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BRIEF REPORT

Molecular phylogeny of equine herpesvirus 1 isolates from onager, zebra and Thomson's gazelle

Y. M. Ghanem · H. Fukushi · E. S. M. Ibrahim · K. Ohya · T. Yamaguchi · M. Kennedy

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Abstract Viruses related to equine herpesvirus type 1 (EHV-1) were isolated from an aborted fetus of an onager (*Equus hemionus*) in 1984, an aborted fetus of Grevy's zebra (*Equus grevyi*) in 1984 and a Thomson's gazelle (*Gazella thomsoni*) with nonsuppurative encephalitis in 1996, all in the USA. The mother of the onager fetus and the gazelle were kept near plains zebras (*Equus burchelli*). In phylogenetic trees based on the nucleotide sequences of the genes for glycoproteins B (gB), I (gI), and E (gE), and teguments including ORF8 (UL51), ORF15 (UL45), and ORF68 (US2), the onager, Grevy's zebra and gazelle isolates formed a genetic group that was different from several horse EHV-1 isolates. Within this group, the onager and gazelle isolates were closely related, while the Grevy's zebra isolate was distantly related to these two isolates. The epizootiological origin of the viruses is discussed.

Equine herpesvirus-1 (EHV-1), a member of the alphaherpesvirus subfamily, is a major pathogen in horses (*Equus caballus*), in which it is associated with respiratory manifestations, epizootic abortion and neurological disorders [1]. EHV-1 has also caused clinical manifestations in wild equids. An onager (*Equus hemionus onager*) fetus was aborted after 10 months of gestation in February, 1984, at the National Zoological Park in Washington, DC, where a 9-month-old male plains zebra (*Equus burchelli*), located in a pen adjacent to the onagers, developed weakness, posterior ataxia and partial rectal prolapse one week after the onager abortion [14]. In October, 1984, at the Lincoln Park Zoo in Chicago, a 5-year-old female Grevy's zebra (*Equus grevyi*) aborted a female fetus [19]. In 1998, severe multi-systemic infection by EHV-1 was also reported in a Grevy's zebra stallion in England [2]. Viruses isolated from these cases were identified by serological assay and DNA fingerprinting. The DNA fingerprints were similar to each other and distinct from that of EHV-1 isolates in domestic horses [2, 14, 19].

Although EHV-1 infections are usually limited to equine species, infections have also been reported in non-equine animals including cattle, alpacas, llamas, fallow deer, antelopes, and a Thomson's gazelle [5, 6, 11, 12, 17]. EHV-1 in these ruminants was associated with abortion, blindness, and encephalitis. Our recent analysis indicated that the cattle isolates were identical to EHV-1s isolated from horse [16]. EHV-1 isolate 94–137 was isolated from a captive Thomson's gazelle (*Gazella thomsoni*) that died after an acute neurologic illness characterized by depression, recumbancy, and seizures [11]. The Thomson's gazelle had been kept at a zoo with a plains zebra (*E. burchelli*). DNA fingerprints of the gazelle isolate were similar to the DNA fingerprints of onager and zebra isolates. Molecular and epizootiological evidence suggested that EHV-1 was transmitted from the zebra to the gazelle.

Y. M. Ghanem · H. Fukushi · E. S. M. Ibrahim · K. Ohya · T. Yamaguchi
Department of Applied Veterinary Sciences, United Graduate School of Veterinary Sciences, Gifu University, Gifu, Japan

H. Fukushi (✉) · T. Yamaguchi
Laboratory of Veterinary Microbiology, Faculty of Applied Biological Sciences, Gifu University, Yanagido 1-1, Gifu 501-1193, Japan
e-mail: hfukushi@gifu-u.ac.jp

