

## Gazelle Herpesvirus 1: A New Neurotropic Herpesvirus Immunologically

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A herpesvirus was isolated from Thomson's gazelle (*Gazella thomsoni*) kept at a zoological garden in Japan during an outbreak of epizootic acute encephalitis. The virus, gazelle herpesvirus 1 (GHV-1), was serologically related to equine herpesvirus 1 (EHV-1). However, DNA fingerprints of GHV-1 were different from those of EHV-1 and other equine herpesviruses. Southern hybridization with probes of cloned *Bam*HI fragments derived from U<sub>L</sub> and U<sub>S</sub> segments of EHV-1 revealed differences in the DNA restriction profiles throughout the entire genome. Nucleotide sequences were determined for a conserved region of an essential envelope glycoprotein B (gB) gene and a type-specific glycoprotein G (gG) homologue gene. The predicted amino acid sequence of GHV-1 gB showed 97, 92, 61, and 57% identity to EHV-1, EHV-4, feline herpesvirus, and pseudorabies virus, respectively, indicating that GHV-1 was closer to EHV-1 than any other herpesvirus. The GHV-1 gG gene showed 93.2, 92.3, and 53% identity to EHV-1, EHV-8, and EHV-4 gGs, respectively. GHV-1 was virulent to suckling mice of the ICR strain by intracerebral inoculation and was virulent to 4-week-old BALB/c mice by intranasal inoculation, causing neurological symptoms and death. We conclude that GHV-1 is a new type of equine herpesvirus with strong neurotropism. © 1997 Academic Press

### INTRODUCTION

Herpesviruses have been isolated from various animals (Roizmann *et al.*, 1992). Their infection in natural hosts is usually latent or mild. Some herpesviruses may cross species barriers and induce severe and fatal diseases in other hosts. An example is an infection of humans with B virus (cercopithecine herpesvirus 1), a monkey herpesvirus, which often has a fatal outcome in humans (Weigler, 1992).

Equine herpesvirus 1 (EHV-1) is a pathogen which causes abortion, respiratory disease, and occasionally neurological disorders in horses (Allen and Bryans, 1986). EHV-1 infections are usually limited to equine species, although infections in nonequine animals including cattle, alpacas, llamas, and antelopes have been reported (Chowdhury *et al.*, 1988; Crandell *et al.*, 1988; Rebhun *et al.*, 1988). Diseases induced by EHV-1 in these ruminants include abortion, blindness, and malignant catarrhal fever-like syndrome. DNA fingerprinting of ruminant isolates showed a high degree of similarity or complete identity to EHV-1 (Chowdhury *et al.*, 1988; Crandell *et al.*, 1988; Rebhun *et al.*, 1988).

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*Gazella thomsoni* is a small ruminant native to Africa. Several zoological gardens in Japan have imported gazelles for display. There have been a few reports on viral and bacterial diseases in *Gazella* spp. (Crandell *et al.*, 1988; Yeruham *et al.*, 1994). We encountered an outbreak of acute fatal disease in a herd of gazelles kept at a zoological garden in September, 1993. A herpesvirus was isolated from the brain of affected gazelles and was determined to be the causative agent. This report concerns the virology, epizootiology, and pathology of this virus.

### MATERIALS AND METHODS

#### *G. thomsoni*

Twelve *G. thomsoni* were first introduced into the zoological garden of Toyohashi city, Aichi prefecture, in February, 1992. Since then, eight normal newborns and one malformed newborn have been delivered. The latter was sacrificed after birth. Ten died of injury following crashing against trees due to fright, biting by stray dogs, reproductive failure, or septicemia. There were 10 gazelles at the end of September, 1993, when the first sudden death was observed. Eight gazelles died over a 2-week period (Table 1). Seven of these had acute encephalitis. The remaining one was a newborn that died of malnutrition, because the sick mother gazelle, P4122, refused nursing. No evidence of infection was found in this foal. The onset

TABLE 1  
Occurrence of Acute Neurological Diseases and Death in Gazelles

No. gazelle	Age <sup>a</sup>	Sex	Disease onset	Death	Nonsuppurative encephalitis <sup>b</sup>
P4121 <sup>c</sup>	47	Female	— <sup>d</sup>	9/26/93	Yes
P4123 <sup>c</sup>	58	Female	—	10/5/93	Yes
P4122	60	Female	10/3/93	10/6/93	Yes
P4124	68	Female	10/5/93	10/9/93	Yes
P-92-12	10	Male	10/6/93	Recovered	N.D. <sup>e</sup>
P-A-24	46	Male	10/7/93	10/9/93	Yes
P4119	47	Male	10/7/93	10/11/93	Yes
P4120	2	Male	10/8/93	10/12/93	Yes
P-A-8	48	Female	—	Recovered	N.D.

<sup>a</sup> Age in months.

<sup>b</sup> Confirmed by histopathology.

<sup>c</sup> Died with no symptoms.

<sup>d</sup> Neurological disorders not observed.

<sup>e</sup> Not examined.

of disease was sudden. Two animals that died of infection had no sign of disease. The main symptoms were neurological in nature, such as rotation movements and spasms. Most of the affected gazelles died within 4 days after the onset of symptoms. Laboratory tests showed positive occult blood and albumin in the urine. Of the two gazelles that did not die, one gazelle had mild disease and recovered 2 days after veterinary treatment, and the other, a pregnant gazelle, did not develop neurological symptoms. This gazelle delivered a normal foal in December, 1993. The etiological investigation was conducted with two animals, P4119 and P4120, involved in the later stage of the episode.

