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Application of molecular techniques on equine herpes virus 1 infection

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<td>AHV-1</td>
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<td>AHV-2</td>
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<td>BAC</td>
<td>Bacterial artificial chromosome</td>
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<tr>
<td>bp</td>
<td>Base pair</td>
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<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>EMEM</td>
<td>Eagle's minimum essential medium</td>
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<tr>
<td>EEEV</td>
<td>Eastern equine encephalitis virus.</td>
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<td>EBV</td>
<td>Epstein-Barr virus</td>
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<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>FEK</td>
<td>Fetal equine kidney</td>
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<td>GST</td>
<td>Glutathione S-transferase</td>
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<td>gp</td>
<td>Glycoprotein</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<td>ICP4</td>
<td>Infected cell protein</td>
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<td>IPTG</td>
<td>Isopropyl-B-D-thiogalactopyranoside</td>
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<tr>
<td>LB</td>
<td>Luria-Bertani broth</td>
<td></td>
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<tr>
<td>MDBK</td>
<td>Madin-Darby bovine kidney</td>
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<td>MEM</td>
<td>Minimum essential medium</td>
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<td>MCMV</td>
<td>Mouse cytomegalovirus</td>
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<td>MOI</td>
<td>Multiplicity of infection;</td>
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<td>NIH</td>
<td>National Institutes of Health</td>
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<tr>
<td>nt</td>
<td>Nucleotide</td>
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<tr>
<td>OD600</td>
<td>Optical density at 600 nm</td>
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<tr>
<td>ORF</td>
<td>Open reading frame</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<tr>
<td>PFU</td>
<td>Plaque forming unit</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PRV</td>
<td>Pseudorabies virus</td>
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<td>RK13</td>
<td>Rabbit kidney 13</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse transcription and polymerase chain reaction</td>
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1- Introduction

Equine herpesvirus-1 (EHV-1) is an important, ubiquitous equine viral pathogen that exerts its major impact by inducing abortion storms or sporadic abortions in pregnant mares, early neonatal death in foals, respiratory disease in young horses and myeloencephalopathy (Jackson et al. 1977; Peet et al. 1978; Ostlund 1993; Van Maanen 2002; Reed and Toribio 2004; Patel and Heldens 2005).

For several decades, the pathogenesis of equid herpesvirus-1 (EHV-1) infection has been studied in the hope that this may lead to improvements in vaccines, diagnostic techniques and methods of control (Allen and Bryans 1986; Mumford et al. 1994; Smith et al. 1997; Allen et al. 1999).

The functional analysis of EHV-1 ORFs or of strain-specific properties was largely performed by generating virus mutants by homologous recombination techniques, thereby introducing marker genes instead of the targeted viral ORFs (Osterrieder and Wolf, 1998). In recent years, manipulation of the large herpesvirus genomes has been facilitated by
the introduction of bacterial artificial chromosome (BAC) cloning and mutagenesis. The genomes of several herpesviruses have been cloned as infectious BACs using this technique (Brune et al. 2000). Targeted and random mutagenesis of herpesvirus genomes cloned as BACs is considerably faster and more reliable than conventional approaches, because mutagenesis is no longer dependent on the growth of viruses in eukaryotic cells but can be performed in Escherichia coli (Messerle et al. 1997; Borst et al. 1999; Wagner et al. 1999; Schumacher et al. 2000).

The unique short (Us) region of most alphaherpesvirus genomes contains an open reading frame called Us9 (ORF76). The Us9 gene was first described and the protein product was first characterized in herpes simplex virus type 1 (HSV-1) (Frame et al. 1986; McGeoch et al. 1985). Sequences homologous to HSV-1 Us9 have been found in the Us regions of HSV-2 (Dolan et al. 1998) and varicella-zoster virus (VZV) (Davison and Scott 1986) as well as in the animal pathogens pseudorabies virus (PRV) (Van Zijlet et al. 1990) bovine herpesvirus 1 (Leung-Tack et al. 1994) equine herpesvirus 1 (EHV-1) (Flowers and O’Callaghan 1992) feline herpesvirus 1 (Willemsen et al. 1995) canine herpesvirus (Haanes and Tomlinson 1998) and simian herpesvirus B (Killeen et al. 1992).

Pseudorabies virus (PRV) Us9 gene product of ORF76 is a small tail-anchored (TA) membrane protein that is essential for axonal sorting of viral structural proteins and is highly conserved among other members of the alphaherpesvirus subfamily (Lyman et al. 2009).

Unlike the gE/gI deletion mutant viruses, which show a small-plaque phenotype, the Us9 deletion mutant of PRV forms wild-type-size plaques in
cell cultures, indicating that Us9 is not involved in cell-to-cell spread of the virus (Brideau et al. 2000 b).

EHV-1 and BHV-1 Us9 were able to fully compensate for the loss of PRV Us9, whereas VZV and HSV-1 Us9 proteins were unable to functionally replace PRV Us9 when they were expressed in a PRV background (lyman et al. 2009).

**Aims of the study:**

Objectives of this study were to investigate the role of EHV-1 ORF 76 and its protein product Us9 in the pathogenesis and virulence of EHV-1 through the following:

1. Construction of an ORF76 deletion mutation and the corresponding revertant virus using the Ab4p BAC.
2. Characterize the ability of the ORF76 deletion mutation and the corresponding revertant virus to replicate in different cell lines in vitro.
3. In vitro growth properties of ORF76-negative mutant in cultured mouse neurons
4. Effect of ORF76 deletion on transcription activities of neighbors ORFs.
5. Characterize the ability of the ORF76 deletion mutation and the corresponding revertant virus to cause a disease after intranasal inoculation in the CBA/N1 mice model.
6. Verify the role of us9 of EHV-1 in pathogenicity and virulence.
8. Production of polyclonal antibody against EHV-1 Us9.
2. REVIEW OF LITERATURE

2.1. Virus

Herpesviruses have been discovered throughout the spectrum of vertebrates and in at least one invertebrate. In nature, each is closely associated with a single host species and the most extensively studied hosts are infected by several distinct herpesviruses (Minson et al. 2000). About 250 herpesviruses have been detected in a wide range of hosts such as human, birds, reptiles, amphibians, fishes and invertebrate (Essbauer and Ahne 2001).

Herpesviruses are among the largest and most complex family of viruses (Davison and Clements 2004). Their virions are 200–250 nm in diameter and consist of a linear double-stranded DNA genome of 125–245 kbp packaged within an icosahedral capsid approximately 125 nm in diameter embedded in a matrix known as the tegument which contains many virus-coded proteins itself wrapped in a lipid membrane containing several viral glycoproteins (Davison 2002).

The family herpesviridae are classified into three subfamilies Alphaherpesvirinae, Betaherpesvirinae, and Gamaherpesvirinae based on genome structure and biological criteria including the type of disease, cell tropism, latency and the rate of growth (McGeoch et al. 1995).

The alphaherpesvirinae which is exemplified by herpes simplex virus type 1(HSV-1), pseudorabies virus (PRV), equine herpesvirus 1 (EHV-1) and varicella- zoster virus (VZV) has a wide range of experimental host and exhibits the capacity to establish latent infections in sensory ganglia
Betaherpesvirinae including human cytomegalovirus (HCMV), human herpes virus 6 (HHV-6) and mouse cytomegalovirus (MCMV) has a narrow range of host and exhibits long growth cycles in cell culture (Eizuru 2006). Gamaherpesvirinae is exemplified by Epstein-Barr virus (EBV), equine herpes virus 2 (EHV-2), and equine herpes virus 5 (EHV-5) (Roizman et al. 1992).

In equids eight herpesviruses have been identified: five belong to the subfamily alpha herpesvirinae and three to the gamma herpesvirinae. The horse is the natural host to alphaherpesvirus types 1 (EHV-1), 3 (EHV-3 coital exanthemavirus), 4 (EHV-4) and gammaherpesvirus types 2 (EHV-2) and 5 (EHV-5) while the donkey is the host to EHV-1 homologue asinine herpesvirus type 3 (AHV-3) and EHV-3 homologue asinine herpesvirus type 1 (AHV-1) and asinine gammaherpesvirus type 2 (AHV-2) (Browning and Studdert 1987; Browning et al. 1988; Crabb and Studdert 1995; Roizman 1996). The (Fukushi et al. 1997) isolation of a new EHV-1 related neurotropic virus from a gazelle (Gazelle herpesvirus-1 EHV-9) indicates that the list of presently known equid herpesviruses is likely to grow (Hartley et al. 1999).

Infection with EHV-1 may result in rhinopneumonitis, abortion in pregnant mares and fatal myeloencephalopathy (Patel and Heldens 2005). Epidemics of the 3 clinical entities may occur separately or concurrently within a horse population and each has the potential for huge attendant economic and equine welfare consequences (Allen et al. 2002).
2.2. Epidemiology of Equine Herpesvirus -1 Infections

EHV-1 infections are ubiquitous in horse populations throughout the world (Allen and Bryans 1986; Wilson 1997).

The primary natural reservoir of EHV-1 is the carrier horse that is latently infected with EHV-1 virus. The reservoir of latently infected adult horses is the source of infection for new horses early in life especially foals. The carrier state is probably life-long. It has been demonstrated in 40 to 60 percent of previously infected animals. Sites of latency are the lympho-reticular tissues associated with the respiratory tract primarily the bronchial submaxillary and retropharyngeal lymphatic glands and also the trigeminal ganglia (EHV-1). Latent virus may be reactivated by certain environmental/pharmacological stimuli and multiply in the respiratory epithelium. Reactivated infectious virus is shed into the respiratory tract for up to 48 hours without any associated signs of respiratory illness. Reactivation of latency in a carrier mare that is pregnant may be the source of virus that ultimately leads to abortion in that individual or in other susceptible mares with which she has close contact (Allen and Bryans 1986; Edington et al. 1994).

Transmission of EHV-1 occurs by the respiratory route through direct or indirect contact with infective nasal/conjunctival secretions, aborted fetuses, placental membranes and fluids and the various secretions and excretions of congenitally infected neonatal foals. EHV-1 virus is very contagious and can spread rapidly among susceptible horses. Clinical cases are most contagious during the first week after infection. Viral shedding by
the respiratory route may be as long as 14 days in the immunologically naïve animal. Transplacental transmission of EHV-1 occurs (Timoney 2006).

Equine herpesvirus respiratory disease occurs annually in areas of horse concentration with the highest morbidity observed in weanlings and yearlings. Since immunity to reinfection with EHV-1 is short-term lasting only a few months reinfection occur many times throughout the life of a horse. While infection in older animals is usually mild or subclinical EHV-1 has been implicated in the “poor performance” syndrome in 2 and 3 year old Thoroughbreds in training in Europe and elsewhere (Timoney 2006).

EHV-1 is the most common viral cause of abortion in mares resulting in major financial losses to the horse-breeding industry. Over the last 20 years, research into the role of EHV-1 in equine abortion revealed that the incidence of this virus varies between 4.5 and 8.9% (data obtained in the UK and the USA) (Léon et al. 2008).

Equine disease monitoring in Central Kentucky over the past 51 years (1957–2008) has confirmed the frequency of EHV-1 induced abortions has declined, even though the number of broodmares has increased threefold (Powell 2008). The majority of such abortions in recent years have been single, sporadic events on individual farms among populations of mares that are routinely vaccinated against disease. In contrast, cases of EHV-1 induced neurologic disease have increased significantly in number since the year 2000 (Allen et al. 2008; Marenzoni et al. 2008; Patel and Heldens 2005). Within the United States and the United Kingdom, the number of reported outbreaks has risen from one occurrence in the early 1970s to 32 during the
years 2001–2005 (Anonymous 2007). The associated case-fatality rate may also be increasing within the United States, ranging from 20% in some instances, to as high as 50% in others (Slater et al. 2006). Additionally, in 2005 significant outbreaks occurred in Canada, South Africa, Switzerland, Ireland and other European nations (Goehring et al. 2006; Slater et al. 2006).

An important contribution was the study reporting that EHV-1 infection of sucking foals can occur as early as 30 days of age (Gilkerson et al. 1997). Therefore, lactating mares may be the primary source of EHV-1 infection of foals and the latter further transmit the virus to other mares and foals (Gilkerson et al. 1997; Gilkerson et al. 1999). Stress activated latent virus is likely to be the source of such transmissions.

Limited published data suggest that EHV-1 is also an important pathogen of wild equids. There has been a case of abortion and perinatal foal mortality in a herd of zebra (Equus grevyi) in a zoo in Chicago (Wolff et al. 1986). A year previously, a zoo in Washington reported a case of late term (10 months) abortion in an onager (Equus hemionus onager) and a week later a nine-month-old male zebra (Equus burchelli) in a pen adjacent to the onagers developed posterior ataxia and rectal prolapse (Montali et al. 1985). Virus isolates from three fallow deer (Dama dama) kept on the same game farm as horses in Alberta, Canada were closely related to EHV-1 (Kinyili and Thorsen, 1979). EHV-1 has also been detected from a field case of zebra stallion (Blunden et al. 1998).
The genome sequence (Telford et al. 1992) of EHV-1 has facilitated molecular epidemiological studies. The genome sequences of two contrasting EHV-1 strains (Tearle et al. 2003) have been compared (Davis-Poynter, Newmarket, UK) allowing sequence variations that may account for phenotypic differences to be identified. Comparison of Ab4 (an endotheliotropic, abortigenic and paralytic virus which causes ‘high level’ viraemia) and V592 (a less virulent virus that does not cause paralysis and is associated with ‘low level’ viraemia and reduced endotheliotropism and abortogenic potential) revealed 0.1% difference in sequence (150 bases in 150,000), mostly single base changes, causing coding changes in 31 open reading frames (ORFs). Of these, ORF 68 (Us2) showed the highest variation rate (2%) and was used as a phylogenetic marker to separate an international collection of viruses from Europe and the USA into six groups. This has allowed molecular epidemiology from a number of outbreaks to be carried out. For example, the five viruses isolated from the Findlay and the Ohio State University outbreak all belonged to the same ORF 68 group other outbreaks in the USA (Pennsylvania and Kentucky) and UK (Kent) were caused by different viruses. This approach provides a means of genetic typing which can be refined by including other ORFs that segregate with ORF 68. There does not appear to be any relationship between the phylogenetic groups generated by ORF 68 analysis and the paralytic potential of strains.