M. Kennedy
Department of Comparative Medicine, University of Tennessee, Knoxville, TN, USA

Table 1 Sequences of primers used for PCR and sequencing

Primer	Sequence	Position
PCR primers used for ORF 33		
Forward	GCAGTATTCTCCTCGGTTTTCCAC	61,310–61,333
Reverse	GAGGTCACACTTTGAGTACGTGTC	64,418–64,395
Sequencing primers for ORF 33		
F1	AGATCGTACCTACCCGGACT	61,360–61,379
R 2	CCTGAATTTGTAGGGAGCGA	61,983–61,964
F3	CCAAGTTTGACCTTGGGAGA	61,904–61,923
R4	GTTCCAGCTGACGGTGACAT	61,983–62,552
F5	AAACTACAAGCCGAAGAGCC	62,503–62,522
R6	CTCGATAGACGACGTGGTT	63,160–63,141
F7	GCAGGTACCGATTCTTCCAA	63,091–63,110
R8	CGCTGTATTTCGCTGTAGTC	63,776–63,757
F9	TTGGAAGACCGCGAGTTTCT	63,688–63,707
R10	GGTCACACTTTGAGTACGTG	64,416–64,397
PCR primers for ORF 73 and 74		
Forward	CAAACGCGACACAGCAAGTAGT	132,869–132,890
Reverse	GCATTCCCTCGGCATAGTTG	136,114–136,095
Sequencing primers for ORF73 and 74		
A	CAAACGCGACACAGCAAGTAGT	132,869–132,890
B	CACTACCACGACTATTTCCGAG	133,234–133,255
C	ATCTGTTGGGACGCACTGGCAAT	133,685–133,707
D	TCCAACCCCCAAACCTTCGA	134,076–134,095
E	TTGGGGCTATGATCGACGGAAG	134,503–134,523
F	AAGAAGCCGCCCAAACAACCG	134,909–134,929
G	AGCGACCAAGATCCTACACCG	135,244–135,264
H	TACATCCGAAGCAACCGTAAGC	135,695–135,716
a	ATATACGCCCCGAATCTGTAG	133,328–133,307
b	ATGTGCAGGCGATGTGGTACGA	133,770–133,749
c	CCCATCTACCCCCACAACTAT	134,189–134,169
d	GGCTCACGCACACCTTCTTAACTG	134,636–134,613
e	GGCTCTACGATATGTGACTCCA	135,090–135,069
f	CGGGTCACGTCAGTAAGCACATTC	135,513–135,490
g	GTTTGGGAGGTGGTGGGTATTC	135,875–135,854
h	GCATTCCCTCGGCATAGTTG	136,114–136,095
PCR primers used for ORF 8		
Forward	AGAGAACTCTGATAGTTGGC	10,262–10,281
Reverse	TTACACCGCAACCAAACTGG	11,037–11,018
PCR primers for ORF 15		
Forward	CGC ATC GGT TTC TCT ATT ACC G	20,471–20,492
Reverse	GTA AAG CAA CAT GGC AGG AGA C	21,159–21,180
PCR primers used for ORF 68		
Forward	CGAACGGGTTGAACAGGTGCTTAC	124,999–125,022
Reverse	GGAGTTGGTTCAACCCACCCATTTG	126,308–126,284
Sequencing primers for ORF 68		
F1	GGTTGAACAGGTGCTTAC	125,00–125,022
R2	CGAATGGTATACGCAGAG	141–124
F3	CGGATGATTATGCTCAAC	220–237
R4	GGTTCAACCCACCCATTTG	126,302–126,284

Another EHV-1-related virus was isolated from Thomson's gazelles kept in a zoo in Japan [8]. All of the nine Thomson's gazelles that were kept in a pen showed neurological symptoms, and seven of them died. Two of the dead gazelles were necropsied, and a virus was isolated from each one. The two viruses were found to be identical. The virus was identified as equine herpesvirus type 9 (EHV-9) based on molecular phylogenic analysis. Our previous study indicated that EHV-9 was distantly related to EHVs isolated from an onager, a zebra, a gazelle in the USA, and horses [9].

Ibrahim et al. [9] investigated the genetic relatedness and pathogenicity of onager, zebra and gazelle herpesvirus isolates based on nucleotide sequencing of the glycoprotein G (gG) gene and experimental infections in Syrian hamster. The gG gene sequences of the viruses isolated from onager and zebra were identical, and the sequence of the gazelle isolate showed 99.5% identity to the sequences of onager and zebra isolates. In a hamster experimental model which has been used to evaluate the virulence of EHV-1 in alien hosts, EHV-1 isolates from onager, zebra and gazelle were much more virulent than those isolated from horses. These data indicated that EHV-1s isolated from the abovementioned onager, zebra and gazelle belong to a genetic group that is distinct from the EHV-1 isolated from horses, although the data were limited to the gG gene. Borchers et al. [3] showed that viruses isolated from a captive Grevy's zebra (T-965) and a blackbuck (*Antelopa cervicapra*) (Ro-1) form a distinct group of equid herpesviruses. Together, these data indicated that the EHV-1s related to zebra exposure are another type of equid herpesviruses.

