Fukushi H, Tomita T, Taniguchi A, Ochiai Y, Kirisawa R *et al.* (1997) Gazelle herpesvirus 1: a new neurotropic herpesvirus immunologically related to equine herpesvirus 1. *Virology*, **227**, 34–44.

Gesser RM, Koo SC (1996) Oral inoculation with herpes simplex virus type 1 infects enteric neurons and mucosal nerve fibers within gastrointestinal tract of mice. *Journal of Virology*, **70**, 4097–4102.

Hagemoser WA, Kluge JP, Hill HT (1980) Studies on the pathogenesis of pseudorabies in domestic cats following oral inoculation. *Canadian Journal of Comparative Medicine*, **44**, 192–202.

Ha-Lee YM, Dillon K, Kosaras B, Sidman R, Revell P *et al.* (1995) Mode of spread to and within the central nervous system after oral inoculation of neonatal mice with AD strain of Theiler's murine strain encephalitis virus. *Journal of Virology*, **69**, 7354–7361.

Hasebe R, Kimura T, Sato E, Okazaki K, Ochiai K *et al.* (2002) Equine herpesvirus-1 induced encephalomyelitis in mice: a comparative study of neuroadapted virus and its parental strain. *Journal of Comparative Pathology*, **127**, 118–125.

Kodama A, Yanai T, Yonemaru K, Sakai H, Masegi T *et al.* (2007) Acute neuropathogenicity with experimental infection of equine herpesvirus 9 in common marmosets (*Callithrix jacchus*). *Journal of Medical Primatology*, **36**, 335–342.

Kritas SK, Nauwynck HJ, Pensaert MB (1995) Dissemination of wild-type and gC-, gE- and gI-deleted mutants of Aujeszky's disease virus in the maxillary nerve and trigeminal ganglion of pigs after intranasal inoculation. *Journal of General Virology*, **76**, 2063–2066.

Laast VA, Pardo CA, Tarwater PM, Queen SE, Reinhart TA *et al.* (2007) Pathogenesis of simian immunodeficiency virus-induced alterations in macaque trigeminal ganglia. *Journal of Neuropathology and Experimental Neurology*, **66**, 26–34.

Moolenbeek C, Ruitenberg EJ (1981) The 'Swiss roll': a simple technique for histological studies of the rodent intestine. *Laboratory Animals*, **15**, 57–59.

Narita M, Uchimura A, Kimura K, Tanimura N, Yanai T *et al.* (2000) Brain lesions and transmission of experimental equine herpesvirus type 9 in pigs. *Veterinary Pathology*, **37**, 476–479.

Plummer G, Coleman P, Cleveland P, Henson D (1973) Neurovirulence of equine herpes type 1 in mice of different ages. *Journal of Infectious Diseases*, **128**, 202–210.

Pusterla N, Mapes S, Wilson WD (2010) Prevalence of equine herpesvirus type 1 in trigeminal ganglia and submandibular lymph nodes of equids examined postmortem. *Veterinary Record*, **67**, 376–379.

Roscic-Mrkic B, Schwender RA, Odermatt B, Zuniga A, Pavovic J *et al.* (2001) Roles of macrophages in measles virus infection of genetically modified mice. *Journal of Virology*, **75**, 3343–3351.

Sasseville VG, Lackner AA (1997) Neuropathogenesis of simian immunodeficiency virus infection in macaque monkeys. *Journal of Neurovirology*, **3**, 1–9.

Schrenzel MD, Tucker TA, Donovan TA, Martin DM, Busch MD *et al.* (2008) New hosts for equine herpesvirus 9. *Emerging Infectious Diseases*, **14**, 1616–1619.

Steiner I, Kennedy PG (1995) Herpes simplex virus latent infection in the nervous system. *Journal of Neurovirology*, **1**, 19–29.

Taniguchi A, Fukushi H, Matsumura T, Yanai T, Masegi T *et al.* (2000a) Pathogenicity of a new neurotropic equine herpesvirus 9 (gazelle herpesvirus 1) in horses. *Journal of Veterinary Medical Science*, **62**, 215–218.

Taniguchi A, Fukushi H, Yanai T, Masegi T, Hirai K (2000b) Equine herpesvirus 9-induced lethal encephalitis in experimentally infected goats. *Archives of Virology*, **145**, 2619–2627.

Villarreal D, Young CR, Storts R, Tinng JW, Welsh CJ (2006) A comparison of the neurotropism of Theiler's virus and poliovirus in CBA mice. *Microbial Pathogenesis*, **41**, 149–156.

Waki K, Freed EO (2010) Macrophages and cell–cell spread of HIV-1. *Viruses*, **2**, 1603–1620.

Wu L (2008) Biology of HIV mucosal transmission. *Current Opinion in HIV and AIDS*, **3**, 534–540.

Yanai T, Fujishima N, Fukushi H, Hirata A, Sakai H *et al.* (2003a) Experimental infection of equine herpesvirus 9 in dogs. *Veterinary Pathology*, **40**, 263–267.

Yanai T, Sakai T, Fukushi H, Hirai K, Narita M *et al.* (1998) Neuropathological study of gazelle herpesvirus 9 (equine herpesvirus 9) infection in Thomson's gazelles (*Gazella thomsoni*). *Journal of Comparative Pathology*, **119**, 159–168.

Yanai T, Tsujioka S, Sakai H, Fukushi H, Hirai K *et al.* (2003b) Experimental infection with equine herpesvirus 9 (EHV-9) in cats. *Journal of Comparative Pathology*, **128**, 113–118.

Zhao YM, Narita M, Kawashima K (1996) Pathologic changes in closed porcine intestinal loops inoculated with Aujeszky's disease virus. *Journal of Veterinary Medical Science*, **58**, 809–810.

Zink MC, Laast AV, Helke KL, Brice AK, Barber SA *et al.* (2006) From mice to macaques – animal models of HIV nervous system disease. *Current HIV Research*, **4**, 293–305.

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ORIGINAL PAPER

The use of different diagnostic tools for *Babesia* and *Theileria* parasites in cattle in Menofia, Egypt

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Abstract Bovine piroplasmosis is caused by tick-borne hemoprotozoans of the genera *Babesia* and *Theileria* and is the most prevalent in tropical and subtropical countries, causing a major economic impact worldwide. In the current study, a total of 405 cattle of different ages, sexes, and breeds were randomly sampled for surveying and diagnosis of babesiosis and theileriosis using three methods: direct microscopy (blood smears), indirect fluorescent antibody test (IFAT) and polymerase chain reaction (PCR). Giemsa-stained blood smears revealed that, out of 405 examined cattle, 33 (8.15 %) were infected with *Babesia* sp. and 65 (16.05 %) with *Theileria* sp. (total number of infected cattle was 98). Mixed infection was seen in 11 (2.72 %) animals. Moreover, application of the three diagnostic assays on 158 randomly sampled cattle indicated that 17 (10.76 %) and 33 (20.89 %) were positive for *Babesia* and *Theileria* spp. by the direct smear technique, 25 (15.82 %) and 33 (20.89 %) by IFAT (fluorescence was greenish yellow for *Babesia* and yellowish for *Theileria*), and 20 (12.66 %) and 38 (24.05 %) by PCR. Using primers specific for *Babesia* and *Theileria* spp., we found that diagnostic bands appeared at ~350 and ~370 bp, respectively indicating the presence of these piroplasms. Statistically, there was a non-significant difference of the positivity in response to the three techniques; thus, any of these methods can be described as useful for diagnosing blood parasites in both domesticated animals and birds. On the basis of the obtained results, it could be concluded that direct microscopy can be used in acute infections, whereas IFAT and PCR are useful in chronicity.