However, the sequence variation of the DNA polymerase gene encoded by ORF 30 does provide a means of identifying paralytic viruses. There are two single base changes between Ab4 and V592: G becomes A at positions 2254 (altering the amino acid at position 752 from D to A) and
The majority of non-paralytic strains (69/71) examined possessed A at position 2254 whilst the majority of paralytic viruses (35/41) possessed G at position 2254. Although virulence may intuitively be expected to be determined multigenically, these data suggest that dimorphism within the DNA polymerase gene correlates with paralytic or non-paralytic potential (Slater et al. 2006).

A range of environmental factors can influence the epidemiology of EHV-1 infections. Included among the more significant are: the type of control measures taken to prevent the introduction of infection onto a premises whether the population of horses at risk is immunized regularly against both infections, how pregnant mares are managed especially after the first trimester of pregnancy and whether steps are taken to minimize the risk of management-related stress especially for pregnant mares and the potential for reactivation of latency in carrier animals (Allen and Bryans 1986).

2.3. Pathogenesis

2.3.1. Respiratory Disease

Exposure to and infection with EHV-1 occurs through inhalation of aerosolized virus-infective respiratory secretions. This virus infects and replicate in nasal and nasopharyngeal epithelium in animals with inadequate mucosal immunity. By 12 hours after infection progeny virus and viral antigen are detectable in infected epithelial cells. Over the course of the following 4 to 7 days this results in development of multiple nasal and nasopharyngeal erosions and shedding of considerable quantities of virus into the respiratory tract. Concurrently virus invades the underlying tissues and can be found in leukocytes in the subepithelial lamina propria beneath
the surface erosions as well as in the endothelial cells of nasal blood vessels and lymphatics. Virus may also spread to the lungs either through surface airway infection or by the bloodstream to the interstitial pulmonary vasculature. Within 12 to 24 hours of the onset of EHV-1 infection virus is transported via infected leukocytes to lymph nodes associated with the respiratory tract (Kydd et al. 1994).

Viral antigen expression can be demonstrated in lymphocytes and macrophages within the afferent lymphatics and in the subcapsular and medullary sinuses and lymphoreticular parenchyma of infected lymph nodes. Following amplification virus migrates via infected leukocytes from the lymph nodes into the bloodstream and lymphatic circulation giving rise to a cell-associated viraemia involving monocytes and CD4+ and CD8+ T-lymphocytes (Allen et al. 1999; Slater et al. 1994a).

This enables EHV-1 to be widely disseminated throughout the body and gain access to certain target sites of the virus namely the pregnant uterus central nervous system testes and endocrine organs where it undergoes a secondary replication cycle in the vascular endothelium especially of end circulation microvessels (Smith 1998). Viral spread by cell-to-cell infection from vascular endothelium in smooth muscle myocytes in infected blood vessels can also occur (Smith et al. 1992; Smith et al. 1993).

Concomitant with transportation of EHV-1 to the lymph nodes associated with the respiratory tract the virus gains access to the neurons of the trigeminal nerve innervating the nasopharyngeal mucosa and conjunctiva reaching the trigeminal ganglia by 48 hours post infection (Slater et al.)
This may precede or coincide with the onset of viraemia.

2.3.2. Abortion/Neonatal Disease

The majority of cases of equine herpesvirus abortion occur between the 7th and 11th months of gestation (Allen and Bryans 1986).

Some mares exposed to infection very late in pregnancy may not abort however but carry to term and give birth to a congenitally infected live foal with a diffuse viral pneumonitis that is almost invariably fatal within the first few days of life. The key event in the pathogenesis of abortion/neonatal disease due to EHV-1 is the cell-associated viraemia that enables the virus to be transported from the lymph nodes associated with its site of entry into the body to the pregnant uterus (Bryans 1969; Mumford et al. 1994).

Once the virus reaches the uterus it infects the endothelial cells of the uterine vasculature causing an endometrial vasculitis. This has been demonstrated as early as 6 days after intranasal infection becoming more widespread over the ensuing 3 to 7 days. The vasculitis in turn gives rise to thrombosis in affected vessels and areas of infarction in the microcotyledons of the placenta (Edington et al. 1991; Mumford et al. 1994; Smith et al. 1992; Smith et al. 1993).

It is worth noting that endothelial cell infection in tissues of the immunologically naïve fetus is rarely thrombogenic (Smith et al. 1993).

The primary determinant of whether abortion supervenes is the extent and
severity of the thrombosis that takes place in the infected uterus. In mares in which the thrombotic lesions are more focal and not generalized, evidence of viral replication has been demonstrated in trophoblast cells and chorionic blood vessels associated with endometrial infarcts. In such cases there is extension of the virus infection to the fetus by day 15 or 16 post exposure viral spread presumably taking place via the vasculature of the allantochorion and the umbilical cord (Edington et al. 1991; Smith et al. 1996; Smith et al. 1993).

Viral invasion leads to the development of necrotic lesions in many tissues throughout the fetus. The outcome is abortion with both fetus and placenta positive for virus. This represents the majority of naturally encountered cases of equine herpesvirus abortion. Where thrombosis in the pregnant uterus is extensive however it results in placental ischemia premature placental separation probably triggered by the local release of prostaglandins from the damaged tissues and expulsion of the fetus (Smith 1994 and Smith et al. 1993).

In such cases of severe thrombo-ischemic necrosis the sequence of events takes place so quickly that there is insufficient time for the virus to invade the fetus, which consequently is virus negative. These atypical cases of equine herpesvirus abortion where the placenta is virus positive but the fetus is uninfected occur infrequently (Timoney 2006).

Aside from the risk of abortion resulting from respiratory exposure of a pregnant mare to EHV-1 there is increasing evidence that reactivation of latent virus can also play a role in outbreaks of abortion (Allen et. al. 1999).
Reactivation of latent virus may occur from lymphocytes associated with the respiratory epithelium and/or the trigeminal ganglia. This results in viral shedding into the respiratory tract sometimes accompanied by fever cell-associated viraemia stimulation of the immune response and the potential for abortion (Edington et al. 1985; Slater et al. 1994a; Slater et al. 1994b).

2.3.3. Neurological Disease

A manifestation of EHV-1 infection that has been reported with increasing frequency over the past 15 years is virus-associated neurological disease that is characterized by a high morbidity and case fatality rate (Allen 2006; Wilson 1997). This important syndrome differs from other arboviral and non-arboviral diseases of the CNS of the horse in that evidence of a true encephalitis and viral replication in neurons is lacking (Borchers et al. 2006). In light of this and since clinical signs of herpesvirus-associated neurological disease primarily reflect involvement of the spinal cord the syndrome is considered a myeloencephalopathy. The majority of cases of the disease result from infection with certain strains of EHV-1 (Borchers et al. 2006).

Equine herpesvirus myeloencephalopathy is usually a sequel to a primary febrile respiratory infection and sometimes also abortion that involves the development of a leukocyte-associated viraemia and transport of the virus to the vasculature of the central nervous system as well as to other sites in the body. The property of endotheliotropism is central to the ability of certain strains of EHV-1 to cause vascular-mediated neurological disease (Edington et al. 1986; Patel et al. 1982).
Our understanding of this disease was significantly advanced by the discovery of a genetic basis for the neuropathogenicity of certain strains of EHV-1. A very extensive genetic analysis study was carried out of isolates of the virus from outbreaks of neurological disease as well as from outbreaks of abortion without neurological involvement. The isolates had been collected over a 35-year period in a total of 8 countries. The study revealed a point mutation among 83 percent of virus strains from neurological disease outbreaks which was absent from 93 percent of strains from outbreaks solely of abortion. The point mutation is located in the catalytic subunit of the gene (ORF 30) encoding the viral DNA polymerase gene (Nugent et al. 2006).

More recent studies have identified a property inherent to neuropathogenic strains of EHV-1 that is believed to play an important role in the ability of these strains to cause neurological disease. Such strains give rise to a leukocyte-associated viraemia of earlier onset greater magnitude and longer duration than strains of the non-neuropathogenic phenotype. As a consequence the vasculature in the central nervous system is exposed to a greater concentration of EHV-1 infected leukocytes thereby enhancing the risk of exposure of the vascular endothelium to viral infection (Allen and Breathnach 2006).

Similar to the pathogenesis of EHV-1 induced abortion viral infection of the vascular endothelium and smooth muscle cells in the small blood vessels of the spinal cord and/or brain gives rise to a vasculitis, thrombosis, hemorrhage and areas of infarction (Platt et al. 1980; Thein 1996; Wilson 1997). These changes result in secondary ischemia of affected regions of the central nervous system which in turn leads to neuronal degeneration, axonal
swelling and foci of malacia. Vascular lesions in the spinal cord and/or brain of horses affected with this disease are consistent with an immune-mediated vasculitis with the formation of antigen-antibody complexes rather than a consequence of direct viral replication (Allen et al. 1999; Edington et al. 1991; Smith et al. 1996; Smith et al. 1992; Smith et al. 1993).

(Borchers et al. 2006) believe that an anamnestic immune reaction is a critical prerequisite for generation of the immune-mediated vascular damage associated with cases of herpesvirus myeloencephalopathy. While vasculitis has been noted in both immunologically naïve as well as primed horses thrombosis appears to occur only in primed animals. Factors that influence the extent and severity of the pathological changes observed in cases of equine herpesvirus myeloencephalopathy include: distribution and severity of ischemia induced lesions, virus strain and genotype, challenge dose of virus level of leukocyte-associated viraemia and the immune status of the infected animal.

2.4. Clinical Features

2.4.1. Respiratory Disease

Respiratory disease caused by EHV-1 is clinically indistinguishable and is primarily an upper respiratory tract infection. In younger horses this takes the form of an acute febrile respiratory illness characterized by a rhinopharyngitis and a tracheobronchitis, which can spread rapidly through a group of, exposed and unprotected animals (Allen and Bryans 1986; Crabb and Studdert 1995).
After an incubation period of 2-8 days affected horses may develop any combination or all of the following clinical signs; fever ranging from 39°C to 42°C that can persist for 1 to 7 days and is frequently biphasic, leukopenia involving both a neutropenia and a lymphopenia, serous to mucopurulent nasal and sometimes conjunctival discharge associated with a variable degree of rhinitis and perhaps conjunctivitis, anorexia, depression pharyngitis cough, not uncommonly enlargement of the submandibular or retropharyngeal lymphatic glands and sometimes abnormal lung sounds. Secondary bacterial infections are common. The severity of disease varies with the age of the horse and the level of pre-existing immunity due to natural exposure and/or vaccination. Subclinical infections with either virus are common even in young animals. In uncomplicated cases of infection clinical recovery is usually complete within a few weeks. The respiratory manifestation of EHV-1 infection is not a life-threatening disease (Timoney 2006).

2.4.2. Abortion

Abortion caused by EHV-1 is seldom preceded by any premonitory signs of illness in the mare (Allen and Bryans 1986). After an incubation period that can range from one week to several months (usually 3 to 4 weeks) abortion takes place with the majority of cases occurring between the 7th and 11th months of gestation. Since infected fetuses are viable until shortly before expulsion they do not exhibit evidence of autolysis at time of abortion. Abortion is not associated with any detectable inflammatory changes in the mare’s reproductive tract or any related adverse effects on her subsequent fertility. Viral antigen cannot be detected in the mare's uterus beyond 48 hours after abortion of an infected fetus (Smith et al. 1993).
While many cases of EHV-1 abortion occur as sporadic isolated events the virus can frequently be responsible for multiple cases of abortion ("abortion storm") in a group of susceptible pregnant mares. In the majority of outbreaks only one or two mares will be involved with maiden mares most often affected (Timoney 2006).

2.4.3. Neonatal Disease

Some mares exposed to EHV-1 very late in gestation may not abort but instead give birth to a congenitally infected foal that is usually ill at birth (Allen and Bryans 1986). Such foals are weak jaundiced exhibit progressive respiratory distress and almost invariably die within a few days of age from severe interstitial pneumonia that is frequently complicated by secondary bacterial infection (Timoney 2006).

2.4.4. Neurological Disease

Frequently but not invariably the occurrence of equine herpesvirus myeloencephalopathy is preceded by an episode of respiratory disease, abortion, neonatal foal pneumonia or a febrile illness among the horses on an affected premises (Platt et al. 1980; Wilson 1997).

The disease, which can occur in horses of either gender and any age or breed, is most frequently seen in late winter, spring or early summer. The number of animals affected in an outbreak can vary greatly; it can range from a single case to multiple cases occurring over several weeks with morbidity rates as high as 90 percent being recorded. Clinical signs are rapid in onset and reach maximal severity within 48 hours of the initial evidence
of neurologic disease, which may or may not be preceded by fever of short-term duration (Allen and Bryans 1986; Platt et al. 1980).