The aim of this report was to analyze the molecular phylogeny of the abovementioned EHV-1 isolates from gazelle, zebra and onager by nucleotide sequencing of viral envelope glycoprotein and tegument genes including gB (ORF33, UL27), gI (ORF73, US7), gE (ORF74, US8), ORF8 (UL51), ORF15 (UL45), and ORF68 (US2).

The EHV-1s used in this study were T-529, isolated from an aborted onager fetus [14], T-616, isolated from an aborted zebra fetus [19], and 94–137, isolated from a Thomson's gazelle that died after an acute neurologic illness [11]. T-529 and T-616 were kindly provided by Dr. P. Allen (University of Kentucky, USA). Ab4p as a reference

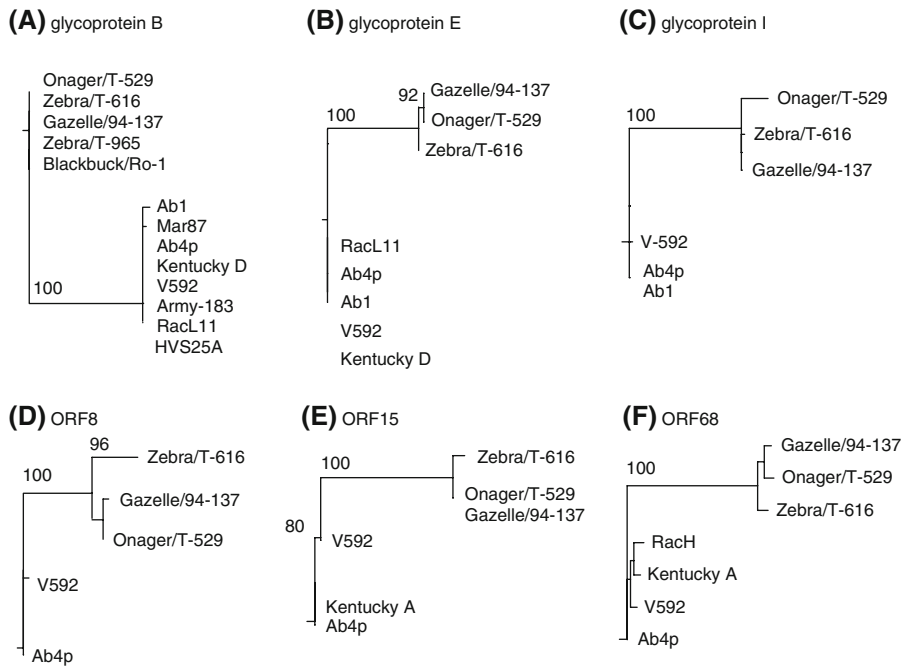

Fig. 1 Phylogenic trees of glycoprotein (**a**–**c**) and tegument genes (**d**–**f**). The phylogenic trees were constructed by DNA maximum likelihood analysis [7]. Only bootstrap values greater than 80% with 100 replicates are shown. *Scale bars* indicate the base substitution rate per site. Accession numbers are as follows: the gB genes for onager, gazelle, and zebra strains were AB280630, AB280624, and AB280634; the gE and gI genes of onager, gazelle, and zebra were AB280631, AB280627, and AB280635; the ORF 8 genes of onager, gazelle, and zebra were AB280628, AB280625, AB280632; the ORF 15 gene of onager was AB281333 (the gene from gazelle has the same sequence) and that of zebra was AB281334; ORF 68 of onager, gazelle, and zebra were AB280629, AB280626, and AB280633. Other accession numbers: genomes of ab4p (AY665713) and V592 (AY464052); glycoprotein B of T-965 (DQ095873), Ro-1 (DQ095872), Ab1 (M36298), Mar87 (DQ095871), Kentucky D (AB279609), Army-183 (M35145), RacL11 (X95374), and HVS25A (D00401); glycoprotein E of RacL11 (AB279608), Ab1 (M36299), and KentuckyD (AB279611); glycoprotein I of Ab1 (M36299); ORF15 of Kentucky A (S57839); ORF68 of RacH (Z67986) and KentuckyA (M80429)

was provided by Dr. A. J. Davison (University of Glasgow, UK) [18]. Viruses were propagated in fetal equine kidney (FEK) cells as described previously [9].

