

# Analysis of Equid Herpesvirus 1 Strain Variation Reveals a Point Mutation of the DNA Polymerase Strongly Associated with Neuropathogenic versus Nonneuropathogenic Disease Outbreaks

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**Equid herpesvirus 1 (EHV-1) can cause a wide spectrum of diseases ranging from inapparent respiratory infection to the induction of abortion and, in extreme cases, neurological disease resulting in paralysis and ultimately death. It has been suggested that distinct strains of EHV-1 that differ in pathogenic capacity circulate in the field. In order to investigate this hypothesis, it was necessary to identify genetic markers that allow subgroups of related strains to be identified. We have determined all of the genetic differences between a neuropathogenic strain (Ab4) and a nonneuropathogenic strain (V592) of EHV-1 and developed PCR/sequencing procedures enabling differentiation of EHV-1 strains circulating in the field. The results indicate the occurrence of several major genetic subgroups of EHV-1 among isolates recovered from outbreaks over the course of 30 years, consistent with the proposal that distinct strains of EHV-1 circulate in the field. Moreover, there is evidence that certain strain groups are geographically restricted, being recovered predominantly from outbreaks occurring in either North America or Europe. Significantly, variation of a single amino acid of the DNA polymerase is strongly associated with neurological versus nonneurological disease outbreaks. Strikingly, this variant amino acid occurs at a highly conserved position for herpesvirus DNA polymerases, suggesting an important functional role.**

Equid herpesvirus 1 (EHV-1), a member of the subfamily *Alphaherpesvirinae*, is a highly prevalent equine pathogen that can cause a range of clinical signs, from respiratory distress to the induction of abortion, neonatal foal death, and occasionally neurological damage resulting in paralysis (9, 14, 19, 33, 37, 61). The severity of disease resulting from EHV-1 infection is likely to be influenced by a number of factors, including the age and physical condition of the host; whether the infection is primary, a reinfection, or a reactivation of latent virus; the immune status of the host; and the pathogenic potential of the strain involved. In order to assess the relative importance of EHV-1 strain variation regarding disease outcome, it is necessary to develop methods enabling precise discrimination between genetic subgroups of interrelated strains. Previous studies have utilized DNA restriction fragment length polymorphism (RFLP) to separate field isolates of EHV-1 into subgroups according to characteristic restriction enzyme site changes and the presence of variable numbers of copies of short sequence repeats. These studies demonstrated a relatively low frequency of genetic polymorphism for EHV-1 and suggested that distinct strains of

EHV-1 do exist in the field (3, 4, 8, 25, 32, 41, 54, 57). However, the relative lack of variation of EHV-1 sequences between strains has resulted in too few RFLP variants to be identified for detailed epidemiological studies. Furthermore, although such analyses may be used for tracing the genetic relatedness of strains, they allow identification only of those genetic changes resulting in restriction fragment variation, rather than the majority of changes that do not affect restriction cleavage sites.

Studies of the pathogenesis of EHV-1 infection, in particular relating to induction of abortion and neurological damage, have demonstrated differences in pathogenic potential correlated with differences in the ability to disseminate to and establish infection at vascular endothelial sites, in particular within the endometrium and central nervous system (CNS) (1, 17, 38, 42, 44, 60). Infection of endothelial cells is associated with vasculitis and thrombosis, with the resulting restricted blood flow leading to ischemic damage at the utero-placental junction and the CNS. Two field isolates of EHV-1 which show consistent differences in pathogenicity have been extensively characterized. Ab4, a neuropathogenic strain, was originally isolated from a case of paralytic disease (14) and V592, a nonneuropathogenic strain, from a multicasel outbreak of abortion, during which no neurological disease was reported (39). In experimental infections of horses, Ab4 infection resulted in severe clinical disease, including a high frequency of abortion and a less frequent occurrence of paralysis. In contrast, disease following experimental V592 infection was largely restricted to a short-lived fever and mild respiratory signs, with infrequent induction of abortion and no neurological damage (38, 51, 52).

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These differences in clinical outcome may be related to observed differences in replication of these strains in vivo in leukocytes and endothelial cells: Ab4 typically caused a prolonged high level of viremia and widespread endothelial cell infection in experimental challenge, whereas V592 typically caused a more short-lived, low-level viremia and restricted or absent endothelial cell infection (38, 52).

In order to identify a genetic basis for the observed differences in pathogenic potential of the prototypic neuropathogenic and nonneuropathogenic isolates described above, we have determined the complete genomic sequence of V592 (16) (GenBank accession number AY464052). The information generated, in conjunction with the previously determined sequence of Ab4 (56) (GenBank accession number AY665713), has provided a valuable resource for identification of multiple loci of sequence variation between two EHV-1 pathotypes, which may be useful for subtyping of EHV-1 field isolates. In this paper, we report on amino acid coding variation identified between EHV-1 strains V592 and Ab4. We further describe the testing of selected genetic markers, comprising either variable-repeat-length regions or nucleic acid point mutations, against a panel of EHV-1 field isolates from various European countries, North and South America, and Australia. The sequence results for the selected markers were compared and analyzed according to the geographical origins and disease classifications (either neuropathogenic or nonneuropathogenic) of the outbreaks from which the field isolates were derived. The most significant finding is that variation of a single amino acid of the DNA polymerase is strongly associated with neuropathogenic versus nonneuropathogenic disease outbreaks.

## MATERIALS AND METHODS

**Viruses and cells.** The majority of EHV-1 field isolates were obtained from archive material held at the Animal Health Trust (United Kingdom isolates) and the Gluck Equine Research Center (U.S. and Canadian isolates). Additional isolates, or purified DNA from isolates, originating from outbreaks in other countries were provided by G. Fortier (Laboratoire Frank Duncombe, France), M. Studdert (University of Melbourne, Australia), S. Raidal (Murdoch University, Australia), C. van Maanen (Animal Health Service, The Netherlands), C. Galosi (Cátedra de Virología, UNLP, Argentina), and K. van der Meulen (University of Ghent, Belgium). The majority of isolates were obtained by culture of diagnostic material (nasal swab or blood or tissue samples) on rabbit kidney (RK-13) or equine dermal (NBL-6) cell lines. Some isolates were obtained by culture on primary equine (equine embryonic lung [EEL] or equine fetal kidney) cell types.

In order to provide a consistent nomenclature, isolates were coded according to their country of origin (C), year of outbreak (Y), a unique identifier for the outbreak (x: 1, 2, 3, etc.), and the pathogenic features of the outbreak (p: 0, attenuated vaccine strain; 1, nonneuropathogenic; 2, neuropathogenic) according to the scheme CCYY\_x\_p. Countries of origin are designated AR (Argentina), AU (Australia), BE (Belgium), CA (Canada), FR (France), GB (Great Britain), NL (The Netherlands), PL (Poland), and US (United States).

**Preparation and shotgun cloning of purified virion DNA from V592.** DNA was prepared from purified virions essentially as previously described (49, 56). Equine fibroblasts (EEL) were infected at low multiplicity and incubated at 37°C until the complete cytopathic effect had developed. The tissue culture medium was harvested and centrifuged at low speed (1,500 × g, 5 min) to remove cell debris, and the supernatant was centrifuged at high speed (17,000 × g, 200 min) to pellet virions. Pellets were resuspended in 5 ml minimum essential medium (Eagle)-2% fetal calf serum and then purified via a sucrose gradient (40% to 60% sucrose in phosphate-buffered saline), with high-speed centrifugation (69,000 × g, 120 min). The virion material present at the sucrose gradient interface was collected and pelleted by centrifugation (17,000 × g, 180 min), and the pellet was resuspended in 1 ml Tris-EDTA buffer (10 mM Tris, 0.1 mM EDTA, pH 8).

Virion DNA was prepared by proteinase K-sodium dodecyl sulfate digestion followed by phenol-chloroform extraction and ethanol precipitation. The DNA (redissolved in Tris-EDTA buffer) was self-ligated (T4 DNA ligase) to remove free ends and then sonicated to generate randomly sheared fragments. Sonicated DNA was gel purified, and fragments with an approximate size range of 300 to 1,000 bp were selected. Fragments were cloned into M13mp19 via blunt-end cloning with a Perfectly Blunt cloning kit (Novagen, Madison, WI). In addition, a panel of semirandom clones was generated by digestion of viral DNA with frequent cutting restriction enzymes (AluI, PvuII, and BalI), followed by cloning into M13mp19 as described above.

**Determination of V592 complete genomic sequence.** Single-stranded M13 templates were prepared and sequenced using proprietary sequencing reagents and methods of the ABI Prism DNA sequencing guide (Applied Biosystems), with sequencing reactions analyzed with either an ABI 377 or an ABI 3700 automated sequencer. Sequence reads were assembled using the Staden sequence analysis programs PREGAP4 and GAP4 (53), run using the LINUX operating system. In the latter stages of assembly, sequence data for the gaps between contigs were obtained via PCR amplification from virion DNA of fragments spanning the gap regions that were then cloned into M13mp19 and sequenced as described above (a minimum of three independent M13 clones were sequenced for each PCR-amplified region). In the final assembly, each nucleotide of the V592 sequence was determined, on average, 5.27 times. The sequences of the genomic termini of V592 were not specifically determined in this study but rather were assumed to be identical to those of the previously sequenced Ab4 strain.