### Viruses and cell cultures

The reference viruses used were strains HH-1 (Kawakami *et al.*, 1970) and 89c25 (Matsumura *et al.*, 1994) for EHV-1 and TH20 (Kawakami *et al.*, 1962) for EHV-4.

Several cell lines were used for the isolation, propagation, and titration of viruses, including Madin and Darby bovine kidney (MDBK) cells, fetal horse kidney (FHK) cells, and porcine kidney (PK-15) cells. Cells were cultured with Eagle's minimal essential medium (MEM) supplemented with 5% fetal bovine serum, 100 units/ml of penicillin, and 100 µg/ml of streptomycin.

Titrations of viruses were done by plaque assay using methylcellulose medium for overlay (Matsumura *et al.*, 1996).

### Antibodies

Antisera were prepared against the HH-1 strain of EHV-1, the TH20 strain of EHV-4, and the isolates by immunization of Japanese White rabbits. For HH-1 and TH20 strains, rabbits were given four intravenous inoculations of 1 ml of sucrose gradient-purified viruses (10<sup>7</sup> plaque-forming units (PFU)/ml) at 10-day intervals. For the isolates, rabbits were given six intravenous inocula-

tions of 1 ml of concentrated cell-free virus preparation (10<sup>6</sup> PFU/ml) at 14-day intervals. Rabbits were bled 7 days after the last inoculation. Sera were stored at -20°. Monoclonal antibodies specific to EHV-1 and EHV-4 were kindly provided by Dr. G. P. Allen (Yeargan *et al.*, 1985). Polyclonal rabbit antisera to bovine herpesvirus 1 (infectious bovine rhinitis virus, IBRV), swine herpesvirus 1 (pseudorabies virus, PRV), and alcelaphine herpesvirus 1 (malignant catarrhal fever virus, MCFV) were kindly provided by Dr. K. Okazaki, by Dr. M. Narita, and by Dr. T. Sugimura.

### Histopathology and immunohistochemistry

Gazelles were necropsied on the day of death or the day after death. Tissue samples of major organs were fixed in 10% buffered formalin, embedded in paraffin, and sectioned for hematoxylin and eosin (H and E) staining. Viral antigens were detected by immunostaining using the avidin-biotin-peroxidase method (Vectastain ABC kit, Funakoshi, Tokyo) with rabbit anti-EHV-1 HH-1 antiserum as described previously (Yanai *et al.*, 1995).

### Isolation of virus

Viral isolation was attempted from brain, spinal cord, kidney, liver, lung, and spleen tissues. Tissues were homogenized to prepare 10% (w/v) suspensions in MEM.

Isolations were performed with a 24-well culture plate. Each well was inoculated with 0.1 ml of tissue samples. The plates were incubated in a humidified 5% CO<sub>2</sub> incubator at 37° and observed daily for 1 week for cytopathic effects (CPE). Cultures showing CPE were harvested and stored at -80°. CPE negative cultures were passaged at least three times.

### Neutralization test

For serological identification of GHV-1, neutralization tests were performed with rabbit antisera against herpes-

viruses including PRV, IBRV, MCFV, EHV-1, and EHV-4. PRV, IBRV, and MCFV are neurotropic viruses in ruminants. EHV-1 causes neurological disorders occasionally in horses and ruminants. EHV-4 is serologically related virus to EHV-1.

The neutralization test was done by the 50% plaque reduction method. An equal volume of 0.2 ml of serial twofold dilutions of heat-inactivated serum and viral suspension containing 200 PFU were incubated overnight at 4° or for 1 hr at 37°. Four wells were inoculated per dilution at 50  $\mu$ l/well. The plates were incubated at 37° for 1 hr with intermittent tilting every 15 min. After absorption, the inocula were removed and replaced with 1 ml of MEM containing 0.75% methylcellulose and 5% fetal bovine serum. The plates were incubated at 37° for 3 days. Plaques were stained with 0.01% neutral red and counted. Antibody titers were expressed as the highest serum dilution producing greater than 50% reduction in plaque counts.

### DNA fingerprinting and Southern analysis

Cell-free virions were sedimented by ultracentrifugation of culture supernatants at 100,000 *g* for 2 hr at 4°. Viral pellets were suspended in 0.5 ml of 10 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA, at pH 8.0. Viral DNA was extracted and dissolved in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0, as described previously (Fukushi and Hirai, 1989).

DNA was digested with 1 unit of enzyme for 0.1  $\mu$ g DNA per microliter. The digests were electrophoresed at 35 to 40 V for 18 to 24 hr on a horizontal 0.7% agarose gel in Tris-phosphate buffer (89 mM Tris, 22 mM phosphate, 2 mM EDTA) (Maniatis *et al.*, 1982).

Vacuum transfer of DNA was done as described previously (Fukushi and Hirai, 1989). Probes were prepared from cloned *Bam*HI A, D, J, and N fragments located at various sites of the EHV-1 genome (Kirisawa *et al.*, 1993b). DNA probes for Southern analysis were labeled with digoxigenin-dUTP by the random priming method. Hybridization was carried out at 65° for 18 hr in 0.75 *M* sodium ion buffer. After hybridization, the sheets were washed two times at room temperature for 5 min in 2 $\times$  SSC (1 $\times$  SSC is 0.15 *M* sodium chloride plus 0.015 *M* sodium citrate, pH 7.0) containing 0.1% SDS, followed by two 15-min washes at 65° in 0.1 $\times$  SSC containing 0.1% SDS. Immunological detection of Southern blots was performed using alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim, Yamanouchi, Tokyo, Japan) and the chemiluminescent substrate Lumi-Phos 530 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) according to the manufacturer's instructions.

