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Variation in Cellular Tropism Between Isolates of Equine Herpesvirus-1 in Foals

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With 5 Figures

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Summary

Subtype-1 isolates of Equine herpesvirus-1 (EHV-1) from a quadriplegic horse and from an aborted foetus were compared with each other and with a subtype-2 respiratory isolate. All 3 isolates were detected in the epithelium and macrophages of the respiratory tract. Both the paresis and foetal subtype-1 isolates replicated in the epithelium of the ileum and this correlated with the recovery of virus from faeces *in vivo*. The paresis subtype-1 isolate also had a predelection for vascular endothelial cells, particularly in the nasal mucosa, but also in the lungs, central nervous system, adrenal and thyroid.

In the 9 foals inoculated with the paresis isolate two developed hind limb dysfunction, four developed diarrhoea, and one of these 4 died with an intussusception.

The differences between these isolates are discussed in relation to other herpesviruses.

Introduction

Infection with Equine herpesvirus-1 (EHV-1) has produced abortion (10), rhinopneumonitis (3), and occasionally paresis (16). The subtype-2 virus of EHV-1 has been associated only with respiratory disease and grows *in vitro* in a narrower range of monolayers than the subtype-1 isolates (1, 2) which have been recovered from the upper respiratory tract, buffy coat, aborted foetuses, and the central nervous system of horses with locomotor dysfunction.

Paresis had not been unequivocally associated with EHV-1 infection in the U.K. until an outbreak in 1979 (7, 11), although subtype-1 virus had been recovered from aborted foetuses in previous years (2). The emergence of the

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paresis syndrome led us to ask whether differences existed between subtype-1 isolates of EHV-1. The paper records part of our investigation into this problem.

Materials and Methods

Experimental Design

Fourteen conventional foals, weaned at 1 week of age, and one gnotobiotic foal were infected when 1—2 weeks of age with one of three isolates (see below and Tables 1 and 2) giving 2.0 ml of inoculum (4.25 TCD_{50}/ml) intranasally. Leucocytes and nasal, conjunctival, and rectal swabs were taken daily while serum samples were collected every 3 days. At least two foals infected with each isolate were autopsied 8 days after inoculation (d.p.i.), and with both subtype-1 isolates also at 4 d.p.i. In addition 2 inoculated with the paresis isolate were monitored over 8 weeks (Table 1). At post-mortem examination tissues were taken for virus isolation on monolayers (see below) for the detection of antigen by IIF (see below), and for routine histology. They included nasal mucosa, trachea, lung, tonsil, thymus, spleen, liver, kidney, duodenum, jejunum, ileum, salivary gland, thyroid, adrenal, conjunctiva, cornea, lachrymal gland, and the submandibular, retropharyngeal, bronchial and mesenteric lymph nodes, as well as cervical, thoracic and lumbar spinal cord, and samples of cerebral cortex and the cerebellar folia. Extensive sections were examined by immunofluorescence and light microscopy from the brain.

Cell Cultures

Equine embryonic kidney (EK) (18) and rabbit kidney (RK-13) cells were grown in Eagle's minimal essential medium (MEM) supplemented with 5 per cent v/v foetal calf serum (FCS), 15 mM bicarbonate and antibiotics. EK cells were used within 4 to 10 passages since their sensitivity to EHV-1 decreased in cells of higher passage. Tubes were seeded with 2.5×10^5 cells in one ml of medium and inoculated as monolayers 16 hours after seeding. After inoculation MEM maintenance medium containing 2 per cent FCS was changed every 3 or 4 days over a period of 10 days.

Viruses

The subtype-1 foetal isolate was supplied by R. Burrows, Pirbright, while the paresis isolate was from a paralyzed mare (11) and the subtype-2 from a nasal swab collected in an outbreak of respiratory disease.

Subtyping was established by growth or lack of growth in EK, equine dermal (ED), calf testis (BT), and rabbit kidney (RK-13) monolayers (2), and by neutralization using the two antisera described below. Each isolate was plaque cloned 3 times in RK-13 or EK cells, and a stock grown in EK cells. To confirm subtyping aliquots of the stocks were titrated in EK and RK-13 cells prior to animal inoculation.

Titration of Infectious Virus

Tissues stored at —70° C were homogenized to obtain 10 per cent (w/v) suspensions and clarified at 250 g for 10 minutes. Leucocytes, collected in heparin, were washed 3 times with phosphate buffered saline (PBS), and resuspended at a concentration of 1×10^7/ml.