**Preparation of DNA from field isolates.** DNA samples were prepared from a panel of isolates originating from a number of different countries, recovered from outbreaks of either neurological or nonneurological disease. DNA was prepared from either a pooled tissue sample or infected cells (rabbit kidney [RK-13], equine dermal [NBL-6], or primary equine [EEL or equine fetal kidney] cells) by standard proteolytic digestion using a High Pure PCR template preparation kit (Roche Diagnostics GmbH, Mannheim, Germany). Briefly, for DNA extraction from pooled tissue samples, approximately 25 mg of tissue was ground in 2 ml of a 2% minimum essential medium (Eagle) solution by using a glass homogenizer; to 200 µl of this solution 200 µl lysis buffer and 40 µl proteinase K were added, and this mixture was incubated for 1 h at 55°C. Binding buffer (200 µl) was added, and the mixture was further incubated for 10 min at 72°C. Isopropanol (100 µl) was added, and the mixture was transferred to the upper reservoir of a combined High Pure filter tube-collection tube assembly. The DNA sample was then purified by a series of rapid wash and spin steps and eluted by the addition of 200 µl prewarmed elution buffer. For DNA extraction from either EEL or RK-13 cells, a 1-ml cell suspension (approximately 10<sup>6</sup> cells/ml) was centrifuged for 5 min at 8,000 rpm and the cells were resuspended in 200 µl phosphate-buffered saline. Binding buffer (200 µl) and proteinase K (40 µl) were added to the resuspended cell pellet, and the mixture was incubated for 10 min at 72°C. Isopropanol (100 µl) was added, and DNA was purified as described above.

**PCR amplification and sequencing of selected variable loci of field isolates.** PCR primers used for amplification and sequencing of selected loci are detailed in Table 1. The PCR mix (50 µl) consisted of 0.3 µM of each primer (Proligo, France), 0.2 mM of each deoxynucleoside triphosphate (Applied Biosystems), 3× PCRx Enhancer solution (Invitrogen, United Kingdom), and 1.25 U AmpliTaq DNA polymerase (Applied Biosystems) in 10 mM Tris-HCl (pH 8.3) solution containing 1.5 mM MgCl<sub>2</sub> (Applied Biosystems). The PCR was denatured for 4 min at 94°C and then cycled for 35 cycles for 30 s at 94°C, 1 min at the annealing temperature of the primers used, and 2 min at 72°C, followed by a final step of 10 min at 72°C. After cycling, 10 µl of each PCR product was analyzed on a 1.0% agarose gel containing ethidium bromide. Following product identification, the PCR products were purified using a QIAquick PCR purification kit (QIAGEN Ltd., Sussex, United Kingdom) and quantified on a 2% agarose gel containing ethidium bromide, with DNA quantification standards (Gibco BRL, Life Technologies, United Kingdom).

**Sequencing reactions and sequence analysis.** The purified PCR products were directly sequenced using ABI sequencing reagents (dRhodamine or Big Dye) according to the manufacturer's instructions and EHV-1 specific sequencing primers (Table 1). The extension products were purified by a series of ethanol washes, and the purified products were analyzed using either an ABI 3700 or an ABI 3100 automated sequencer. Sequences were assembled using the DNASTAR program SeqManII (version 4.03; DNASTAR, Inc.). Following sequence assembly, further analysis, in particular, identification of coding changes and multiple alignment of homologous sequences, was carried out using the OMIGA software package (version 2.0; Oxford Molecular Ltd., United Kingdom), BioEdit (version 7.0.1.4 [21]), or MEGA2 (26).

TABLE 1. PCR amplification and sequencing primers

ORF	Amplification primers		Sequencing primer(s)	
	Name	Sequence (5'–3')	Name	Sequence (5'–3')
8	8f 8r	AATTATTGGTGGGCAGAACC TTCTCCAGCACAGCAACG	8s	CCATCACCACGCTATTCTC
11	11f 11r	CTGTAACGATGCTAACGCTACG TTCAGAAACCTGTCCAGGTCC	11sf1	TAGCCCAAACGACGACAAAAG
15	15f 15r	CAAAGCGCAAAGCAAAATGC GGGCCTTGTAGATCAGCTCTCC	15sf1	ATCCAACACACGCAACATC
30	30f1 30r1 30f2 30r2	GCGCTACTTCTGAAAACG CCACAACTTGATAAACACG AAACCACAAGCCTTACTCC GCGCAACTATCACATACG	30-1s 30-1sr1 30-2s 30-2sr1	TACCCAAGCATTATCCAG GATAACCCTGACGGAGTAAG GAGAAGACCTTTCAGCGAC CTCAGCAGTCATAACGAAC
31	31f 31r	GGACTTACCGTCGAACAGACC ACCCAGTACGTAGCCATGAAGC	31sr1 31sr	GGAGACAGGTTGAGAGAAG CAGCCTCGCAAGCGCGGGTGAGGT
32	32f 32r	TGATTACTACTACCCCCCTCC AGCCAGATTACGTCATCC	32sf1	TATTTCTGACACCCACCAC
37	37f 37r	ACATACTCCGCCATCTCC ACAGCTACATGACTCGTGC	37s	CCATCTATAACGCCAGCC
40	40f 40r	ATCACAAACGCATCCTCG CACAGCTACCCAAAATACC	40s	ACTCGCCGAATCCCAGATG
42	42f1 42r1	ATCCAGCGCAACTCTTAC GTCCGTAGAGCTGAGTAGAG	42fs1 42rs1	GGCAAGTATAACCTGAACGG TGAAACAGCGAACACGGGTC
45	45f 45r	TGGCACGAAAGCACTACG AAACCTGCACGAATGTTGG	45sr1	TGATCTATCAGAGCCAAC
50	50f 50r	ACTCGACTCAGACTACATTGG ATAGCAGAATGGGGATAGC	50sr1	GCTCACTAAATAGCCAAAC
52	52f 52r	CCAACAGCTTTCACATCACC TTCAAATTCCTCGCTCACC	52s	GCTACTTCGTTCACTTCC
57	57f1 57r1	GGTGCTGAACGTAAACCTG CTCAGGGCTGCTAATATATGC	57fs1 57rs1	CGTAAACCTGAGCGAG GTAGAAACCTTGACGACACGG
67	67f 67r	CAAACTCCAACACCACG GAGAAGAGGTTCTACAAGCAGC	67s	GAAGAAGCATTCGCCAAG
68	68f 68r	AGCATTGCCAAACAGTTCC CAAGAAACCACTGCTCAACC	68s1 68s3 68r2 68r3	GAAGATAGAATGGGTGTGAG ACCGTTGAGCATAATCATCC GTCCCTACCTTTTAACG CGGCGGCCGTGGACCGA
73	73f 73r	GAACATATGAGCATGTACC GACCAGTAGAACATCTTCC	73s	ACGGACAACGCTTACCCAC

**Statistical methods.** Fisher's exact test was used to test the null hypotheses that there were no differences in the relative proportions of open reading frame 68 (ORF68) groups between (i) isolates originating from either Europe or North America and (ii) isolates that were from outbreaks characterized clinically as either neurological or nonneurological. The same test was also used to test the null hypothesis that there was no difference in the relative proportions of specific single nucleotide polymorphisms (SNPs) between isolates that were from neurological or nonneurological outbreaks.

**Nucleotide sequence accession numbers.** The nucleotide sequences determined in this study were submitted to GenBank and assigned accession numbers as follows: for the EHV-1 strain V592 complete genome, AY464052; for the EHV-1 isolate ORF30 sequences, DQ180606 to DQ180738; and for the EHV-1 isolate ORF68 sequences, DQ172308 to DQ172415.

## RESULTS

**Comparison between the genomic sequences of Ab4 and V592.** The V592 genomic sequence (149,430 bp) was determined via shotgun cloning and sequencing of DNA prepared from purified virions. The V592 stock used to generate DNA for sequencing had not been plaque purified and contained a mixture of DNA populations at certain sites. It should be noted that the determined sequence is the consensus for the majority of sequence reads. Sites with heterogeneity between individual sequencing templates are shown in Table 2. These sites are all

TABLE 2. Sequence heterogeneity between individual sequence templates of V592

Position <sup>a</sup>	ORF	Variation
105–308	NA <sup>b</sup>	10–13 copies of 17-bp repeat element
366–707	NA	14–19 copies of 18-bp repeat element
1959	2	Substitution of A to G in minority of reads (2/9)
73803–74090	NA	8–9 copies of 32-bp repeat element
108895–108984	NA	4–5 copies of 18-bp repeat element
112316–112419	NA	8–9 copies of 13-bp repeat element
123549–123656	NA	2–4 copies of 27-bp repeat element
129328–129552	71	17–18 copies of 15-bp repeat element

<sup>a</sup> Nucleotide positions are numbered according to the V592 genomic sequence.