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Introduction

Babesia and *Theileria* species are apicomplexan–hemoprotozoan parasites transmitted by Ixodidae ticks (Preston 2001; Silva et al. 2010) and are viewed to be devastating parasites affecting the production of livestock, mainly cattle and small ruminants. They pose a significant problem for veterinary authorities, occasionally emerging in conjunction with other disease conditions, and thus being difficult to pinpoint (Altay et al. 2008). These infections are of worldwide importance and are characterized by anemia, icterus, hemoglobinuria, and death, and as a result, they have a high economic impact in several parts of the world, including tropical and temperate countries (Wagner et al. 2002).
Bovine babesiosis is caused by multiple species: *Babesia bigemina*, *Babesia divergens*, *Babesia bovis*, and *Babesia major*. *Babesia* species have the potential for wide

distribution wherever their tick vectors are encountered. Two species, *B. bigemina* and *B. bovis*, have a considerable impact on cattle health and productivity in tropical and subtropical countries. Cattle suffering from theileriosis demonstrate varying clinical signs that range from lymphoproliferative changes with high morbidity and mortality, as seen with *Theileria annulata* and *Theileria parva*, to benign or mild disease, as seen with *Theileria orientalis* (Altay et al. 2008; Safieldin et al. 2011).

Detection of these blood parasites is highly beneficial in early diagnosis. Traditionally, microscopy using Giemsa-stained blood smears has been considered the "gold standard" for detecting *Babesia* and *Theileria* organisms in the blood of infected animals, particularly in acute cases, but not in carriers, where the parasitemia is low, with small numbers of the protozoa in the peripheral blood (Friedhoff and Bose 1994; Bose et al. 1995). Therefore, serological techniques were proposed for detecting circulating antibodies against these parasites, particularly in subclinical infections during epidemiological investigations. In addition, serological diagnosis using the indirect fluorescent antibody test can be used to detect antibodies against the *Theileria* species (Leemans et al. 1997). One disadvantage of such tests is the occurrence of false-positive and false-negative results, involving cross-reactions or improper specific immune response (Passos et al. 1998), while another is the inability to differentiate between previous and current infections, making sensitive and highly specified diagnostic techniques for *Babesia* and *Theileria*. Therefore, the application of PCR-based techniques is imperative in order to detect these hemoparasites in carrier animals (Criado-Fornelio et al. 2009). These methods were used for diagnosis of babesiosis and theileriosis in several species of related countries, showing similar climatic conditions, including Tunisia (M'ghirbi et al. 2008), United Arab Emirates (Jaffar et al. 2010) and Iran (Zaeemi et al. 2011).

The present study was performed for the purpose of surveying and diagnosing both *Babesia* and *Theileria* spp. in cattle in Menofia province, Egypt, using Giemsa-stained blood films, indirect fluorescent antibody test (IFAT)-serological test, and PCR assay.

Materials and methods

Animals and the study area

A total of 405 cattle of different ages, sexes, and breeds were clinically examined for diagnosis of *Babesia* and *Theileria* spp. during field trips in Menofia province (coordinates: 30 °03′00″ N 31 °15′00″ E), Egypt. Animals suffered from signs of blood parasites that were typical indications of babesiosis and theileriosis. Blood smears and blood samples were collected to confirm clinical diagnosis of both diseased and carrier animals.

Blood smears

Thin smears were prepared from EDTA-whole blood on clean and dry slides, fixed in methanol, stained with Giemsa stain, and microscopically examined for the detection of intra-erythrocytic forms of both *Babesia* and *Theileria* spp. piroplasms at 100× objective magnification. The smears were recorded as negative for piroplasms if no parasites were detected in 50 oil-immersion fields (Moretti et al. 2010).

Blood samples

Blood samples were collected for both PCR (with anticoagulant, sodium salt of EDTA) and IFAT (without anticoagulant). Blood was collected from the jugular vein and immediately preserved in Eppendorf tubes containing a few drops of EDTA.

Laboratory assays

Indirect fluorescent antibody test

Available parasite-coated slides for both *Babesia* and *Theileria* antigens were kindly provided by the Veterinary Serum and Vaccine Research Institute, Abassia, Egypt, ready for the indirect fluorescent antibody test as described by Leeflang and Perie (1972). A positive reaction is indicated by a bright fluorescence (Papadopoulos et al. 1996).

Polymerase chain reaction

DNA extraction and amplification DNA extraction was performed according to the Manual Chemical Method (rapid isolation of mammalian DNA) using cell lysis buffer (pH 8.0), ethanol (70 %), isopropanol, potassium acetate solution, red blood cell lysis buffer, and proteinase K (20 mg/ml) as described by Sambrook and Russell (2001).

PCR amplification was performed in a final reaction volume of 50 μl containing 200 μM of each dNTPs, 0.2 uM of each primer, 2.5 U of Taq DNA polymerase (Fermentas, Germany), 10 mM of TBE (tris, boric acid, and EDTA) buffer pH 8.0 containing 1.5 mM $MgCl_2$ and 5 μl of the DNA template. The designated primers were obtained from Bioneer Corporation (064550), Korea. The oligonucleotide sequences of the primers used were forward strand primer BAB GF2 (5′-GTC TTG TAA TTG GAA TGA TGG-3′) and reverse strand primer BAB GR2 (5′-CCA AAG ACT TTG ATT TCT CTC-3′) (Adaszek and Winiarczyk 2008) under the following conditions: an initial denaturation at 95 °C for 5 min followed by 40 cycles of

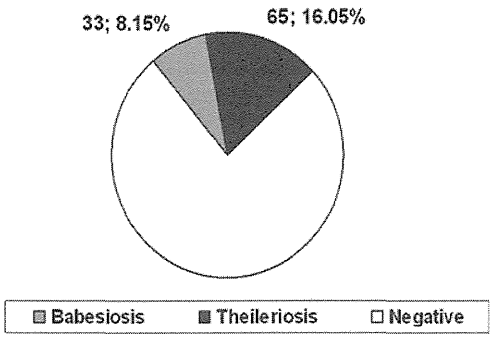

Fig. 1 Diagrammatic scale showing the overall prevalence of *Babesia* and *Theileria* parasites among the examined cattle in Menofia province

denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min, followed by final extension at 72 °C for 10 min.

The amplification reactions were carried out in a PCR thermal cycler Biometra T- personal/Germany S/N 1205334 and the corresponding amplicons were checked on 1.5 % agarose gel, stained with ethidium bromide, examined under UV transilluminator, and photographed using a digital camera.

Statistical analysis

Data were analyzed using multiple comparisons between different diagnostic methods for *Babesia* and *Theileria* parasites, including direct microscopy, IFAT, and PCR using the general linear model, Tukey test. This was carried out using Minitab statistical software (MTW13) (Raza et al. 2007). $P<0.05$ was accepted to be statistically significant.