The viruses were inoculated to FEK cells at a multiplicity of infection of 0.01. Total DNA was extracted as described previously [8]. Primers used are listed in Table 1. PCR products of the glycoprotein genes (gB, gE and gI) were directly sequenced. Tegument protein genes (ORF 8, 15 and 68) were cloned into the pGEM-T Easy plasmid and sequenced using standard procedures. The plasmids were cloned as a part of a separate project on functional analyses of teguments. Nucleotide sequences were determined by Dragon Genomic (Dragon Genomic, Inc., Japan). Sequences were analyzed using GENETYX-MAC ver.12 (Software Development Co., Ltd, Japan). Sequence alignments were performed using the MAFFT program [10]. Phylogenic trees were constructed by the DNA maximum likelihood program of the PHYLIP package [7]. Branching was confirmed by bootstrapping with 100 sets of data. The other sequences of EHV-1 from horses, antelope and zebra were obtained from the NCBI database for comparative analyses. Accession numbers are shown in the legend of Fig. 1.

Sequences of glycoprotein and tegument genes of onager, zebra and gazelle isolates, as well as those of several horse strains from Japan were determined in order to clarify phylogenic relationship among the viruses. The gB gene sequences of onager, zebra and gazelle isolates were identical to each other, forming their own unique phylogenetic branch including other EHV-1 isolates from