Serial ten-fold dilutions were made of the virus stocks, tissue samples and leucocyte suspensions. These were absorbed for 2 hours on to confluent monolayers of RK-13 or EK cells in tubes (subtype-1 isolates or subtype-2 isolates respectively). Subsequently cultures were examined daily over 7 days for CPE and the median infective dose ($TCID_{50}$) calculated by the SPEARMAN-KÄRBER-method (4).

Infected Cells in Tissues (IIF Test)

Frozens 8 μm sections of tissues were fixed in acetone at room temperature for 10 minutes, and air dried. A rabbit antiserum to EHV-1 subtype-1 (13) was added for 1 hour

at 37° C. A sheep antiserum to rabbit immunoglobulin G (IgG) which had been conjugated to fluorescein isothiocyanate (FITC) (13) was then incubated on sections for 30 minutes at 37° C and the preparations were counterstained with 0.1 per cent Evans blue in PBS. Sections were rinsed after each stage with PBS. Preparations were examined using a Vicker's incident light (M41 Photoplan) microscope fitted with a 200 watt mercury vapour lamp, and with BG12, BG38 and OG.9 and OG.4 barrier filters. Controls were (a) tissues from uninfected foals tested as above, (b) tissues from infected foals tested with a preimmune rabbit serum. Infected cell controls were (a) sections of brains from EHV-1 infected mice (13) and (b) EK monolayers infected with EHV-1 (18). At least 400 sections taken at 200 μm intervals from cervical, thoracic and lumbar spinal cord and from cerebrum and cerebellum were examined for virus infected cells but only 30 to 50 sections of other tissues were tested.

Virus Antisera

The preparation of hyperimmune rabbit serum to the subtype-1 paresis isolate, P1, has been described previously (13). A convalescent antiserum from a gnotobiotic foal infected with a subtype-2 isolate (18) was also used.

Complement Fixation (CF) Test

Foal sera were tested for antibody to EHV-1 using the method of Bradstreet and Taylor, adapted by THOMSON *et al.* (18).

Virus Neutralizing Antibody (VN) Test

A quantal assay using EK cell monolayers in 96-well "Multi dish" (Linbro Scientific Co. Inc., Hamden, Conn.) was used (18). Hyperimmune rabbit serum to the paresis isolate and a gnotobiotic foal serum were used as positive standards to subtypes-1 and -2 of EHV-1 respectively. The standard antisera and test sera were inactivated at 60° C for 30 minutes before use.

Results

Clinical Symptoms

Two of the nine foals inoculated with the paresis isolate developed abnormal gaits involving the right hind leg at 8 and 10 d.p.i. respectively; another died from an intussusception at 7 d.p.i. and three others developed diarrhoea. Eight of the 9 had temperatures >103.0° F for at least 24 hours and 5 had a bilateral mucopurulent nasal discharge and conjunctivitis. Symptoms associated with the foetal and respiratory isolates were restricted to a rhinitis and conjunctivitis, but without encrustations, and while pyrexia occurred in 3/4 of the foals given the foetal isolate none occurred following inoculation of the respiratory isolate. Clinical symptoms, beginning at about 4 d.p.i. progressed until 8 d.p.i. in animals infected with the paresis isolate, but had resolved in the other two groups by this time.

Virus Excretion

The recovery of virus from swabs is summarized in Table 1. Virus was isolated from nasal swabs in each group of animals but was only isolated from the buffy coat of foals infected with the foetal and paresis subtype-1 isolates. In addition faecal swabs yielded virus at 6 and 7 d.p.i. in 2 foals given the paresis isolate and at 6 d.p.i. in one foal given the foetal isolate. Conjunctival shedding of virus was

Table 1. *A comparison of the recovery of 3 isolates of EHV-1 from samples taken from foals infected at 1—2 weeks of age*

Isolate of EHV-1	Route	Duration in days 10 20 30	No. of foals: Virus isolation	No. of foals: t>103.0°F
Subtype-1 "Paresis"	Nasal	[a]	7/9	
	Buffy coat	[a]	9/9	8/9
	Faecal		2/9	
	Ocular		2/9	
Subtype-1 "Foetal"	Nasal		3/4	
	Buffy coat		4/4	3/4
	Faecal		1/4	
	Ocular		0/4	
Subtype-2 "Respiratory"	Nasal [b]		2/2	
	Buffy coat		0/2	0/2
	Faecal		0/2	
	Ocular [b]		1/2	

| Day of post-mortem examination
[a] Death following infection with EHV-1
[b] Gnotobiotic foal

detected over a similar period in 2 foals with the paresis isolate and 1 gnotobiotic foal infected with the respiratory isolate. In the two foals inoculated with the paresis isolate and kept for observations virus was detected in nasal swabs up to 21 and 10 d.p.i. respectively, while viraemia was observed up to 10 and 8 d.p.i.