Results

In this study, parasitological examination of 405 randomly selected cattle, by direct microscopy using Giemsa-stained thin blood films, revealed that 98 had intra-erythrocytic stages of piroplasms of both *Babesia* and *Theileria* spp. with an overall prevalence of 24.2 %. Among those, 33 (8.15 %) were infected by *Babesia* sp., 65 (16.05 %) had *Theileria* sp., and 11 cases showed mixed infection (Fig. 1). These results were consistent with the clinical signs and previous case histories taken from the farmers and owners of animals that were collected from different districts in Menofia province. As a result of chronic infection, the developing antibodies reacted positively using the IFAT, producing a fluorescence that was clearly distinct and greenish yellow (Fig. 2) or yellowish (Fig. 3) in color and indicated the presence of the intra-erythrocytic stages of both *Babesia* and *Theileria* piroplasms, respectively. PCR findings showed that diagnostic bands were produced at ~350 and ~370 bp, and the fragments were specific for both *Babesia* and *Theileria* spp., respectively (Fig. 4). This

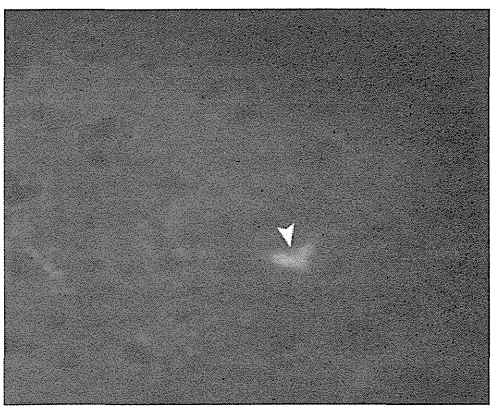

Fig. 2 Greenish yellow fluorescence (*arrow*) indicating intra-erythrocytic stages of *Babesia* sp. piroplasms using IFAT (×100)

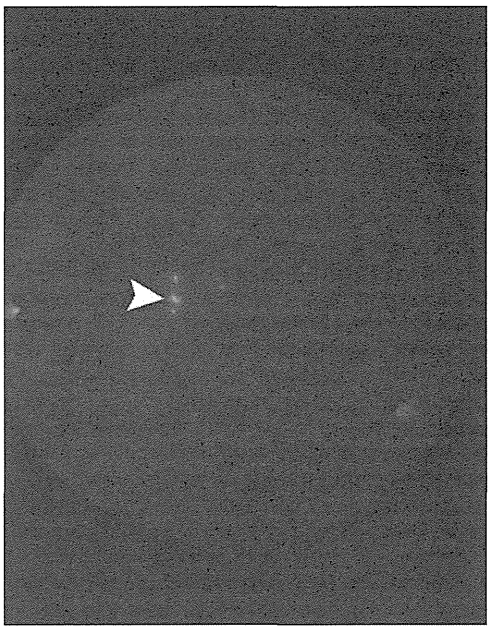

Fig. 3 Yellowish fluorescence of *Theileria* sp. piroplasms (*arrow*) indicating positive reaction with IFAT (×100)

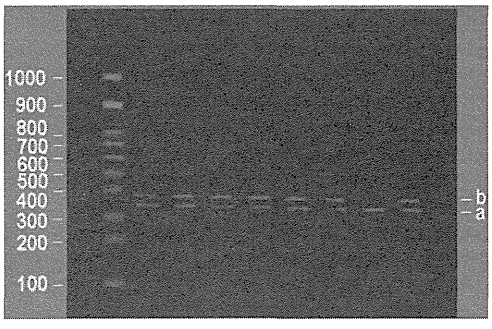

Fig. 4 PCR results showing diagnostic bands at 350-bp for *Babesia* (**a**) and 370bp for *Theileria* (**b**). *Lane M* 100-bp ladder=DNA marker (from Fermentas, Germany)

Table 1 Comparative detection of *Babesia* and *Theileria* parasites in cattle using direct microscopy, IFAT, and PCR

Parasite	Assay (n=158)						*P value*
	Direct microscopy		IFAT		PCR		
	Infected	Percent	Infected	Percent	Infected	Percent	
Babesia sp.	17	10.76	25	15.82	20	12.66	NS
Theileria sp.	33	20.89	33	20.89	38	25.05	NS

finding revealed the presence of both hemoparasites in the blood of examined cattle.

Furthermore, the seroprevalence of *Babesia* and *Theileria* species in cattle using IFAT revealed that out of 158 serum samples examined for the presence of antibodies, 25 (15.82 %) and 33 (20.89 %) were positive for *Babesia* spp. and *Theileria* spp., respectively. Furthermore, PCR findings indicated that 20 (12.66 %) and 38 (24.05 %) were positive for *Babesia* and *Theileria* spp., respectively (Table 1).

The findings in the present study confirmed that the use of direct microscopy, IFAT, or PCR is valuable in the diagnosis of *Babesia* and *Theileria* spp. Based on the confidence intervals calculated using the Tukey method (Minitab), it has been found that the differences between the three methods were not statistically significant, as there was no pairwise variation between infected animals (Fig. 5). This means that in some instances, mainly acute infections, the use of any of these methods may be applicable, although IFAT and PCR are preferable in chronic infections. It is worth mentioning that the methodology in the present investigation is based on collecting a considerable number of animals regardless of studying the effects of sex, age, or season on the infection level.

Discussion

Babesiosis and theileriosis have extensive prevalence and mortality rates with high economic losses in several countries (Shahnawaz et al. 2011). In Egypt, large numbers of cattle are infected with subclinical piroplasmosis (Adham et al. 2009). In such cases, in addition to the parasitological examination of stained blood films for detecting the *Babesia* protozoan parasites, low parasitemia necessitates the use of more advanced diagnostic tools, rather than conventional ones, to detect specific antibodies. In addition, negative microscopic examination does not exclude the possibility of infection (Weiland and Reiter 1988). Subclinical babesiosis and theileriosis lead to the affected livestock, including cattle and small ruminants, becoming chronic carriers of the piroplasms and in turn sources of infection for tick vectors, and cause natural transmission of the disease. Therefore, latent infections are the target in the epidemiology of the diseases.

In the present study, the results obtained from Giemsa-stained blood smears revealed that 33 smears (8.1 %) were positive for *Babesia*. Similar results were obtained by Sevnc et al. (2001) in Turkey and Mazyad and Khalaf (2002) in Egypt. On the other hand, Jeon (1978) in Korea and Osaki et al. (2002) in Brazil detected infection rates of 23 and 64 %, respectively. Fluctuation in the prevalence rates might be due to the variation of environmental conditions that affect both parasites and vectors. For *Theileria* sp., the present investigation showed that 65 (16.05 %) of 405 cattle were positive. These results agreed with those obtained by Jeon (1978) in Korea (17 %), Acici (1995) in Turkey (17 %), and those obtained in Egypt, which included Abu El-Magd (1980) in Quena province (11.1 %) and Adel (2007) in Gharbia province (11.31 %). Conversely, our results opposed a number of reports in Egypt, among them El Bahy (1986), who revealed prevalence rates of 65 and 53 % in cattle and buffaloes, respectively, and Gamal EI-Dien (1993) in El-Behera province, who found that the prevalence of *T. annulata* was 65.4 % using stained blood films.

