ
EHV-2.T2           YDSESGDDCPTLPTSLPHMLHELRAAFSRVKTFFQMKDQLDNMLLDGSLLEDFKGYLGCQ
                   ****.************************************************************

EHV-2.141          ALSEMIQFYLEEVMPQAENHSTDQEKDKVNSLGEKLKTLRVRLRRCHRFLPCENKSKAVE
EHV-2.5FN          ALSEMIQFYLEEVMPQAENHSTDQEKDKVNSLGEKLKTLRVRLRRCHRFLPCENKSKAVE
EHV-2.86           ALSEMIQFYLEEVMPQAENHSTDQEKDKVNSLGEKLKTLRVRLRRCHRFLPCENKSKAVE
EHV-2.T2           ALSEMIQFYLEEVMPQAENHSTGQEKDKVNSLGEKLKTLRVRLRRCHRFLPCENKSKAVE
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EHV-2.141          QVKSAFSKLQEKGVYKAMSEFDIFINYIEAYMTTKMKN
EHV-2.5FN          QVKSAFSKLQEKGVYKAMSEFDIFINYIEAYMTTKMKN
EHV-2.86           QVKSAFSKLQEKGVYKAMSEFDIFINYIEAYMTTKMKN
EHV-2.T2           QVKSAFSKLQEKGVYKAMSEFDIFINYIEAYMTTKMKN
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Fig. 4. Multiple sequence alignment of the deduced amino acid sequence of the EHV2.86/67 vIL-10 gene with the amino acid sequences of EHV2.5FN, EHV2.141 and EHV2.T-2 using the program ECLUSTALW. Identical amino acids are marked with an asterisk and conserved amino acids are marked with a dot

Discussion

Comparison of the predicted amino acid sequence of gB from four strains of EHV2 using the computer program BESTFIT showed them to have 94–96% identity. Three regions of variation were observed and located between amino acids 30–49 (Site I), 130–165 (Site II) and 415–448 (Site III). Variability at Site I encompassed the region previously identified as containing the epitope for seven neutralising MAbs [14]. The exact boundaries of the binding sites of the seven neutralising MAbs were not determined although comparison of the variability in the amino acid sequence of the four strains indicated this to be most likely between amino acids 30–49. Our previous results suggested that the non-neutralising MAb 15C7, recognized a linear epitope located in the 89 kDa subunit of gB outside of Site I [14]. The epitope of MAb 15C7 was not determined but the reduced reactivity with EHV2.141, EHV2.T-2 and EHV2.5FN in ELISA would suggest the epitope of MAb 15C7 lies within the first 480 amino acids of EHV2.86/67 gB showing amino acid variability from these strains of EHV2. Comparison of the gB amino acid alignment for all four strains of EHV2 suggests that the epitope could lie within the Site II or Site III regions of variability.

The results of the IFA and sequence analysis of Site I from different strains of EHV2 suggested that EHV2 isolates would form two groups, EHV2.86/67-like and EHV2.141-like. The importance of Site I was suggested by the failure of the seven MAbs that recognise an epitope in the N-terminal region of gB to neutralise EHV2.141 and EHV2.T-2 in MAb neutralisation tests. In this respect, our findings of a variation in serum neutralisation of different strains of EHV2 using MAbs is

similar to findings obtained previously using rabbit polyclonal serum produced against whole virus [22]. Previous studies of HSV-1 [17], pseudorabies virus [29] and human cytomegalovirus (HCMV) [19] have identified the presence of MAb neutralisation epitopes located within the first 100 amino acids of the N-terminal region of gB. A human MAb which had potent, complement independent neutralising activity has been mapped to an area of HCMV gB located between amino acids 68–77 [18]. This region is positionally homologous to the location of EHV2 neutralising MAbs although there is no conservation of amino acid sequence between HCMV and EHV2 gB at this site. Additionally, an antigenic domain in the amino terminus of HCMV gB between amino acids 50–54 has been shown to be strain-dependent when examined with murine MAbs [19]. The importance of this N-terminal region of gB is unknown although the finding that MAbs directed against this region are neutralising suggests that the region of gB may have a function in virion attachment or penetration. Variation in the amino acid sequence of this region may be related to different receptor types used by the different herpesviruses. It is believed that antigenic variation among virus strains serves to evade the immune system of the host although other regions of EHV2 gB may contain functionally important epitopes that are conserved among strains. Based on our previous results of Western blot analysis with a EHV2 gB-GST fusion protein, EHV2.86/67 specific equine sera contains antibodies with binding specificity for Site I [14].

