Equine herpesvirus 4 DNA in trigeminal ganglia of naturally infected horses detected by direct in situ PCR

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Neuronal and lymphoid tissues of 15 randomly selected horses were analysed post mortem by liquid nested-PCR to study the tropism of equine herpesvirus 4 (EHV-4). In four animals the trigeminal ganglia and in one case the lung were positive. Using a direct in situ PCR the EHV-4 genome was localized in the nuclei of neurons and in the bronchiolar as well as alveolar epithelium of the lung. In none of these tissues could infectious virus or viral antigens be detected. Applying the more sensitive liquid RT–PCR, however, an acute infection was demonstrated in one of the trigeminal ganglia by amplification of viral transcripts coding for glycoprotein B. The failure to detect these transcripts in the other trigeminal ganglia and the lung indicates a latent infection. This report formally proves that, like other members of the Alpha-herpesvirinae, EHV-4 establishes latency in the trigeminal ganglia.

Equine herpesviruses 1 and 4 (EHV-1 and -4) are important and widespread pathogens in the horse population. EHV-1 infections result in respiratory disease, abortion and, more rarely, paresis (Allen & Bryans, 1986). EHV-4 is mainly, but not exclusively, involved in respiratory tract infections (Studdert, 1974).

Although EHV-1 and -4 are members of the Alpha-herpesvirinae, they have been regarded as being latent not in the classic neuronal sites but in lymphoid tissues and peripheral blood leukocytes (PBL). Therefore, they would represent an exception to the rule (Edington, 1991). The lymphotropism of EHV-1 seemed to be in good accordance with the occurrence of viraemia (Dutta & Campbell, 1977; Slater et al., 1994a), which was believed to be a central step in the development of abortion and neurological dysfunction. The fact that EHV-1 may cause neurological disease in horses was explained by ischaemic injuries to nervous tissue following endothelial infection and thrombosis without direct infection of neurons (Patel et al., 1982; Edington et al., 1986). This theory, however, neglects the fact that EHV-1 has been found by PCR in the trigeminal ganglia of 1/5 experimentally infected horses (Welch et al., 1992). Recently, we also demonstrated latent EHV-1 in trigeminal ganglia of experimentally infected specific pathogen-free (SPF) foals by applying a nested PCR and the cocultivation technique (Slater et al., 1994b).

Regarding the tropism of EHV-4, no reliable data are available. On the one hand, EHV-4 has been found in a latent form in lymphoid tissues draining the respiratory tract and in PBL (Welch et al., 1992; Edington et al., 1994). In contrast to EHV-1, however, EHV-4 infections only seldom lead to viraemia (Matsumura et al., 1992). On the other hand, EHV-4 has also been suggested to be a neurotropic virus, based on single previous reports (Allen & Bryans, 1986; Edington et al., 1994) where EHV-4 was recovered from trigeminal ganglia by cocultivation or was detected by PCR, respectively. Moreover, EHV-4 has been found in association with neurological diseases in cerebrospinal fluid and brain tissues (Meyer et al., 1987; Rösch et al., 1992; Thein et al., 1993).

In this study, we have used a liquid nested-PCR to analyse the tissue distribution of EHV-4. Fresh specimens (cerebrum, cerebellum, bulbus olfactorius, medulla oblongata, trigeminal ganglion, spinal cord, ischiadic nerve, optic nerve, lung, liver, spleen, bronchial lymph nodes, tonsils) of 15 randomly selected horses, which died of different diseases (Table 1), were collected post mortem. Tissue DNA was extracted and analysed by the EHV-1 and the EHV-4 gB-specific nested-PCR (Borchers & Slater, 1993). EHV-4 DNA was detected in trigeminal ganglia of four horses (27%) and once in the lung (data not presented), but not in other tissues, indicating that the EHV-4 strains detected here are non-lymphotropic. None of the tissues contained EHV-1 DNA (data not presented).

In order to localize EHV-4 DNA in paraffin embedded tissues a direct in situ PCR was established. The direct in situ PCR was chosen because it is much faster than the indirect one, which includes a hybridization step (Duealand et al., 1995; Mehta et al., 1995). Furthermore, we used digoxigenin labelled
primers instead of incorporation of dUTP (Gressens & Martin, 1994) to circumvent polymerase repair mechanisms, which are known to produce background staining (O’Leary et al., 1996).