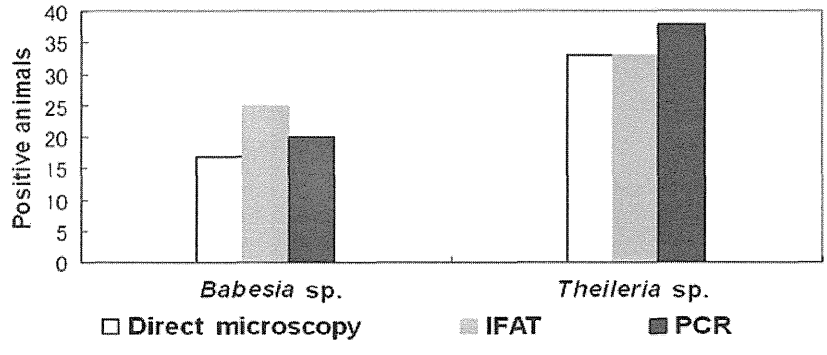

Fig. 5 Comparison of the positivity of *Babesia* and *Theileria* spp. using three diagnostic techniques: direct microscopy, IFAT, and PCR

Variation in prevalence rates could possibly be attributed to an abundance of the vectors as a result of high temperature and humidity. The present investigation showed that mixed infection, using Giemsa-stained blood smears, of *Babesia* and *Theileria* spp. appeared in 11 (2.72 %) of 405 examined cattle. These results coincided with those obtained by Dumanli and Ozer (1987) in Turkey, who found a mixed infection rate for *B. bigemina* and *T. annulata* of 1.5 % in cattle.

Using IFAT, the present study revealed an infection rate of 15.82 % (25/158) for *Babesia* sp. This finding was in accordance with that obtained by Terkawi et al. (2012), who examined a herd of cattle in the central region of Syria using IFAT and found infection rates of 18.36 and 21.74 % for *B. bovis* and *B. bigemina*, respectively. Moreover, Jaffar et al. (2010) in Dubai, UAE recorded infection rates of 10.5 and 33.3 % for equine babesiosis and theileriosis. On the other hand, Singh et al. (2009) recorded a rate of 56.11 % for *B. bigemina* in India, Iseki et al. (2010) noted rates of 68.8 and 75.8 % for *B. bovis* and *B. bigemina* in Thailand, and Sevgili et al. (2010) recorded an infection rate of 43.9 % for *B. bigemina* in Sanliura, Turkey. The variation in infection rates could be due to differences in climatic conditions and species of cattle. Concerning *Theileria* sp., the current investigation revealed that out of 158 bovine serum samples, 33 (20.89 %) was found to be positive for *Theileria* antibodies. This result agreed with that obtained by Sayin et al. (2003), who noted that 34 (21 %) of 155 examined cattle were found to be seropositive for *T. annulata*. Moreover, Adel (2007) in Gharbia province, Egypt reported that the incidence peaks of *T. annulata* seropositive native breed cattle using IFAT were 27.8 and 22 % in spring and summer, respectively. On the other hand, Gamal EI-Dien (1993) in EI-Behera province, Egypt detected that the prevalence of *T. annulata* was 71.9 %, and Caille (1987) in Somalia found that 71.2 % of cattle had antibodies against *T. annulata*. This discrepancy may be due to variation in the susceptibility of the animal species and could also be due to variance in animal locale.

Results of PCR revealed that 20 (12.66 %) of 158 animals were positive for *Babesia* sp. These findings were similar to those mentioned by Oliveira-Sequeira et al. (2005), who recorded a 10 % infection rate for *Babesia* using PCR and M'ghirbi et al. (2008) who revealed an infection rate of 11.11 % for bovine *Babesia* spp. Similar results were obtained by Figueroa et al. (1993) and Gubbels et al. (2002). On the other hand, De Vos and Potgieter (1994) in France found a 20 % infection rate, Costa-Junior et al. (2006) in Brazil found that 8 of 30 cattle (26.7 %) were positive for *Babesia* infection using PCR, and Rania (2009) in Egypt mentioned that the infection rate of *Babesia* sp. was 25.33 %. These discrepancies could be due to changes in locale. The infection rate of *Theileria* using PCR indicated that 60 animals (21.8 %) were positive. This result was similar to those cited by Ogden et al. (2003), Aktas et al. (2005), Altay et al. (2007) and M'ghirbi et al. (2008). Ogden et al. found an infection rate of 23.4 % for *Theileria* sp. in cows in Tanzania using PCR.

The authors concluded that the three techniques, direct microscopy, IFAT, and PCR, are all methods of choice with slight variation in their results, and this was confirmed by obtaining non-significant findings among them. Consequently, they are used in the detection of prevalence of blood parasites, primarily *Babesia* and *Theileria*. Direct microscopy with Giemsa-stained blood films is the conventional and the more practical method on farms, as it is rapid and inexpensive, but it is useful only in acute infections. Moreover, IFAT and PCR are modern assays that help veterinarians detect protozoan parasites in chronic carrier animals, and they circumvent the problem of false negative results obtained under direct microscopy.

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References

Abu El-Magd MM (1980) Some epidemiological studies about theileriosis at Quna Governorate. PhD. thesis, Faculty of Veterinary Medicine, Assuit University, Assuit, Egypt

Acici M (1995) Prevalence of blood parasites in cattle in the Samsun region, Turkey. Etlik Vet Mikrob Derg 8:271–277

Adaszek L, Winiarczyk S (2008) Molecular characterization of *Babesia canis canis* isolates from naturally infected dogs in Poland. Vet Parasitol 152:235–241

Adel EM (2007) Studies on some blood parasites infecting farm animals in Gharbia
governorate, Egypt. PhD thesis, Faculty of Veterinary Medicine, Cairo University, Cairo, Egypt

Adham FK, Abd-El-Samie EM, Gabre RM, El Hussein H (2009) Detection of tick blood parasites in Egypt using PCR assay I—*Babesia bovis* and *Babesia bigemina*. Parasitol Res 105:721–730

Aktas M, Altay K, Dumanli N (2005) Development of a polymerase chain reaction method for diagnosis of *Babesia ovis* infection in sheep and goats. Vet Parasitol 133:277–281

Altay K, Dumanli N, Aktas M (2007) Molecular identification, genetic diversity and distribution of *Theileria* and *Babesia* species infecting small ruminants. Vet Parasitol 147:161–165

Altay K, Fatih Aydin M, Dumanli N, Aktas M (2008) Molecular detection of *Theileria* and *Babesia* infections in cattle. Vet Parasitol 158:295–301

Bose R, Jorgensen WK, Dalgliesh RJ, Friedhoff KT, De Vos AJ (1995) Current state and future trends in the diagnosis of babesiosis. Vet Parasitol 57:61–74

Caille JY (1987) Serological survey of the prevalence and seasonal incidence of haemoprotozoa in livestock in Somalia. Thesis, Freie Universitat Berlin, Berlin, Germany

Costa-Junior LM, Rabelo EML, Filho OAM, Ribeiro MFB (2006) Comparison of different direct diagnostic methods to identify *Babesia bovis* and *Babesia bigemina* in animals vaccinated with live attenuated parasites. Vet Parasitol 139:231–236

Criado-Fornelio A, Buling A, Pingret JL, Etievant M, Boucraut-Baralon C, Alongi A, Agnone A, Torina A (2009) Hemoprotozoa of domestic animals in France: prevalence and molecular characterization. Vet Parasitol 159:73–76

De Vos AJ, Potgieter FT (1994) Bovine babesiosis. In: Coetzer JAW, Thomson GR, Tustin RC (eds) Infectious diseases of livestock. Oxford University Press, Cape Town, pp 278–294

Dumanli N, Ozer E (1987) Elaziğ yöresinde sığirlarda görülen kan parazitleri ve yayihs¸jan üzerinde araştirmalar. Selcuk Ü nin. Vet Fak Derg 3:159–166