Severity of disease will vary by individual; it can range from mild incoordination, posterior paresis and ataxia to posterior paralysis, recumbency, bladder atony with urinary incontinence, fecal retention, sensory deficits of the perineal area which may extend also to the inguinal region and even parts of the hind limbs, decreased tail tone and limb edema. Neurologic deficits may be symmetric or asymmetric. Although the hind limbs are most frequently affected some horses may exhibit cranial nerve deficits and develop a head tilt, tongue weakness, nystagmus and even blindness. The disease may be rapidly progressive in some animals. Severely affected cases become paraplegic or even quadriplegic, recumbent and die. It is important to emphasize that the range and severity of clinical signs develop within 1 to 2 days of the onset of illness. How a particular horse is affected will depend on the location and extent of the lesions in the spinal cord and brain. Some horses will make full clinical recoveries from the disease in a matter of days to several months whereas others will only make a partial recovery and be left with permanent neurological deficits. Case fatality rates ranging from 1 percent to as high as 50 percent have been reported (Timoney 2006).

2.5. Latency

Latency by alphaherpesviruses is an important epidemiological strategy ensuring survival and spread within the natural host population (Whitley and Gnann 1993).
For EHV-1 latency has been demonstrated in lymphoid as well as in neural tissues (Welsh et al. 1992; Gibson et al. 1992; Edington et al. 1994; Slater et al. 1994a; Baxi et al. 1995; Borchers et al. 1999).

Experimentally EHV-1 has been reactivated from both naturally reared (Edington et al. 1994) and experimentally infected conventional (Edington et al. 1985) and specific pathogen free (SPF) (Slater et al. 1994b) horses upon immunosuppression but with differing results. Infectious virus was recovered principally from leukocytes and only occasionally from nasal mucus after corticosteroid treatment of conventional horses (Edington et al. 1985) whereas considerable shedding in nasal mucus without recurrence of viraemia both after corticosteroid and cyclophosphamamide treatment was recorded in SPF horses (Slater et al. 1994b).

The CD5+/CD8+ T lymphocytes were defined as the predominant site of EHV-1 latency, which was independently and indirectly activated, by both IL-2 and equine chorionic gonadotrophin (Smith et al. 1998). It is assumed that reactivated latent virus shed in nasal mucus plays a key role in the epidemiology of EHV-1 infections.

The physiological factors responsible for the activation of latent virus and the relative importance of the neural and lymphoid latency sites under natural conditions remain undefined but it is well recognized that spontaneous shedding may follow weaning, castration, relocation and terminal illness (Burrows et al. 1984).
Experimental reactivation of EHV-1 in SPF foals was asymptomatic despite significant shedding in nasal mucus (Slater et al. 1994b). It is however unknown if reactivation of latent EHV-1 in pregnant mares will result in an abortion. We consider this quite likely since in experimental EHV-1 challenges viraemia is only detectable by co-cultivation of viable leukocytes but not if the cells are frozen and thawed first (Gleeson and Coggins 1980) indicating that there is no infectious virus in lysed cells and the fact that abortions could occur months after challenge and after the disappearance of leukocyte-associated viraemia (Gleeson and Coggins 1980).

Clearly latently infected leukocytes are invisible to immune surveillance and elimination. While recrudescent virus shed in nasal mucus is considered the common source of EHV-1 transmission, contact with an EHV-1 infected aborted fetus constitutes another potential transmission route that is particularly important during abortion epizootics (Timoney 2006).

In common with other herpesviruses, EHV-1 can establish a latent infection, which can become reactivated after the primary infection has resolved (Allen and Bryans, 1986). Experimental reactivation by administration of immunosuppressive agents has resulted in shedding EHV-1 into nasal mucus and in some cases, viraemia has been induced (Edington et al., 1985; Gibson et al., 1992). In healthy horses previously infected with EHV-1 but not currently shedding virus, co-cultivation of explanted trigeminal ganglia yielded infectious virus (Slater et al., 1994a) and viral DNA has been detected by PCR in trigeminal ganglia, olfactory nerve,
spleen, lymphoid tissue associated with the respiratory tract and peripheral blood leucocytes (PBL) (Welch et al., 1992; Edington et al., 1994; Slater et al., 1994). Latency-associated transcripts (LATs) have been detected using in situ hybridisation in low numbers of neurones of the trigeminal ganglia, which demonstrated that some of the cells which contain EHV-1 transcribe regions of the viral genome during latency (Baxi et al., 1995).

Virus can also be reactivated following various stimuli in mice previously infected with EHV-1, with infectious virus isolated from nasal turbinates and PBL (Field et al., 1992). Consistent with this, viral DNA was detected by PCR in trigeminal ganglia (Baxi et al., 1996). Also, low levels of virus DNA could still be detected in trigeminal ganglia, olfactory bulbs and PBL in mice in which infectious virus was no longer detectable (Baxi et al., 1996). In that series of experiments, LATs could not be detected using in situ hybridisation, which may indicate either that they were not present or were below the level of detection. However, using in situ PCR and expression of a lacZ reporter gene, Marshall and Field (1997) have shown that the mitral/tufted neurons within olfactory bulbs were probable sites of EHV-1 latency in the mouse.

2.6. Diagnosis

Preliminary diagnosis can be made based on the rapid spread of the disease and the presence of compatible clinical signs. Early hematological abnormalities include mild anemia and lymphopenia, which are followed a few days later by mild hyperfibrinogenemia. Azotemia and hyperbilirubinemia may occur secondary to dehydration and anorexia, respectively. If affected horses develop secondary bacterial
bronchopneumonia, their blood work will often be characterized by leukocytosis with a mature neutrophilia, marked hyperfibrinogenemia, and hyperglobulinemia. In more severe cases, neutropenia and a left shift may be observed. Traditionally, virus isolation has been the gold standard for diagnosing EHV-1 infection. The sample of choice is a nasopharyngeal swab which should be taken early in the febrile phase of the disease. The swabs should be kept in viral transport medium and shipped on ice to a veterinary diagnostic laboratory. Due to the lymphotropism of EHV-1, virus isolation can also be attempted from citrated or heparinized whole blood. Virus isolation requires the maintenance of specific cell culture lines, making this process relatively expensive and time-consuming. EHV-1 can be propagated in a broad range of cell lines; however, Virus isolation is often hampered by the fragility of the virus, intermittent viral shedding, and the interference with local antibodies. (Harless and Pusterla 2006).

PCR offers an alternative to virus isolation and has proven to be a sensitive method of detecting EHV-1 in respiratory secretions, peripheral blood lymphocytes, and other tissues (Lawrence et al. 1994). Many conventional PCR assays have been established to study the pathophysiology and improve the diagnosis of this virus. Conventional single or nested PCR assays do have inherent risks of carry-over contamination due to postamplification steps required to detect the PCR products. Novel molecular platforms such as the real-time PCR have strongly reduced the risk of contamination. PCR assays used in routine diagnostics are based on the detection of viral genomic DNA and are therefore unable to distinguish between lytic, dead, or latent virus. Alternative molecular approaches have recently been established using the
real-time TaqMan PCR to allow discrimination between the different viral states (Pusterla et al. 2005).

Postmortem diagnosis of EHV-1 can be made by virus isolation, immunohistochemistry, polymerase chain reaction (PCR) or histopathological examination on appropriate tissues collected during necropsy: lung, liver, spleen and thymus from aborted fetuses and newborn foals; spinal cord and brain tissue from cases of neurological disease. Detailed procedures for diagnosis of EHV-1 infections in the horse have been reported recently (Allen 2000).

While not conclusive and often difficult to interpret in vaccinated horses serological evidence for recent infection by EHV-1 can be obtained by laboratory examination of single collections of serum by complement-fixation (McCarten et al. 1995) or recombinant glycoprotein-G ELISA tests (Crabb and Studdert 1955). Retrospective serodiagnosis of EHV-1 infection can be accomplished by demonstration of a 4-fold rise in antiviral titre between acute- and convalescent-phase serum samples.

2.7. Treatment

Specific details of the veterinary medical and nursing procedures that may be used for salvaging individual horses affected with clinical EHV-1 disease have been thoroughly described (Paradis 1996; Cutler and MacKay 1997; Donaldson and Sweeney 1997; Goehring and Sloet van Oldruitenborgh-Oosterbaan 2001; Olsen 2001). The rationale for such treatment regimens is the use of symptomatic and supportive care for
maintaining the nutritional needs and reducing distress or the likelihood of death or permanent impairment of the affected horse.

Antipyretics are given to pyrexic animals, fluid and electrolyte replacement therapy to horses with inappetence and antibiotics given for controlling bacterial superinfection in newborn foals suffering from systemic EHV-1 infection acquired in uterus. Nonsteroidal anti-inflammatory agents are indicated in the management of EHV-1-associated vasculitis respiratory tract disease or other soft-tissue injuries. The use of antiviral chemotherapy with acyclic nucleoside analogues may have some merit in reducing the mortality rate during outbreaks of EHV-1 perinatal disease (Murray et al. 1998).

The administration of dimethlysulphoxide and a corticosteroid is a standard treatment for horses with EHV-1-associated paralysis. Horses with bladder wall or sphincter paralysis associated with EHV-1 myeloencephalopathy may require urinary catheterization, postcatheter flushing of the bladder with povidoniodine solution and antibiotic treatment for cystitis. Because mares aborting EHV-1 infected foals remain asymptomatic treatment is rarely indicated. Horses exhibiting prolonged recumbency from EHV-1 myeloencephalopathy require intensive round-the-clock nursing care. Light tranquilization thick bedding with frequent repositioning of the patient daily cleansing and topical care of decubital ulcers and urine scalding and the use of mechanical slings for limited periods for assisted support of recumbent or paretic animals are often indicated (McConnico et al. 1991).
Euthanasia should be considered in laterally recumbent horses or sling-supported animals failing to show improvement after a few days or in animals developing severe systemic complications from recumbency. The prognosis for full neurological recovery of EHV-1 infected horses so severely affected as to require prolonged sling-assistance is not generally favourable (van Maanen et al. 2001).

2.8. Vaccines

Control of respiratory diseases due to EHV-1 through vaccination began in the early 1940s in the USA using antigens produced in vivo. These preparations turned out to be ineffective (Doll et al. 1955; Doll and Bryans 1963). Growth of EHV-1 isolates in tissue culture led in the 1970s to an inactivated vaccine containing an adjuvant (Mayr et al. 1978).

In parallel a tissue culture grown inactivated vaccine was developed in the USA and initially appeared to be effective against EHV-1 challenge (Bryans 1978). In later years it became apparent that none of these vaccines could protect against EHV-1 mediated abortions (Burrows et al. 1984; Bürki et al. 1990; Mumford et al. 1991).

Following the realization that EHV-1 and EHV-4 were distinct viruses newer inactivated vaccines included both virus types (Heldens et al. 2001).

Currently there are at least 12 multivalent EHV-1 and EHV-4 and 2 EHV-1 only multi-dose inactivated and two multi- doses live EHV-1 (one monovalent and one multivalent) vaccines marketed (Table 1). All 16 commercially available vaccines are for parenteral application only.
However most of these vaccines only claim protection against respiratory diseases due to EHV-1 and EHV-4. Currently in the EU only one product is licensed: an inactivated EHV-1 plus EHV-4 vaccine containing a carbomer as an adjuvant that claims protection against EHV-1 mediated abortions if administered during pregnancy at fifth, seventh and ninth months of gestation (Heldens et al. 2001).

Nonetheless much effort has also gone into developing live deletion mutant vaccines mostly using EHV-1 strains largely spurred on by the success achieved for other alphaherpesviruses notably bovine herpesvirus 1 (Kaashoek et al. 1994) and pseudorabiesvirus (Mettenleiter et al. 1994).

Table 2.1. Commercially available equine vaccines for control of diseases caused by EHV-1.

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Manufacturer (market)</th>
<th>Vaccine components and type</th>
<th>Protection claim</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duvaxyn EHV-14</td>
<td>Fort Dodge (Europe)</td>
<td>EHV-1 and EHV-4 inactivated</td>
<td>Abortion and respiratory disease</td>
</tr>
<tr>
<td>Equiffa</td>
<td>Merial (Europe)</td>
<td>EHV-1 EIV-1 and EIV-2 inactivated</td>
<td>Respiratory disease</td>
</tr>
<tr>
<td>Equi Guard</td>
<td>Boehringer Ingelheim (USA)</td>
<td>EHV-1 and EHV-4 inactivated</td>
<td>Respiratory disease</td>
</tr>
<tr>
<td>Equi Vac EHV-1/4</td>
<td>Fort Dodge (USA)</td>
<td>EHV-1 EIV-1 and EIV-2 inactivated</td>
<td>Respiratory disease</td>
</tr>
<tr>
<td>Fluvac EHV-4/1</td>
<td>Fort Dodge (USA)</td>
<td>EHV-4 EHV-1 EIV-1 and EIV-2 inactivated</td>
<td>Respiratory disease</td>
</tr>
<tr>
<td>Pnemabort K+1B</td>
<td>Fort Dodge (USA)</td>
<td>EHV-1 inactivated</td>
<td>Abortion and respiratory disease</td>
</tr>
<tr>
<td>Prestige</td>
<td>Intervet (USA)</td>
<td>EHV-1 and EHV-4</td>
<td>Respiratory disease</td>
</tr>
<tr>
<td>Product</td>
<td>Manufacturer</td>
<td>Constituents</td>
<td>Indications</td>
</tr>
<tr>
<td>-------------</td>
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<td>--------------------------------------</td>
</tr>
<tr>
<td>Prestige II</td>
<td>Intervet (USA)</td>
<td>EHV-1 EHV-4 EIV-1 and EIV-2 inactivated</td>
<td>Respiratory disease</td>
</tr>
<tr>
<td>Prestige V</td>
<td>Intervet (USA)</td>
<td>EIV-2 EEEV WEEV and tetanus inactivated</td>
<td>Respiratory disease plus</td>
</tr>
<tr>
<td>Equigard-Flu</td>
<td>Boehringer Ingelheim (USA)</td>
<td>EHV-1 EHV-4 EIV-1 and EIV-2 inactivated</td>
<td>Respiratory disease</td>
</tr>
<tr>
<td>Double-EFT EHV</td>
<td>Fort Dodge (USA)</td>
<td>EHV-1 EHV-4 EIV-1</td>
<td>Respiratory disease</td>
</tr>
<tr>
<td>Prodigy</td>
<td>Intervet (USA)</td>
<td>EHV-1 inactivated</td>
<td>Abortion</td>
</tr>
<tr>
<td>Resequin</td>
<td>Intervet (Europe)</td>
<td>EHV-1 and EHV-4 inactivated</td>
<td>Respiratory disease</td>
</tr>
<tr>
<td>Resequin Plus</td>
<td>Intervet (Europe)</td>
<td>EHV-1 EHV-4 EIV-1 and EIV-2 inactivated</td>
<td>Respiratory disease</td>
</tr>
<tr>
<td>Rhinomune</td>
<td>Pfizer (USA)</td>
<td>EHV-1 modified live</td>
<td>Respiratory disease</td>
</tr>
<tr>
<td>Rhino-Flu</td>
<td>Pfizer (USA)</td>
<td>EHV-1 modified live EIV-1 and EIV-2 inactivated</td>
<td>Respiratory disease</td>
</tr>
</tbody>
</table>

a: Abbreviations:
EIV: equine influenza virus types 1 (H7N7) and 2 (H3N8).
EEEV: Eastern equine encephalitis virus.
WEEV: Western equine encephalitis virus.
b: All vaccines administered parenterally twice 3–4 weeks apart and six monthly boosters (respiratory disease claim) and for each single pregnancy at the fifth, seventh and ninth months of gestation (abortion claim).

