Replication of Equid Herpesvirus-1 (EHV-1) in the Testes and Epididymides of Ponies and Venereal Shedding of Infectious Virus


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Summary

Six Welsh Mountain pony colts were infected intranasally with the Ab4 isolate of EHV-1. Clinical and virological monitoring demonstrated mild upper respiratory tract disease, with nasal shedding of virus and establishment of a cell-associated viraemia. Detailed pathological examination of the urogenital tract was performed post mortem on days 4–9 post-infection (PI). EHV-1 was isolated from the epididymis on day 8 and the testis on day 9 PI, with viral replication in endothelial cells of these organs and an associated necrotizing vasculitis and thrombosis. Productive viral infection of germinal epithelium was not observed.

In a further study, three Welsh Mountain pony stallions were infected intranasally with Ab4, which again resulted in mild upper respiratory tract disease and the establishment of a cell-associated viraemia. Semen samples were collected up to day 60 PI. Two stallions showed a decrease in the proportion of morphologically normal sperm. Significant numbers of inflammatory cells were observed in the sperm-rich fraction of ejaculates collected from one stallion between days 16 and 28 PI; infectious virus was recovered from the semen of this animal between days 17 and 25 PI, after the cessation of viraemia. The affected stallion appeared clinically normal over the period of venereal EHV-1 shedding.

Introduction

Equid herpesvirus 1 (EHV-1) causes equine respiratory disease, abortion and occasional cases of paralysis (reviewed by Allen and Bryans, 1986). EHV-1 neurological disease has been observed in stallions and geldings (Saxegaard, 1966; Crowhurst et al., 1981; Meyer et al., 1987; McCartan et al., 1995). Field observations have shown that stallions may exhibit scrotal oedema and loss of libido, in addition to neurological disorders, during outbreaks of the paralytic form of EHV-1 infection on studs during the covering season (Greenwood and Simson, 1980; McCarten et al., 1995). However, because major outbreaks are rare, little is known about virus-specific effects on the reproductive tract of stallions, or whether venereal spread is of importance.

EHV-1 was shown to replicate in the vaginal tunics of colts if injected
directly into the cavum vaginale of the scrotum (Smith et al., 1993a), but the infection was self-limiting and did not extend to the deep genital organs. In this paper we describe the results of experimental infection of 2-year-old pony colts and mature pony stallions with EHV-1 by the respiratory route, to establish whether (1) the virus replicates in the genital tract, and (2) venereal shedding occurs and fertility is likely to be reduced.

**Materials and Methods**

*Experimental Infection and Sampling of Colts and Stallions*

Six 2-year-old Welsh Mountain pony colts were infected intranasally by direct inoculation with a high dose (10^5.5TCID_{50}/ml) of a paralytic isolate (Ab4) of EHV-1 (Edington et al., 1986). The method used has been described elsewhere for studies on the pathogenesis of abortion and neurological disease (Mumford et al., 1994). The animals were selected for challenge on the basis of low and stable complement-fixing and virus-neutralizing antibody titres to EHV-1 and EHV-4 (Thomson et al., 1976). In two of the colts, a disparity in testicular size was noted on pre-infection clinical examination, the left testis being smaller than the right.

Daily clinical and virological monitoring included the recording of rectal temperatures and collection of nasopharyngeal swabs and heparinized blood for virus isolation (VI). The colts were sedated and killed (by shooting) singly on each of days 4–9 PI (colts numbered 4–9 respectively). Samples of the penis and prepuce, testes, epididymides, accessory sex glands (bulbo-urethral glands, seminal vesicles and prostate), genital lymph nodes (superficial inguinal, sublumbar, external and internal iliac), scrotum, kidneys and bladder were collected into virus transport medium (VTM) for VI and the polymerase chain reaction (PCR), and into 10% neutral buffered formalin (NBF) for routine histopathology and immunoperoxidase staining (IP).