Detection of Virus in Tissues

Virus Isolation

The results are summarized in Table 2, in which the number of tissues is restricted to those from which isolations were made (cf. Materials and Methods). Whilst with all three isolates virus was recovered from the upper respiratory tract including the submandibular and retropharyngeal lymph nodes, only foals infected with the paresis and foetal isolates showed evidence of virus in the thymus and small intestine. The titre of virus in the ileum was higher than in any other tissue, reaching $10^{6.0}$ $TCID_{50}$/gm in one foal. In other tissues virus ranged from $10^{2-4.7}$ $TCID_{50}$/gm (Table 2). Only in animals receiving the paresis isolate was virus recovered from the central nervous system. While 9 of the 11 foals given the paresis or foetal isolate were viraemic at necropsy the majority of tissues, including such vascular organs as spleen and liver were negative for virus.

Table 2. *Recovery of the isolates of EHV-1 from infected foals at post-mortem examination*

		Isolate of EHV-1 Subtype-1								Subtype-2	
		Paresis				Foetal				Respiratory	
		4 d.p.i.		8 d.p.i.		4 d.p.i.		8 d.p.i.		8 d.p.i.	
System	Tissue	N	V	N	V	N	V	N	V	N	V
Respiratory	Nasal mucosa	3/3	$10^{2.7}$	3/4	$10^{2.0}$	1/2	$10^{2.0}$	2/2	$10^{3.0}$	2/2	$10^{3.2}$
	SMLN	1/3	$10^{2.0}$	2/4	$10^{2.0}$	1/2	$10^{2.0}$	1/2	$10^{2.0}$	1/2[a]	$10^{3.0}$
	RPLN	—	—	2/4	$10^{2.0}$	1/2	$10^{2.0}$	—	—	1/2	$10^{2.0}$
	Trachea	—	—	—	—	—	—	1/2	$10^{2.0}$	1/2	$10^{2.0}$
	Lung	2/2	$10^{3.0}$	1/4	$10^{3.0}$	—	—	1/2	$10^{2.0}$	1/2	$10^{4.0}$
	Conjunctiva	ND	ND	—	—	—	—	1/2	$10^{3.0}$	1/2	$10^{2.0}$
	Cornea	ND	ND	—	—	—	—	—	—	1/2	$10^{2.0}$
Lympho-reticular	Thymus	2/3	$10^{3.0}$	2/4	$10^{3.5}$	—	—	2/2	$10^{2.5}$	—	—
	Leucocytes	2/3	+	4/4	+	2/2	+	1/2	+	—	—
Alimentary	Small intestine	—	—	3/4	$10^{4.0}$	—	—	1/2	$10^{3.0}$	—	—
Central nervous system	Spinal cord	—	—	—	—	—	—	—	—	—	—
	Cerebellum	1/3	$10^{2.0}$	2/4	$10^{3.0}$	—	—	—	—	—	—
Others	Kidney	—	—	1/4	$10^{2.0}$	—	—	—	—	—	—
	Thyroid	—	—	1/4	$10^{2.0}$	—	—	—	—	—	—
	Adrenal	—	—	1/4	$10^{2.0}$	—	—	—	—	—	—

N Number of foals from which virus was isolated/number of foals examined
V Mean of $TCID_{50}$/gm of wet tissue
[a] Gnotobiotic foal
ND Not examined
+ Isolation of virus from 10^6 leucocytes

Virus by Immunofluorescence

Detection of antigen by immunofluorescence correlated with virus isolation; location in the respiratory tract occurring with all three isolates; in the thymus and small intestine with paresis and foetal isolates; and in the central nervous system with only the paresis isolate. Antigen was evident in both cytoplasm and nucleus and characteristic intranuclear inclusion bodies were often present (Fig. 1). Examination of cellular localization indicated that all three isolates infected nasal mucosa (Fig. 1), bronchial epithelium and alveolar cells; these latter had the appearance of pneumonocytes. Sections of cornea, conjunctiva and lachrymal gland showed that the virus replicated in the conjunctival epithelium (Fig. 2). In the small intestine virus was predominantly in the epithelial cells of the crypts (Fig. 3) while in the animal with an intussusception many desquamated cells showed positive fluorescence. Occasionally single or foci of positive lymphoid