### Polymerase chain reaction for amplifying glycoprotein genes

Primers for the glycoprotein B (gB) gene and conditions for PCR have been described elsewhere (Kirisawa

*et al.*, 1993a). The glycoprotein G (gG) homologue gene was amplified with primers GG1 (5'-CACACTCAGTTA-TTACAGAC-3') and GG2 (5'-CTGTAAAGCGGTAGTAAG-CTGC-3') using TaKaRa LA PCR kit (TaKaRa BIOMEDICALS, Shiga, Japan) and the shuttle PCR program (30 cycles of 98° for 10 sec and 65° for 5 min) in a thermal cycler (GeneAmp PCR System 9600, Perkin-Elmer-Cetus, ABI, Japan). The amplified region corresponded to 127,654 to 128,973 of the EHV-1 Ab4p strain genome sequence (GenBank Accession No. M86664). PCR products were purified for DNA cloning by chloroform extraction and ethanol precipitation.

### Cloning and sequencing of DNA

Molecular cloning was performed by the method of Maniatis *et al.* (1982). The bacterial hosts were *Escherichia coli* DH5a, MV1184, and LE392. The plasmid and phage vectors were pUC19, pTV119, pT7Blue(R) (TaKaRa Biomedicals, Shiga, Japan), and lambda EMBL3. Sequencing reactions were examined with the AutoRead and AutoCycle sequencing kits (Pharmacia Japan, Tokyo, Japan). The labeled DNA was sequenced with an ALFred sequencer (Pharmacia Japan).

### Comparative sequence analysis

Sequence data were transferred into and edited by the Wisconsin Sequence Analysis Package (Genetics Computer Group, Inc., Madison, WI) (Devereux *et al.*, 1984). The partial sequence of the gB homologue gene and the complete sequence of the gG homologue gene of the isolates were submitted to the DNA Data Bank of Japan (DDBJ, National Institute of Genetics, Mishima, Japan). Accession numbers are D49800 for the gB homologue and D85905 for the gG homologue. Other DNA sequences were obtained from DDBJ for the gB gene of the EHV-1 Ab4p strain (Accession No. M86664) and Ab1 strain (Accession No. M36298), EHV-4 1942 strain (Accession No. M26171), feline herpesvirus (FHV) (Accession No. S49775), and pseudorabies virus (PRV) Becker strain (Accession No. M17321), and for the gG gene of EHV-1 Ab4p (Accession No. M86664), EHV-8 (asinine herpesvirus 3, AHV3) (Accession No. U24184), EHV-4 405/76 (Accession No. S44796), FHV (Accession No. S72415), and PRV (Accession No. M10986). Multiple alignments were examined by ClustalW (Thompson *et al.*, 1994) on a SUN workstation. Genetic distances were calculated by the Jukes and Cantor method (Jukes and Cantor, 1969) and the gamma-distribution method (Jin and Nei, 1990) for nucleotide sequences and by the PAM method (Dayhoff *et al.*, 1978) for predicted amino acid sequences. The phylogenetic tree was constructed by the neighborhood-joining method (Saitou and Nei, 1987). These molecular phylogenetic analyses were examined by the program package ODEN (Ina, 1994) at the National Institute of Genetics, Mishima, Japan; the Phylip package

version 3.572 (Felsenstein, 1989); and the GCG Wisconsin package (Devereux *et al.*, 1984) on a SUN workstation at the Gifu University Computing Center, Gifu, Japan.

## Mice

Slc:ICR (ICR) and BALB/c Cr Slc (BALC/c) mice were obtained from Japan SLC, Inc., Shizuoka, Japan. Suckling ICR mice were inoculated intracerebrally with  $10^3$  to  $10^5$  PFU of cell-free viral suspensions. The infected mice were nursed by their own mothers and observed for at least 14 days. Four-week-old BALB/c mice were inoculated with  $10^2$  to  $10^5$  PFU of viruses via several routes, including oral, intraperitoneal, intravenous, subcutaneous, and intranasal routes. To observe viral transmission, uninoculated mice of the same age were kept with inoculated mice in the same cage. Major organs including brain and lung were taken for histopathology and viral isolation after death.

## RESULTS

### Pathological findings of gazelles

No gross abnormalities were observed in seven gazelles at autopsy. Most of the organs, except brains and spinal cords, did not show apparent histological changes. The major findings of the central nervous system were ischemic changes of the neurons in the cerebral cortex. Acidophilic or amphophilic intranuclear inclusion bodies were seen frequently in the neurons (data not shown). The spongiosis consisted of diffuse or nodular hyperplasia of microglia and perivascular infiltration of mononuclear cells was observed in the cerebral cortex. These findings are characteristic of nonsuppurative encephalitis.