There was a good correlation of our DNA sequencing data and MAb neutralisation data with the cross-neutralisation data of Plummer et al. [22], suggesting that EHV2.86/67 was antigenically related but distinct from EHV2.141. The results of the IFA and sequence analysis of Site I from different strains of EHV2 suggested that EHV2 isolates would form two groups, EHV2.86/67-like and EHV2.141-like (Table 2). The functional consequences of variation in EHV2 gB at Site I are unknown although variation in the size of plaques and time to full cytopathic effect (CPE) exists for different EHV2 isolates [6]. A direct correlation was not established between plaque size and gB type as to whether plaques were similar in size in strains that were EHV2.86/67-like EHV2.141-like. It is likely that other genes besides gB may influence plaque size. Furthermore, adaptation to cell culture conditions by multiple cell culture passage may influence the time of onset of CPE for herpesviruses and the number of passages for each strain of EHV2 used in the present study varied considerably.

The basis for antigenic variation in EHV2 gB is undetermined. One possibility is that random mutations arising in the EHV2 genome would periodically result in antigenically variant viruses that are then rapidly dispersed throughout an immunologically naive equine population. In this respect genomic variation would be considerably greater than antigenic variation. However, the establishment of lifelong latency in B-lymphocytes by EHV2 may prevent the replacement of existing strains of EHV2. As such, several strains of EHV2 would be expected to be found in the equine population concurrently. Such a theory is in accordance with clinical observations of the isolation of several genomically different isolates from an individual horse [4]. By comparison with EHV2 gB the EHV2 vIL-

10 homologue showed minimal amino acid variability between strains possibly reflecting a positive selection against mutation in a gene with potential survival advantages for the virus.

An alternative explanation for the genetic differences in gB seen between the EHV2 strains might be that they are not related to immunological selection, but rather reflect selection related to independent mechanisms such as the ability of the mutated virus to replicate in different cell populations. It might be expected that mutations preventing the normal folding of EHV2 gB would be selected against. Similarly, mutations in regions of gB important in binding to receptors or membrane fusion might also be selected against. In this regard there is little information on important structural domains of gB among the gammaherpesviruses. For the betaherpesvirus HCMV the region between amino acids 560 and 640 of gB is highly conserved among different strains of HCMV and comparatively, amino acids 560 to 680 appear to be highly conserved among the four strains of EHV2 examined. This region showed considerable homology to EHV2 gB and contained several conserved amino acid sequences including the sequence GQLG which has been reported to be conserved among all examined herpesviruses [12]. Additionally, in this region four cysteine residues and the predicted site of N-linked glycosylation were conserved among all the gammaherpesviruses and HCMV, suggesting these regions are functionally important. This region of HCMV has been suggested to be important in the early folding of HCMV gB and mutations in this sequence result in a misfolded molecule which is not transported in the cell [3]. The conservation of this region among different strains of EHV2 suggests a similar important function in maintaining correct conformation of EHV2 gB.

The genetic variation of gB among different strains of EHV2 also has implications for studies in which primers based on the EHV2.86/67 sequence were used [23]. From our analyses previous gB based PCR primer sets would not amplify all strains of EHV2 and as such give minimal estimates of the number of EHV2 infected horses. The complete sequence of 3 additional EHV2 isolates presented here will facilitate the design of primers for future epidemiological studies intended to amplify all EHV2 strains.

Based on the analysis of Site I of 10 strains of EHV2 using sequencing and IFA, approximately 40% of EHV2 strains might be expected to be EHV2.141-like. Further analysis of the amino acid sequence of additional EHV2 glycoproteins may provide further insight into antigenically variable regions of EHV2 important with regard to strain variation and explain the observation of multiple infections with genomically variable strains of EHV2. Such information may improve our understanding of the clinical importance of this common equine virus.