El Bahy NMA (1986) Some studies on ticks and tick borne disease among ruminants in Fayom Governorate. M.V.Sc. thesis (Parasitology), Faculty of Veterinary Medicine, Cairo University, Cairo, Egypt

Figueroa JV, Chieves LP, Johnson GS, Buening GM (1993) Multiplex polymerase chain reaction based assay for the detection of *Babesia bigemina*, *Babesia* bovis and *Anaplasma marginale* DNA in bovine blood. Vet Parasitol 50:69–81

Friedhoff K, Bose R (1994) Recent developments in diagnostics of some tick-borne diseases. In: Uilenberg, G., Permin, A., Hansen, J.W. (eds). Use of applicable biotechnological methods for diagnosing haemoparasites. Proceedings of the Expert Consultation, Merida, Mexico, October 4–6, 1993. Food and Agriculture Organization of the United Nations (FAO), Rome, Italy, pp 46–57

Gamal EI-Dien HY (1993) Studies on *Theileria* protozoan among cattle in Behera Province. M.V.Sc. thesis, Faculty of Veterinary Medicine, Alexandria University, Alexandria, Egypt

Gubbels MJ, Yin H, Bai Q, Liu G, Nijman IJ, Jongejan F (2002) The phylogenetic position of the *Theileria buffeli* group in relation to other *Theileria* species. Parasitol Res 88:S28–S32

Iseki H, Zhou L, Kim C, Inpankaew T, Sununta C, Yokoyama N, Xuan X, Jittapalapong S, Igarashi I (2010) Seroprevalence of *Babesia* infections of dairy cows in northern Thailand. Vet Parasitol 170:193–196

Jaffar O, Abdishakur F, Hakimuddin F, Riya A, Wernery U, Schuster RK (2010) A comparative study of serological tests and PCR for the diagnosis of equine piroplasmosis. Parasitol Res 106:709–713

Jeon Y (1978) A survey of babesiosis and theileriosis in Korean cattle. Korean J Vet Res 18:23–26

Leeflang MJ, Perie CD (1972) Duration of serological response to the indirect fluorescent antibody test of cattle recovered from *Theileria parva* infection. Res Vet Sci 14:270–271

Leemans I, Hooshmand-Rad P, Uggla A (1997) The indirect fluorescent antibody test based on schizont antigen for study of the sheep parasite *Theileria lestoquardi*. Vet Parasitol 69:9–18

M'ghirbi Y, Hurtado A, Brandika J, Khlif K, Ketata Z, Bouattour A (2008) A molecular survey of *Theileria* and *Babesia* parasites in cattle, with a note on the distribution of ticks in Tunisia. Parasitol Res 103:435–442

Mazyad SA, Khalaf SA (2002) Studies on Theileria and Babesia infecting live and slaughtered animals in Al Arish and El Hasanah, North Sinai Governorate, Egypt. J Egypt Soc Parasitol 32 (2):601–610

Moretti A, Mangili V, Salvatori R, Maresca C, Scoccia E, Torina A, Moretta I, Gabrielli S, Tampieri MP, Pietrobelli M (2010) Prevalence and diagnosis of *Babesia* and *Theileria* infections in horses in Italy: a preliminary study. Vet J 184:346–350

Ogden NH, Gwakisa P, Swai E, French NP, Fitzpatrick J, Kambarage D, Bryant M (2003) Evaluation of PCR to detect *Theileria parva* in field-collected tick and bovine samples in Tanzania. Vet Parasitol 112(3):177–183

Oliveira-Sequeira TC, Oliveira MC, Araujo JP Jr, Amarante AF (2005) PCR-based detection of *Babesia* bovis and *Babesia bigemina* in their natural host *Boophilus microplus* and cattle. Int J Parasitol 35:105–111

Osaki SC, Vidotto O, Marana ERM, Vidotto MC, Yoshihara E, Pacheco RC, Igarashi M, Minho AP (2002) Occurrence of antibodies against *Babesia bovis* and studies on natural infection in Nelore cattle, in Umuarama municipality, Paraná State, Brazil. Rev Bras Parasitol Vet 11:73–83

Papadopoulos B, Marie Perie N, Uilenberg G (1996) Piroplasms of domestic animals in the Macedonia region of Greece. 1. Serological cross-reactions. Vet Parasitol 63:41–56

Passos LM, Bell-Sakyi L, Brown CG (1998) Immunochemical characterization of in vitro culture-derived antigens of *Babesia bovis* and *Babesia bigemina*. Vet Parasitol 76:239–249

Preston PM (2001) Theilerioses. In: Wallingford MW (ed) Encyclopedia of arthropod-transmitted infections of man and domesticated animals. CABI, Wallingford, pp 487–502

Rania YE (2009) Some studies on diagnosis on babesiosis. M.V.Sc. thesis, Faculty of Veterinary Medicine, Benha University, Benha, Egypt

Raza MA, Iqbal Z, Jabbar A, Yaseen M (2007) Point prevalence of gastrointestinal helminthiasis in ruminants in southern Punjab, Pakistan. J Helminthol 81:323–328

Safieldin M, Abdel Gadir A, Elmalik Kh (2011) Factors affecting seasonal prevalence of blood parasites in dairy cattle in Omdurman locality, Sudan. J Cell Anim Biol 5(1):17–19

Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, New York

Sayin F, Dincer S, Karaer Z, Cakmak A, Inci A, Yukari BA, Vatansever Z, Nalbantoglu S (2003) Studies on the epidemiology of tropical theileriosis (*Theileria annulata* infection) in cattle in central Anatolia, Turkey. Trop Anim Health Prod 35(6):521–539

Sevgili M, Cakmak A, Gokcen A, Altas MG, Ergun G (2010) Prevalence of *Theileria annulata* and *Babesia bigemina* in cattle in the vicinity of Sanliurfa. J Anim Vet Adv 9(2):292–296

Sevnc F, Sevnc M, Brdane FM, Altnoz F (2001) Prevalence of *Babesia bigemina* in cattle. Rev Med Vet 152(5):395–398

Shahnawaz S, Ali M, Aslam MA, Fatima R, Chaudhry ZI, Hassan MU, Ali M, Iqbal F (2011) A study on the prevalence of a tick-transmitted pathogen, *Theileria annulata*, the hematological profile of cattle from Southern Punjab (Pakistan). Parasitol Res 109:1155–1160

Silva MG, Marques PX, Oliva A (2010) Detection of *Babesia* and *Theileria* species infection in cattle from Portugal using a reverse line blotting method. Vet Parasitol 174:199–205

Singh H, Mishra AK, Rao JR, Tewari AK (2009) Comparison of indirect fluorescent antibody test (IFAT) and slide enzyme linked immunosorbent assay (SELISA) for diagnosis of *Babesia bigemina* infection in bovines. Trop Anim Health Prod 41:153–159

Terkawi MA, Alhasan H, Huyen NX, Sabagh A, Awier K, Cao S, Goo Y, Aboge G, Yokoyama N, Nihikawa Y, Kalb-Allouz A, Tabbaa D, Igarashi I (2012) Molecular and serological prevalence of *Babesia bovis* and *Babesia bigemina* in cattle from central region of Syria. Vet Parasitol. doi:10.1016/j.vetpar.2011.12.038

Wagner GG, Holman P, Waghela S (2002) Babesiosis and heartwater: threats without boundaries. Vet Clin Food Anim 18:417–430