### Isolation of gazelle herpesviruses

Histopathological findings indicated viral encephalitis. Bacterial cultures were negative. Therefore, two gazelles (P4119 and P4120) were examined in more detail, because fresh samples were available. Viral isolation was attempted in MDBK and PK-15 cell cultures with tissue homogenates. CPEs, i.e., swelling, rounding, multinuclear cell formation, and detachment of cells, were observed in MDBK cell cultures 2 days after inoculation of brain tissue from P4119. This gazelle had marked histopathological changes in the brain. CPE did not develop until the second passage with P4120. This gazelle had a milder brain lesion. Isolation from other organs from these two gazelles was negative. When the same samples were inoculated into PK-15 cells, no CPE was observed in three blind passages. These two isolates were labeled as gazelle viruses P19 and P20 for gazelles P4119 and P4120, respectively.

CPEs of P19 and P20 were prevented by incorporation of BrdU in culture medium and treatment of the inoculum with chloroform, indicating that the CPE agents were

enveloped DNA viruses (data not shown). Electron microscopic examination showed immature capsids of herpesvirus-like particles in the nucleus of infected MDBK cells (data not shown). Furthermore, negative staining of concentrated specimens showed typical herpesvirus-like virions. These findings suggested that the CPE agents P19 and P20 were herpesviruses. Because these isolates had marked characteristics of herpesvirus, they were named gazelle herpesvirus 1 (GHV-1). GHV-1 P19 was used for further analyses.

### Serological identification of GHV-1

Neutralization of GHV-1 was observed only with rabbit anti-EHV-1, anti-EHV-4, and anti-GHV-1 antisera. The titers of the anti-EHV-1 were 1:1280 for GHV-1 and greater than 1:2560 for EHV-1. Antisera to PRV, IBRV, and MCFV did not neutralize GHV-1. GHV-1 reacted with monoclonal antibodies specific to EHV-1 but not with EHV-4, indicating that GHV-1 was related to EHV-1 serologically.

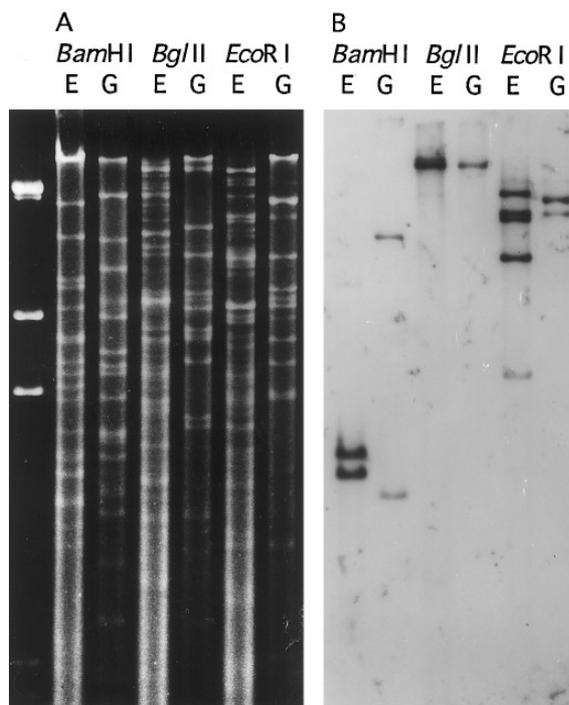


FIG. 1. DNA fingerprints (A) and Southern blots (B) of GHV-1 (G) and EHV-1 HH-1 strains (E). DNAs from cell-free virions were digested with *Bam*HI, *Bgl*II, or *Eco*RI. Digested DNAs were run in a 0.7% agarose gel followed by staining with ethidium bromide (0.5  $\mu$ g/ml). DNAs were vacuum-transferred onto nylon membrane and hybridized with the cloned HH-1 *Bam*HI N fragment labeled with digoxigenin as probe. The lane at the far left is  $\lambda$  DNA digested with *Hind*III. The *Bam*HI N fragment probe hybridized with 4.95 and 4.6 kb of *Bam*HI, 37.9 kb of *Bgl*II, and 17.9, 16.9, 9.5, and 6.6 kb of *Eco*RI fragments of EHV-1 and with 14.5 and 4.3 kb of *Bam*HI, 37.9 kb of *Bgl*II, and 17.7 and 16.9 kb of *Eco*RI fragments of GHV-1. The *Bam*HI N fragment (4.95 kb) of HH-1 containing a part of IR<sub>S</sub>/TR<sub>S</sub> is shown to hybridize with a P fragment (4.6 kb) of genomic DNA digested with *Bam*HI as well as with the N fragment itself.



TABLE 2

Genetic Distances of Glycoprotein B Homologue Gene among GHV-1, EHV-1, EHV-4, FHV, and PRV

	GHV-1	EHV-1	EHV-4	FHV	PRV
GHV-1		0.046	0.170	0.582	0.500
EHV-1	0.024		0.164	0.582	0.490
EHV-4	0.073	0.059		0.553	0.554
FHV	0.509	0.499	0.485		0.662
PRV	0.645	0.626	0.607	0.532	

*Note.* Numbers to the right of the diagonal space indicate genetic distances (substitution rate of base per site) determined from nucleotide sequences according to Jukes and Cantor (1969). Numbers to the left of the diagonal indicate genetic distances (substitution rate of amino acid per site) determined from amino acid sequences using the PAM table (Dayhoff *et al.*, 1978).

to identify similarities between GHV-1- and EHV-1-related viruses, including EHV-1, EHV-4, and EHV-8, indicating that GHV-1 did not belong to any equine herpesvirus type.