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References

1. Agius CT, Nagesha HS, Studdert MJ (1992) Equine herpesvirus 5: Comparisons with EHV2 (equine cytomegalovirus), cloning and mapping of a new equine herpesvirus with a novel genome structure. Virology 191: 176–186
2. Blakeslee JR Jr, Olsen RG, McAllister ES, Fassbender J, Dennis R (1975) Evidence of respiratory tract infection induced by equine herpesvirus, type 2, in the horse. Can J Microbiol 21: 1940–1946
3. Britt WJ, Mach M (1996) Human cytomegalovirus glycoproteins. Intervirology 39: 401–412
4. Browning GF, Studdert MJ (1987) Epidemiology of equine herpesvirus 2 (equine cytomegalovirus). J Clin Microbiol 25: 13–16
5. Browning GF, Studdert MJ (1987) Genomic heterogeneity of equine betaherpesviruses. J Gen Virol 68: 1 441–1 447
6. Browning GF, Studdert MJ (1988) Equine herpesvirus 2 (equine cytomegalovirus). Vet Bull 58: 775–790
7. Browning GF, Studdert MJ (1989) Physical mapping of a genome of equine hepesvirus 2 (equine cytomegalovirus). Arch Virol 104: 77–86
8. Browning GF, Studdert MJ (1989) Physical mapping of the genomic heterogeneity of isolates of equine herpesvirus 2 (equine cytomegalovirus). Arch Virol 104: 87–94
9. Crabb BS, Allen GP, Studdert MJ (1991) Characterization of the major glycoproteins of equine herpesviruses 4 and 1 and asinine herpesvirus 3 using monoclonal antibodies. J Gen Virol 72: 2 075–2 082
10. Crabb BS, Studdert MJ (1990) Comparative studies of the proteins of equine herpesviruses 4 and 1 and asinine herpesvirus 3: antibody response of the natural hosts. J Virol 71: 2 033–2 041
11. Felsenstein J (1989) PHYLIP. Phylogeny Inference Package (Version 3.2). Cladistics 5: 164–166
12. Goltz M, Broll H, Mankertz A, Weigelt W, Ludwig H, Buhk HJ, Borchers K (1994) Glycoprotein B of bovine herpesvirus type 4: its phylogenetic relationship to gB equivalents of the herpesviruses. Virus Genes 9: 53–59
13. Holloway SA, Lindquester GJ, Studdert MJ, Drummer HE (1999) Identification, sequence analysis and characterisation of equine herpesvirus 5 glycoprotein B. Arch Virol 144: 288–307
14. Holloway SA, Studdert MJ, Drummer HE (1998) Characterisation of equine herpesvirus 2 glycoprotein B. J Gen Virol 79: 1 619–1 629
15. Jolly PD, Tu ZF, Robinson AJ (1986) Viruses associated with respiratory disease of horses in New Zealand: An update. N Z Vet J 34: 46–50
16. Kemeny L, Pearson JE (1970) Isolation of herpesvirus from equine leukocytes: comparison with equine rhinopneumonitis virus. Can J Comp Med 34: 59–65
17. Kousoulas KG, Arsenakis M, Pereira L (1989) A subset of type specific epitopes map in the amino terminus of herpes simplex virus 1 glycoprotein B. J Gen Virol 70: 735–741
18. Meyer H, Mashuso Y, Mach M (1990) The g58/116 complex of human cytomegalovirus represents the amino terminal part of the precursor molecule and contains a neutralizing epitope. J Gen Virol 71: 2 443–2 450
19. Meyer H, Sundqvist VA, Pereira L, Mach M (1992) Glycoprotein gp 116 of human cytomegalovirus contains epitopes for strain common and strain specific antibodies. J Gen Virol 73: 2 375–2 383
20. Palfi V, Belak S, Molnar T (1978) Isolation of equine herpesvirus type 2 from foals, showing respiratory symptoms. Zbl Veterinarmed B 25: 165–167

21. Pereira L (1994) Function of glycoprotein B homologues of the family Herpesviridae. Infect Agents Dis 3: 9–28
22. Plummer G, Goodheart CR, Studdert MJ (1973) Equine herpesviruses: antigenic relationships and deoxyribonucleic acid densities. Infect Immun 8: 621–627
23. Reubel GH, Crabb BS, Studdert MJ (1995) Diagnosis of equine gammaherpesvirus 2 and 5 infections by polymerase chain reaction. Arch Virol 140: 1 049–1 060
24. Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA 74: 5 463–5 467
25. Schlocker N, Gerberbretscher R, Vonfellenberg R (1995) Equine herpesvirus 2 in pulmonary macrophages of horses. Am J Vet 56: 749–754
26. Studdert MJ (1971) Equine herpesviruses. 4. Concurrent infection in horses with strangels and conjunctivitis. Aust Vet J 47: 434–436
27. Telford EA, Watson MS, Aird HC, Perry J, Davison AJ (1995) The DNA sequence of equine herpesvirus 2. J Mol Biol 249: 520–528
28. Wilks CR, Studdert MJ (1974) Equine herpesviruses. 5. Epizootiology of slowly cytopathic viruses in foals. Aust Vet J 50: 438–442
29. Zaripov MM, Morenkov OS, Fodo N, Brown A, Schmatchenko VV, Fodor I (1999) Distribution of B-cell epitopes on the pseudorabies virus glycoprotein B. J Gen Virol 80: 537–541

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