<sup>b</sup> NA, not applicable.

regions of variable repeat length, apart from one position of single-nucleotide heterogeneity.

The V592 sequence was compared with that of Ab4 (56) to identify all positions of sequence variation and to determine amino acid coding changes for known ORFs. Overall, there was a nucleotide variation rate of approximately 0.1%, with 50 regions of insertion or deletion of 1 or more nucleotides and 110 single nucleotide substitutions, of which 43 resulted in amino acid coding changes. A total of 31 of 76 ORFs had amino acid variations between the two strains, as shown in Table 3. Two ORFs (ORF24 and ORF71) showed variable copy numbers of short nucleotide direct repeat elements, in agreement with previous studies that had identified variation in repeat element copy numbers for ORF24 and ORF71 among EHV-1 field isolates (8, 22, 25, 32). Such regions are relatively unstable and therefore of limited use for epidemiological studies. This instability is apparent from the variable copy numbers of several of these repeat regions identified during sequencing of V592 (Table 2). Furthermore, PCR amplification of several regions of variable lengths, including those present in ORF24, demonstrated variable lengths of PCR products upon comparison of individual isolates derived from the same outbreak (data not shown).

ORF14 of V592 had a 9-bp duplication, resulting in the insertion of three amino acids. The majority of field isolates tested encoded an equivalent 9-bp repeat (J. Nugent and N. Davis-Poynter, unpublished data). ORF68 (homologous to herpes simplex virus type 1 [HSV-1] Us2 and related genes of other alphaherpesviruses) displayed the most significant coding change, with a single nucleotide deletion in V592 (G<sub>8</sub> in Ab4, G<sub>7</sub> in V592) resulting in a frameshift and hence multiple coding changes and premature truncation compared with Ab4. Such a frameshift had previously been noted from analysis of several other EHV-1 isolates (34). The other variable ORFs possessed minor coding changes between Ab4 and V592, usually comprising a single amino acid substitution.

**Evaluation of sequence variation of multiple loci for a panel of EHV-1 field isolates.** As noted above, EHV-1 infection results in outbreaks of various levels of severity, which may either be restricted to respiratory disease or include abortion and/or neurological disease. Previous evidence had indicated genetic variability of EHV-1 isolates but had not demonstrated conclusively the cocirculation of genetically distinct strains of EHV-1. We assembled DNA samples prepared from a panel of

TABLE 3. Nonsynonymous differences between V592 and Ab4

ORF	Position <sup>a</sup>	Variation(s) in:	
		Ab4 sequence	V592 sequence
2	59	G	D
5	114	G	V
8	114	D	N
11	189	Q	K
13	305	S	L
	460	A	T
14	619–621	* <sup>c</sup>	PSR (9-bp duplication resulting in 3-amino-acid insertion)
15	166	D	N
22	430	S	P
24	2567–2568	(GPPPTLPPAPPL PQSTSKAAS) <sub>2</sub>	*(126-bp deletion [2 copies of 63-bp repeat element] resulting in 42-amino-acid deletion)
	2586	G	S
	2855–2862	*	DQAKDQAK (24-bp insertion [2 copies of 12-bp repeat element] resulting in 8-amino-acid insertion)
	2904	E	K
	2914–2928	*	TGAVPENTPLPDDSP (45-bp insertion resulting in 15-amino-acid insertion)
	3099	T	A
29	12	T	K
30	752	D	N
	990	E	K
31	90	N	S
32	42	S	L
33	15	N	H
	976	N	D
34	66	D	G
36	47	S	R
37	265	A	V
39	440	S	L
40	196	R	H
42	1275	K	R
45	427	E	G
46	140	F	S
50	367	P	S
52	386	A	V
57	804	K	R
64	73	T	A
	648	T	S
67	261	S	F
68	210	R	H
	247	D	M <sup>d</sup>
71	226–227	SS	TA
	231–299	*	TAATTTAATTSSATTAATTSS (TTTAA) <sub>6</sub> TTT (additional copies of 15-bp repeat elements and nucleotide substitutions, resulting in 69-amino-acid insertion <sup>b</sup> )
73	122	A	V
76	128	F	S

<sup>a</sup> ORF amino acid positions are numbered according to the V592 amino acid sequence.

<sup>b</sup> Repeat element with variable copy number in V592; sequence present in majority of reads is shown.

<sup>c</sup> \*, gap in sequence.

<sup>d</sup> Due to a single C deletion resulting in frameshift relative to Ab4. The resulting polypeptide is 303 amino acids long (cf. 418 for Ab4).



TABLE 4. EHV-1 isolate groups according to ORF68 sequence

Group no.	Neurological isolate(s)	Nonneurological isolate(s)
1	GB80_1_2, GB83_4_2	GB83_1_1, GB83_3_1, US85_1_1, AR85_1_1
2	AR91_2_2, AU82_1_2, GB79_1_2, GB99_1_2, GB03_2_2, US85_2_2, US86_2_2, US86_3_2, US03_1_2, US03_2_2, US03_3_2, US03_4_2, US03_7_2, US70_1_2, US03_5_2	AR79_1_1, AR90_1_1, AR90_2_1, AR91_1_1, AR91_3_1, AR96_1_1, AR00_1_1, FR97_1_1, FR99_1_1, FR99_2_1, FR99_5_1, GB86_2_1, US76_1_1, US79_1_1, US83_2_1, US84_1_1, US85_4_1, US89_1_1, US90_1_1, US99_1_1, US00_1_1
3	BE95_1_2, BE99_2_2, BE03_1_2, FR02_1_2, GB88_2_2, GB03_3_2, GB03_4_2, GB03_5_2, GB04_10_2, GB04_11_2, NL95_1_2, NL99_1_2	AU02_1_1, BE94_1_1, BE97_1_1, BE99_1_1, GB83_2_1, GB89_1_1, GB89_2_1, GB91_2_1, GB93_2_1, GB00_2_1, GB01_1_1, GB03_1_1, GB02_1_1, GB04_1_1, GB04_3_1, GB04_5_1, GB04_14_1, GB05_1_1, US86_1_1, US90_2_1, US90_3_1, US92_1_1
4	GB04_12_2, GB04_13_2, GB04_15_2, US86_4_2	FR00_1_1, GB81_2_1, GB81_3_1, GB00_1_1
5	CA89_1_2, US41_1_2, US82_1_2, US83_3_2, US99_2_2, US99_3_2, US01_1_2, US02_1_2, US03_6_2, US03_9_2, US03_8_2, US03_10_2, US03_11_2	PL68_1_0, US72_1_0, US75_1_1, US81_1_1, US85_3_1, US01_2_1
6		GB85_1_1, GB91_1_1, GB01_2_1
Not assigned	GB86_3_2	GB87_1_1

field isolates recovered from outbreaks that occurred in various countries, collected over the course of approximately 30 years. PCR procedures to amplify selected regions of ORFs found to vary in predicted amino acid sequence between Ab4 and V592 were developed by using the amplification primers listed in Table 1. The purified PCR products were then sequenced using the respective sequencing primers (Table 1).

**ORF68 as the primary strain grouping marker.** A region of ~600 bp in ORF68 was particularly polymorphic and so was tentatively adopted as a marker system for efficiently distin-

guishing isolates without having to type multiple loci. ORF68 sequences from 106 field isolates (each from a different outbreak) and two tissue culture attenuated vaccine strains (RacH [PL68\_1\_0] [30, 31, 35] and the component of the vaccine Rhinoquin [US72\_1\_0] [Jensen-Salsbury] [45, 46, 47]) could be categorized into six common strain groups (Table 4). A sequence alignment of representative isolates for the various ORF68 sequences determined is shown in Fig. 1. Ab4 (GB80\_1\_2) is located in group 1, and V592 (GB85\_1\_1) is located in group 6. Group 1 is distinguished from group 2 only