Weiland G, Reiter I (1988) Methods for measurement of the serological response to Babesia. In: Ristic M (ed) Babesiosis of domestic animals and man. CRC, Boca Raton, pp 143–162

Zaeemi M, Haddadzadeh H, Khazraiinia P, Kazemi B, Bandehpour M (2011) Identification of different *Theileria* species (*Theileria lestoquardi*, *Theileria ovis*, and *Theileria annulata*) in naturally infected sheep using nested PCR–RELP. Parasitol Res 108:837–843

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DISEASE IN WILDLIFE OR EXOTIC SPECIES

Spontaneous T/NK-cell Lymphoma associated with Simian Lymphocryptovirus in a Japanese Macaque (*Macaca fuscata*)

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Summary

A 5-year-old female Japanese macaque (*Macaca fuscata*) was humanely destroyed because of severe anaemia with poor response to treatment. At necropsy examination, marked splenomegaly and systemic enlargement of lymph nodes were observed. Microscopical examination revealed diffuse proliferation of neoplastic lymphoid cells in the spleen and lymph nodes with infiltration of the liver, lung, gastrointestinal tract, kidney and bone marrow. Immunohistochemically, the neoplastic cells expressed CD3 and CD4, but not CD20, CD79α or CD8, consistent with a T helper phenotype. A portion of neoplastic cells expressed the natural killer (NK) cell marker CD56. In-situ hybridization detected Epstein-Barr virus (EBV)-encoded small RNAs in the neoplastic cells, indicating the involvement of simian lymphocryptovirus (LCV). This is the first report of simian LCV-associated T/NK-cell lymphoma with the predominant expression of T-cell antigens in non-human primates.

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Keywords: Japanese macaque; lymphocryptovirus; T/NK-cell lymphoma

Lymphomas are common, spontaneously arising tumours in non-human primates (Beniashvili, 1989). Certain viruses are associated with the development of lymphoma in non-human primates as well as in man. These include simian lymphocryptovirus (LCV) and simian T-cell lymphotropic virus (STLV). These viruses are closely related to Epstein-Barr virus (EBV) and human T-cell lymphotropic virus type I (HTLV-I), respectively. A significant percentage of Japanese macaques (*Macaca fuscata*) are seropositive for simian LCV (Ishida *et al.*, 1993) and STLV (Hayami *et al.*, 1984); however, there are only a few reports of lymphomas in Japanese macaques and it remains unclear whether those viruses are associated with the development of lymphoma.

A female Japanese macaque was born and reared at the Primate Research Institute of Kyoto University and had been kept in an outdoor group. At 5 years of age, the macaque suddenly exhibited an unsteady gait and a swelling of the left cheek that was due to a large abscess of the upper gingiva. Severe anaemia (red blood cell count 0.8×10^{12}/l, haemoglobin 2.0 g/dl and haematocrit 6.6%; normal reference intervals $4.5–6.0 \times 10^{12}$/l, 12–15 g/dl and 40–45%, respectively) and low platelet count (124×10^{9}/l; reference interval $200–300 \times 10^{9}$/l) were detected by means of haematological testing. The white blood cell count was not elevated (8.2×10^{9}/l; reference interval $7.0–12.0 \times 10^{9}$/l). Increases in alanine

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aminotransferase (69 IU/l; reference interval 15—45 IU/l), blood urea nitrogen (11.4 mmol/l; reference interval 3.6—8.9 mmol/l) and C-reactive protein (1.3 mg/dl; reference interval <0.3 mg/dl) were detected by means of serum biochemistry. This macaque had not been examined serologically for simian LCV infection. After 5 days of treatment, the animal was humanely destroyed because of a poor prognosis and necropsy examination was performed immediately.

There was marked diffuse splenomegaly (the spleen measured 7 × 13 cm in length and width, respectively; normal size of spleen is 2—3 × 4—5 cm in length and width, respectively, in age-matched Japanese macaques) and enlargement of superficial and visceral lymph nodes (2.2 cm maximum dimension; normal lymph nodes are approximately 0.5 cm maximum dimension). The spleen was uniformly red in colour and the lymph nodes were cream in colour with loss of corticomedullary differentiation on cut surface.

Samples of the major organs and enlarged lymph nodes and a sample of sternal bone marrow were fixed in 10% neutral buffered formalin and processed routinely. Sections were stained with haematoxylin and eosin (HE). Microscopically, there was a diffuse proliferation of neoplastic lymphoid cells in the white pulp, cords and red pulp sinuses of the spleen (Fig. 1A). In the lymph nodes, the same neoplastic cells replaced the normal architecture and invaded the surrounding adipose tissues. Extensive necrosis was observed in some lymph nodes. Bone marrow infiltration was found and was thought likely to be the cause of the severe anaemia. In the liver, there was periportal and sinusoidal infiltration of neoplastic cells. Neoplastic cells were also observed in the lamina propria of the gastrointestinal tract and the interstitium of the kidneys. In addition, circulating neoplastic cells were frequently found in the blood vessels of various organs. In the lung, there were numerous neoplastic cells in the alveolar capillaries. The neoplastic infiltrates were composed of two types of mononuclear cell (Fig. 1B). The predominant population was of small, mature cells with hyperchromatic, round or oval nuclei and scant cytoplasm. These cells were similar to normal lymphocytes, but often contained irregular nuclei. Larger neoplastic cells were also observed. These had medium to large nuclei with a distinct, large nucleolus and a moderate amount of pale, eosinophilic cytoplasm.

Serial sections were subjected to immunohistochemistry (IHC) using polyclonal antibody specific for human CD3 (Dako, Glostrup, Denmark) and monoclonal antibodies specific for human CD4 (clone 1F6; Nichirei Biosciences Inc., Tokyo, Japan), CD8 (clone C8/144B; Nichirei Biosciences Inc.), CD20 (clone L26; Dako), CD56 (clone 1B6; Nichirei Biosciences Inc.), CD79α (clone HM57; Dako) and mouse Ki-67 antigen (clone TEC-3; Dako). For 'visualization' of immunoreactivity, the EnVision System (Dako) was used in conjunction with 3-amino-9-ethylcarbazole (ACE) or 3, 3′-diaminobenzidine (DAB) and sections were counterstained with haematoxylin.

The majority of the neoplastic cells, irrespective of the cell type, were positive for CD3 (Fig. 1C) and CD4, but negative for CD20, CD79α and CD8, exhibiting the T helper cell phenotype. The larger neoplastic cells were also positive for the natural killer (NK) cell marker CD56 (Fig. 1D). Both cell types expressed the proliferation marker Ki-67 (Fig. 1E).

To confirm the presence of simian LCV in neoplastic cells, in-situ hybridization (ISH) was performed on formalin-fixed and paraffin wax-embedded sections using peptide nucleic acid (PNA) probes complementary to EBV-encoded small RNAs (EBERs) (Epstein-Barr Virus PNA Probe, Dako). Signals for EBERs were 'visualized' using the PNA ISH detection Kit (Dako) and sections were counterstained with nuclear fast red. EBER transcripts were detected more frequently in the larger neoplastic cells than in the smaller neoplastic cells. The EBER-positive cells were scattered throughout the tumour tissues (Fig. 1F).