*Pneumabort K is no longer on the market.*
Following intranasal administration to young horses EHV-1 strain Kentucky A with at least six gene deletions produced poor or no VN antibody and the horses were not significantly protected when challenged four weeks later (Matsumara et al. 1996). This was also the case with thymidine kinase deficient (TK−) strain of EHV-1 (Cornick et al. 1990). Similar findings were reported for a double (glycoproteins E and I) deletion mutant of EHV-1 (Matsumara et al. 1998).

A possible downside in the development of live EHV-1 vaccines is the study (Rappocciolo et al. 2003) with strain Ab4, which concluded that attenuated EHV-1 vaccines are likely to induce MHC, class 1 down-regulation and result in incomplete protection. Clearly we must question whether this applies to all EHV-1 strains. It is also noteworthy that a canarypox recombinant virus expressing EHV-1 gB, gC and gD provided only partial protection to horses against respiratory disease and virus shedding but not viraemia following EHV-1 challenge (Audonnet et al. 1999). A DNA expression vector encoding EHV-1 gD afforded partial protection against abortion in a mouse model (Walker et al. 2000). Hamsters were used to test efficacy of a vaccinia virus expressing EHV-1 gB and gC (Guo et al. 1990) and a gC subunit vaccine (Stokes et al. 1991). Mice have been used as a model by other workers (Tewari et al. 1994; Zhang et al. 1998; Ruitenberg et al. 1999).

That not all EHV-1 deletion mutants are over attenuated is suggested by the finding that a naturally occurring TK− isolate of strain Ab4 replicated after intranasal inoculation and partially protected SPF foals (Slater et al. 1993). While the latter data give hope for the development of a deletion
mutant EHV-1 vaccine a different approach has given promising results. Hence a clone of EHV-1 isolated following classical mutagenesis in vitro and having temperature sensitive and a host range phenotype typically found in EHV-4 strains significantly protected pregnant mares for up to six months after a single intranasal inoculation against febrile respiratory disease virus shedding in nasal mucus and abortions due to EHV-1 challenge. This live vaccine also significantly cross-protected adult horses against EHV-4 (Patel et al. 2003).

A further significant characteristic of this live vaccine is its ability to afford a significant virological and clinical protection against EHV-1 to sucking foals with maternally derived neutralizing antibody to EHV-1 (Patel et al. 2004). Serological evidence (Wilson and Rossdale 1999; Breathnach et al. 1999) indicates that multidoses inactivated vaccines are unlikely to be effective in significantly protecting sucking foals with maternal antibodies. As sucking foals are now considered an important reservoir in the transmission of EHV-1 (Gilkerson et al. 1999) it has been suggested that sucking foals with maternal antibodies ought to be the target for immunoprophylaxis in order to eventually eliminate or significantly reduce EHV-1 in the field (Foote et al. 2002).

2.9. Prevention and control
The potential for equine losses during an outbreak of EHV-1 disease is related directly to herd size. No infection control strategy has so great a bearing on the success in controlling the magnitude of epidemic EHV-1 disease as that of the subdivision of the at-risk population of horses into
smaller groups and the maintenance of those groups as closed physically separated units (Bryans 1981; Bryans and Allen 1986).
Horses should if feasible be segregated into like groups that avoid the mixing of yearlings with older animals, pregnant with nonpregnant mares, horses with donkeys etc. For pregnant mares it is important that foaling groups (a) be established early in gestation to allow for the establishment of social hierarchies prior to the increased vulnerability to EHV-1 abortion during late gestation; (b) are assembled on the basis of similar expected due dates; and (c) do not combine first-foal mares with older broodmares (Powell 1992; Anon 2000; Vickers and Powell 2001).

The high prevalence of EHV-1 latency in horses precludes the practice of identifying and separating latent carrier horses from the herd. Measures to control the frequency of reactivation of latent EHV-1 are aimed at minimizing stress experienced by horses stress caused by crowding, poor nutritional state, heavy parasite infestation, lengthy transport, disruption of established social groups, inclement weather, en masse weaning and other disease states (Bryans 1981).

Euthanasia should be considered in laterally recumbent horses or sling-supported animals failing to show improvement after a few days or in animals developing severe systemic complications from recumbency. The prognosis for full neurological recovery of EHV-1 infected horses so severely affected as to require prolonged sling-assistance is not generally favourable (Van Maanen et al. 2001).

Swift decontamination of the environmental surfaces of facilities in which an epidemic of EHV-1 has occurred can be achieved by thorough
cleaning with detergent and water followed by rigorous chemical
disinfection with phenolic- or iodophor-type compounds (Dwyer 1992).

2.10. Genomic organization of equine herpes virus 1 and bacterial
artificial chromosome (BAC) technology

An overview of EHV-1 gene regulation was provided by Dennis
O’Callaghan (LA USA). The 155 KB genome encodes 76 genes of which 63
are located in the unique long (UL) region 9 in the unique short (Us) region
and 4 in the internal repeat (IRs) regions. Gene transcription and regulation
is tightly and sequentially regulated into three phases: immediate early (IE)
early (E) and late (L). The virus encodes a single IE gene 55 E genes and 20
L genes. Six of these have regulatory functions and are responsible for the
tightly controlled cascade of virus gene regulation (Salter et al. 1993).
Fig. 2.1. EHV-1 Ab4p genome map.
The genes of herpesvirus are classified as essential or nonessential for growth in cultured cells; for example, ICP4 and ORF30 are essential genes of EHV-1, while gI and gE are nonessential. Analysis of essential genes is difficult and time consuming with traditional methods that use the homologous recombination in eukaryotic cells, which constitutively express the target essential gene product. An alternative approach using bacterial artificial chromosome (BAC) has recently become the preferred method (Brune et al. 2000). This approach allows rapid and efficient alteration of herpes viral genome in Escherichia coli. In principle, any essential and non-essential genes on BAC can be modified for deletion, alternation, and replacement with other genes. By establishing a BAC system, researchers can easily perform recombination of any genes including essential and nonessential genes using genetics of E. coli (Smith et al. 2005).

Until today, herpesvirus genomes have been cloned as BAC including pseudorabies virus (Smith and Enquist, 2000), human cytomegalovirus (Yu et al. 2002), herpes simplex virus type 1 (Tanaka et al. 2003), varicella-zoster virus (Brune et al. 2000), Epstein-Barr virus (Kanda et al. 2004), rhesus cytomegarovirus (Chang and Barry 2003) and EHV-1 (Goodman et al. 2007; Hansen et al. 2006; Rudolph et al. 2002). Osterrieder and his colleagues have cloned the EHV-1 genome using the KyA, RacL11 and Ab4p strains (Goodman et al. 2007; Rudolph et al. 2002) and Hansen et al. (2006) used the HVS25A strain as sources of BAC. KyA, RacL11 and Ab4p BAC were constructed by insertion of BAC vector sequences into the ORF71 (gp2 gene) in the viral genome. Although the ORF71 is a nonessential gene, its product, gp2, seems to contribute in EHV-1 virulence and pathogenesis (Smith et al. 2005). Therefore, BAC sequences need to be
reverted to the original sequences to use these BACs prior to pathogenicity evaluation (Goodman et al. 2007).

The HVS25A strain BAC, which was inserted a BAC vector to the intergenic region between ORF62 and ORF63, appeared similar growth to wild type in cell culture (Hansen et al. 2006). HVS25A strain was isolated from an aborted foal (Whalley et al. 1981) and used in a murine model of respiratory disease (Csellner et al. 1998). However, there is no data about the neuropathogenicity on HVS25A strain.

The Ab4p strain is a neurovirulent strain that was isolated from a case of equine paresis (Gibson et al. 1992). Adding with a whole genome sequence (Telford et al. 1992), Ab4p has been confirmed to cause neurological disorders in experimental infection of horses, hamsters and mice (Fukushi, et al. 2000; Frampton et al. 2004; Awan et al. 1990).

Therefore Ab4p appears to be the suitable strain for analysis of neuropathogenicity of EHV-1. The BAC sequence could be efficiently removed from the viral genome by using a lambda recombination system, resulting in Ab4p strain without BAC sequence (Ab4p attB). The Ab4p attB showed neurological symptoms in mice and its growth kinetics in cultured cells was the same as that of the wild type Ab4p. This Ab4p BAC and Ab4p attB will be significant tools for the analyzing the neuropathogenesis of EHV-1. Using this Ab4p BAC, an ORF37 deletion mutation and the corresponding revertant virus were constructed to characterize the ability of the virus to replicate in different cell lines in vitro and cause a disease after intranasal inoculation in the CBA/N1 mice model (Kasem et al. 2010).
2.11. Us9 in other veterinary alphaherpesviruses

The unique short (Us) region of most alphaherpesvirus genomes contains an open reading frame called Us9. The Us9 gene was first described and the protein product was first characterized in herpes simplex virus type 1 (HSV-1) (Frame et al. 1986; McGeoch et al. 1985).

Sequences homologous to HSV-1 Us9 have been found in the Us regions of HSV-2 (Dolan et al. 1998) and varicella-zoster virus (VZV) (Davison and Scott 1986) as well as in the animal pathogens pseudorabies virus (PRV) (van Zijlet et al. 1990) bovine herpesvirus 1 (Leung-Tack et al. 1994) equine herpesvirus 1 (EHV-1) (Flowers and O’Callaghan. 1992) feline herpesvirus 1 (Willemsen et al. 1995) canine herpesvirus (Haanes and Tomlinson. 1998) and simian herpesvirus B (Killeen et al. 1992).

Pseudorabies virus (PRV) Us9 gene product is a small tail-anchored (TA) membrane protein that is essential for axonal sorting of viral structural proteins and is highly conserved among other members of the alphaherpesvirus subfamily (Lyman et al. 2009).

Bovine herpesvirus 5 (BHV5) Us9 is essential for the anterograde spread of the virus from the olfactory mucosa to the bulb (Chowdhury et al. 2002).

Bovine herpesvirus 1 (BHV-1) Us9 not only complemented for BHV-5 Us9 and rescued the anterograde-spread defect of the BHV-5 Us9-deleted virus but conferred increased neurovirulence and neuroinvasiveness in rabbit seizure model (Chowdhury et al. 2006).
3. MATERIALS AND METHODS

3.1. Virus strain and cell lines.

EHV-1 Ab4p strain, which was kindly provided by Dr. A. J. Davison, Glasgow University, Scotland, was used. The virus was propagated in fetal equine kidney (FEK) cells. Other cells used in this study were Madin-Darby bovine kidney (MDBK) and Rabbit kidney 13 (RK-13) cells. All of these cells were cultivated with Eagle's minimum essential medium (EMEM) (Nissui, Tokyo, Japan) supplemented with 5-10% fetal bovine serum (FBS) and 100 units/ml penicillin and 100 μg/ml streptomycin.

To compare viral growth in the neurons, CX (M) cells (Sumitomo Bakelite, Tokyo, Japan) derived from mouse cerebral cortices were cultured in neuron culture medium (Sumitomo Bakelite, Tokyo, Japan).

3.2. Construction of Ab4pΔORF76 (Us9 deletion).

An EHV-1 BAC clone, pAb4p BAC, based on the neuropathogenic strain Ab4p (Previously established by Veterinary Microbiology laboratory, Faculty of Applied Biological Sciences, Gifu University.) was used to delete ORF76 in pAb4p BAC. For modification of pAb4p BAC, Red mutagenesis was used (Datsenko and Wanner, 2000). Briefly, competent E. coli DH10β harboring pAb4p BAC and the Red/ET plasmid pKD46 [DH10β (pAb4p, pKD46)] were grown in Luria-Bertani broth (LB) with tetracycline (30 μg/ml), ampicillin (50 μg/ml), and L-arabinose (0.1% final concentration) at 30°C to an optical density at 600 nm (OD600) of 0.6 and then made electrocompetent exactly as previously described (Datsenko and Wanner, 2000). To delete ORF76 in pAb4p BAC, ORF76 was replaced with the
rpsL-neo cassette (rpsL-neo gene) conferring streptomycin sensitivity and kanamycin resistance, resulting in recombinant BAC termed pAb4pΔORF76 BAC as follows. A pair of primer-1 (5'- TTT CCC TCT CAG CGA TCA CTT TTC ACC ACC GAA GAA CAG GCC CTC ATC GGG GCC TGG TGA TGA TGG CGG GAT CG -3') and primer-2 (5'-GGG CTG TTG TGG GGT AAA AGG TGG TGT TAC GGA AAC ACG CGT GCC AAG AAT CAG AAG AAC TCG TCA AGA AGG CG-3') containing 50-nucleotide homology arms bordering the desired deletion from position 136687 to 137468 of gene 76 and 24 nucleotides (in boldface) for amplification of the rpsL-neo cassette sequences was designed to be used for amplification of the insertion fragment with using rpsL-neo template DNA (Gene Bridges) as a template DNA (fig.3.1.).