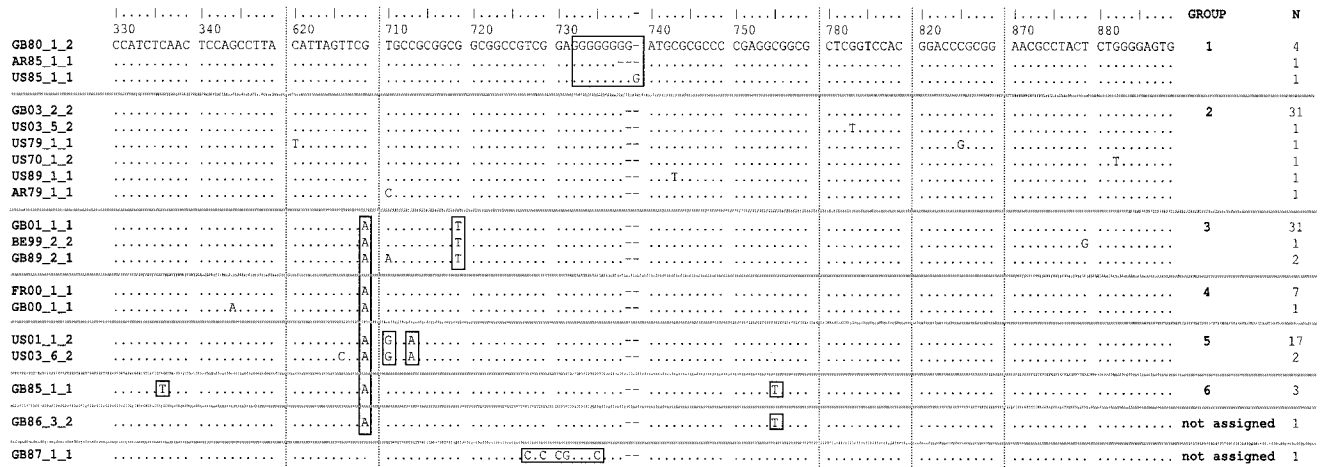


FIG. 1. Regions of sequence variation for ORF68 are shown, with a representative isolate for each different sequence type displayed. N indicates the number of independent isolates identified with each particular sequence. Sequences are aligned with reference to the prototype Ab4 (GB80\_1\_2) sequence (GenBank accession number AY665713), with dots indicating sequence identity and dashes indicating gaps in the alignment. Nucleotide positions are indicated above the alignment, in accordance with the Ab4 (GB80\_1\_2) sequence. Vertical dotted lines indicate breaks between contiguous blocks of sequence. Nucleotide positions characteristic of each ORF68 group are indicated by boxes (further details in text). Isolate GB86\_3\_2 has one of the two nucleotides (T<sub>755</sub>) which distinguish between group 4 and group 6 and cannot be assigned to either of these groups. Isolate GB87\_1\_1 has a stretch of five nucleotide substitutions (boxed), none of which are seen with any other isolate. This may be a representative of a separate group, but in the absence of other isolates sharing the signature five substitutions, this isolate has not been assigned to a group.

TABLE 5. ORF68 group distribution according to outbreak origin and disease characteristics

ORF68 group	No. of isolates					
	Geographical origin				Outbreak disease characteristics	
	North America	South America	Australia	Europe	Neurological	Nonneurological
1	1	1	0	4	2	4
2	19	8	1	8	15	21
3	4	0	1	29	12	22
4	1	0	0	7	4	4
5	17	0	0	0	13	4
6	0	0	0	3	0	3

on the basis of the number of G residues in a homopolymeric tract (nucleotides [nt] 732 to 739). Within group 1, isolates encode G<sub>6</sub> (AR85\_1\_1), G<sub>8</sub> (Ab4 [GB80\_1\_2], GB83\_1\_1, GB83\_3\_1, and GB93\_4\_2), or G<sub>9</sub> (US85\_1\_1) in this region, whereas group 2 and all other groups encode G<sub>7</sub>. Polymorphism in this region was originally identified by Meindl and Osterrieder (34), who demonstrated that Ab4 (G<sub>8</sub>) expressed a longer form of the protein than isolates with G<sub>7</sub>. Our data indicate that the ORF68 sequence for Ab4 is atypical and that the majority of EHV-1 isolates encode a shorter form of the protein than that expressed by Ab4. Isolates in groups 3 to 6 all encode A<sub>629</sub>, compared with G<sub>629</sub> for groups 1 and 2. Additional SNPs characteristic of each group are indicated in Fig. 1, namely, group 3 (T<sub>719</sub>), group 5 (G<sub>710</sub> and A<sub>713</sub>), and group 6 (T<sub>336</sub> and T<sub>755</sub>). Isolate GB86\_3\_2 encodes A<sub>629</sub> and T<sub>755</sub> and has not been assigned a group, since it may be considered a member of either group 4 or group 6, differing by a single substitution from the consensus sequence for either group. Isolate GB87\_1\_1 is similar to isolates from group 2 but has five substitutions in the region from nt 727 to 735, none of which are seen with any other isolate. This isolate may represent a group distinct from groups 1 to 6; however, in the absence of other isolates sharing the signature 5 substitutions it has not been assigned a group.

Analysis of the ORF68 strain distribution according to geographical origin of the isolates provided evidence for geographical restriction of certain strain groups (Table 5). Isolates from 104 disease outbreaks from each of the six major strain groups were analyzed; the tissue culture attenuated vaccine strains RacH (PL68\_1\_0) and the component of the vaccine Rhinoquin (US72\_1\_0) and the two isolates not assigned to a group (GB86\_3\_2 and GB87\_1\_1) were excluded from the analysis. There was a statistically significant heterogeneity between North America and Europe in the assignment of outbreaks to groups ( $P < 0.001$  by Fisher's exact test): all 17 group 5 field isolates came from outbreaks in North America, whereas the majority (36/42) of group 3 and group 4 isolates were from outbreaks in Europe. No single strain group was found to be responsible for outbreaks of neurological disease, with neurological disease represented in five of the six groups. Overall, 45% of the outbreaks presented neurological disease, and this is reflected in the proportion of neurological outbreaks in most of the groups (30 to 50% for groups 1 to 4). Group 5 had the highest proportion of neurological outbreaks (76%), while group 6 (the smallest group, with three outbreaks) included only nonneurological outbreaks.

**Comparison of ORF68 grouping with SNPs of other ORFs.** A panel of field isolates recovered from 12 neurological and 13

nonneurological disease outbreaks, and with representatives from each major strain group, was analyzed for SNPs for a number of ORFs distributed across the genome (Table 6). Each ORF was sequenced across the region with a coding difference between Ab4 and V592 by use of the primers detailed in Table 1. Extra SNPs were detected in some ORFs, and these are shown if present in more than one isolate. Notably, where multiple isolates from the same outbreak were analyzed, consistent sequences were found for all markers tested (data not shown).

Data from ORF68 and the genome-wide SNP typing is presented as a network (Fig. 2) (5). The available data set was incomplete, and sequencing of further isolates and SNPs is under way to conduct a more thorough analysis, but the following preliminary inferences about the evolution and epidemiology of EHV-1 may be drawn. First, the pooled data are consistent with a tree-like genealogy (with the exception of a single repeated substitution of ORF30 [see below]), indicating that there is currently no evidence for recombination among the isolates in the representative panel. Second, the genome-wide data substantiate the ORF68-based classification, except for groups 1 and 2, which are not distinguished by any further SNPs and are superimposed on the network. Third, group 6 is quite divergent from the other strains analyzed, with nine further distinctive substitutions, namely, with ORF8 (A<sub>340</sub>), ORF11 (A<sub>565</sub>), ORF30 (A<sub>2968</sub>), ORF32 (T<sub>125</sub>), ORF40 (T<sub>498</sub> and A<sub>587</sub>), ORF42 (G<sub>3824</sub>), ORF67 (T<sub>782</sub>), and ORF73 (T<sub>365</sub>). Other groups had their characteristic SNPs, but none was as divergent as group 6. V592 (GB85\_1\_1), a member of group 6, had an extra substitution in ORF50 (T<sub>1099</sub>) that was not observed with any other isolate. Analysis of two independent isolates from the same outbreak confirmed that this SNP is characteristic of the outbreak, rather than being an adventitious mutation of V592 (data not shown). Three isolates could not be reliably assigned to groups or were reassigned based on combined data. Based on ORF68, isolate GB86\_3\_2 had differed by a single substitution from the consensus sequence of either group 4 or group 6 and by two substitutions from that of group 3. Interestingly, genome-wide data placed this isolate nearest to group 3 isolates. Using SNP data, two group 2 isolates (GB99\_1\_2 and US99\_1\_1) could be distinguished from the rest of groups 1 and 2.