Three mature pony stallions (stallions 1, 2 and 3) were infected with EHV-1 Ab4 and clinically and virologically monitored in a manner identical with that used for the colts. In addition, whole ejaculates from stallions 1 and 3 and split ejaculates (sperm-rich and gel fractions separated) from stallions 2 and 3 were collected at intervals (see below) by means of a Cambridge artificial vagina (Sampford Veterinary Products, Saffron Walden, UK) and an ovarioctomized mare kept in oestrus by administration of oestradiol benzoate (7.5 mg every 3 days; Intervet, UK). To avoid cross-contamination with blood or nasal mucus, semen samples were collected before blood samples and nasopharyngeal swabs. Whole ejaculates were collected from stallion 1 on days 5–8, 14–18 and 30 PI. Split ejaculates were collected from stallion 2 daily on days 5–25 PI and less frequently (eight occasions) thereafter until day 60 PI. Split ejaculates were collected from stallion 3 daily from days 5–10 PI, and whole ejaculates less frequently (22 occasions) from days 28–60 PI. Semen was therefore collected on 10 days from stallion 1, on 29 days from stallion 2 and on 28 days from stallion 3.

**Detection of Virus in Tissues of Colts**

Tissues for VI were homogenized and resuspended to give 10% suspensions. After clarification by centrifugation (2000 g for 15 min), 200 μl of the clarified supernate were inoculated on to rabbit kidney (RK-13) monolayers in duplicate, and examined daily for a cytopathogenic effect (CPE), as previously described (Smith et al., 1992).

Viral DNA from tissue homogenates was amplified by the PCR, by a method previously described (Smith et al., 1992), with modifications. Primers 9/91 (GCA TGT TGA CTA ATC CTG GGT GGC) and 10/91 (TTC GGT AGA AGA CGG AGA CGG CTT CTT), binding to the gC gene homologue of EHV-1, were used to amplify DNA fragments. A 60-μl sample of the amplification product was subjected to
Electrophoresis in a 3% agarose gel run at 95 V, and the bands were "visualized" with ethidium bromide.

Formalin-fixed tissues were processed by routine methods to paraffin wax, sectioned at 6 μm and stained with haematoxylin and eosin (HE). Duplicate sections were attached to slides with poly-L-lysine for indirect immunoperoxidase staining (Whitwell et al., 1992), with a hyperimmune polyclonal rabbit antiserum raised against Ab4 grown in RK-13 cells. The serum was cross-adsorbed with equine embryonic lung cells, erythrocytes, peripheral blood mononuclear cells, fetal calf serum and RK-13 cells to remove irrelevant antibodies binding to tissue proteins.

Microscopical and Microbiological Evaluation of Semen

Immediately after collection, semen was assessed for total and progressive sperm motility by placing a drop on a pre-warmed glass slide for light microscopy. One millilitre of the ejaculate was fixed with an equal volume of formol citrate and stained with nigrosin and eosin for morphological examination of sperm, and with Giemsa for the identification of other cell types.

After microscopical assessment of morphology and motility, the remainder of the ejaculate was put on ice pending VI and PCR. When processed on the day of collection, a sample of the ejaculate was ultrasonically disintegrated (3 × 15 s) and centrifuged to remove germ cells and debris; the seminal plasma was then diluted 1 in 100 for inoculation on to RK-13 monolayers, this being the most concentrated dilution which was not itself cytotoxic (data not shown). Monolayers were examined daily for CPE as described for tissue VI. When VI was not performed on the day of collection, semen samples were stored at −70°C.

Semen samples for examination by the PCR were subjected to a proteinase K/phenol/chloroform DNA extraction. The PCR was performed, with 20 μl of the resuspended DNA extract, as described for tissue PCR. Re-amplifications of the PCR product were carried out on negative samples.

Semen samples from stallions 2 and 3 were cytocentrifuged, fixed in acetone and kept at −70°C. Immunofluorescent staining was performed as described previously (Smith et al., 1993a).

Results

Clinical Outcome of Infection in Colts and Stallions

All six colts developed a mild upper respiratory tract infection, with serous nasal discharge and nasal shedding of virus on days 2–4 PI. Pyrexia, anorexia and mild submandibular lymphomegaly were noted on days 1–3. In colt 5, transient gravitating oedema of the scrotum was observed on day 2. Viraemia was recorded in colts 4, 5, 7, 8 and 9 from day 3 PI until the animals were killed; colt 6 was viraemic on days 3 and 4. Ataxia was not observed.

All three stallions became pyrexic, shed virus from the nasopharynx for 3–6 days between days 1 and 7 PI, and were viraemic for 1 to 6 days between days 3 and 10 PI. Scrotal oedema was not observed. Stallion 1 exhibited mild hindlimb ataxia on days 9–13, which prevented semen collection over this period. In stallion 3, a sudden-onset head tilt caused by accidental trauma rendered semen collections impossible from days 10–28 PI.