Like many other species of non-human primates, Japanese macaques are latently infected with the EBV-related herpesvirus, simian LCV (Carville and Mansfield, 2008). Previous studies have demonstrated that almost all Japanese macaques are seropositive for simian LCV (Ishida and Yamamoto, 1987; Ishida *et al.*, 1993). However, simian LCV infection is almost always subclinical and there is little evidence for the oncogenic potential of simian LCV in Japanese macaques. In the present case, ISH revealed EBER transcripts in the neoplastic lymphoid cells, indicating the role of simian LCV in the development of lymphoma. At present, it is impossible to detect EBER transcripts with specific probes for Japanese macaque LCV due to the lack of available sequence data, but we were able to detect the viral RNA with a probe used for the detection of EBV. Previous studies have shown that EBER genes are relatively conserved between EBV and simian LCVs (73 and 42% nucleotide identities between EBV and Rhesus macaque LCV in the EBER1 and EBER2 genes, respectively) and contain highly conserved regions (Rao *et al.*, 2000). In addition, it is unlikely that the probes detected transcripts from EBV, rather than simian LCVs in the neoplastic cells, because EBV is not pathogenic

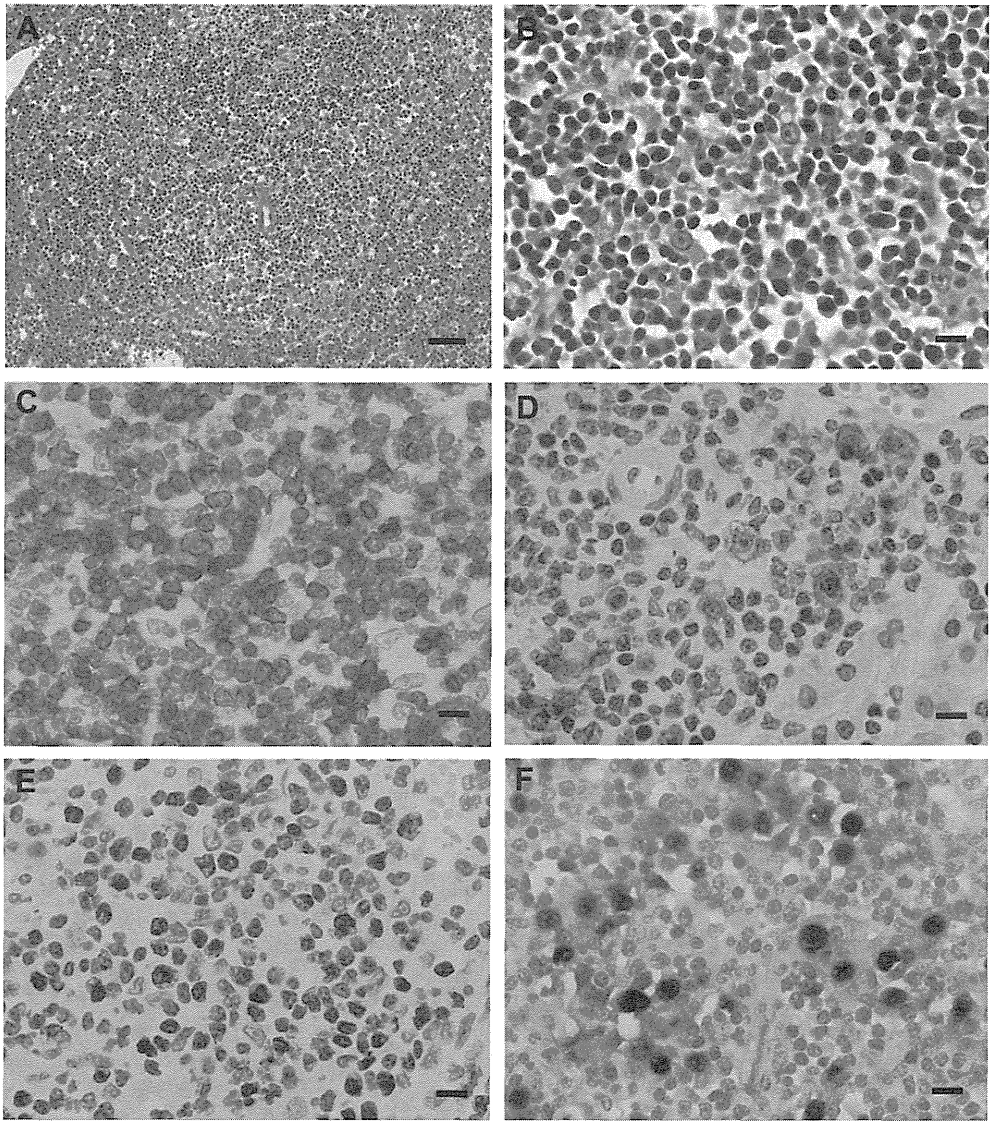

Fig. 1. Histopathology, IHC and ISH of the neoplastic lesions found in the spleen (A, B, C, D and F) and liver (E) of a Japanese macaque. (A) Neoplastic cells in the red and white pulp of the spleen. HE. Bar, 50 μm. (B) Higher magnification of (A). The lesions are composed of two types of neoplastic cells. HE. Bar, 10 μm. (C) The majority of the neoplastic cells express CD3. IHC. Bar, 10 μm. (D) The larger neoplastic cells show membrane expression of CD56. IHC. Bar, 10 μm. (E) Ki-67 is expressed by both types of neoplastic cells. IHC. Bar, 10 μm.(F) EBER transcripts are seen mainly in the larger neoplastic cells. ISH. Bar, 10 μm.

for macaques, as shown in experimental infection studies (Frank *et al.*, 1976; Levine *et al.*, 1980). Therefore, this diagnostic technique would facilitate the detection of simian LCV in histological sections of tissue from Japanese macaques and contribute to a better understanding of the pathogenicity of the virus.

In people, EBV has been implicated in the development of a wide range of B cell malignancies (Carbone *et al.*, 2008). Similarly, simian LCVs have been shown to be associated with B-cell lymphomas in non-human primates and this provides an important animal model for studying EBV-associated lymphoma (Feichtinger *et al.*, 1992; Pingel *et al.*, 1997; Carville and Mansfield, 2008). However, whereas EBV has been reported to be associated with several types of T/NK-cell lymphomas (Carbone *et al.*, 2008; Piccaluga *et al.*, 2011) it remains unclear whether simian LCVs also contribute to the development of these malignancies. Therefore, it is noteworthy that the present case clearly exhibited the immunophenotypes of T/NK cells that were positive for CD3 and CD56. To date, there has been only one report of T/NK-cell lymphoma associated with simian LCV. Interestingly, the previous case was also found in a Japanese macaque and was

diagnosed as the nasal type of NK/T-cell lymphoma based on the involvement of the nasal cavity, the absence of expression of the T-cell antigen CD3 and the predominant expression of the NK-cell antigen CD16 (Suzuki *et al.*, 2005). There was a distinct difference in CD3 expression between the present and previous cases and, therefore, this is the first report of simian LCV-associated T/NK-cell lymphoma with predominant expression of CD3 in non-human primates. It remains difficult to determine whether simian LCV preferentially induces T/NK-cell lymphoma in Japanese macaques. Further studies are needed to define the cell tropism of simian LCV in Japanese macaques, but the Japanese macaque may serve as a potential animal model of EBV-associated T/NK-cell lymphomas in man.

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References

Beniashvili DS (1989) An overview of the world literature on spontaneous tumors in nonhuman primates. *Journal of Medical Primatology*, **18**, 423–437.