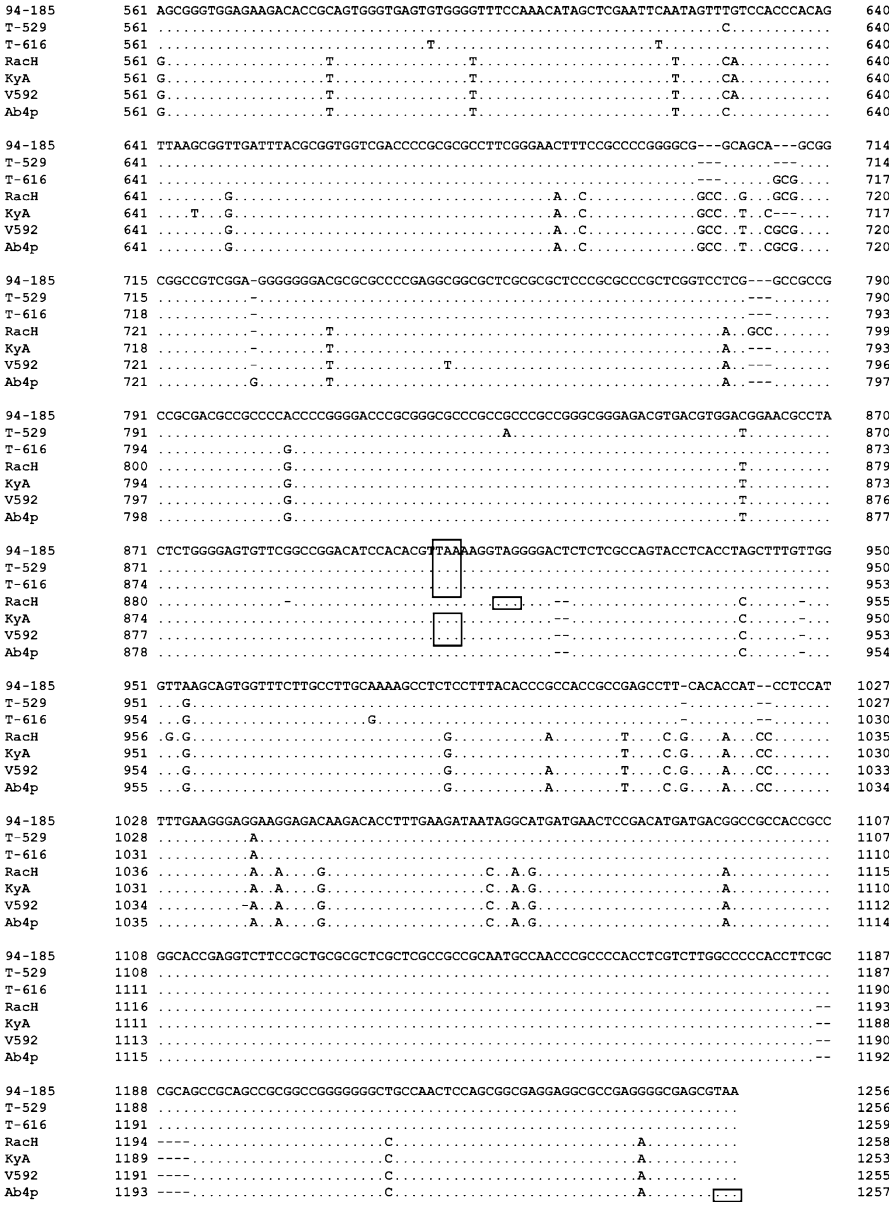

Fig. 2 Multiple alignment of a part of the ORF68 nucleotide sequences, which are from position 561 to the end of the putative open reading frame. The *boxes* indicate termination codons. Only nucleotides that differ from the 94–185 sequence are shown. *Dashes* indicate deletions

antelope (Ro-1) and zebra (T-965) isolates (Fig. 1a). The gE sequences of the onager and gazelle isolates differed by two bases from the sequence of the zebra isolate, while the amino acid sequences were identical among the three isolates. The glycoprotein I sequences of the zebra and gazelle isolates were similar and differed by several bases from the onager isolate.

Sequencing of tegument genes ORF8, ORF15, and ORF68 indicated that the onager and gazelle isolates were almost identical to each other, while the zebra isolate was different from the two viruses (Fig. 1d, e). These three viruses were distantly related to EHV-1 isolates of horses.

The ORF68 sequences of most EHV-1 isolates include several deletions of nucleotides that resulted in frameshifts. Therefore, the predicted amino acid sequences of ORF 68 varied in length from strain to strain. However, by using just the nucleotide sequence corresponding to the open reading frame of Ab4p, it was possible to compare the entire putative ORF68 sequences (Fig. 2). In the region of the putative ORF68 sequences, 94–137 and T-529 were similar to each other and distantly related to T-616 (Fig. 1f).

The gazelle, zebra and onager examined in this study were each kept close to zebras (*E. bruchelli* and *E. grayvi*). The EHV-1 isolates from onager, zebra and gazelle (T-529, T-616 and 94–137, respectively) were distinguishable from horse isolates, as shown in our previous work on gG sequence analysis [9].

The present study showed that the onager isolate (T-529) was closely related to the gazelle isolate (94–137). EHV-1 94–137 was isolated from a Thomson' gazelle, a non-equine species that was kept in an enclosure with a plains zebra (*E. bruchelli*) [11]. The onager, from which T-529 was isolated, was also reported to be kept with *E. bruchelli* at the zoo. Molecular phylogenic analysis of *Equus* spp. [15] has indicated that *E. h. onager* is more closely related to the horse, (*E. caballus*) than to zebras (*E. burchelli* and *E. grevyi*). Herpesviruses are generally regarded to have co-evolved with their hosts [13]. If the EHV-1 in onager evolved with its host, T-529, the onager isolate, should be closer to EHV-1 in horse than EHV-1 in zebras. Our present data did not support this hypothesis. The genetic distance between the onager isolate and horse isolates was almost identical to the genetic distance between the zebra isolate and the horse isolates (Fig. 1). The close relatedness of 94–137 and T-529, together with their association with *E. burchelli*, suggests that the isolates of the gazelle and onager were transmitted from *E. bruchelli* that were kept near them.

Other EHV-1 isolates from non-equine species except cattle were not available to us. Recently, Borchers et al. [4] reported antibodies against equine herpesviruses in *E. burchelli* in the Serengeti and we found zebras to be their natural host of EHV-9 (Borchers et al. in press), indicating that zebra species possess multiple equine herpesviruses which could be transmitted to other species as severe pathogens. Therefore, further molecular analyses are needed to determine how specific the viruses are to their host species.

In summary, sequence analysis of glycoprotein- and tegument-encoding genes has indicated that gazelle EHV-1, 94–137, is more closely related to onager EHV-1 (T-529) than to zebra EHV-1 (T-616). The group containing these three EHV-1 isolates is distantly related to horse-derived EHV-1s.

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