Pathological and Microbiological Findings in Colts

The results of IP, VI and PCR on the tissues of the colts are summarized in Table 1.
Table 1
Detection of EHV-1 and associated vascular lesions in the genital tract of six pony colts on days 4 to 9 post-infection

<table>
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<tr>
<th>Technique</th>
<th>Results in colts killed singly on post-infection days</th>
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<td>Virus isolation</td>
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<td>Histopathology</td>
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<td>Immunoperoxidase staining</td>
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+, Positive result; -, negative result; NSAD, no significant abnormality detected; LN, lymph node; Ecs, endothelial cells.
No significant gross abnormalities of the urogenital tract were recognized in colts 4, 6 and 8 at necropsy. Mild oedema of the vaginal tunics was noted in colt 5. In colts 7 and 9, in which a disparity in testicular size had been noted before inoculation, the left testis was approximately half the size of the contralateral organ and soft and flabby in texture. Low-grade oedema of the superficial inguinal and caudal sublumbar lymph nodes was noted in colts 4, 6, 7 and 8. In all six colts, the accessory sex glands were small and immature.

No significant histological lesions were detected in the urogenital tract of colt 4; there were occasional small lymphocytic perivascular or interstitial aggregates, some associated with free spermatozoa (sperm granulomas) in the testis and epididymal body. IP staining for viral antigen proved positive in endothelial cells of the kidney.

In colt 5, scattered lymphohistiocytic perivascular cuffs or peritubular lymphoid nodules and sperm granulomas were observed in the testes and epididymides. Viral antigen was not demonstrable in association with these changes. There was mild oedema of the vaginal tunics, with occasional extravasated mononuclear cells, but local vascular lesions were not evident.

No significant histological lesions or foci of antigen expression were detected in colt 6.

In colt 7, endothelial-cell swelling in association with mild lymphohistiocytic vasculitis or perivascular cuffing was observed in the epididymides, bulbourethral glands and spermatic cords, but IP staining proved negative.

Viral antigen was demonstrated in endothelial cells of the epididymides in colt 8, with an associated segmental lymphohistiocytic vasculitis or marked perivascular cuffing and focal thrombosis (Fig. 1). Some small arterioles demonstrated fibrinoid mural necrosis (Fig. 2), and there was patchy mild interstitial oedema; areas of ischaemic necrosis were not identified. Occasional intra-epithelial lymphocytes were noted in the epididymal ducts adjacent to vascular lesions (Fig. 3). Patchy lymphohistiocytic vasculitis was noted in the testes, with associated endothelial-cell swelling (Fig. 4). There was mild lymphocytic perivascular inflammation, not associated with viral antigen expression, in the vaginal tunics of the scrotum.

Viral antigen was detected in endothelial cells of the testes, epididymides, kidneys and superficial inguinal lymph nodes of colt 9, in association with mild lymphohistiocytic vasculitis or perivascularitis. A microabscess containing neutrophils and degenerate sperm was noted in the right seminal vesicle, but this was not associated with antigen expression or local vascular lesions.

Spermatogenesis was proceeding in all six colts. Scattered groups of hypoplastic or dilated inactive tubules were found, predominantly in the small left testes of colts 7 and 9. Viral antigen was not detected in cells of the germinal epithelium or in the lumina of seminiferous tubules or epididymal ducts.

Infectious virus was not isolated from the urogenital tract of colts 4–7. EHV-1 was recovered from the epididymis of colt 8 and from the testis of colt 9 at the first passage (Table 1).

Viral DNA was not detected in the urogenital tract of colts 5–7. EHV-1 DNA sequences were amplified from the kidney of colt 4, from the epididymis
Fig. 1. Peroxidase-positive endothelial cells, some sloughed, in thrombosed interstitial arteriole of epididymis. Right caput epididymidis on day 8 post-infection (PI). Indirect immunoperoxidase. × 66.

Fig. 2. Acute fibrinoid necrosis of small epididymal arteriole. Right caput epididymidis on day 8 PI. Haematoxylin and eosin (HE). × 132.
Fig. 3. Perivascular lymphocytic infiltrate in epididymal interstitium. Note intra-epithelial lymphocytes in adjacent epididymal ducts. Right caput epididymidis on day 8 PI. HE. × 66, reproduced here at 80%.