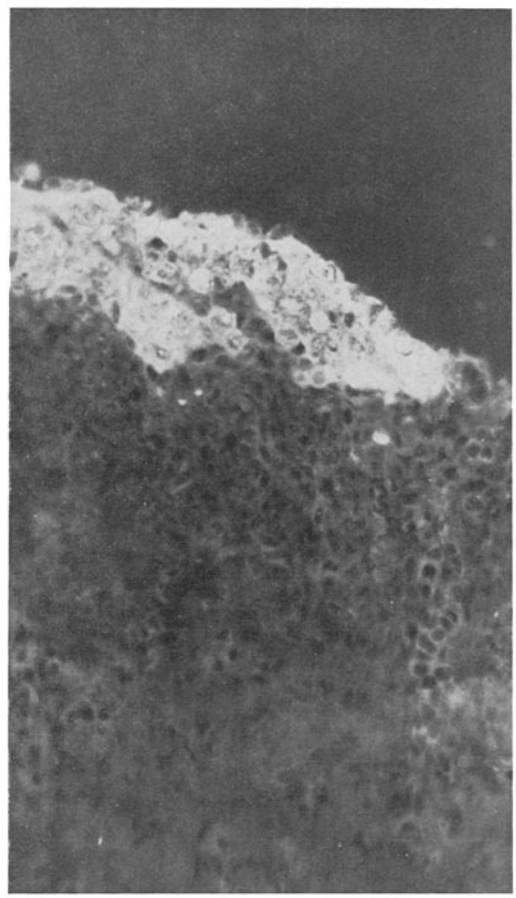

Fig. 1. Turbinate epithelium from a foal 8 days after intranasal administration of subtype-2 EHV-1. The focus of antigen positive cells show fluorescence both diffusely in the cytoplasm and within the intranuclear inclusion bodies. ($\times 480$)

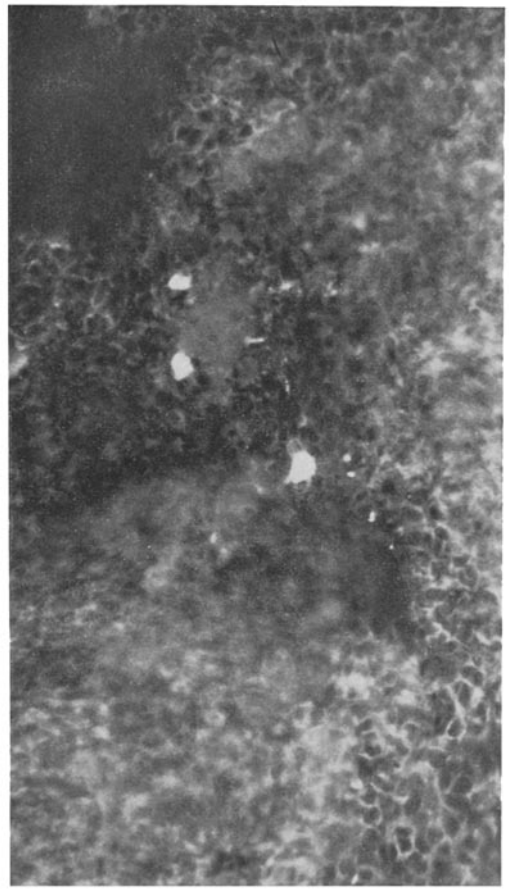

Fig. 2. Isolated epithelial cells in the conjunctival epithelium of a foal killed 8 days after infection with the paresis subtype-1 isolate show diffuse fluorescence. (×480)

cells could also be seen in Peyer's patches. Cortical lymphoblastoid cells were most often infected in the thymus, while in lymph nodes macrophages and lymphoblasts showed fluorescence.

The paresis isolate showed a tropism for endothelial cells and this was observed predominantly in arteries and capillaries of the nasal mucosa, lung and central nervous system. Foci of infection were seen in the rete arteriosus of the nasal mucosa (Fig. 4) and in the same section these were occasionally associated with thrombi. Infected circulating lymphocytes were often seen in these sections. A similar endothelial distribution occurred in the pulmonary arteries but was less frequently detected while endothelial tropism in vessels of the spinal cord occurred both in the white and grey matter of the foals with locomotor dysfunction (Fig. 5) but was infrequent. Virus was not detected in neurones or other neural cells.