PCR amplification was performed in 50 µl volumes containing DNA (100 ng), 200 µM of each dNTP, 0.2 µM of each dNTP, 0.2 µM of each primers, 5 µl 10X Ex Taq Buffer (Mg2+ plus) and 1.25U Takara Ex Taq™ DNA Polymerase (5 units/µl) (TAKARA, JAPAN). The PCR conditions were: 5 cycles of 5 s at 98 °C, 30 s at 58 °C and 2 min at 74 °C, and 30 cycles of 5 s at 98 °C and 74 °C for 2min. The PCR product was separated on agarose gel (0.9%) and stained with ethidium bromide.

The resulting 1420-bp PCR fragment was purified from agarose gel (QIAquick gel extraction kit; QIAGEN) and electroporated into DH10β (pAb4p, pKD46) using 0.1-cm cuvettes (Bio-Rad Laboratories) under standard electroporation conditions (1.35 kV/cm, 600 Ohms, 10 µF). After electroporation, cells were grown in 1 ml of LB for 70 min at 37°C and plated onto LB agar plates containing 50µg/ml of ampicillin, 30µg/ml
tetracycline and 15µg/ml of kanamycin. Resistant colonies were picked into liquid LB medium, grown at 37°C, and small-scale preparations of mutant pAb4p-DNA (pAb4pΔORF76 BAC) were obtained by alkaline lyses of E. coli (Sambrook et al. 1989) to be confirmed by PCR and nucleotide sequencing.

3.3. Construction of Ab4pΔORF76R (Revertant virus).

To replace the rpsL-neo gene with the ORF76 gene in the pAb4pΔORF76 BAC, DH10β (pAb4pΔORF76, pKD46) were grown in Luria-Bertani broth (LB) with tetracycline (30µg/ml), ampicillin (50µg/ml), kanamycin (15µg/ml) and L-arabinose (0.1% final concentration) at 30°C to an optical density at 600 nm of 0.6 and then made electrocompetent as previously described (Datsenko and Wanner 2000). ORF76 was amplified by PCR with a pair of primer-3 (5'-TTT CCC TCT CAG CGA TCA CTT TTC ACC ACC GAA GAA CAG GCC CTC ATC GG-3') and primer-4 (5'-GGG CTG TTG TGG GGT AAA AGG TGG TGT TAC GGA AAC ACG CGT GCC AAG AA -3') including 50-nucleotide homology arms bordering the desired deletion from position 136687 to 137468 of gene 76.

PCR amplification was performed in 50 µl volumes containing DNA (100 ng), 200 µM each dNTP, 0.2 µM of each dNTP, 0.2 µM of each primers, 5 µ1 10X Ex Taq Buffer (Mg2+ plus) and 1.25U Takara Ex Taq™ DNA Polymerase (5 units/µl) (TAKARA, JAPAN). The PCR conditions were: 5 min at 94 °C (initial denaturation), 30 cycles of 5 s at 98 °C, 30 s at 68 °C and 90 s at 72 °C and finally 7min at 72 °C (final extension). The PCR product was separated on agarose gel (0.9%) and stained with ethidium bromide.
The resulting 782bp PCR fragment was purified by agarose gel electrophoresis (QIAquick gel extraction kit; QIAGEN) and electroporated into DH10β (pAb4pΔORF76, pKD46) using 0.1-cm cuvettes (Bio-Rad Laboratories) under standard electroporation conditions (1.35 kV/cm, 600 Ohms, 10 µF). After electroporation, cells were grown in 1 ml of LB for 70 min at 37°C and plated onto LB agar plates containing 50µg of ampicillin/ml, 50µg of streptomycin. Double resistant colonies were picked into liquid LB medium, grown at 37°C. Small-scale preparations of mutant DNA of pAb4pΔORF76R BAC were obtained by alkaline lyses of E. coli (Sambrook et al. 1989), to be confirmed by PCR and nucleotide sequencing (fig.3.1.).

Fig. 3.1. Construction of Ab4pΔORF76 and its revertant Ab4pΔORF76R mutants.
3.4. Regeneration of infectious Ab4p attB, Ab4pΔORF76 and Ab4pΔORF76R viruses.

DNA was extracted by using a Nucleo Bond BAC 100 kit (MACHEREY-NAGEL, USA) from each BAC culture. For Ab4p attB, Ab4pΔORF76 and Ab4pΔORF76R, the DNAs were constructed by LR clonase reaction, which excises the BAC fragment from each BAC DNA. Then transfected into RK-13 cells in a 24-well plate by lipofectamine 2000 (Invitrogen, Tokyo, Japan) and incubated at 37 °C. After 5-7 days cultivation, supernatant was collected. The supernatant was inoculated to
MDBK cells. After 60 min of adsorption, the MDBK cells were covered by MEM containing 1.5% of carboxymethylcellulose and incubated for 4-5 days at 37 °C. Using GFP fluorescence as a marker, the desired virus plaques identified and selected under fluorescent microscopy. The Ab4p attB, Ab4pΔORF76 and Ab4pΔORF76R viruses were purified by three rounds of plaque purification.

3.5. Construction of Ab4pΔORF76 non sens mutant.

The same technology used for Construction of Ab4pΔORF76 and its revertant was used for the Construction of Ab4pΔORF76 non sens mutant. ORF76 ORF76 in pAb4p BAC was replaced with the rpsL-neo cassette (rpsL-neo gene) conferring streptomycin sensitivity and kanamycin resistance, resulting in recombinant BAC termed pAb4pΔORF76 BAC as follows. A pair of primer-1 (5’- TTT CCC TCT CAG CGA TCA CTT TTC ACC ACC GAA GAA CAG GCC CTC ATC GG GCC TGG TGA TAG TGG CGG GAT CG -3’) and primer-2 (5’-GGG CTG TTG TGG GGT AAA AGG TGG TGT TAC GGA AAC ACG CGT GCC AAG AA AAG AAC TCG TCA AGA AGG CG-3’) containing 50-nucleotide homology arms bordering the desired deletion from position 136687 to 137468 of gene 76 and 24 nucleotides (in boldface) for amplification of the rpsL-neo cassette sequences was designed to be used for amplification of the insertion fragment with using rpsL-neo template DNA (Gene Bridges) as a template DNA. To replace the rpsL-neo gene with the ORF76 gene in the pAb4pΔORF76 BAC introducing three stop codons after the first methionine (in boldface) in the frame of ORF 76 (Us9) the following primers were used: Upper US9- non sens 5’T TT CCC TCT CAG CGA TCA CTT TTC ACC ACC GAA GAA CAG GCC CTC ATC GG ATG TAG
TAG TAG GAG GCT GCC GCA 3’ Lower US9-non sens 5’GGG CTG TTG TGG GGT AAA AGG TGG TGT TAC GGA AAC ACG CGT GCC AAG AA TTA CGG AAA CAC GCG TGC CAA GAA3’. Regeneration of infectious Ab4pΔORF76 non sens mutant was done as previously described for Ab4pΔORF76 and its revertant.

3.6. Time course of viral growth.

Monolayers of MDBK, RK13 and FHK cells prepared in 24-well plates were inoculated with Ab4pattB, Ab4pΔORF76 and Ab4pΔORF76R at a m.o.i. of 0.1 plaque-forming unit (PFU)/cell. After 1.5 h adsorption, cells were washed three times with MEM and incubated at 37°C in a 5% CO2 atmosphere in 0.5 ml/well of MEM with 5% FCS. At 0, 6, 12, 24, 36 and 48 hrs intervals after inoculation, culture fluids with scraped cells were centrifuged to sediment the infected cells. The supernatants were used as the extracellular samples. Following two washes with MEM, the cell pellets were resuspended in 0.5 ml of MEM and subjected to three freeze–thaw cycles. After centrifugation, the resulting supernatants were used as the intracellular samples. Both extracellular and intracellular samples were titrated for viral infectivity.

3.7. Plaque size measurement.

Plaque areas were measured after plating of the viruses on MDBK cells and 3 days of incubation at 37 °C under a 0.6% methylcellulose overlay. For each virus, plaque areas of at least 50 plaques for each experiment were determined in triplicate using the ImageJ 1.28 software that is freely available from the National Institutes of Mental Health webpage.
3.8. Virus growth kinetics in mouse neurons.

To compare viral growth in the neurons, CX (M) cells (Sumitomo Bakelite, Tokyo, Japan) derived from mouse cerebral cortexes were cultured in 24-well plates coated with poly-L-lysine (Sumitomo Bakelite) in neuron culture medium (Sumitomo Bakelite, Tokyo, Japan). Titers of the various viruses were determined by infecting CX (M) cells at a multiplicity of infection MOI of 1. The supernatant and cells were separately harvested at the indicated timing (0, 24, 48, 72 and 96 h) and virus titers were determined by plaque assay on MDBK cells after freeze and thaw cycles as described previously (Yamada et al. 2008).

3.9. Analysis of transcription kinetics by real-time RT-PCR.

For analysis of transcription activity of ORF76, MDBK cells were infected with Ab4pattB, Ab4pΔORF76 and Ab4pΔORF76R, at a multiplicity of infection MOI of 1. Total RNA was extracted by using Nucleospin RNA kit (MACHEREY-NAGAL, USA) from the infected and uninfected MDBK cells harvested at 0, 4, 8 and 12 h post infection. Then 1.5 µg of RNA was heated at 95 °C for 5 min for denaturation, combined with reverse transcriptase master mix consisting of 4 µl of 5 × RT buffer (TOYOBO, Osaka, Japan), 5mM of dNTP (TAKARA), 25 pmole of random primer (TOYOBO), 40 U of RNase inhibitor (TOYOBO) and 50 U of reverse transcriptase (TOYOBO). The reaction mixture was incubated at 30 °C for 10 min, 42 °C for 40 min followed by incubation at 99 °C for 5 min.
to stop the reaction. Real-time PCR assay were carried out by using 12.5 µl of SYBR Premix Ex Taq (TAKARA), 10 µM of specific primers and 10 ng of cDNA in the Thermal Cycler Dice Real Time System (TAKARA). Primers sequences are for ORF76 (ORF76A 5’-CTA CCG TGG AAG CGG TAT GT-3’ and ORF76B 5’-ATT CTC AGA AGC AGC GGT GT-3’), ORF75 (ORF75A 5’-CAA CCC TGT CAG AAA CAG CA -3’ and ORF75B 5’- GGG GGA GGT AGA GTT TCC AG-3’) and ORF67 (ORF67A 5’-TCG GCC CTT ATG TAA TAG CG -3’ and ORF67B 5’-CTC CTA CTT CAG GCG GTG TC-3’) Relative quantities were measured by the ΔCt Method (Livak and Schmittgen 2001).

3.10. Production of anti-EHV-1 Us9-specific polyclonal guinea pigs serum.

To produce antibody against EHV-1 Us9, the entire EHV-1 Us9 open reading frame (ORF) was expressed in bacteria as a glutathione S-transferase (GST) fusion protein followed by immunization of guinea pigs with the recombinant Us9 protein.

3.10.1 Cloning of EHV-1 Us9.

For this, the entire EHV-1 Us9 ORF coding region was amplified by long PCR using a forward primer that introduced a EcoRI site directly upstream of the start codon, allowing for an in-frame fusion with the GST open reading frame and a reverse primer that introduced an NotI site directly downstream of the Us9 termination codon. (ECOR1+ US9 F5’ CCGGAATTC ATGGAGAAGGCGGAGGCTGCCGA3’ and NOT1+
PCR amplification was performed in 50 µl volumes containing DNA (100 ng), 200 µM of each dNTP, 0.2 µM of each dNTP, 0.2 µM of each primers, 5 µl 10X LA Taq Buffer (Mg2+ plus) and 1.25U Takara LA Taq™ DNA Polymerase (5 units/µl) (TAKARA, JAPAN). The PCR conditions were: initial denaturation at 94 C for 5 Min., 30 cycles at 98 C for 5 s, 60 C for 30 s, 72 C for 2 min and a final step of 72 C for 7 min.

The 687-bp EcoRI- NotI PCR-generated fragment containing the complete EHV-1 Us9 ORF was ligated into pGEX-6P-1 (GE Healthcare Bio-Sciences AB), generating the pGST-US9 expression vector. The PCR-amplified portion of this construct was sequenced to confirm that no mutations had been introduced into the cloned Us9 gene.

3.10.2. Us9-specific polyclonal antiserum.

Expression of the GST-Us9 fusion protein was induced by the addition of IPTG (isopropyl-B-D-thiogalactopyranoside) to a culture of BL21 cells transformed with the pGST-US9 plasmid. The fusion protein was then bound to glutathione-Sepharose beads (GE Healthcare Bio-Sciences AB), and the recombinant Us9 protein was isolated as a completely soluble form and used to immunize guinea pigs (Japan SLC Corporation). Sera collected before and after four subcutaneous applications of 100-µg protein at 1-week intervals were analyzed.

3.10.3. Western blot analysis for reactivity against Us9.
For Western blot analyses, RK13 cells were infected with Ab4pattB, Ab4pΔORF76 and Ab4pΔORF76R at a multiplicity of 5 p.f.u. per cell and incubated at 37 °C for 1–18 h. Cell lysates were harvested. After centrifugation at 14,000 rpm for 1 min in an Eppendorf centrifuge, cells were washed twice with phosphate-buffered saline (PBS), resuspended in 100 µl of PBS, and mixed with the same volume of sample buffer.