Southern hybridization was used to estimate the genetic relatedness between GHV-1 and EHV-1. Probes were prepared from cloned *Bam*HI A, D, J, and N fragments of EHV-1 HH-1 strain, fragments of which were located at various sites of the EHV-1 genome (Kirisawa *et al.*, 1993b). Hybridization and washing were performed under stringent conditions: hybridization at 65° for 16 hr followed by washing at room temperature for 5 min in 2× SSC containing 0.1% SDS and two 15-min washes at 65° in 0.1× SSC containing 0.1% SDS. All EHV-1 probes tested hybridized with GHV-1 DNA (Fig. 1B and data not shown). The sizes and numbers of hybridized fragments were different between the two. Conversely, cloned GHV-1 DNA fragments hybridized with EHV-1 DNA (data not shown).

A segment of the GHV-1 glycoprotein B gene, consisting of 1181 of 2942 base pairs corresponding to positions 1477 to 2657 of the gB gene of the Ab4p isolate of EHV-1, was sequenced for quantitative evaluation of phylogenetic relatedness (Fig. 2). The gene segment analyzed was from a relatively conserved region of the gB gene. This region had been used for differentiation of EHV-1 and EHV-4 by PCR (Kirisawa *et al.*, 1993a). The results showed 95.5% nucleotide sequence homology between GHV-1 and EHV-1 and 84.7% homology between GHV-1 and EHV-4 (Table 2). Genetic distances were estimated as base substitution rate per site by the Jukes–Cantor method for nucleic acid sequence and as amino acid substitution rate per site by Dayhoff's method. Genetic distances of the nucleic acid sequences were 0.046 between GHV-1 and EHV-1 and 0.170 between GHV-1 and EHV-4. The homologies and genetic distances of predicted amino acid sequences of GHV-1 were 97.5% and 0.024 for EHV-1 and 92.6% and 0.073 for EHV-4.

The sequence of another glycoprotein gene, the glyco-

protein G homologue gene, was determined to compare it with EHV-1, EHV-4, and EHV-8 to study its relatedness to alphaherpesviruses (Fig. 3). The GHV-1 gG gene consists of 1233 bp and codes for a predicted protein of 411 amino acids. The size of this homologue was identical to that of the EHV-1 and EHV-8 gG gene and was 6 amino acids longer than the EHV-4 gG gene (Fig. 4). The GHV-1 gG homologue gene shared 92.4 and 93.2% homology with EHV-1, 93.5 and 92.3% with EHV-8, and 65.3 and 66.1% with EHV-4 in nucleotide and predicted amino acid sequences, respectively (Table 3). Genetic distances were estimated as base substitution rate per site by the gamma distance method for the nucleotide sequences and as amino acid substitution rate per site by Dayhoff's method for predicted amino acid sequences. Estimated genetic distances from GHV-1 were 0.085 and 0.091 to EHV-1, 0.071 and 0.091 to EHV-8, and 0.0645 and 0.0418 to EHV-4 for nucleotide and predicted amino acid sequences, respectively. The gG amino acid sequences revealed a conserved region at the N-terminus and a highly variable region at the C-terminus. Phylogenetic analyses using the gG nucleotide and amino acid sequences showed equal genetic distances among GHV-1, EHV-1, and EHV-8 (Fig. 5).

#### Experimental infection of mice with GHV-1

*Suckling mice.* Intracerebral inoculation of suckling mice with GHV-1 induced growth retardation and neurological disorders including spasm and ataxia. All mice died within 8 days after inoculation. The pathological changes observed in brains were degeneration and necrosis of neurons, glial reaction, and perivascular infiltration of mononuclear cells in thalamus and cerebral cortex. Acidophilic intranuclear inclusion bodies were observed frequently in the neurons. Immunohistochemical staining with anti-GHV-1 antiserum showed positive cells in the degenerated foci (data not shown).

*Young mice.* Young mice intranasally inoculated with GHV-1 developed neurological symptoms including clamorous behavior, convulsions, and weight loss 4 to 6 days postinoculation. Symptoms developed sooner with higher doses than with lower doses. All mice inoculated with 10<sup>4</sup> PFU of GHV-1 became comatose and died 6 to 8 days postinoculation. Mortalities were 100% with 10<sup>4</sup> PFU and 33% with 10<sup>2</sup> PFU. The LD<sub>50</sub> was less than 10<sup>3</sup> PFU. Mice inoculated by other routes did not develop disease. No clinical symptoms were observed in uninoculated mice kept in the same cage with inoculated mice for the 12 days of observation.

Virus was recovered from the brain of intranasally inoculated mice only. Histopathologic findings included bilateral degeneration and necrosis of neurons in the cerebral cortex and hippocampus. Several degenerated nerve cells containing acidophilic intranuclear inclusion bodies were found. Immunohistochemical staining with

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1 ctgtaaagcggtagtaagctgcagtt ATG TTG ACT GTC GCA GCA GCT CTG AGT CTG CTC AGC TTG 65
1 M L T V A A A L S L L S L 13

66 CTT ACG AGC GTC GCC GGA CGG CTC GCC CCA GAT GAA CTC TGT TAT GCC GAA CCA CGC AAG 125
14 L T S V A G R L A P D E L C Y A E P R K 33