Organ samples were fixed in 4–10% buffered formalin, embedded in paraffin wax and cut into 3–4 µm sections, mounted on silane-coated slides (Sigma) and dried overnight at 37 °C. Paraffin was removed by incubation in fresh xylene for 10 min, followed by washings in 100% ethanol and PBS. Tissue sections were then digested with proteinase K (50 µg/ml). After incubation in sterile distilled water and subsequently in 100% ethanol, the air dried slides were placed on an 85 °C heat block (MWG thermocycler) for 5 min. The PCR mix (100 µl) consisted of 50 pmol of each primer, 8 mM MgCl₂, 200 mM of each NTP, 1 × PCR buffer II (In situ PCR Core Kit, Applied Biosystems), 0.1% BSA (Bio Labs), 10% glycerol (Sigma), 100 ng salmon sperm DNA. After 2 min at 85 °C 20 U of IS Taq polymerase (In situ PCR Core Kit, Applied Biosystems) was added. We used the 20-mer inner primer pairs of the solid phase EHV-4 gB-specific nested-PCR (Borchers & Slater, 1993), which were 5’ end labelled with digoxigenin. After the preheated PCR mix had been added, the sections were covered with mineral oil (Sigma). The PCR reaction consisted of an initial step of 1 min incubation at 94 °C, 15 cycles of 94 °C, 67 °C, 72 °C, each for 40 s, and a final step of 5 min at 72 °C. After cycling, the slides were cooled and kept at 4 °C for 10 min. The mineral oil was removed and a 5 min hot wash (45 °C) in 1 × SSC–0.2% BSA followed. Sections were then blocked in PBS–6% BSA for 30 min at room temperature. Colorimetric detection of the PCR products with alkaline phosphatase-conjugated antidigoxigenin antibody (Boehringer Mannheim) was performed according to the manufacturer’s protocol. Reactions were stopped after 1–5 min when a signal was evident on a microscopically viewed control. Finally, sections were mounted with glycerol–Moviol and covered with a cover-slip.

Several types of control were included in these experiments. Sections of paraffin embedded EHV-1 infected and non-infected mouse lungs as well as horse tissues tested negative by liquid nested EHV-4 PCR served as negative controls. None of the negative controls gave positive results, proving the specificity of the system.

Applying this technique to paraffin embedded horse trigeminal ganglia tested positive by liquid-nested PCR, EHV-4 DNA could be clearly localized in the nuclei of neurons. To

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**Table 1. Virus recovery in tissues of EHV-infected horses**

<table>
<thead>
<tr>
<th>Animal data</th>
<th>Clinical and pathological findings</th>
<th>Tissue specimen</th>
<th>Method of virus detection</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>G 7</td>
<td>Colic; follicular hyperplasia of larynx</td>
<td>Trigeminal ganglion</td>
<td>nPCR</td>
<td>Positive EHV-4</td>
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<tr>
<td>Frisian, male, 2 years</td>
<td></td>
<td></td>
<td>in situ PCR</td>
<td>Positive EHV-4</td>
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<td></td>
<td></td>
<td></td>
<td>ABC</td>
<td>Negative EHV-1/4</td>
</tr>
<tr>
<td>G 8</td>
<td>Chronic obstructive bronchitis (COPD)</td>
<td>Trigeminal ganglion</td>
<td>nPCR</td>
<td>Positive EHV-4</td>
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<tr>
<td>Pony, female, 17 years</td>
<td></td>
<td></td>
<td>in situ PCR</td>
<td>Positive EHV-4</td>
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<td></td>
<td></td>
<td></td>
<td>ABC</td>
<td>Negative EHV-1/4</td>
</tr>
<tr>
<td>G 12</td>
<td>Fracture of phalanx proximalis; acute bronchitis and interstitial pneumonia; cardiovascular collapse</td>
<td>Lung</td>
<td>nPCR</td>
<td>Positive EHV-4</td>
</tr>
<tr>
<td>Trotter, male, 7 years</td>
<td></td>
<td></td>
<td>in situ PCR</td>
<td>Positive EHV-4</td>
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<td></td>
<td></td>
<td></td>
<td>ABC</td>
<td>Negative EHV-1/4</td>
</tr>
<tr>
<td>G 15</td>
<td>Hindlimb ataxia; paralysis of bladder and rectum; serologically positive for EHV-1 and EHV-4; necrosis of neurons in cerebellum and medulla oblongata; interstitial pneumonia and bronchitis</td>
<td>Trigeminal ganglion</td>
<td>nPCR</td>
<td>Positive EHV-4</td>
</tr>
<tr>
<td>Thoroughbred, castrated male, 6 years</td>
<td></td>
<td></td>
<td>in situ PCR</td>
<td>Positive EHV-4</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>ABC</td>
<td>Negative EHV-1/4</td>
</tr>
<tr>
<td>G 17</td>
<td>Chronic obstructive bronchopneumonia (COPD), rhinitis chronic, laryngitis follicularis</td>
<td>Trigeminal ganglion</td>
<td>nPCR</td>
<td>Positive EHV-4</td>
</tr>
<tr>
<td>Haflinger, castrated male, 23 years</td>
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<td>in situ PCR</td>
<td>Positive EHV-4</td>
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<td></td>
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<td>ABC</td>
<td>Negative EHV-1/4</td>
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</table>