Then separated by SDS-PAGE and electrotransferred to nitrocellulose membranes (Millipore). Blots were blocked with 5 % low-fat milk in Tris-buffered saline (TBS-T; 150 mM NaCl, 10 mM Tris/HCl, pH 8.0, 0.25 % Tween 20) and incubated for 1 h with guinea pigs antisera against the US9 gene products at dilutions of 1: 1 000 in TBS-T. Bound antibody was detected with peroxidase-conjugated anti-guinea pigs antibodies (Bethyl) and visualized by chemiluminescence (Amersham) recorded on X-ray films.

3.11. Animal experiments.

Animal experiments were conducted as described previously (Ho and Mocarski 1988; Osterrieder et al. 1996; Fukushi et al. 2000). Briefly, 3 groups each consists of 26 animals four-week-old specific pathogen free (SPF) male CBA/N1 mice (Japan SLC Corporation) were inoculated with a virus preparation by the intranasal route at 1x10^4 PFU per head in addition to one control group of 26 animals inoculated with MEM only. Behavior and body weight of each mouse were observed from 3 days before the inoculation to the end of the period. Body weights were evaluated by analysis of variance and multiple comparisons of the groups. Two mice from each group were euthanized every day from 1 to 10 dpi for virus isolation and DNA detection. Lungs, olfactory bulbs and the brains were used for
virological assay.

Table 4.1. Mice experiment design.

<table>
<thead>
<tr>
<th>Group /Treatment</th>
<th>Ap4battB</th>
<th>Ab4pΔORF76R</th>
<th>Ab4pΔORF76</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>Body weight and virus inoculation</td>
<td>Body weight and virus inoculation</td>
<td>Body weight and virus inoculation</td>
<td>Body weight and MEM inoculation</td>
</tr>
<tr>
<td>Day 1- Day 10</td>
<td>Body weight and Sacrifice 2 animals</td>
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<td>Body weight and Sacrifice 2 animals</td>
<td>Body weight and Sacrifice 2 animals</td>
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<tr>
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<tr>
<td>Day 12</td>
<td>Body weight</td>
<td>Body weight</td>
<td>Body weight</td>
<td>Body weight</td>
</tr>
<tr>
<td>Day 13</td>
<td>Body weight and Sacrifice of the remaining animals</td>
<td>Body weight and Sacrifice Of the remaining animals</td>
<td>Body weight and Sacrifice Of the remaining animals</td>
<td>Body weight and Sacrifice Of the remaining animals</td>
</tr>
</tbody>
</table>

All experiments were conducted under the guidelines for animal experiments in Gifu University with certification by the committee of Faculty of Applied Biological Sciences, Gifu University.

3.11.1. Preparation of olfactory bulbs, brains and lungs for PCR and virus titeration.

Tissues were homogenized in MEM at 10% (w/v). The homogenates were centrifuged at 3,000 rpm for 10 minutes to remove the cellular debris. Supernatant was serially 10-fold diluted in MEM. A volume of 0.1 ml per well was inoculated onto a confluent MDBK monolayer in 24-well plates. Virus titers were determined by plaque assay. The detection limit in the organ homogenates was 1x10^2 pfu per gram of a mouse organ. DNA was extracted with a Sepagene kit for virus DNA detection in mice organs (Sanko Junyaku, Japan). Viral DNA was detected by using primers for ORF76 and primers for rpsL-neo gene for the mutant virus as previously
described.

### 3.11.2. Histopathology and Immunohistochemistry.

The brain, nasal cavity, lungs and olfactory bulbs were collected and fixed in 10% buffered formalin. After fixation, tissues were dehydrated and embedded in paraffin wax by routine methods, sectioned at 5 μm, stained with hematoxylin and eosin (HE), and examined by light microscopy.

Paraffin wax sections of the nasal mucosa, brain and lungs were immunolabelled with EHV-1 rabbit antiserum by the avidin-biotin-complex (ABC) immunoperoxidase method, as described previously (Yanai et al. 1998), with ABC kits (Vector Laboratories, Burlingame, CA, USA). The primary antibody was rabbit EHV-1 antiserum (1:1000, Veterinary Microbiology Laboratory at Gifu University) followed by application of a secondary antibody (biotinylated anti-rabbit IgG, DAKO Cytomation, USA) with Liquid DAB Substrate Chromogen System (DAKO Cytomation, USA) used as the chromogen and haematoxylin counterstain. Tissue sections from an EHV-1-infected mice and sera from a nonimmunized rabbit and goat were used as controls.

### 3.11.3 Immunofluorescence.

Immunofluorescence was performed on paraffin wax sections of the nasal mucosa, bulb and brain as described previously (Robertson et al. 2008), with little modification of the technique for antigen retrieval, proteinase k was used 5 minutes. The primary antibody was polyclonal Us9 guinea pig serum (1:500, prepared during this study) followed by application of a secondary antibody (anti guinea pig FITC sigma Aldrich, USA). The reaction was examined under Keyence Biozero system.
4. Results

4.1. Construction of ORF76 deletion mutant and in its revertant.

The roles and significance of ORF76 (Us9) of EHV-1 were investigated by using molecular recombination of Ab4p BAC. To construct an ORF76 (Us9) deletion mutant, the ORF76 of pAb4p BAC was replaced with a prokaryotic selection marker, the rpsL-neo gene conferring streptomycin sensitivity and kanamycin resistance, by Red mutagenesis in E. coli. The resulting ORF76 negative Ab4p BAC mutant was termed pAb4pΔORF76 BAC (Fig. 4.1).

The correct insertion of the rpsL-neo gene and deletion of ORF76 was confirmed by PCR and nucleotide sequencing. pAb4pΔORF76 BAC DNA, isolated from E. coli, was treated with LR clonase enzyme to excise the pZC320-GFP fragment and transfected into RK13 cells to reconstitute the virus with ORF76-deletion, designated Ab4pΔORF76. To restore ORF76, homologous recombination of amplified PCR product of ORF76 and pAb4pΔORF76 BAC in DH10β resulted in replacement of rpsL-neo gene with ORF76-encoding sequence and reconstitution of a revertant BAC, pAb4pΔORF76R BAC. The ORF76 rescuant pAb4pΔORF76R BAC DNA, isolated from E. coli, was reacted with LR clonase to excise the BAC fragment and transfected into RK13 cells to reconstitute the ORF76 rescuant virus, Ab4pΔORF76R.

The genotypes of all generated viruses were confirmed by nucleotide sequencing and PCR. When the ORF76 was present, it resulted in a PCR product of 782bp. The 782bp product was detected in cells infected with
Ab4p attB and Ab4pΔORF76R viruses (Fig. 4.1, lanes 1 and 2). Insertion of the rpsL gene instead of ORF76 resulted in a product of 1420bp in size in cells infected with ORF76 deletion mutant Ab4pΔORF76 (Fig. 4.1, lane 3).

Fig. 4.1. PCR analysis of the generated recombinant viruses using primers ORF76-1 and ORF76-2. Intact ORF76 yields a fragment of 782 bp, whereas virus DNA containing the rpsL gene results in a fragment of 1420bp. The molecular size marker is the 100-bp ladder (TOYOBO, Japan). PCR products from the different viruses were electrophoresed in 1% agarose gel. Markers (lane M) were included to assess the sizes of the PCR products. Lane 1: Ab4p attB, lane 2: Ab4pΔORF76R, lane 3: Ab4pΔORF76, M: Molecular weight marker.
4.2. Characterization of In vitro growth properties of ORF 76-negative mutants in cultured cell line.

To characterize the Ab4pΔORF76 mutant virus it was compared with Ab4p attB and the revertant virus Ab4pΔORF76R in cell cultures in respect of plaque size and titre. To assess a possible contribution of ORF76 to the plaque formation of EHV-1, plaque areas of Ab4pΔORF76 were quantified and compared to those of Ab4p attB and Ab4pΔORF76R. In three independent experiments in MDBK cells, Ab4pΔORF76 relative plaque sizes was about 100% while the relative plaque size of Ab4p attB and Ab4pΔORF76R were 120% (Fig. 4. 2.).

![Fig. 4. 2. Plaque size of the generated recombinant viruses. Viruses were titrated by plaque formation on MDBK cells. The experiments were performed in triplicate. B:](image-url)
Relative plaque sizes of 50 randomly selected plaques of the Ab4p attB, Ab4pΔORF76R and Ab4pΔORF76. Error bars are standard errors.

The extracellular Ab4pΔORF76 Virus titers in MDBK cells were similar with all tested viruses, while its intracellular titers were one log lower at 24 hr. (Fig. 4. 3.).

![Fig. 4. 3. Comparison of the in vitro growth curve of mutant viruses generated by BAC technology. MDBK Cells were infected at a MOI of 0.1. At the indicated times after infection, cells and supernatant were harvested separately as described in Materials and Methods. Intracellular (A) and extracellular (B) viruses were titrated by plaque formation on MDBK cells. The experiments were performed in duplicate. Error bars are standard errors.](image_url)
For RK13 cells no differences were observed in between Ab4pΔORF76, Ab4p attB and Ab4pΔORF76R virus titers (Fig. 4.4).

**Fig. 4.4.** Comparison of the in vitro growth curve of mutant viruses generated by BAC technology. Rk 13 Cells were infected at a MOI of 0.1. At the indicated times after infection, cells and supernatant were harvested separately as described in Materials and Methods. Intracellular (A) and extracellular (B) viruses were titrated by plaque formation on MDBK cells. The experiments were performed in duplicate. Error bars are standard errors.
Ab4pΔORF76 extracellular Virus titers in FHK were one log lower at 24 hr and 36 hr. While intracellular titers were three logs lower at 6hr and one log lower at 12 hr and 24 hr than Ab4p attB and Ab4pΔORF76R. (Fig. 4, 5.).

**Fig. 4, 5.** Comparison of the in vitro growth curve of mutant viruses generated by BAC technology. FHK Cells were infected at a MOI of 0.1. At the indicated times after
infection, cells and supernatant were harvested separately as described in Materials and Methods. Intracellular (A) and extracellular (B) viruses were titrated by plaque formation on MDBK cells. The experiments were performed in duplicate. Error bars are standard errors.

4.3. In vitro growth properties of ORF 76-negative mutant in cultured mouse neurons.

The intracellular and extracellular virus titers of Ab4pΔORF76 were one order of magnitude lower than those of Ab4p attB and Ab4pΔORF76R in mouse neuronal cells (Fig. 4. 6.), indicating that the deletion of ORF76 affected EHV-1 multiplication in neuronal cells.
Fig. 4. Growth curve of mutant viruses by using mouse neurons, CX (M) Cells. The neuron cells were infected at a MOI of 1. At the indicated times after infection, cells and supernatant were harvested separately as described in Materials and methods. Intracellular (A) and extracellular (B) viruses were titrated by plaque formation on MDBK cells. The experiments were performed in duplicate. Error bars are standard errors.

4.4. Effect of ORF76 deletion on transcription activities of ORFs 75, and 67.

To evaluate the effects of the deletion of ORF76 on transcript levels of the neighboring ORFs. Transcript levels of ORFs 75 and 67 were measured in MDBK cells infected with Ab4p attB, Ab4pΔORF76 and
Ab4pΔORF76R. β-actin levels in cells infected with the different strains were the same. ORF76 transcripts were not detected in cells infected with the deletion mutant, (Fig. 4. 7A). Deletion of ORF76 did not affect transcription levels of ORF75 (Fig. 4. 7B), and ORF67 (Fig. 4. 7C).
Fig. 4. Analysis of transcription activity of ORF76, ORF75 and ORF67 by real–time RT-PCR. Real-time RT-PCR analysis was performed by using RNAs from MDBK cells infected with Ab4p–attB, Ab4pΔORF76 and revertant virus at different times 0, 4, 8 and 12 hours post infection. The figure compares the transcription levels of these viral genes in MDBK cells. Transcription activity of ORF76 (A), ORF75 (B) and ORF67 (C) were examined by real–time RT-PCR. Relative quantity was evaluated by crossing point method using β-actin gene control.
4.5. Pathogenicity of ORF76 deletion mutant virus in mice

4.5.1. ORF76 deletion mutant virus is nonneurovirulent.

To evaluate the role of ORF76 (Us9) in the neuropathogenicity of EHV-1, CBA/N1 mice were inoculated with Ab4p attB, Ab4pΔORF76, and Ab4pΔORF76R. Mice that were inoculated with Ab4p attB and Ab4pΔORF76R showed nervous signs such as hyperactivity, arching the back and paralysis. These symptoms started from 5 and 6 dpi in the Ab4p attB and Ab4pΔORF76R inoculated groups respectively. Mice inoculated with Ab4pΔORF76 did not show any nervous signs and gained body weight throughout the observation period. The body weights of mice inoculated with Ab4p attB and Ab4pΔORF76R decreased at 7 and 8 dpi, then start to increase again at the 9th day. From 7 to 13 dpi, mean body weights of mice inoculated with Ab4pΔORF76 were larger than those of mice inoculated with Ab4p-attB or Ab4pΔORF76R (Fig. 4. 8.).
Fig. 4. 8. Mean body weight curves of mice inoculated with mutant viruses. Mice in groups of twenty six were infected intranasally with $1 \times 10^4$ PFU of the indicated virus. Mean body weights were measured from 3 days before inoculation (-3 dpi) to 13 dpi. Each data represents the mean of the body weight for the indicated group. Error bars indicate standard errors.