126 ACT GGC CAC CCA CCA AAC ACC CAG CCC GAA CGT CCA CCC GTA ATA TTT GAG CCC CCA ACA 185
34 T G H P P N T Q P E R P P V I F E P P T 53

186 ATT GCG ATT AAG GCT GAA TCC AAG GGT TGC GAG CTA ATT TTA TTA GAG CCA CCC ATA GAT 245
54 I A I K A E S K G C E L I L L E P P I D 73

246 GTA AGC TAT CGC AGA GAA GAT AAG GTG AAC GCG TCC GTT GCT TGG TTC TTT GAC TTT GGG 305
74 V S Y R R E D K V N A S V A W F F D F G 93

306 ACT TGC CAA ATG CCC ATC GCA TAC AGA GAG TAT TAC GGT TGT ATT GGC AAT GCT ATT CCC 365
94 T C Q M P I A Y R E Y Y G C I G N A I P 113

366 TCC CCC GAG ACC TGT GAT GCT TAC TCA TTT ACT CTC CTT AGG ACA GAG GGT ATT GTG GAG 425
114 S P E T C D A Y S F T L L R T E G I V E 133

426 TTT ACC ATC GTA AAC ATG AGC CTA CTG TTT CAG CCT GGA ATA TAC GAT AGT GGC AAT TTT 485
134 F T I V N M S L L F Q P G I Y D S G N F 153

486 ATC TAC AGC GTT CTC CTG GAC TAC CAC ATA TTT ACA GGA CGT GTA ACG TTG GAT GTG GAA 545
154 I Y S V L L D Y H I F T G R V T L D V E 173

546 AAG GAC ACA AAC TAT CCA TGT GGC ATG ATT CAT GGA CTT ACT GCT TAC GGA AAC ATC AAC 605
174 K D T N Y P C G M I H G L T A Y G N I N 193

606 GTA GAT GAG ACC ATG GAC AAC GCC AGC CCG CAC CCG CGT GCC GTG GGG TGC TTT CCC GAG 665
194 V D E T M D N A S P H P R A V G C F P E 213

666 CCC ATC GAC AGC GAA GGG GGG GCA AAC GTT ACA TTT ACC GAA TTG GGG ATA CCA GAC CCA 725
214 P I D S E G G A N V T F T E L G I P D P 233

726 AAC TCA TTT CTT GAT GAC GAG AGT GAT TAC CCG AAT ATA TCA GAC TGT CAC TCG TGG GAG 785
234 N S F L D D E S D Y P N I S D C H S W E 253

786 TCA CAC ACT TAC CCA AAT ACG CTG AGG CCG GCC ACC GGA CCA CAG ACA TTG TTG GTG GGT 845
254 S H T Y P N T L R P A T G P Q T L L V G 273

846 GCA GTT GGA CTC AGA ATC CTG GCC CAG GCA TGG AAG TTT GTC GGT GAT GAA ACG TAC GAC 905
274 A V G L R I L A Q A W K F V G D E T Y D 293

906 ACC ATC CGC GCA GAA GCA AAA AAT CTA GAG AAC CAC GTA CAC TCC AAT GCA GCA GAT TCG 965
294 T I R A E A K N L E N H V H S N A A D S 313

966 CCT CCC GAA ACT CCA TCG CCG CAG GAA AAC TTG AAC GAC CCC GAA GTT GCC CAC CTG CGA 1025
314 P P E T P S P Q E N L N D P E V A H L R 333

1026 AGC GGC CAA AAC GAA GAC AAC ACA CAC ACG GGG GGT GCG TCA AAC GGC ATC CAG GAC TGT 1085
334 S G Q N E D N T H T G G A S N G I Q D C 353

1086 GAC AGT CAG CTC AAA ACT GTG TAT GCC TGC TTG GCT CTC ATT GGA CTC GGC ACA TGT GCC 1145
354 D S Q L K T V Y A C L A L I G L G T C A 373

1146 ATG ATA GGA CTG ATA GTT TAC ATC TGT GTA TTG AGG TCA AAA CTA TCC TCC CGG GAT TTT 1205
374 M I G L I V Y I C V L R S K L S S R D F 393

1206 TCG CGC GCG CAA AAT GTA AAA CAT AGA AAT TAC CAG CGA CTT GAG TAC GTT GCT TAA cacc 1266
394 S R A Q N V K H R N Y Q R L E Y V A * 412