**ORF30 sequence variation is associated with pathogenic potential.** Strikingly, variation at a single nucleotide position among the typed SNPs was inconsistent with the simple pattern of rare unique mutations seen across the EHV-1 genome. Using the network analysis described above, approximately six

TABLE 6. Analysis of SNPs of 15 ORFs for a panel of EHV-1 field isolates

SNP of ORF at nt position <sup>a</sup>																				
Isolate	Group	SNP of ORF at nt position <sup>a</sup>																		
		ORF8 340	ORF11 565	ORF15 496	ORF30 2254 2279		ORF31 269	ORF32 125	ORF37 794	ORF40 498 <sup>c</sup> 587		ORF42 3824	ORF45 1280	ORF50 1099	ORF52 1157	ORF57 2411 2426 <sup>c</sup>	ORF67 759 <sup>c</sup> 782		ORF73 365	
GB80_1_2	1	G	C	G	G	A	G	A	C	C	C	A	A	C	C	A	T	G	C	C
GB93_4_2		G	C	G	G	A	G	A	C	C	C	A	A	ND	C	C	A	T	G	C
GB83_1_1		G	C	G	A	A	G	A	C	C	C	A	A	A	C	C	A	T	G	C
US85_1_1		G	C	G	A	A	G	A	ND <sup>b</sup>	C	C	C	A	A	C	C	A	T	G	C
US70_1_2	2	G	C	G	G	A	G	A	C	C	C	A	A	C	C	A	T	ND	C	C
US85_2_2		G	C	G	A	A	A	G	A	C	C	C	A	A	C	C	A	T	G	C
GB99_1_2		G	C	A	G	A	ND	ND	ND	C	ND	ND	A	G	C	ND	A	T	ND	C
US99_1_1		ND	C	C	ND	A	A	G	A	C	T	C	A	A	C	ND	ND	G	G	C
GB81_4_1		G	C	G	A	A	A	A	A	C	C	C	A	A	C	C	T	G	C	C
NL95_1_2	3	G	C	A	G	A	G	G	C	T	C	C	A	G	T	G	T	ND	C	C
GB88_2_2		G	C	A	G	A	G	G	C	T	C	C	A	G	T	G	T	G	C	C
GB89_1_1		G	C	A	A	A	G	G	C	T	C	C	A	ND	T	G	T	G	C	C
GB91_2_1		G	C	ND	A	A	G	ND	ND	T	C	C	ND	ND	ND	ND	ND	G	C	ND
US86_4_2	4	G	C	A	G	A	G	A	C	T	C	C	A	G	T	A	C	G	C	C
FR00_1_1		G	ND	ND	G	A	ND	A	C	ND	ND	A	A	G	C	A	C	ND	C	C
GB81_3_1		G	C	A	A	A	G	A	C	T	C	C	A	G	C	A	C	G	C	C
GB00_1_1		G	C	A	A	A	G	A	C	T	C	C	A	G	C	ND	C	ND	C	C
US99_2_2	5	G	C	A	G	A	G	A	C	T	C	C	A	G	C	A	T	A	C	C
US99_3_2		G	C	A	A	A	G	A	C	T	C	C	A	G	C	T	A	A	C	C
US02_1_2		G	C	A	A	A	G	A	C	T	C	C	A	G	C	T	A	A	C	C
US75_1_1		G	C	A	A	A	G	A	A	C	T	C	C	A	C	T	A	ND	ND	C
GB91_1_1	6	A	A	A	A	A	A	G	T	T	T	ND	ND	G	T	T	T	G	T	T
GB01_2_1		A	A	A	A	A	A	A	ND	T	T	T	C	G	T	T	T	ND	ND	T
GB85_1_1		A	A	A	A	A	G	G	T	T	T	T	G	G	T	T	T	G	T	T
GB86_3_2		Not assigned	G	C	A	ND	ND	G	G	C	T	C	A	G	T	G	T	G	C	C

<sup>a</sup> For each ORF, the nucleotide position of the SNP is indicated relative to the start of the ORF, according to the Ab4 (GB80\_1\_2) sequence (56). Nucleotides differing from the Ab4 sequence are highlighted in bold.

<sup>b</sup> ND indicates the sequence was not determined.

<sup>c</sup> Three SNPs (nt 498 of ORF40, nt 2426 of ORF57, and nt 759 of ORF67) did not result in amino acid coding changes.

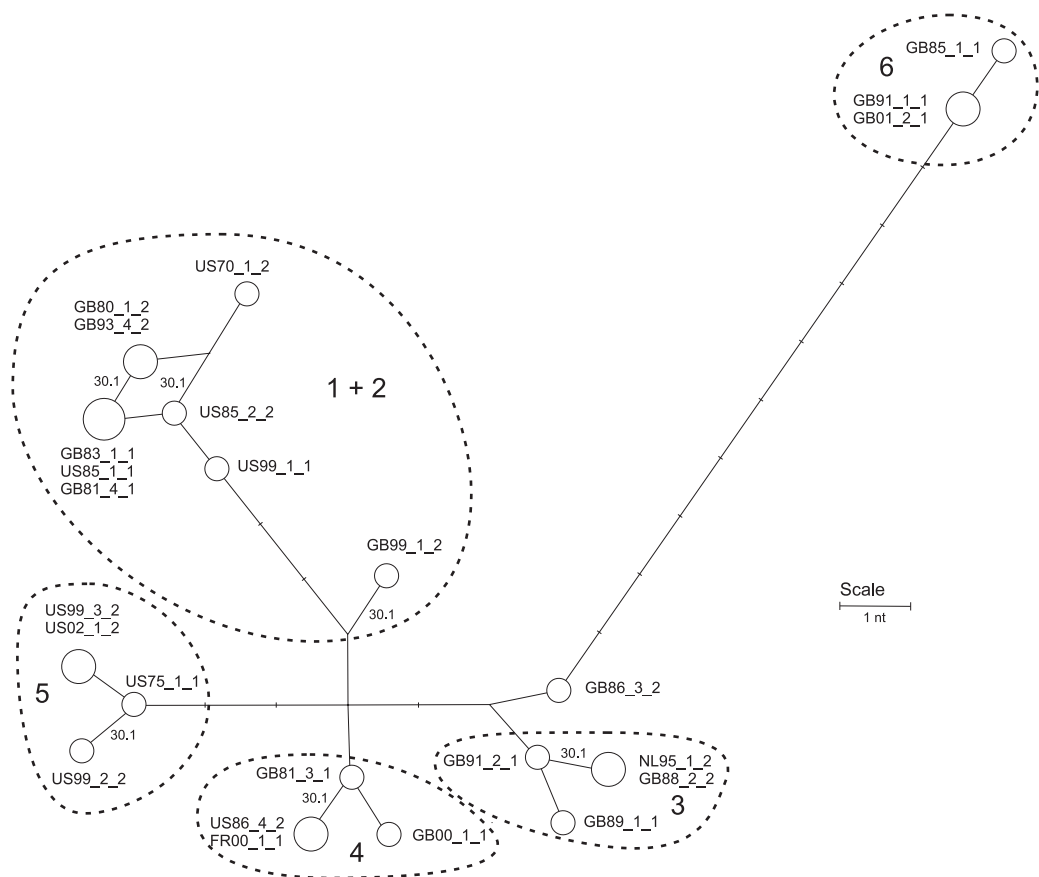


FIG. 2. Reduced median network for panel of EHV-1 isolates. Polymorphism data were derived from a set of ORFs containing at least one nonsynonymous substitution and from ORF68 sequences in a panel of 25 isolates representing neuropathogenic and nonneuropathogenic outbreaks and all six ORF68 groupings. Isolates are labeled as shown in Table 6, and the area of each circle is in proportion to the frequency of isolates sharing the same genotype. The positions of substitutions at ORF30 nt 2254 are shown (30.1'), and other substitutions are to scale but unlabeled. The parallelogram at top left indicates the alternative mutational paths involving two variable sites. Groupings based on ORF68 are indicated by dashed lines.

distinct, independent mutations can be inferred at nt 2254 of ORF30 (encoding the catalytic subunit of the DNA polymerase). In contrast to all of the other SNPs detected, disease severity was more closely related to the genotype of an isolate at this position than to its genetically more stable ORF68 group membership. Fisher's exact test was used to assess the statistical association with neurological disease of a total of 51 SNPs from 22 ORFs (including the ORFs specified in Table 6 and seven additional ORFs: ORF2, -5, -13, -14, -29, -34, and

-36) and of the six ORF68 groups. Each SNP had data available from at least 10 neuropathogenic and 10 nonneuropathogenic outbreaks. Only ORF30 at nt 2254 had a significant association ( $P < 0.01$ ) with neurological/nonneurological outbreak status. ORF30 sequences (nt 2200 to 2450) were analyzed for a panel of 131 field isolates, comprising 82 from outbreaks where no neurological disease was reported and 49 from outbreaks including at least one case of neurological disease (Table 7). Seventy-eight (95%) of the nonneurological

TABLE 7. Change in amino acid coding identified for ORF30 that correlates with distribution of isolates according to EHV-1 disease severity

Significant amino acid in ORF30 sequence at position 752 <sup>a</sup> (D/N)	Amino acid encoded at position <sup>b</sup> :			No. of isolates from outbreaks of EHV-1 disease severity		
	753 (Y/S)	760 (D/G)	769 (H/N)	Neurological	Nonneurological	Attenuated
D	Y	D	H	42	4	1
D	S	D	H	0	0	1
N	Y	D	H	4	76	0
N	Y	G	H	3	1	0
N	Y	D	N	0	1	0

<sup>a</sup> Amino acid coding change D/N<sub>752</sub> corresponds to nucleotide change G/A<sub>2254</sub>.  
<sup>b</sup> Amino acid coding change Y/S<sub>753</sub> corresponds to nucleotide change A/C<sub>2258</sub>, D/G<sub>760</sub> corresponds to nucleotide change A/G<sub>2279</sub>, and H/N<sub>769</sub> corresponds to nucleotide change C/A<sub>2305</sub>.