Viruses were consistently recovered from the lungs from 1 to 5 dpi of mice inoculated with Ab4p attB and from 1 to 4 dpi of mice inoculated with Ab4pΔORF76R, respectively. On the other hand, the virus was recovered from 1 to 3 dpi in Ab4pΔORF76 inoculated mice. The viruses were recovered from olfactory bulbs and brains of mice inoculated with Ab4p attB and Ab4pΔORF76R from 3 to 7 dpi. While the virus was not recovered from Ab4pΔORF76 inoculated mice olfactory bulbs and brains. Viruses DNA was detected in the lungs, olfactory bulbs and brains of mice inoculated with Ab4p attB. and Ab4pΔORF76R from 1 dpi. While Ab4pΔORF76 DNA was detected in the lungs from 1 dpi but not detected at all in olfactory bulbs and brains of mice inoculated with Ab4pΔORF76.
Table 4.1. Virus titration and DNA detection in mice organs inoculated with Ab4p attB, Ab4p ΔORF76 and Ab4p ΔORF76R.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Day post inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organ</td>
<td>0</td>
</tr>
<tr>
<td>Ab4p attB</td>
<td></td>
</tr>
<tr>
<td>Olfactory bulbs</td>
<td>−/−</td>
</tr>
<tr>
<td>Brain</td>
<td>−/−</td>
</tr>
<tr>
<td>Lung</td>
<td>−/+</td>
</tr>
<tr>
<td>Ab4pΔORF76^6</td>
<td></td>
</tr>
<tr>
<td>Olfactory bulbs</td>
<td>−/−</td>
</tr>
<tr>
<td>Brain</td>
<td>−/−</td>
</tr>
<tr>
<td>Lung</td>
<td>−/+</td>
</tr>
<tr>
<td>Ab4pΔORF76^6R</td>
<td></td>
</tr>
<tr>
<td>Olfactory bulbs</td>
<td>−/+</td>
</tr>
<tr>
<td>Brain</td>
<td>−/+</td>
</tr>
<tr>
<td>Lung</td>
<td>−/+</td>
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</table>

*: Virus titer in pfu per gram of organ/virus DNA detection.
+: Virus DNA was detected.
−: Virus titer was less than 1 × 10^2 pfu per gram or virus DNA was not detected.

The histopathological findings of the lungs showed that interstitial pneumonia in the lungs of all mice examined but not in mock inoculated mice. The olfactory bulbs and brains of mice infected with Ab4pΔORF76
did not show any histopathological changes or signs of encephalitis nor meningitis (Fig. 4.9.), while the olfactory bulbs and brains of mice infected with Ab4p attB and Ab4pΔORF76R showed non-suppurative encephalitis and meningitis (Fig. 4.10.-4.15.). The histopathological lesions consisted of degeneration and necrosis of the neurons, lymphocytic cell infiltration, perivascular cuffing, meningitis and gliosis.

Fig. 4.9. Brain; mice, infected with Ab4pΔORF76 via the nasal route. There were no lesions observed in the cerebrum.
Fig. 4. 10. Brain; mice, infected with Ab4p attB. Neuronal necrosis (arrows) with Perivascular cuff (arrowhead) was observed in the cerebrum.
Fig. 4. 11. Brain; mice, infected with Ab4pΔORF76R. Perivascular cuff (arrowhead) of neutrophils, lymphocytes and plasma cells were observed in the cerebrum.

Fig. 4. 12. Brain; mice, infected with Ab4p attB. Gliosis (arrowhead) was observed.
Fig. 4. 13. Brain; mice, infected with Ab4pΔORF76R. Meningitis (arrowhead) was observed in the cerebrum.
Fig. 4. 14. Lung; mice, infected with Ab4p attB. Interstitial pneumonia, characterized by thickened alveolar septa with varying numbers of macrophages, lymphocytes, and plasma cells.
Fig. 4. 15. Lung: mice, infected with Ab4pΔORF76R strain via the nasal route. Interstitial pneumonia, characterized by thickened alveolar septa with varying numbers of macrophages, lymphocytes, and plasma cell.

4.5.2. **ORF76 deletion mutant virus infects the olfactory epithelium but failed to get transported to the olfactory bulbs.**

Nasal cavity of mice infected with Ab4p attB, Ab4pΔORF76R and Ab4pΔORF76 showed very mild rhinitis, multiple foci of necrosis of the olfactory epithelial cells (Fig. 4.16.) along with inflammatory cells infiltrates within the mucosa and admixed with the desquamated epithelial cells (Fig. 4. 17.).

The olfactory bulbs of mice inoculated with Ab4p attB and Ab4pΔORF76R showed typical encephalitis at 4 and 6 days post infection respectively while no pathological changes were recorded in mice inoculated with Ab4pΔORF76.
Fig. 4. 16. Olfactory epithelium; mice, infected with Ab4p attB strain, showed necrosis.
Fig. 4. 17. Olfactory epithelium; mice, infected with Ab4pΔORF76, showed necrosis, together with desquamation of clusters of neutrophils and epithelial cells in the lumen.
Fig. 4. 18. Olfactory bulb; mice, infected with Ab4pΔORF76R. Neuronal necrosis (arrows) was observed in the olfactory bulb in the glomerular (G), mitral (M) and Granular (GR) cell layers.

Fig. 4. 19. Higher magnification of fig 18 showed neuronal necrosis (arrows) in the olfactory bulb in the glomerular(G) cell layer.

There were positive immunohistochemical (using antibodies against EHV 1) and immunofluorescence (using antibodies against us9) reactions for EHV-1 antigen in the cytoplasm of degenerating olfactory epithelial cells (Fig. 4.21.- 4.24.) in the nasal cavity of mice infected with Ab4p attB, Ab4pΔORF76R and Ab4pΔORF76. While the reactions were positive in brains of mice inoculated with Ab4p attB and Ab4pΔORF76R (Fig. 4. 25. - 4.28.) only.
Fig. 4. Brain mice infected with Ab4pΔORF76 strain. EHV-1 antigen not detected. ABC method for EHV-1 antigen, counterstained with Mayer’s hematoxylin.
Fig. 4. Olfactory epithelium; mice, infected with Ab4pΔORF76 strain. EHV-1 antigen (arrow) is detected in the cytoplasm in some of the neurons in the olfactory epithelium. ABC method for EHV-1 antigen, counterstained with Mayer’s hematoxylin.
Fig. 4. 22. Olfactory epithelium; mice, infected with Ab4p attB strain. EHV-1 antigen is detected in the cytoplasm of some neurons in the olfactory epithelium. ABC method for EHV-1 antigen, counterstained with Mayer's hematoxylin.

Fig. 4. 23. Ab4p attB olfactory epithelium, positive signaling by indirect immunofluorescent technique. x 100.
Fig. 4. 24. Ab4pΔORF76R Olfactory epithelium, positive signaling by indirect immunofluorescent technique. x100.

Fig. 4. 25. Brain; mice, infected with Ab4pΔORF76R strain. EHV-1 antigen (arrow) is detected in the cytoplasm and dendrites of neuronal cells. ABC method for EHV-1 antigen, counterstained with Mayer's hematoxylin.
Fig. 4. 26. Brain; mice, infected with Ab4p attB strain. EHV-1 antigen is detected in neuronal cells in an area showed neuronal degeneration, perivascular cuffing, mild gliosis and meningitis. ABC method for EHV-1 antigen, counterstained with Mayer's hematoxylin.

To identify EHV-1 Us9, the Us9 ORF coding region was expressed as a GST fusion protein. Bacterially expressed EHV-1 Us9 was purified as described in Materials and Methods and used for generating guinea pig antisera. As shown in Fig. 4. 29, the EHV-1 Us9-specific antibody precipitated proteins with approximate molecular masses of 35 to 42-kDa in Ab4p attB (Lane 2), Ab4pΔORF76R (Lane 3) and Ab4pORF76 non sens mutant (Lane 5). The EHV-1 Us9-specific bands were absent in RK13 cells mock (Lane 1) and infected with the EHV-1 Us9-deleted mutant (Lane 4).
Fig. 4. 29. Western blotting analysis of the generated recombinant viruses using guinea pig anti Us9 serum detects Us9 polypeptides with relative molecular masses of between 35 and 42 kDa. Lane 1: RK 13 Mock, lane 2: Ab4p attB, lane 3: Ab4pΔORF76R, lane 4: Ab4pΔORF76 lane 5: Ab4p ORF76 non sens mutant M: Molecular weight marker.
5. Discussion

The functional analysis of EHV-1 ORFs or of strain-specific properties was largely performed by generating virus mutants by homologous recombination techniques, thereby introducing marker genes instead of the targeted viral ORFs (Osterrieder and Wolf 1998).

A number of herpesvirus genes have been shown to be nonessential for growth in cultured cells. However, when viral mutants were tested in certain animal models, several of these genes proved to be important in promoting viral replication and disease in vivo (Subak-Sharp and Dargan 1998; Visalli and Brandt 2002; Ward and Roizman 1994).

In recent years, manipulation of the large herpesvirus genomes has been facilitated by the introduction of bacterial artificial chromosome (BAC) cloning and mutagenesis. The genomes of several herpesviruses have been cloned as infectious BACs using this technique (Brune et al. 2000). Targeted and random mutagenesis of herpesvirus genomes cloned as BACs is considerably faster and more reliable than conventional approaches, because mutagenesis is no longer dependent on the growth of viruses in eukaryotic cells but can be performed in Escherichia coli (Messerle et al., 1997; Borst et al., 1999; Wagner et al., 1999; Schumacher et al. 2000).

The Ab4p strain is a neurovirulent strain that was isolated from a case of equine paresis (Gibson et al. 1992). Adding with a whole genome sequence (Telford et al. 1992), Ab4p has been confirmed to cause neurological disorders in experimental infection of horses, hamsters and
mice (Fukushi, et al. 2000; Frampton et al. 2004; Awan et al. 1990). Therefore Ab4p appears to be the suitable strain for analysis of neuropathogenicity of EHV-1.

The BAC sequence could be efficiently removed from the viral genome by using a lambda recombination system, resulting in Ab4p strain without BAC sequence (Ab4p attB). The Ab4p attB showed neurological symptoms in mice and its growth kinetics in cultured cells was the same as that of the wild type Ab4p. This Ab4p BAC and Ab4p attB will be significant tools for the analyzing the neuropathogenesis of EHV-1. Using this Ab4p BAC, an ORF37 deletion mutation and the corresponding revertant virus were constructed to characterize the ability of the virus to replicate in different cell lines in vitro and cause a disease after intranasal inoculation in the CBA/N1 mice model (Kasem et al. 2010).

Laboratory of Veterinary Microbiology, Faculty of Applied Biological Sciences, Gifu University, Japan. established an EHV-1 BAC clone, pAb4p BAC, based on the neuropathogenic strain Ab4p. This pAb4p BAC has no deletion of genes, because the BAC vector (pZC320-GFP sequence) was inserted into the intergenic region between ORF2 and ORF3 of Ab4p using the lambda insertion-excision system. Thus, pAb4p BAC should maintain the complete original genetic information of Ab4p.

Ab4p attB behaved like the wild type Ab4p in terms of in vitro growth and neuropathogenicity in mouse, the pathogenicity of Ab4p attB was similar to that of the wild type Ab4p in mice. Especially, the same nervous symptoms were observed in each mice inoculated with Ab4p and Ab4p attB,
respectively. Ab4p attB, which contains attB sequence, can be used to evaluate the neuropathogenesis of EHV-1. (Kasem et al. 2010). Therefore Ab4p attB can be regarded as equivalent to the wild type Ab4p which was not included in this work.

In this study, EHV-1 BAC clone, pAb4p BAC used as a base to construct an ORF76 deletion mutation and the corresponding revertant virus to characterize the ability of the virus to replicate in different cell lines in vitro and cause a disease after intranasal inoculation in the CBA/N1 mice model.

This work described the construction of an ORF76 replacement mutant that is viable in vitro yet shows significant attenuation in mice models.

The rpsL-neo gene replacement, which was used to delete the ORF76 (Us9), had no obvious effect on expression or function of ORF75 or ORF67. Therefore phenomena observed in this work could be regarded to be caused by the deletion of ORF76 itself.

The ability of the EHV-1 ORF76 deletion mutant to replicate in cultivated mouse neural cells derived from cerebral cortex was significantly impaired. These results suggested that the ORF76 product (US9) plays a role in the multiplication of EHV-1 in neural cell.

EHV-1 ORF76 deletion mutant virus shows different growth kinetics in
different ordinary cell cultures, for RK13 no differences were observed in the end-point virus titers between Ab4pΔORF76 and Ab4p attB and Ab4pΔORF76R viruses. While for MDBK intracellular Ab4pΔORF76 titer was one log lower at 24hr but resume the same titer of other viruses at 48 hr. For FHK extracellular Virus titers were one log lower at 24hr and 36hr. While intracellular titers were three logs lower at 6hr and one log lower at 12 hr and 24hr than Ab4p attB and Ab4pΔORF76 and resumed nearly the same titers at 48 hr of other viruses extra and intracellular. The causes of Ab4pΔORF76 different growth kinetics may be related to time and expression level of Us9, which may be different in different cell culture. The causes of Ab4pΔORF76 different growth kinetics need more study and investigation.

The ORF76 product (Us9) is dispensable for viral replication in cell cultures in other alphaherpesviruses, the PRV Us9 null viruses are indistinguishable from wild-type PRV Becker in single-step growth release and plaque size (Brideau et al. 2000 b). The deletion of EHV1 ORF76 had only a slight effect on viral growth in cell culture, indicating that the EHV1 ORF76 gene product is not essential for growth in ordinary tissue culture cells. However, ORF76 also had smaller plaque size and altered plaque morphology. It is possible that EHV 1 US9 mutant produces smaller plaques because they produce fewer progeny.