1267 tgtaaaataaaaaaagtttcaaatcgaaaacattgttctgtaataactgagtgtg 1323

```

FIG. 3. Nucleotide and predicted amino acid sequences of glycoprotein G homologue gene of GHV-1. An open reading frame and amino acids conceptually translated from the sequence are in uppercase letters.

anti-GHV-1 antiserum showed positive cells in the de-generated foci (data not shown).

## DISCUSSION

We reported an epizootic encephalitis in gazelles in the zoological garden. The diagnosis was made on the

basis of autopsy in which 7/9 animals showed histological evidence of encephalitis and a herpes-like virus was isolated from the brains of two gazelles. Epidemiological, clinical, and laboratory studies indicated that this outbreak originated within the gazelle herd in the zoological garden. The microbiological, immunological, genetic, and ultrastructural studies determined

	1					60
GHV-1	MLTVAALSL	LSLLTSVAGR	LAPDELCYAE	PRKTGHPPNT	QPERPPVIFE	PPTIAIKAES
EHV-1	---L---	-----AT--	-----	--R--S---	-----	-----
EHV-8	---L---	-----T--	-----	--R--S---	-----	-----
EHV-4	--A-G-T-C-	--F--GAT--	-----D---	-----PM-RS	K-KHQ-LL--	A-KV-LT---
	61					120
GHV-1	KGCELILLEP	PIDVSYRRED	KVNASVAWFF	DFGTCQMPIA	YREYYGCIGN	AIPSPETCDA
EHV-1	-----D-	-----	-----I---	-----A-R---	-----	-V-----
EHV-8	-----D-	-----K--	-----	-----A-R---	-----	V-----
EHV-4	---Q---D-	---MG--L--	-I--I---	---N-R---	---D-V--	-----G
	121					180
GHV-1	YSFTLLRTEG	IVEFTIVNMS	LLFQPGIYDS	GNFIYSVLLD	YHIFTGRVTL	DVEKDTNYPC
EHV-1	-----I-	-----	-----	-----	-----	E-----
EHV-8	-----I-	-----	-----	-----	-----	-----
EHV-4	-----VK--	V-----	--L-----	-S---A---	MDVL---I-	N--N-----
	181					240
GHV-1	GMIHGLTAYG	NINVDETMND	ASPHPRAVGC	FPEPIDSEGG	ANVTFTELGI	PDPNSFLDDE
EHV-1	-----	-----	-----	-----N-AW	-----	-----
EHV-8	-----	D-----	-----	-----NN-AW	-----	-----
EHV-4	--T-----D-	-----T.H	TT-----	---L-NFDAW	E---E-M--	-----
	241					300
GHV-1	SDYPNISDCH	SWESHTYPNT	LRPATGPQTL	LVGAVGLRIL	AQAWKFGVDE	TYD...TIR
EHV-1	G-----	---Y-----	---Q-----	-----	-----	-----
EHV-8	-----D-	---Y-----	---Q-----	-----	-----	-----
EHV-4	-----TM--Y	--DLY---KS	-KQ-E----	-I-----	-----EN-	--SQHTR-YT
	301					360
GHV-1	AEAKNLENHV	HS.NAADSPP	ETPSPQENLN	.....DP..	.....EVAH	LRSGQNE...
EHV-1	-----T--	P-.S--E-SL	-NQ-T--ES-	.....S...	-----	---VNSD...
EHV-8	-----T--	--.S---SS	-NQLS---V-	.....N...	-----	---C-----
EHV-4	RD--EVDVTQ	P-PVQ---VL	AKKRTSMKN-	PIYSEGK-HA	KPFSTIDSI-	TEGMK-NPVY
	361					420
GHV-1	.....	.DNTHTGAS	NGIQDCDSQL	KTVYACLALI	GLGTCAMIGL	IVYICVLRSK
EHV-1	.....	..S-----	-----	-----	-----	-----
EHV-8	.....	..A---VL	--L---N--	---I----	-----	-----
EHV-4	SESMLNVQH	S-SIT---VL	H-L---N--	---I----	--AHVP....	.....
	421		444			
GHV-1	LSSRDFSRAQ	NVKHRNYQRL	EYVA			
EHV-1	---N---	-----	---			
EHV-8	---E---	-----	---			
EHV-4	.....	.....	.....			

FIG. 4. Multiple alignment of predicted amino acid sequences of glycoprotein G homologue of GHV-1, EHV-1, EHV-8, and EHV-4. Multiple sequence alignment was examined by ClustalW (Thompson *et al.*, 1994). Amino acids that match the GHV-1 gG sequence are represented by dashes in individual gG sequences. Gaps are represented by dots.

that this gazelle virus (GHV-1) was a new type of herpesvirus.

GHV-1 possessed distinct characteristics from EHV-1 and other EHV-1 related viruses (Table 4). DNA fingerprints of GHV-1 were different from those of EHV-1, EHV-4, and EHV-8, although GHV-1 cross-reacted with EHV-1 and EHV-4 serologically. Southern analysis indicated that GHV-1 shared sequence homology with EHV-1. Sequences of gB and gG homologue genes of GHV-1 were closer to EHV-1 than to other equine herpesviruses including EHV-8. These data suggested that GHV-1 should be recognized as a new member of equine herpesvirus.

The phylogenetic analyses based on the gG homologue gene suggested that GHV-1, EHV-1, and EHV-

8 had branched almost simultaneously from an ancestral virus. Hence the present data would not support the hypothesis that EHV-8 or another closely related virus might be the progenitor of EHV-1 (Browning *et al.*, 1988; Ficorilli *et al.*, 1995; Studdert *et al.*, 1992). Therefore the origin and derivation of EHV-1 and other related viruses are still open questions.

GHV-1 was more virulent than other equine herpesviruses. GHV-1 caused lethal infection in natural and experimental animals including gazelle and mouse, while EHV-1 and other related viruses are not always lethal to the natural and experimental animals (Allen and Bryans, 1986). GHV-1 seemed to specifically prefer neurons and have distinct tissue tropism. Histopathological changes in the affected gazelles and mice were