Virus	Accession		
<i>Alphaherpesviruses</i>			
EHV-1 V592	Q6S6P1	738	ALDEVDLAQLQPS--VNYSTFEVGDQK-LFFVHAHIRESL
EHV-1 Ab4	P28858	738	ALDEVDLAQLQPS--VDYSTFEVGDQK-LFFVHAHIRESL
EHV-4	AAC59546	739	ALNEVDLAGLQPC--VDYSTFEVGDQK-LFFVHAHIRESL
BoHV-1	CAB01595	770	VRREAPAGLTPG--ADYATFDVGGRA-LHFVRAHVRESLL
BoHV-2	AAD55134	739	ALDAEAVGGLAAG--RDYMEITVGGDT-VYFVKAHVRESLL
FeHV-1	CAA12264	727	TTERQSLETLRPG--IDFSEFDVGGHK-LYFVDSHVREEP
HHV-3 (VZV)	P09252	702	TLNFETVKRLNP---SDYATFTVGGKR-LFFVRSNVRESLL
CeHV-9 (SimVZV)	AAG27201	688	AFDIESVKHLGS---NDYSFNVGGQQ-LFFVHAHIRESL
SuHV-1 (PRV)	AAA74383	593	ALAR--PAGLRE---DEFSAFEVNGER-LYFVHAHVRESLL
HHV-1 (HSV-1)	P09854	737	SLRADAVAHLEAG--KDYLEIEVGGRR-LFFVKAHVRESLL
HHV-2 (HSV-2)	P07918	742	SLRPEAVAHLEAD--RDYLEIEVGGRR-LFFVKAHVRESLL
GaHV-2 (MDV-1)	AAG14223	718	VHDDTNLSNLRPQ--DDYLEINVQGKL-LRFVKPHIRESLL
GaHV-3 (MDV-2)	BAA78719	690	INDDRKLADLRPR--DDYMEIDVQGKS-LHFVAKPHIRESLL
MeHV-1 (HVT)	AAG45768	703	LPAGTIINDLRG--DDYIEIDVQGSI-LRFVKPHIRESLL
<i>Betaherpesviruses</i>			
HHV-5 (HCMV)	P08546	737	LVPGEYVPVDP---ADVYSVTLENGVTHRFVRASVRVSVL
MuHV-1 (MCMV)	AAA45940	644	LVE--GSPEVP---EKDVLRLVEIGDQC-HRFVRENVHRSLL
MuHV-2 (RCMV)	Q85428	670	LVD--GSPPVP---DEDVLEVVGEATRYRFVREHVRRESLL
PorcCMV	AAF80109	587	ILNDEDVTGID---EKDILTIVHNKNTVYRFVRSVRESML
CeHV-8 (RhCMV)	AAC05256	611	VAPGGESPPE-----SDVLTVELESGLSYRFVKNVTRNSVL
GPCMV	Q69025	666	LPL--GRDDG--LSDDVFLLEFDGTRYGFVREHVRKSIL
ElephHV-1	AAG41999	625	ITDNYVASLR---EEDITMTVTNTGRVHRFVKPHVRKSIL
HHV-6	NP_042931	592	VLDERQIAGLS---ESDILTIVKLGD-ETHRFVKPCIRESVL
HHV-7	AAC40752	592	VVDENAVIGLH---ADDILTIVHVGPF-VTHRFVKKTVRESIL
<i>Gammaherpesviruses</i>			
HHV-4 (EBV)	NP_039908	604	ITPGEHRLAGLRPGEDYESFRLTGGV-YHFVKKKHVESFL
CalHV-3	AAK38212	601	VTPGEEGKLRDLRPGEDYESFSLSGGT-FHFVKKKHHSKFL
BoHV-4	AAK07928	600	IQDQNLHLH-HLKP-DYETFHLSTGP-IHFVKQHKTKSLL
AlHV-1	AAC58060	622	IKQQDLPKFTNLTA-DYETFMISGGP-VHFVKKKHKTESLL
MuHV-4 (MHV-68)	AAF19277	601	IPDNKLQMFNLTTPA-DYETFTLPSGT-VHFVKKKHKCSLL
SaHV-2 (HVS)	DJBEM2	594	IPHHALHNYPHLKSS-DYETFMLSGGP-IHFVKKKHQASLL
AtHV-2	AAC95533	594	IPHNSLHNYPYLKSS-DYETFMLSGGP-IHFVKKKHQISLL
HHV-8	AAB62593	605	IPGDSLHLHPLSPD-DYETFVLSGGP-VHFVKKKHKRESLL
PorcLTHV-1	AAF16520	592	IHHEDLHKYPQLKEE-DYETFLISSGP-VHFVKKKHISESLL
EHV-2	NP_042605	602	IPGDRCLLHPLGPG-DYETFELASGP-VHFVKKKHKAESLL
<i>Unclassified herpesviruses</i>			
HawGrTurtHV	AAC26681	662	TRRAETLK--ELKAGEDYEEFKVQGM-SLFYVKKPHVRRSLL
TortHV	BAB40430	14	TRNPESLK--DLKAGKDYVSFNVQGH-TLYYVLNHNKQSL

FIG. 3. An alignment of amino acid sequences for various herpesvirus DNA polymerases is shown, generated using ClustalW (run using the BioEdit software [21]). For each sequence, the name of the virus, database accession number, and residue number of the first amino acid are shown (the residue number in italics refers to a partial polymerase sequence). The conserved aspartate residue, corresponding to D/N<sub>752</sub> of the EHV-1 polymerase, is highlighted by gray shading. BoHV-1, bovine herpesvirus 1; FeHV-1, feline herpesvirus 1; HHV-3, human herpesvirus 3; CeHV-9, cercopithecine herpesvirus 9; SimVZV, simian VZV; SuHV-1, suid herpesvirus 1; PRV, pseudorabies virus; GaHV-2, gallid herpesvirus 2; MDV-1, Marek's disease virus type 1; MeHV-1, meleagrid herpesvirus 1; HVT, herpesvirus of turkeys; MuHV-1, murid herpesvirus 1; MCMV, mouse cytomegalovirus; RCMV, rat cytomegalovirus; PorcCMV, porcine cytomegalovirus; RhCMV, rhesus monkey cytomegalovirus; GPCMV, guinea pig cytomegalovirus; ElephHV-1, elephantid herpesvirus 1; EBV, Epstein-Barr virus; CalHV-3, callitrichine herpesvirus 3; AlHV-1, alcelaphine herpesvirus 1; MHV-68, mouse herpesvirus strain 68; SaHV-2, saimiriine herpesvirus 2; HVS, herpesvirus saimiri; AtHV-2, ateline herpesvirus 2; PorcLTHV-1, porcine lymphotropic herpesvirus 1; EHV-2, equine herpesvirus 2; HawGrTurtHV, Hawaiian green turtle herpesvirus; and TortHV, tortoise herpesvirus.

isolates encoded A<sub>2254</sub> (amino acid N<sub>752</sub>), whereas 42 (86%) of the neurological isolates encoded G<sub>2254</sub> (amino acid D<sub>752</sub>). This distribution was found to be highly significant statistically ( $P < 0.0001$  by Fisher's exact test). Although we cannot exclude the possibility that other SNPs (alone or in combination) may show an association with disease severity, it is clear that alleles at the SNP of ORF30 at nt 2254 are strongly associated with disease phenotype but are not associated with group membership defined by ORF68 and supported by other SNPs.

Interestingly, EHV-1 ORF30 amino acid 752 was found to correspond to a highly conserved position for herpesvirus DNA polymerases, as shown in Fig. 3. Remarkably, this position was found to be conserved as an acidic residue (aspartate, with the exception of pseudorabies virus [glutamate]), followed by a hydrophobic residue, for all mammalian herpesvirus polymerases evaluated, including representatives of alpha-, beta-, and gammaherpesviruses. This conservation also extended to unclassified herpesviruses from nonmammalian species (tor-

toise and turtle). The presence of a polar, neutral residue (asparagine) at this position for the majority of EHV-1 isolates tested is therefore highly unusual.

Two attenuated vaccine strains were also analyzed for their ORF30 sequences (Table 7). The component of the vaccine Rhinoquin (US72\_1\_0) was found to have an ORF30 sequence in this region that was identical to that of Ab4 and the majority of isolates from neurological disease outbreaks. The second vaccine strain, RacH (PL68\_1\_0), was found to encode a sequence not seen in any other field isolate tested (D<sub>752</sub> S<sub>753</sub>). Additional sequence variation was noted to occur at nt 2279 (A/G) and 2305 (C/A) in a minority of the isolates tested, corresponding to amino acid coding changes G<sub>760</sub> and N<sub>769</sub>, respectively (Table 7). The four isolates encoding G<sub>760</sub> were from different ORF68 strain groups, two from group 3 (BE95\_1\_2 and GB04\_8\_1) and two from group 5 (US99\_3\_2 and US02\_1\_2). Three of these isolates were from outbreaks of neurological disease, and one was from a single case of abortion without neurological signs. The variant N<sub>769</sub> was identified for isolates from an outbreak of abortion without neurological signs (GB01\_2\_1, group 6).