The unique short (Us) region of most alphaherpesvirus genomes contains an open reading frame called Us9. The Us9 gene was first described and the protein product was first characterized in herpes simplex virus type 1 (HSV-1) (Frame et al. 1986; McGeoch et al. 1985).
Sequences homologous to HSV-1 Us9 have been found in the Us regions of HSV-2 ([Dolan et al. 1998](#)) and varicella-zoster virus (VZV) ([Davison and Scott 1986](#)) as well as in the animal pathogens pseudorabies virus (PRV) ([van Zijlet et al. 1990](#)) bovine herpesvirus 1 ([Leung-Tack et al. 1994](#)) equine herpesvirus 1 (EHV-1) ([Flowers and O’Callaghan. 1992](#)) feline herpesvirus 1 ([Willems et al. 1995](#)) canine herpesvirus ([Haanes and Tomlinson. 1998](#)) and simian herpesvirus B ([Killeen et al. 1992](#)).

The only alphaherpesviruses sequenced to date that do not contain an Us9 gene are the oncogenic avian Marek’s disease herpesvirus ([Brunovskis and Velicer 1995](#)) and herpesvirus of turkeys ([Zelnik, et al. 1993](#)). The fact that the gene is conserved among the alphaherpesviruses, nonessential in tissue culture for HSV-1 and PRV, and absent from several attenuated strains of PRV ([Lomniczi et al. 1984](#), [Mettenleiter et al. 1985](#), [Mettenleiter et al. 1985](#), [Petrovskis, et al. 1986](#)) implies that the Us9 protein plays a role in virus-host interactions.

The Us9 protein is highly conserved among the various alphaherpesviruses. This observation and the fact that its absence causes no obvious phenotype in standard tissue culture cell lines imply a conserved function in natural animal infections with a positive selective advantage. The fact that some naturally attenuated PRV strains have deletions of Us9 provides a hint that the protein affects viral pathogenesis. Consequently, in this study, we produced a Us9-specific polyclonal antiserum to identify and characterize the protein product of the EHV 1 Us9 gene.
The protein product of the HSV-1 Us9 gene was identified by Frame et al. 1986. by using an antiserum made against a synthetic oligopeptide whose sequence was deduced from the DNA sequence. These workers provided evidence that the HSV-1 Us9 protein was a virion structural protein localized to the tegument. Moreover, they demonstrated that the protein was phosphorylated and, using immunogold electron microscopy, found it to be associated with nucleocapsids in the nuclei of infected cells. The HSV-1 Us9 protein was predicted to have a molecular mass of 10 kDa, yet 12 distinct polypeptides ranging from 12 to 20 kDa were observed and characterized as phosphoforms of Us9.

The EHV1 Us9 amino acid sequence predicts a protein of 219 amino acids and 22.287kDa.
Using Western blot analysis with Us9 polyclonal antiserum and EHV1 RK 13-infected cell lysates, we found Us9 several polypeptides with relative molecular masses between 35 and 42 kDa. The EHV-1 Us9-specific bands were absent in RK13 cells mock and infected with the EHV-1 Us9-deleted mutant (fig 27). These results demonstrated that the antibody specifically recognized Us9 encoded by EHV-1. The respective EHV-1 Us9-specific bands recognized by the Us9-specific antibody were of a higher molecular mass, suggesting that the respective Us9 proteins are processed posttranslationally to a higher-molecular-mass protein.

Us9 gene homologues are found in most of the alphaherpesviruses, including PRV and HSV-1, BHV-1 and BHV-5. PRV, HSV-1, BHV-1 and BHV-5 Us9 ORFs are predicted to encode proteins with an approximate molecular mass of 10.8 kDa, 10.0 kDa, 14.7 kDa and 13.7 kDa respectively. However, Us9-specific polyclonal serum precipitated several proteins with molecular masses of from 17 to 20 kDa in PRV, between 12 to 25 kDa in HSV-1, 28-32 kDa in BHV-1 and 15-20 kDa in BHV-5. In PRV, HSV-1, BHV-1 and BHV-5 the Us9 is phosphorylated (Brandimarti and Roizman, 1997, Brideau, et al. 1998, and Chowdhury et al. 2006). Our experiments support the notion that the Us9 proteins in alphaherpesviruses are phosphorylated, suggesting that phosphorylation of Us9 has a functional role.
The data from non-sens mutagenesis experiments (We stopped the first start codon with three stop codons) cancelled the idea that these polypeptides may resulted from the first in-frame methionine found at the beginning of the predicted open reading frame. Moreover, these data suggested that the second methionine is the major site of translation initiation consistent with the finding of Brideau, et al. 1998 on PRV.

The mouse model has been considered to be suitable for investigation of virological and histopathological aspects of EHV-1-induced disease in the horse (Walker et al., 1999). CBA mice have been used as a model for the study of neuropathogenicity of EHV-1 KgI/gE/75 strain and showed brain lesions similar to those observed in EHV-1 infected horses exhibiting neurological signs (Frampton et al., 2004). Additionally, much is known about the genetic and the biological characteristics of the CBA mice. Therefore, CBA mice seem to be a good model for evaluating the neuropathogenicity of the Ab4p BAC system.

The role of the ORF76 (U9) gene in vivo was assessed by intranasal inoculation of Ab4p attB, Ab4pΔORF76 (Us9) and Ab4pΔORF76R into CBA/N1 mice. Following infection of mice, our ORF 76 (Us9) deletion mutant is not neurovirulent and failed to invade the CNS indicated by absence of neurological signs and the normal body weight gain, together with no mortalities and normal histopathological finding of the bulb and brain. Intranasal inoculation of Ab4pΔORF76 (Us9) deletion and ORF 76 (Us9)-revertant mutant viruses revealed that the ORF 76 (Us9)-revertant mutant displayed the wild-type level of neurovirulence and neural spread in olfactory pathway. In contrast, the Ab4pΔORF76 (Us9) deletion mutant is virtually avirulent in similarly infected mice, and its spread within the CNS
was not detected by immunostaining. In the nasal mucosa the ORF 76 (Us9) deletion mutant replicated with equal efficiency when compared with the ORF 76 (Us9) revertant mutant of EHV-1. To spread via the olfactory pathway to the deeper tissues of the CNS, the virus must infect the cell bodies of first-order olfactory receptor neurons located within the olfactory epithelium and spread anterogradely to the second-order neurons in the bulb. Immunostaining data indicated that ORF 76 (Us9) deletion infects the olfactory receptor neurons (first-order neurons), but it is not transported to the bulb.

These findings indicate that EHV 1 ORF 76 (Us9) is necessary for interograde spread of EHV1. **Chowdhury et al. 2006** have proven that the alphaherpesvirus envelope protein Us9 is a type II viral membrane protein that is required for anterograde spread of bovine herpesvirus 5 (BHV-5) infection from the olfactory receptor neurons to the brain. **Brideau, et al. 2000a** explained that at least three viral envelope proteins, Us9, gE, and gI, function together to promote efficient anterograde transneuronal infection by PRV in the rat central nervous system.

Our result supports the suggestion of **Chowdhury et al. 2006** that Us9 has a universal role in alphaherpesvirus neuropathogenesis and/or anterograde neuronal transport.

Mice infected with ORF 76(Us9) deletion did not have viral DNA in the bulb or brain, indicating that the virus did not invade the bulb using PCR experiments for the presence of virus-specific nucleic acid.
The histopathological findings showed no lesion of the brains of the mice inoculated with Ab4pΔORF76 mutant, while that of bulbs and brains of the mice inoculated with Ab4p attB and revertant show degeneration and necrosis of the neurons, lymphocytic cell infiltration, perivascular cuffing, meningitis and gliosis. Moreover, the in vivo virus replication showed that the replication of ORF76 deletion mutant in the mice tissues was impaired; the virus titer in the lungs was lower than those of other viruses, indicating the ORF76 mutant had some effect on the virus replication in lungs.

In summary, the gene product of ORF 76 (Us9) plays important role in anterograde spread of EHV 1 from the first-order olfactory receptor neurons located within the olfactory epithelium to the second-order neurons in the bulb. The deletion of ORF 76 (Us9) resulted in smaller plaque size and different growth kinetics in ordinary tissue cultures and impaired growth rate in mouth neuron cells.

In addition, an EUS4 (ORF 71) (Marshall et al., 1997) and UL24 (ORF 37) (Kasem et al. 2010) deletion mutant generated from the pathogenic Ab4 strain of EHV-1 were attenuated in mice. Thus, virulence certainly is a multigene process, and several EHV-1 genes known to be dispensable for growth in cell culture contribute to the constellation of genes essential for virulence.

7. Summary

Based on Equine herpesvirus 1 (EHV-1) bacterial artificial chromosome clone (neuropathogenic strain Ab4p BAC) ORF76 encoding US9 was replaced with a selection cassette, rpsL-neo gene, to produce an
ORF76 deletion mutant (Ab4pΔORF76). Transfection of RK-13 cells with Ab4pΔORF76 genome DNA produced infectious viruses.

Deletion of ORF76 had no effect on the transcript expression of neighboring genes, ORF75 and ORF67.

EHV-1 ORF76 deletion mutant virus shows different growth kinetics in different ordinary cell cultures, for RK13 no differences were observed in the end-point virus titers between Ab4pΔORF76 and Ab4p attB and Ab4pΔORF76R viruses. While for MDBK intracellular Ab4pΔORF76 titer was one log lower at 24 hr but resume the same titer of other viruses at 48 hr. For FHK extracellular Virus titers were one log lower at 24hr and 36hr. While intracellular titers were three logs lower at 6hr and one log lower at12 hr and 24hr than Ab4p attB and Ab4pΔORF76 and resumed nearly the same titers at 48 hr of other viruses extra and intracellularly.

The deletion of EHV1 ORF76 had only a slight effect on viral growth in cell culture, indicating that the EHV1 ORF76 gene product is not essential for growth in ordinary tissue culture cells. However, ORF76 also had smaller plaque size and altered plaque morphology.

Following intranasal infection in a CBA/N1 mice model, the ORF76 revertant mutant of EHV-1 displayed a parental Ab4pattB level of neurovirulence and neural spread in the olfactory pathway, but the ORF76 deletion mutant of EHV-1 was virtually avirulent and failed to invade the CNS. In the olfactory mucosa containing the olfactory receptor neurons, the ORF76 deletion mutant virus replicated with efficiency similar to that of the
ORF76 revertant mutant of EHV-1. However, the Us9 deletion mutant virus was not transported to the olfactory bulbs.

The growth of Ab4pΔORF76 in cultivated neural cells was one order of magnitude lower than that of parental and revertant viruses. These results indicated that the ORF76 (Us9) is essential for the anterograde spread of the virus from the olfactory mucosa to the olfactory bulbs.

The EHV1 Us9 amino acid sequence predicts a protein of 219 amino acids and 22.287kDa. Using Western blot analysis with Us9 polyclonal antiserum and EHV1 RK 13-infected cell lysates, Us9 several polypeptides were noticed with relative molecular masses between 35 and 42 kDa. The EHV-1 Us9-specific bands were absent in RK13 cells mock and infected with the EHV-1 Us9-deleted mutant. The respective EHV-1 Us9-specific bands recognized by the Us9-specific antibody were of a higher molecular mass, suggesting that the respective Us9 proteins are processed posttranslationally to a higher-molecular-mass protein. The second methionine seems to be the major site of translation initiation.
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الملخص العربي

تم استبدال الجين رقم 76 لفيروس هربس الخيول رقم 1 (العزلة BAC) المصعوم بكتيرى قم في موقع بين الجين رقم 2 والجين رقم 3 للعزلة Ab4p مما يسهل التعامل مع جينات الفيروس و الموجودة داخل بكتيريا إي كولاوي Ab4pΔORF76 والذي أزيل للحصول على الطفرة Ab4pΔORF76R بحيث يسهل التعامل مع جينات الفيروس و موجود داخل بكتيريا إي كولاوي Ab4pΔORF76 وذلك لاعادة الطفرة الى اصلها الأول للحصول على الطفرة Ab4pΔORF76R بينما شائذ البلازميد باستخدام بلازميد BAC من البكتيريا إي كولاوي وتم إزالة البلازميد BAC من الفيروسات المطفرة وانفاتها على خلايا RK13

لم تؤثر إزالة الجين رقم 76 على الجينات المجاورة رقمي 75 و 67. أظهرت الطفرة Ab4pΔORF76 أنماط نمو مختلفة في خلايا الاستزراع النسيجي المختلفة مقارنة بفيروسات Ab4pΔORF76R و Ab4p Ab4pΔORF76R و Ab4p.

أدت إزالة الجين رقم 76 إلى تأثير قليل على نمو الفيروس في خلايا الاستزراع النسيجي. عند عدوى فئران التجربة CBA/N1 أدى العدوى بفيروس Ab4pΔORF76R و Ab4p إلى ظهور أعراض عصبية وانخفاض أوزان الفئران في حين لم تؤدى العدوى بفيروس المطفر Ab4pΔORF76 والتي أزيلت الجين رقم 76 من الفيروسات واستمرت اوزان الفئران المحقونة في الزيادة ولم تنتقل العدوى من الخلايا الطلائية الشمية الى العصب الشمي مما يؤكد على دور
This gene and the protein it produces are involved in the Herpes virus spreading from the epithelial cells to the nervous system via anterograde spread.

A reduced growth rate of Ab4pΔORF76 was observed in neural tissue cultures, which confirms the importance of ORF76 (Us9) in virus spreading within neurons.

According to the nitrogen metabolic rules, it was expected that the Us9 protein would have a mass of 22,287 kilodaltons. However, Western blotting results, which used immune bodies produced against Us9, showed that the protein size varied between 35 and 42 kilodaltons, indicating that Us9 protein is subject to some modifications after translation, which include phosphorylation.
تطبيقات التقنيات الجزيئية على العدوى بفيروس هربس الخيول

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