Fig. 4. Endothelial-cell swelling and sub-intimal and adventitial accumulation of lymphocytes, consistent with early vasculitis, in small testicular artery. Left testis on day 8 PI. HE × 33, reproduced here at 80%.
Table 2
Detection of EHV-1 in nasal swabs, blood and semen during days 1–60 post-inoculation (PI)

<table>
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<tr>
<th>Stallion number</th>
<th>Period (days PI) during which EHV-1 was detected in nasal swabs</th>
<th>Period (days PI) during which EHV-1 was detected in blood</th>
<th>Period (days PI) during which EHV-1 was detected in semen</th>
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Leucocytes were present in the semen of stallions 1 and 2 on PI days 30 and 16–28 respectively. VI, virus isolation; PCR, polymerase chain reaction.

of colt 8, and from the testis, epididymis, kidney and external iliac lymph node of colt 9 (Table 1).

Effects of Infection on Semen

The duration of nasal shedding and cell-associated viraemia in relation to the detection of EHV-1 in the semen of the stallions is summarized in Table 2. Ejaculate volumes varied considerably (5–200 ml), but, allowing for seasonal variation, were consistent in each individual animal. No significant changes in sperm motility were observed. In stallions 1 and 3, a significant decrease in the proportion of morphologically normal sperm was observed. In stallion 1, the average proportion of normal sperm collected pre-infection and between days 5–8 was 68%, but between days 14 and 30 this fell to 44% as a result of abnormalities of the mid-piece and acrosome. In stallion 3, morphologically normal sperm fell from 64% (pre-infection) to 34% (days 8–10 PI), with subsequent fluctuations (22–45%) between days 29 and 48, associated with a large increase in tail and midpiece defects.

In stallion 2, sperm morphology did not show any variation up to day 28 PI, but 5–8% of sperm consisted of immature forms thereafter.

Lymphocytes were observed in the ejaculate collected on day 30 from stallion 1. Neutrophils, plasma cells and lymphocytes were present in the sperm-rich fraction of ejaculates collected from stallion 2 on days 16, 17, 18, 22, 24 and 28 PI. These cells were not observed in the gel fraction. Leucocytes were not observed in any ejaculate from stallion 3.

Infectious virus was isolated from the semen of stallion 2 on days 17, 19, 23, 24 and 25 PI. All other semen samples from this animal and stallions 1 and 3 were virologically negative.

EHV-1 DNA sequences were amplified from semen samples from stallion 2 collected on days 10, 15, 22, 23, 24 and 25. All other semen samples from this animal and stallions 1 and 3 were negative.

All semen cytocentrifugates from stallions 2 and 3 were negative for EHV-1 antigen on immunofluorescence.
**EHV-1 Infections in Colts and Stallions**

**Discussion**

These results demonstrate that after primary infection of the respiratory tract, EHV-1 can specifically replicate in the testes and epididymides and be shed in the semen. Infectious virus was recovered from the epididymis on day 8 PI and the testis on day 9 in two colts, and was shed in semen samples over days 17–25 PI in one stallion. Immunolabelling identified viral antigen in endothelial cells of the infected genital organs in association with a lymphocytic or necrotizing vasculitis and focal microthrombosis.

The endotheliotropism of certain isolates of EHV-1 is well documented (Patel et al., 1982), and associated thrombo-ischaemic necrosis in the central nervous system and pregnant uterus is essential to the pathogenesis of paralytic and abortigenic disease (Edington et al., 1986, 1991; Smith et al., 1992, 1993b). The finding of necrotizing vasculitis and thrombosis at sites of endothelial cell infection in the testes and epididymides of colts in the present study suggests that subsequent venereal shedding in stallions may be facilitated by focal tissue ischaemia and leakage of free virus or infected cells across the disrupted blood–semen barrier. These herpetic vascular lesions, which were associated with EHV-1 antigen expression, are distinct from the interstitial or perivascular lymphocytic foci common in the equine testis in areas of tubular degeneration (Ladds, 1993), which were noted as an incidental finding. Deep genital thrombosis also presents a possible mechanism for the scrotal oedema sometimes observed in EHV-1-infected male horses, particularly if the vascular disease is exacerbated in the older working animal. The absence of clinically detectable oedema in the EHV-1-shedding stallion in this project indicates that this change is not a consistent marker of productive genital infection.