In the foal which died with an intussusception fluorescence in endothelial cells of arterioles was also detected in the thyroid, adrenal, kidney and lymph nodes as well as the lungs and nasal mucosa.

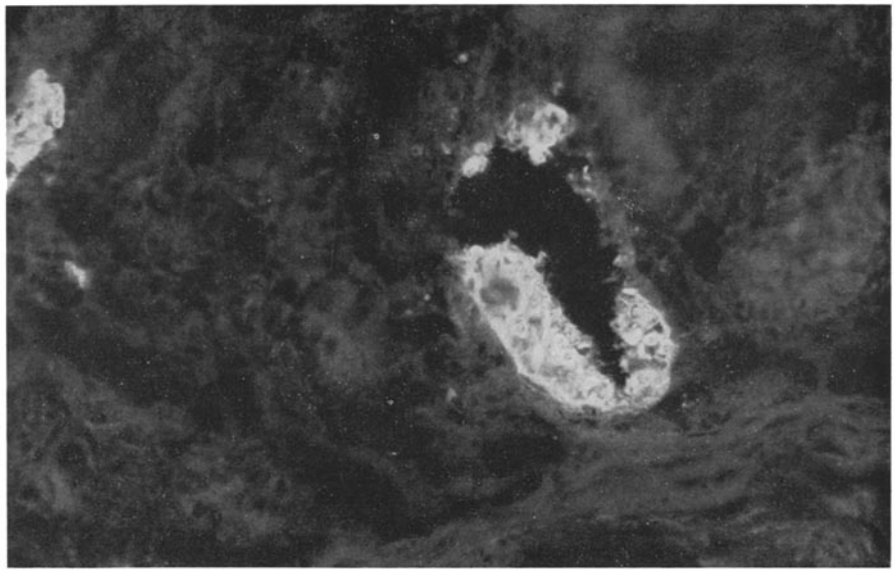

Fig. 3. The entire epithelium of the crypt of the ileum is positive for viral antigen in this foal which died 8 days after infection with the paresis isolate of EHV-1. The foal had an intussusception and bronchopneumonia at post mortem examination. (×480)

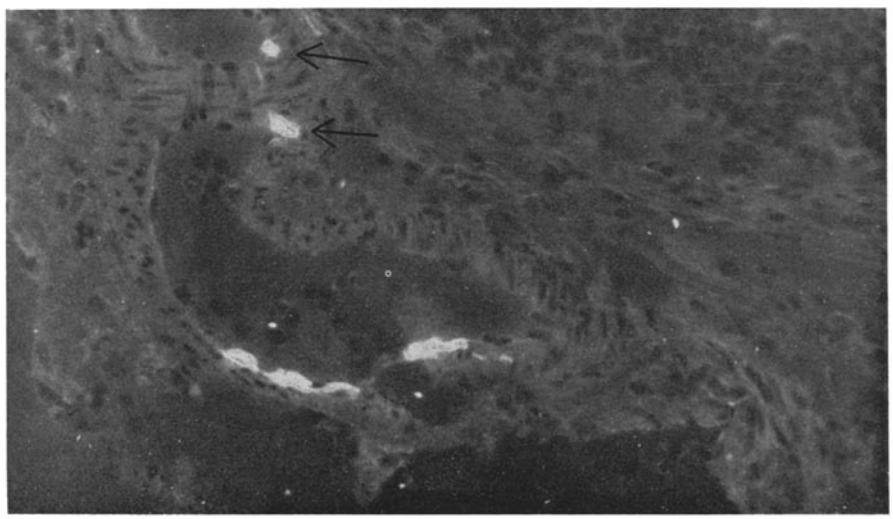

Fig. 4. Fluorescing intranuclear inclusions and a diffuse distribution of viral antigen in the cytoplasm is visible in a focus of endothelial cells lining an arteriole of the nasal mucosa from a foal killed 4 days after intranasal infection with the paresis isolate of EHV-1. Isolated infected cells are also visible (↑). (×480)

Serology

Preinoculation neutralizing and complement fixing titres were <1:8 against the homologous isolate and did not rise in 13 foals during the four or eight days