## DISCUSSION

EHV-1 is associated predominantly with outbreaks of abortigenic disease and more rarely with outbreaks of neurological disease. For many years, it has been suspected that distinct strains of EHV-1 may be responsible for outbreaks of neurological disease. However, previous molecular epidemiology studies, although demonstrating genetic heterogeneity (e.g., via RFLP analyses), had failed to identify genetic markers associated with neuropathogenic disease outbreaks. The starting point for this study was the determination of all nucleotide differences between two strains of EHV-1, Ab4 (high frequency of abortion, neuropathogenic) and V592 (low frequency of abortion, nonneuropathogenic), which have been shown by experimental infection studies with ponies to exhibit markedly different pathogenicities. Following determination of the complete genomic sequence of V592 and comparison with the previously determined sequence of Ab4 (56), we sought to test the following hypotheses. (i) Positions of sequence variation between Ab4 and V592 are indicative of regions of sequence variability among EHV-1 field isolates and will therefore provide markers enabling discrimination between EHV-1 strains. (ii) Ab4 is representative of a distinct group of genetically closely related EHV-1 strains capable of causing neurological disease. (iii) One or more specific sequence markers that vary between Ab4 and V592 are indicative of strains capable of causing neurological disease.

Overall, there was relatively low variation between the Ab4 and V592 sequences, with approximately one variable position per 1,000 bp. The majority of changes were point mutations, involving single nucleotide substitutions. There were also several small deletions or insertions, involving 1 or 2 nucleotides, together with larger deletions and insertions involving a contraction or expansion of short direct repeat elements. Analysis of the larger regions of variable repeat length were of limited value for typing of strains, due to their inherent instability, agreeing with previous studies (2, 8, 22, 25, 32).

On the assumption that the pathogenic differences between

Ab4 and V592 are the result of sequence changes in one or more viral proteins, we evaluated SNPs for several ORFs, found to vary in coding sequence between Ab4 and V592, for a panel of EHV-1 isolates. A region of ORF68 was found to be the most variable, with 19 distinct sequences observed, and was used as the primary marker for grouping isolates into six major strain groups (Table 4; Fig. 1). A frameshift in ORF68 had previously been identified (34), due to the presence of either 7 or 8 G residues in a homopolymeric tract, with Ab4 (G<sub>8</sub>) expressing a longer form of the protein (418 amino acids) than other isolates (303 amino acids). In this study, additional variants were identified with either G<sub>6</sub> or G<sub>9</sub> in this region. Isolates encoding G<sub>6</sub>, G<sub>8</sub>, or G<sub>9</sub> were all assigned to ORF68 group 1 and were closely related in ORF68 sequence (and other SNPs [see below]) to group 2 isolates. The majority of field isolates were found to encode G<sub>7</sub> and are therefore predicted to express the 303-amino-acid form of the polypeptide. ORF68 is homologous to Us2 of HSV-1 and related genes in other alphaherpesviruses (56). The region of homology between ORF68 and Us2 lies upstream of the frameshift region, suggesting that all of the forms expressed by EHV-1 isolates are likely to retain important functional domains.

Analysis of SNPs for several other ORFs (Table 6; Fig. 2) supported the groupings assigned according to ORF68 sequence variation. Groups 1 and 2 appear to be closely related, with characteristic SNPs for ORF15, -37, -45, and -52, which were not observed with other strain groups. Group 1 is distinguished from group 2 solely on the basis of the number of Gs in a homopolymeric run, likely to be a relatively unstable marker. Groups 3, 4, and 5 shared SNPs for most of the ORFs analyzed, but SNPs distinguishing between these groups were also observed. Group 6 has unusual SNPs in several ORFs and, on the basis of the ORFs analyzed, is the most highly diverged from the other groups. Since the ORFs analyzed were selected on the basis of differences between Ab4 (group 1) and V592 (group 6), it is uncertain whether the high proportion of SNPs specific to group 6 reflects relatively early divergence of group 6 from the other groups or is due to sampling bias. These findings support the first hypothesis presented above and suggest that genetically distinct strains of EHV-1 are in circulation.

Studies of other herpesviruses have demonstrated differing degrees of sequence diversity. Clinical isolates of human cytomegalovirus (HCMV) show significant sequence diversity by RFLP analysis and sequencing of selected ORFs (10, 13, 15, 43). Furthermore, it is difficult to assign HCMV isolates into strain groups, due to a relatively high frequency of recombination, indicated by variation of gB via intragenic recombination and relative rarity of genetic linkages between nine separate variable loci (20, 48). Similarly, a study of sequence variation between clinical isolates for genes within the Us region of HSV-1 (gG, gI, and gE) concluded that homologous recombination (intra- and intergenic) was a common event (40). In contrast, varicella-zoster virus (VZV) is relatively genetically stable and has been estimated to have a sequence diversity approximately 10 times lower than that of HSV and 40 times lower than that of HCMV (36, 50, 62). Analysis of VZV strains indicates significant geographical/ethnic restriction of the circulation of strain groups and shows that recombination between different strain groups is a relatively rare event (28, 36, 58). These differences in the various herpesvi-

ruses are likely to reflect differences in their biological properties, in particular relating to their modes of transmission and frequency of reactivation.

The low level of sequence divergence (0.1%) between the Ab4 and V592 genomes and the generally good agreement between the ORF68 grouping and SNPs in other ORFs suggest that EHV-1 genomes are relatively stable, in agreement with previous assessments of EHV-1 sequence diversity (4, 32). The current study has uncovered no evidence for recombination in the isolates and SNPs so far, although more data would be required to reach a definitive conclusion about the potential significance of recombination in the virus' life cycle. The relatively low degree of sequence diversity observed for EHV-1 is similar to that of VZV. EHV-1 is also similar to VZV in showing geographical restriction of certain strain groups. Thus, EHV-1 group 5 isolates were isolated predominantly from outbreaks in North America and group 3 isolates predominantly from outbreaks in Europe. Notably, there was no obvious association between any particular ORF68 strain group and outbreaks of neurological disease, with five of the six major groups including both neuropathogenic and nonneuropathogenic isolates. It is clear that Ab4 is not representative of a group of related strains capable of causing neurological disease, thereby disproving the second hypothesis presented above.

In contrast to all of the other markers tested, an SNP in ORF30 (G/A<sub>2254</sub>) did not cosegregate according to the ORF68 strain grouping (Table 6 and unpublished data). Both forms were present in five of the six major strain groups (the exception being group 6, which comprises only three members, all of which encoded A<sub>2254</sub>). The ORF30 SNP (G/A<sub>2254</sub>), corresponding to amino acid variation D/N<sub>752</sub> of the DNA polymerase (GAC→AAC is the only single nucleotide substitution that will result in a D-to-N change), instead showed a very strong association with neuropathogenic/nonneuropathogenic isolates, respectively. This result is in agreement with the third hypothesis presented above and demonstrates that one of the Ab4 specific alleles (ORF30 G<sub>2254</sub>) shows strong predictive value as a marker of isolates with a propensity for causing neurological disease. It was also noted that three of the seven isolates from neurological outbreaks that encoded ORF30 A<sub>2254</sub> encoded an additional nucleotide change, A/G<sub>2279</sub>, resulting in the coding change D/G<sub>760</sub>. The single outbreak involving an isolate encoding G<sub>2279</sub> that did not include neurological disease occurred on premises with only two pregnant mares (of which one aborted), where there were no other horses in contact with the infected horses. There was no statistically significant association of the G<sub>2279</sub> SNP with neuropathogenicity ( $P = 0.066$ ), although considering this allele occurred infrequently (4/131 isolates), analysis of additional isolates encoding this allele is required to determine whether this may be an uncommon ORF30 variant associated with increased pathogenic potential.

It is important to consider whether differences in the techniques used to generate DNA from the two different outbreak types (neuropathogenic and nonneuropathogenic) may have affected the results, in particular, the potential selection of ORF30 sequence variants in tissue culture. In this study, the majority of DNA samples were analyzed from isolates which had been prepared by limited passage (typically two to five passages) in tissue culture, with only 8/131 samples (all from

abortigenic outbreaks) prepared directly from tissue samples without cell culture. There was not, therefore, a consistent difference in the techniques used to generate the panels of neuropathogenic and nonneuropathogenic samples within each laboratory submitting the samples (although the choices of cells used for virus isolation differed between the various laboratories). Where ORF30 sequences were compared between DNA samples prepared directly from tissues and samples prepared from tissue culture isolates (two neuropathogenic outbreaks), the sequences were found to be identical. Combined with the observation that where samples from more than one horse in a given outbreak were analyzed (seven neuropathogenic and five nonneuropathogenic outbreaks), all samples yielded the same ORF30 sequence, we are confident that the ORF30 D/N<sub>752</sub> sequence variation is not an artifact of tissue culture selection.