It is noteworthy that, despite acute vascular damage in the testes and epididymides of colts on days 8 and 9 PI, inflammatory cells and infectious virus were not detected in the semen until days 16 and 17 respectively, although viral DNA sequences were detected as early as day 10. This delay in shedding may be accounted for by the passive movement and storage of maturing sperm in the epididymis for 6–14 days before ejaculation (Amann, 1981a,b), and further supports the hypothesis that infection of the semen occurs in the testes and epididymides, rather than in the accessory glands or epithelial surfaces of the lower genital tract. Failure to demonstrate viral antigen expression in cytocentrifugates of infected semen may reflect either the relative insensitivity of the immunofluorescence method, or a small number of productively infected cells, or the shedding of latently infected cells (Edington, 1992). Small numbers of intra-epithelial lymphocytes and macrophages occur in the epididymal ducts of normal animals (Wrobel and Dellmann, 1993), and were noted in the EHV-1 infected colts; these leucocytes may assist in the transfer of virus across the blood–semen barrier. More sensitive methods such as in-situ hybridization for latency-associated mRNA transcripts (Edington, 1992) or the novel in-situ PCR assay (Chiu et al., 1992) are needed to screen such cells for latent EHV-1 infection.

The parallel use of VI, IP and PCR on a large number of tissue samples in this study permitted the sensitivity of the three techniques to be compared...
and demonstrated a good concordance between IP and PCR, which were more sensitive than VI in detecting small foci of productive cellular infection. A recent survey of EHV-1 abortions corroborated these findings (Rimstad and Evensen, 1993). PCR assays suitable for the detection of tissues latently infected with EHV-1, as reported by Welch et al. (1992) and Borchers and Slater (1993), were based on either re-amplification of DNA extracts or the use of nested primer sets. The use of such assays in our acutely infected colts would have presented a potential problem in interpretation, owing to probable contamination of tissue samples by viraemic blood. The absence of a simple genital surface infection to account for venereal EHV-1 shedding in this study constitutes a difference from the venereal diseases of man and animals caused by herpes simplex virus, bovine herpesvirus-1, caprine herpesvirus, canine herpesvirus and equine herpesvirus-3 (Poste and King, 1971; Pascoe and Bagust, 1975; Gibbs and Rweyemamu, 1977; Taussig, 1984; Tarigan et al., 1990). In these diseases, infection of the epithelial surfaces of the penis and prepuce results in mechanical transfer of virus to the female genital tract through abrasions produced during coitus. The role of venereal shedding of pseudorabies virus in boars is less well understood, owing to the difficulty of inducing infection of the genital tract by a variety of respiratory and local inoculation routes in this species (Hall et al., 1984; Miry et al., 1987; Miry and Pensaert, 1989).

The high affinity of EHV-1 for the pregnant uterus (Edington et al., 1991; Smith et al., 1993b), and our current findings of viral replication in the male genital organs suggest a potential role for hormones or other micro-environmental factors in determining these sites of secondary replication. There are precedents for an endocrine effect on herpesvirus replication: murine cytomegalovirus replication is increased by exposure to oestrogen, progesterone and corticosteroid (Chong and Mims, 1984), and enhanced replication of herpes simplex virus occurs in the presence of dexamethasone in cells bearing appropriate receptors (Dreyer et al., 1989).

In addition to venereal shedding of EHV-1, the current study has confirmed the non-specific effects of pyrexia on semen quality in stallions, previously documented by Friedman et al. (1991). These authors demonstrated decreases in the number of morphologically normal and progressively motile sperm in pyrexic stallions due to disruption of temperature-sensitive spermatogenesis. Although no significant changes in sperm motility were observed in our study, a marked fall in the number of morphologically normal sperm was observed in the absence of detectable venereal shedding in two of the EHV-1-infected stallions, and immature sperm appeared in samples collected after the cessation of shedding in the third animal. Changes in sperm morphology have also been ascribed to pyrexia in pseudorabies virus-infected boars (Larsen et al., 1980; Hall et al., 1984).

Potential venereal shedding of EHV-1 is an important consideration in the management and epidemiology of outbreaks. Discretionary guidelines on the cessation of covering once paralytic or abortigenic EHV-1 infection has been diagnosed on a thoroughbred stud are in operation in the United Kingdom (Horserace Betting Levy Board, 1994), and these may have to be strengthened.
in view of our current findings, particularly as the pony in which infectious virus could be detected in semen samples appeared clinically normal over the period of venereal shedding. Clearly, further experimental and field data on venereal EHV-1 shedding are needed to assess the practical implications of this work, especially if artificial insemination regimes become more widely used in equine breeding programmes (Philpott, 1993).

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