TABLE 3

Genetic Distances of Glycoprotein G Homologue Gene among GHV-1, EHV-1, EHV-4, and AHV3

	GHV-1	EHV-1	EHV-8	EHV-4	FHV	PRV
GHV-1		0.085	0.071	0.645	1.875	2.292
EHV-1	0.091		0.092	0.639	1.817	2.347
EHV-8	0.091	0.079		0.642	1.846	2.509
EHV-4	0.418	0.385	0.365		1.890	2.959
FHV	1.151	1.134	1.158	1.167		3.259
PRV	1.421	1.413	1.371	1.518	1.668	

Note. Numbers to the right of the diagonal space indicate the number of nucleotide substitutions per site determined by the gamma distance method with  $a = 1$  (Jin and Nei, 1990). Numbers to the left of the diagonal space indicate genetic distances (substitution rate of amino acid per site) determined from amino acid sequences using the PAM table (Dayhoff *et al.*, 1978).

localized in brain tissues with degeneration of neurons and without evidence of respiratory infection. In the mouse model, intranasal inoculation of EHV-1 caused infection of the respiratory tract including nasal mucosa, trachea, and lung but did not cause infection of the nervous system (Awan *et al.*, 1990; Inazu *et al.*,

1993). Therefore GHV-1 has distinct virulence and tissue tropism in contrast to other equine herpesviruses. Experimental infection of mice by GHV-1 may be a useful model for studying the neuropathology of herpesvirus infection.

The range of the natural host(s) of GHV-1 is unknown. The host range of EHV-1 is generally believed to be limited to horses, although several EHV-1 isolates have been isolated from nonequine hosts including fallow deer (Kinyili and Thorsen, 1979; Thorsen *et al.*, 1977), cattle (Crandell *et al.*, 1979; Crandell *et al.*, 1988), antelope (Chowdhury *et al.*, 1988), and alpacas and llamas (Rebhun *et al.*, 1988). Fallow deer isolates were derived from several deer injected with dexamethasone. Others were derived from sporadic and epizootic occurrences. These indicated that EHV-1 can induce apparent and latent infection in nonequine hosts. It is interesting that a zebra kept in the same field with the gazelles and tested several months after the episode had neutralizing antibody to GHV-1 but not to EHV-1 (H. Fukushi and Y. Ochiai, unpublished data). Recent data have indicated that GHV-1 also infected and caused diseases in horses (Taniguchi *et al.*, in preparation). Therefore, it was unclear whether the zebra was infected by GHV-1 before the episode in the

TABLE 4

Characteristics of GHV-1, EHV-1, EHV-8, and EHV-4

	GHV-1	EHV-1	EHV-8	EHV-4
Restriction pattern <i>Bam</i> HI, <i>Bgl</i> II, and <i>Eco</i> RI	Different DNA fingerprints; few or no common restriction sites			
Sequence homology <sup>a</sup>				
Glycoprotein B				
GHV-1		95.5	n.d.	84.8
EHV-1	97.5		n.d.	85.3
EHV-8	n.d.	n.d.		n.d.
EHV-4	92.6	93.9	n.d.	
Glycoprotein G				
GHV-1		92.4	93.5	65.3
EHV-1	91.3		91.8	65.5
EHV-8	91.3	92.2		65.4
EHV-4	66.1	68.3	68.6	
Natural host	Gazelle	Horse	Donkey	Horse
Pathogenicity to hosts	Encephalitis	Abortion, paresis	Rhinitis	Pneumonia
Virulence to				
Suckling mice (ic) <sup>b</sup>	Yes	Yes	Unknown	No
Young mice (in) <sup>c</sup>	Encephalitis	Pneumonia	Unknown	No
Cell culture susceptibility				
Equine cells	Yes	Yes	Yes	Yes
MDBK cells	Yes	Yes	n.d.	No
PK-15 cells	No	Yes	n.d.	Yes

<sup>a</sup> Upper right of diagonal square is nucleotide sequence similarity, and lower left of diagonal space is the predicted amino acid sequence similarity. n.d., not determined, because sequence data of EHV-8 glycoprotein B were not available.

<sup>b</sup> ic, intracerebral inoculation.

<sup>c</sup> in, intranasal inoculation.

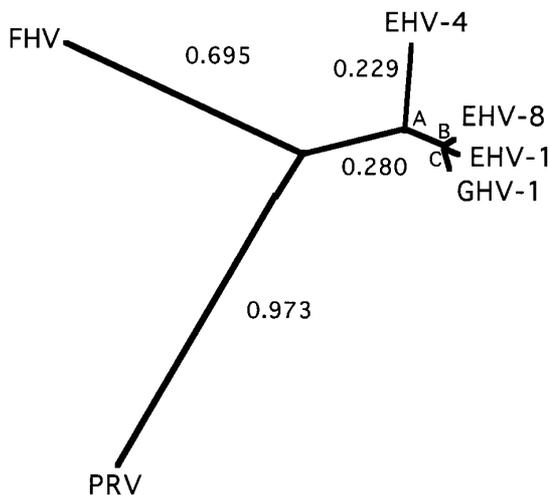


FIG. 5. A phylogenetic tree constructed from the predicted amino acid sequences of GHV-1, EHV-1, EHV-8, EHV-4, FHV, and PRV gG homologues by the neighbor joining method (Saitou and Nei, 1987). The branch distances were 0.114 from node A to B, 0.010 from node B to C, 0.030 from node B to EHV-8, 0.037 from node C to EHV-1, and 0.05 from node C to GHV-1. Another phylogenetic tree based on the nucleotide sequences was almost identical to the tree in this figure.

herd of gazelles or after the episode. Seroepidemiological and virological surveillance should be conducted in gazelles and other animals to determine the prevalence of infection with GHV-1.

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