Two tissue culture attenuated vaccine strains were characterized for their DNA polymerase sequence. One of these strains, the component of the vaccine Rhinoquin (47), was found to encode the DNA polymerase D<sub>752</sub> sequence typical of neuropathogenic strains. Although found to be attenuated in safety and efficacy studies (45, 46), Rhinoquin was associated with paralytic disease when used as a vaccine in the United States and as a consequence was withdrawn from the market (27). It is difficult to verify whether the cases of neurological disease were directly related to administration of Rhinoquin or coincidental to the vaccine trial, but the cases showed a strong temporal link, having occurred 8 to 11 days after administration of the vaccine. It can be speculated that either Rhinoquin reverted the attenuating mutations and was then able to express neuropathogenicity or the Rhinoquin DNA polymerase sequence was introduced into wild-type field strains via recombination, resulting in neurological disease. A second tissue culture attenuated strain used as the progenitor of live vaccines (RacH [30, 31, 35]) also encoded DNA polymerase D<sub>752</sub>. In contrast to Rhinoquin, vaccines derived from RacH have not been associated with neurological disease, and such vaccines are in current use in the United States (RhinoMune; Pfizer Animal Health [6, 7]) and in Europe (Prevaccinol; Intervet [30]). It may be significant, therefore, that RacH encodes a second amino acid coding change, Y/S<sub>753</sub>, that was not identified in any of the field isolates. In this regard, it should be noted that the position corresponding to Y/S<sub>753</sub> is conserved in other herpesviruses as a hydrophobic residue (Fig. 3); hence, the S<sub>753</sub> mutation of RacH is a nonconservative change.

The strong association of DNA polymerase D/N<sub>752</sub> with neurological versus nonneurological disease outbreaks suggests that the viral genotype is a major contributory factor in determining whether a disease outbreak will include animals presenting neurological signs. This does not mean that infection with neuropathogenic strains inevitably results in clinical signs of neurological damage, since the clinical outcome of infection for individual animals within an outbreak will be modulated by other factors, which may include age, host immunity, challenge dose, and hormonal status. Consequently, the proportions of animals that ultimately show neurological signs when infected with a neuropathogenic strain vary for each outbreak. For example, during a large outbreak of neurological EHV-1 disease in the United Kingdom, the neurological attack rate (number of cases presenting neurological



signs/number of horses confirmed to be infected) was 20% (33). In four recent, large outbreaks of EHV-1 neurological disease in horses in the United States, for which complete records were kept (G. Allen, unpublished data), the mean neurological attack rate was 33% (range of 22% to 50%) and the mean neurological case fatality rate was 40% (range of 20% to 50%). All five outbreaks described above involved EHV-1 strains carrying the neuropathogenic-associated form of the DNA polymerase (D<sub>752</sub>).

Notably, the D/N<sub>752</sub> variable position of EHV-1 DNA polymerase corresponds to a highly conserved position in other herpesviruses. Alignment of herpesvirus DNA polymerase sequences from diverse host species indicated that the equivalent position was conserved as an acidic residue (almost invariably D) in other herpesviruses (Fig. 3). This suggests that the ancestral EHV-1 virus probably encoded D<sub>752</sub> and that variants expressing N<sub>752</sub> arose subsequently, possibly due to a selective advantage. The observation that the D/N<sub>752</sub> SNP is unlinked to any of the other SNPs tested suggests that either the D/N<sub>752</sub> mutation has arisen relatively recently and independently in five of the six major strain groups or this position is relatively unstable. Outbreaks of EHV-1 neurological disease are relatively rare compared with outbreaks of nonneurological disease, which suggests that the majority of EHV-1 strains in circulation encode N<sub>752</sub>. It may further be proposed that D<sub>752</sub> variants arise spontaneously but may have a selective disadvantage for long-term maintenance in the population. Comparison between herpesvirus DNA polymerase sequences and those of other related polymerases (from organisms as diverse as bacteriophages and mammals) has revealed a number of conserved domains (23, 59). The variable D/N<sub>752</sub> residue lies between the conserved domains designated II and VI, which comprise regions essential for DNA polymerase activity. Mutations proximal to this position (mostly within domains II and VI), which influence antiviral drug sensitivity for herpes simplex virus, varicella-zoster virus, and human cytomegalovirus, have been noted previously (11, 12, 18, 24, 29). Thus, although the variable D/N<sub>752</sub> residue is not within one of the domains known to be critical for function, it does lie within the core, catalytic region of the enzyme and may therefore influence enzymatic activity.

Considering the strong association of D/N<sub>752</sub> with neuropathogenic potential, it is reasonable to speculate that the D/N<sub>752</sub> coding change affects functional properties of the DNA polymerase that play a direct role in the etiology of EHV-1 neurological disease. Replication studies with various cell types in tissue culture (fibroblast and endothelial) have shown no consistent differences between V592 and Ab4 (55; R. Chiam, K. Smith, and N. Davis-Poynter, unpublished data). It is possible, however, that alterations of DNA polymerase activity affecting replication may be apparent only in specific cell types or in the context of tissues in vivo. Other possible (albeit speculative) modes of action may include variation in the efficiency with which different forms of the DNA polymerase stimulate immune responses (e.g., cytotoxic T lymphocyte) against EHV-1-infected target cells, thereby affecting the efficiency of clearance of the infection. Alternatively, the neuropathogenic form of EHV-1 DNA polymerase may promote damage to blood vessels supplying the CNS, either directly via effects upon infected endothelial cells or indirectly via immu-

nopathology. In order to test the hypothesis that the D/N<sub>752</sub> substitution of the DNA polymerase affects neuropathogenicity, recombinant EHV-1 with the substitution at nt 752 engineered on a syngeneic background has been generated (L. Goodman and N. Osterrieder, unpublished data) and will be used in future in vivo studies to determine the influence of DNA polymerase sequence variation upon EHV-1 disease outcome.

The strong association of a naturally occurring SNP of a single gene with pathogenic potential, observed for EHV-1 DNA polymerase in this study, is to our knowledge unprecedented for a herpesvirus. The implication is that mutation of a single nucleotide has a significant effect upon pathogenicity, although this hypothesis has not yet been tested. This raises an important question concerning the origin of EHV-1 strains giving rise to neurological disease outbreaks, namely, whether they usually originate from nonneuropathogenic strains, via spontaneous mutation of the DNA polymerase (from N<sub>752</sub> to D), or whether they are usually the result of reactivation from animals previously exposed to neuropathogenic strains. Currently, we have no data regarding the relative frequency of latent carriage of EHV-1 strains encoding D/N<sub>752</sub>. DNA polymerase activity may conceivably influence the establishment of, or reactivation from, latency. Thus, establishment of latency may be promoted by abortive replication, if DNA polymerase activity is insufficient to promote full late gene expression prior to the genome entering a quiescent state; conversely, during the early stages of reactivation, DNA polymerase activity may be critical in triggering full lytic cycle gene expression. If EHV-1 DNA polymerase sequence variation does have an impact upon latency/reactivation, this may provide a source of selective pressure acting upon the DNA polymerase D/N<sub>752</sub> sequence. In the absence of a reliable system for evaluating the efficiency of EHV-1 latency establishment and reactivation, it will be difficult to test the abovementioned hypothesis.

In conclusion, determination of the complete genomic sequence of a nonneuropathogenic strain of EHV-1 (V592) and comparison with the previously published genomic sequence of a neuropathogenic strain (Ab4) has led to two important findings. First, a method for subtyping EHV-1 isolates recovered from the field via multilocus typing, which will be useful in tracing the transmission of virus strains between outbreaks, has been developed. Second, an SNP of ORF30 (G/A<sub>2254</sub>), resulting in an amino acid coding change of the DNA polymerase (D/N<sub>752</sub>), shows strong predictive value as a marker of isolates with a propensity to cause neurological disease. If a causal link between DNA polymerase sequence variation and neuropathogenic potential is established, determination of the molecular mechanisms underlying the link will assist future diagnostic, prophylactic, and therapeutic strategies designed to reduce the incidence and severity of neurological EHV-1 disease. These findings may also have implications for molecular epidemiological studies of other herpesviruses. In the case of EHV-1, variation of a single gene was found to be associated with differences in pathogenic potential, whereas none of the other SNPs tested showed significant association with disease. If single gene polymorphisms similarly have a significant association with disease manifestation for other herpesviruses, such strain-related differences may not have been apparent from previous analyses of a limited subset of variable gene se-

quences. The availability of methods for whole-genome SNP analysis will facilitate more-comprehensive studies and may reveal previously unrecognized associations between herpesvirus strain variation and disease.

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