**Fig. 1.** Detection of EHV-4 DNA by direct *in situ* PCR in paraffin embedded trigeminal ganglia. (a) Serial section showing positively (arrow) and negatively (arrowhead) stained neuronal nuclei of trigeminal ganglia of animal G 7 (× 6.3). (b) One of the 27 cells containing EHV-4 genome (× 40). (c) EHV-4 positive (arrow) and negative (arrowhead) neuronal nuclei in the trigeminal ganglia of animal G 17 (× 6.3). (d) One of the 32 cells showing positive staining (× 40). (e) In the trigeminal ganglion of a horse tested negative by solid nested EHV-4 PCR no labelled structures were seen (× 6.3). (f) One of the cells showing no staining (× 40).
Fig. 1. For legend see facing page.
estimate how many neurons were labelled, we examined five to ten serial sections of each ganglion, each section of which contained about 20–200 neurons with visible nuclei. In the case of ganglion G 7 48% of neurons were positive; for G 8, G 15 and G 17 about 65% were positive (Fig. 1a, b, c, d). In the lung tissues of animal G 12, the bronchial as well as the alveolar epithelial cells contained EHV-4 DNA (Fig. 2a, b).

Trigeminal ganglia and lung sections of horses tested negative by liquid nested EHV-4 PCR gave no signals (Fig. 1c, d). To examine whether the detected viral DNA derived from an acute or latent infection, the following methods were applied. Pieces of lung from animal G 12 were minced with scissors, homogenized and titrated on equine dermal (ED) cells as described elsewhere (Slater et al., 1994b). The cell cultures were maintained for 7 days at 37 °C and then repassed three times at weekly intervals. Because of the limited amount of tissue material, none of the trigeminal ganglia could be analysed for infectious virus.

The avidin–biotin complex (ABC) method was applied to lung and trigeminal ganglia sections to detect viral antigen. After unmasking of antigen by incubation with a 0.1% pronase solution type XXV (Pronase E, Sigma), anti-EHV rabbit hyperimmune serum was used as primary antibody. For antibody detection the Vectastain ABC-Kit ‘Elite’ (Cämön, Wiesbaden, Germany) and for colour development the Histoprime Kit (Cämön) were used according to the manufacturer’s recommendations.

In order to detect viral transcripts, RNA was extracted from lung and trigeminal ganglia samples using TRIzol reagent (Life Technologies) according to the manufacturer’s instructions. Isolated RNA was further treated with DNase I (Boehringer Mannheim) at 37 °C for 20 min to digest any possible contaminating viral DNA. The enzyme was removed by applying the sample to an RNeasy spin column (RNeasy Kit, Qiagen). Expand Reverse Transcriptase (Boehringer Mannheim) and random-hexamer-primer with oligo(dT)20 at the 5’ end were used for cDNA synthesis from 2 μg RNA. The resulting cDNA was subjected to the EHV-4 specific gB PCR (Borchers & Slater, 1993). Expression of β-actin mRNAs in all tissue samples was used as a control of RNA quality.