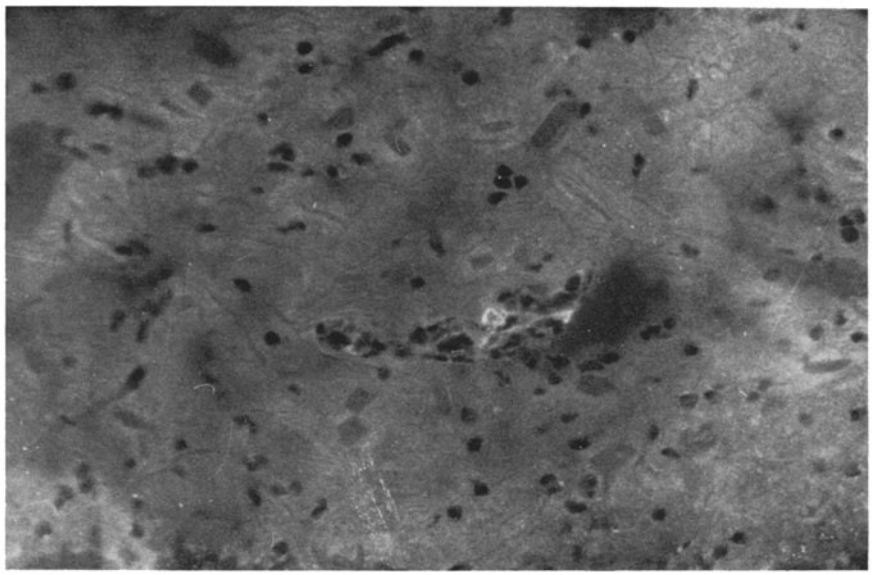

Fig. 5. An isolated capillary endothelial cell in the ventral horn of the lumbar spinal cord is positive for viral antigen. The foal showed a persistent lateral deviation of the right hock 7 days after intranasal infection with the paresis isolate. Isolated haemorrhages were seen in thoracic and lumbar spinal cord. (×480)

of observation. Increases >4-fold occurred by 14 d.p.i. in both of the foals kept for a further period of observation.

Discussion

Variation in virulence between serologically indistinguishable strains of herpesviruses has been recorded with Marek's disease virus (MDV) (14), infectious bovine rhinotracheitis virus (IBRV) (20) and Aujeszky's disease (9). While the virulence of MDV has been shown to vary with the strain of bird the relative virulence remained constant between viruses (14), yet no markers for virulence appear to have been determined (12, 17). Similarly with IBRV strains of differing organ tropism *i.e.* genital and respiratory strains have been identified although they cannot be distinguished serologically. With EHV-1 the predelection is complex in that all isolates replicate in the epithelium of the respiratory tract and conjunctiva, but only subtype-1 isolates infect lymphocytes, thus initiating a viraemia (2). The present work extends the sites of replication to show that both subtype-1 isolates also infected epithelial cells of the small intestine and that this was associated both with recovery of virus from the faeces and with diarrhoea. Moreover the paresis subtype-1 isolate also replicated in vascular endothelial cells of at least the respiratory tract and central nervous system and this in turn was linked to locomotor dysfunction in two foals. The predelection of the paresis isolate for the vessels of particularly the respiratory and central nervous system (CNS) is not understood and is the subject of further investigation. Since the epithelium of nasal mucosa is the site of primary replication it is perhaps not

unexpected to find the plexus of nasal vessels infected. Detection in endothelial cells in the central nervous system was much less frequent and may represent a sampling bias as at least ten times as many sections were examined in the process of investigating the pathogenesis of EHV-1 induced paresis. That the predelection is not restricted solely to respiratory and CNS is supported by the detection of endothelial infection in a wider range of tissues (adrenal, thyroid, kidney and lymph nodes) in the single foal that died 7 d.p.i. with an intussusception. The affinity for endothelial cells is not an age linked tropism of the sort seen with human and porcine cytomegaloviruses (5, 8) since it has been observed in adult horses infected with this paresis isolate of EHV-1 (6).

SABINE *et al.* (15) compared isolates of EHV-1 using restriction endonucleases and identified differences between subtypes-1 and -2, but did not find significant differences between isolates examined within the subtype. TURTINEN *et al.* (19) confirmed this observation suggesting absence of virion protein 8a and reduction of virion protein 19 as the difference between subtype-2 and subtype-1 isolates. They also reported minor unspecified differences in protein profile between isolates within the subtype. However, there was no evidence in either study that the isolates within subtype-1 or -2 expressed any biological differences. The identification of isolates of subtype-1 which differ in their cell tropism would justify further examination to determine whether related differences in biochemical profile can be distinguished. The identification of such differences must be of potential use in the diagnosis, prognosis and prevention of EHV-1 infection.

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