Neither in the lung nor in the trigeminal ganglia could infectious virus or viral antigen, respectively, be detected. Using the more sensitive RT–PCR viral transcripts specific for glycoprotein B could be amplified in the ganglion of animal G 8 only.

Since all five horses, that were positive by PCR had respiratory tract diseases (rhinitis, follicular laryngitis, bronchitis or pneumonia) we assumed an acute EHV-4 infection. In the case of horse G 12 which suffered from an acute bronchitis and pneumonia, we particularly expected to isolate infectious virus, detect viral antigen or at least viral transcripts from the lung, but failed in our attempts. Therefore, we conclude that the lung is latently infected and the preceding acute respiratory disease of animal G 12 was caused by another infectious agent.

The fact that a latent infection by EHV-4 can be established in the lung supports earlier observations (Welch et al., 1992; Edington et al., 1994).

Furthermore, we presumed a relationship between the acute neurological disease of horse G 15 and the detection of EHV-4 DNA in its trigeminal ganglion. The lack of viral envelope antigen and transcripts coding for gB, however, pointed to a latent infection.

In horses G 7, G 8 and G 17, the detection of EHV-4 DNA by liquid nested-PCR and in situ PCR in trigeminal ganglia might be explained by a linkage to subacute or chronic changes of the respiratory tract. As viral infections are known to precede chronic obstructive pulmonary disease (COPD) (Robinson et al., 1996), the presence of EHV-4 DNA suggests an early aetiological participation and therefore a latent infection. In animals G 7 and G 17 the absence of viral antigen and viral transcripts supported this prediction. In contrast, the detection of viral mRNA in the ganglion of animal G 8 indicated an acute infection or reactivation.

Sections of trigeminal ganglia from specific pathogen-free (SPF) ponies which were experimentally infected with EHV-1 were recently analysed by in situ hybridization using a HSV-1 equivalent LAT probe (Baxi et al., 1995). Of 30 000 neurons six (0.02%) were positive for EHV-1 LAT. In the trigeminal ganglia analysed in our experiments by direct in situ PCR about 50–70% of neurons were EHV-4 positive. This relatively high number of positive cells might be due to the higher sensitivity of the in situ PCR compared to the in situ hybridization. As described by others, in situ PCR gave percentages of cells positive for latent HSV that were about ten times higher than indicated by in situ hybridization (Ramakrishnan et al., 1994; Mehta et al., 1995). Additionally, not all of the neurons containing latent EHV-1 DNA might express LATs. Thus, the number of neurons positive for genomic DNA might be much higher than for LATs. Furthermore, under natural conditions many more neurons might become latently infected because of repeated recurrences compared with animal models treated experimentally.

In summary, liquid and in situ PCR results clearly demonstrate that EHV-4 is a neurotropic herpesvirus and that its genome can be found in trigeminal ganglia – exclusively in the nuclei. These findings complement those for other members of the Alphaherpesvirinae and furthermore suggest that this is a site for EHV-4 latency. Moreover, our studies provide evidence that the lung is an additional tissue where EHV-4 can become latent, in the epithelial cells. Studies are in progress to detect latency associated transcripts and establish an in situ site for EHV-4 latency. Moreover, our studies provide evidence that the lung is an additional tissue where EHV-4 can become latent, in the epithelial cells. Studies are in progress to detect latency associated transcripts and establish an in situ site for EHV-4 latency.
Fig. 2. Detection of EHV-4 DNA by direct in situ PCR in the paraffin embedded lung tissue of animal G 12. (a) Positively stained bronchial epithelial cells are indicated by an arrowhead and alveolar epithelial cells by arrows (× 6·3). (b) EHV-4 genome containing bronchus (× 40). (c) In the lung of a horse tested negative by solid nested EHV-4 PCR, no labelled structures were seen (× 6·3). (d) Bronchus showing negative staining (× 40).
References


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