Carbone A, Gloghini A, Dotti G (2008) EBV-associated lymphoproliferative disorders: classification and treatment. *Oncologist*, **13**, 577–585.

Carville A, Mansfield KG (2008) Comparative pathobiology of macaque lymphocryptoviruses. *Comparative Medicine*, **58**, 57–67.

Feichtinger H, Li SL, Kaaya E, Putkonen P, Grunewald K *et al.* (1992) A monkey model for Epstein Barr virus-associated lymphomagenesis in human acquired immunodeficiency syndrome. *Journal of Experimental Medicine*, **176**, 281–286.

Frank A, Andiman WA, Miller G (1976) Epstein-Barr virus and nonhuman primates: natural and experimental infection. *Advances in Cancer Research*, **23**, 171–201.

Hayami M, Komuro A, Nozawa K, Shotake T, Ishikawa K *et al.* (1984) Prevalence of antibody to adult T-cell leukemia virus-associated antigens (ATLA) in Japanese monkeys and other non-human primates. *International Journal of Cancer*, **33**, 179–183.

Ishida T, Suzuki J, Yamamoto K (1993) Serological features of infection with an Epstein-Barr-virus-like agent in Japanese macaques (*Macaca fuscata*). *Folia Primatologica (Basel)*, **61**, 228–233.

Ishida T, Yamamoto K (1987) Survey of nonhuman primates for antibodies reactive with Epstein-Barr virus (EBV) antigens and susceptibility of their lymphocytes for immortalization with EBV. *Journal of Medical Primatology*, **16**, 359–371.

Levine PH, Leiseca SA, Hewetson JF, Traul KA, Andrese AP *et al.* (1980) Infection of rhesus monkeys and chimpanzees with Epstein-Barr virus. *Archives of Virology*, **66**, 341–351.

Piccaluga PP, Gazzola A, Agostinelli C, Bacci F, Sabattini E *et al.* (2011) Pathobiology of Epstein-Barr virus-driven peripheral T-cell lymphomas. *Seminars in Diagnostic Pathology*, **28**, 234–244.

Pingel S, Hannig H, Matz-Rensing K, Kaup FJ, Hunsmann G *et al.* (1997) Detection of Epstein-Barr virus small RNAs EBER1 and EBER2 in lymphomas of SIV-infected rhesus monkeys by in-situ hybridization. *International Journal of Cancer*, **72**, 160–165.

Rao P, Jiang H, Wang F (2000) Cloning of the rhesus lymphocryptovirus viral capsid antigen and Epstein-Barr virus-encoded small RNA homologues and use in diagnosis of acute and persistent infections. *Journal of Clinical Microbiology*, **38**, 3219–3225.

Suzuki J, Goto S, Kato A, Hashimoto C, Miwa N *et al.* (2005) Malignant NK/T-cell lymphoma associated with simian Epstein-Barr virus infection in a Japanese macaque (*Macaca fuscata*). *Experimental Animals*, **54**, 101–105.

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An Ocular Infection Model Using Suckling Hamsters Inoculated With Equine Herpesvirus 9 (EHV-9): Kinetics of the Virus and Time-Course Pathogenesis of EHV-9–Induced Encephalitis via the Eyes

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Abstract

By using a new member of the neurotropic equine herpesviruses, EHV-9, which induced encephalitis in various species via various routes, an ocular infection model was developed in suckling hamsters. The suckling hamsters were inoculated with EHV-9 via the conjunctival route and were sacrificed after 6, 12, 24, 36, 48, 72, 96, 120, and 144 hours (h) post inoculation (PI). Three horizontal sections of the brains, including the eyes and cranial cavity, were examined histologically to assess the viral kinetics and time-course neuropathological alterations using a panoramic view. At 6 to 24 h PI, there were various degrees of necrosis in the conjunctival epithelial cells, as well as frequent mononuclear cell infiltrations in the lamina propria and the tarsus of the eyelid, and frequent myositis of the eyelid muscles. At 96 h PI, encephalitis was observed in the brainstem at the level of the pons and cerebellum. EHV-9 antigen immunoreactivity was detected in the macrophages circulating in the eyelid and around the fine nerve endings supplying the eyelid, the nerves of the extraocular muscles, and the lacrimal glands from 6 h to 144 h PI. At 96 h PI, the viral antigen immunoreactivity was detected in the brainstem at the level of the pons and cerebellum. These results suggest that EHV-9 invaded the brain via the trigeminal nerve in addition to the abducent, oculomotor, and facial nerves. This conjunctival EHV-9 suckling hamster model may be useful in assessing the neuronal spread of neuropathogenic viruses via the eyes to the brain.

Keywords

EHV-9, eye, suckling hamsters, neuropathogenesis

EHV-9, the newest member of the equine herpesvirus family, is a highly neurotropic herpesvirus first isolated in an outbreak of encephalitis in a herd of Thomson's gazelles (*Gazella thomsoni*) kept at a zoological garden.[13,36] Although the natural host of EHV-9 and the complete host range are still unknown, a member of the equidae is suspected to be a natural host of EHV-9, as domestic horses (*Equus caballus*) inoculated intranasally with EHV-9 exhibited only mild encephalitis and there were no resulting deaths.[34] In addition, a high seroprevalence for EHV-9 without any signs was detected among Burchell's zebras (*Equus burchelli*) in Serengeti National Park in Tanzania.[2]

Recently, it has been reported that the host range of EHV-9 has expanded to include Grevy's zebras (*Equus grevysi*) and polar bears (*Ursus maritimus*),[7,31] as well as a giraffe (*Giraffa camelopardalis reticulata*).[18] EHV-9 is most closely related to the recently re-emergent neurotropic pathogen, EHV-1; however, cleavage by restriction enzymes showed that the DNA fingerprint of EHV-9 is different from that of EHV-1 and other equine herpesviruses.[13]

Emerging EHV-9 infections are of special interest because there is a wide range of susceptible hosts, which includes mice and rats,[13] hamsters,[12] goats,[35] pigs,[26] dogs and cats,[37,38] and common marmosets.[21] Recently, EHV-9 was used to induce encephalitis in hamsters by different routes of inoculation, including the oral (25%), peritoneal (25%), and ocular (75%) routes; however, the intravenous route failed to induce

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encephalitis and there were discrepancies in the distribution of EHV-9 antigen immunoreactive cells in the location and severity of the cerebral lesions. This suggests the possibility of transmission of the virus through regional nerves (the non-olfactory route), after initial propagation at the site of viral entry.[9] The exact pathway through which the virus travels from the eye, which proved to have a high incidence of infection (75% of the animals became infected) in comparison to the other routes, to the brain is still unclear.

Thus, the aim of the present study is to elucidate the pathogenesis of EHV-9 ocular-induced encephalitis. Ocular transmission of viruses to the central nervous system (CNS) has been suspected in many viral diseases such as Cercopithecine herpes virus 1 (B-virus) in humans[5] and herpes simplex virus in children with labial vesicles after rubbing of the eye.[14] Fatal infection by B-virus in humans via ocular exposure from biological fluid from macaque monkeys has been reported.[5] A number of viruses have been studied following intraocular inoculation. In general, intraocular inoculation has been found to be effective in inducing CNS infection, with or without inducing specific ocular pathological lesions. Many viruses, including poliomyelitis virus,[10,22] rabies virus,[29] vesicular stomatitis virus,[30] fox encephalitis virus,[15] and equine encephalomyelitis,[20] successfully induced brain infection following intraocular inoculation. The ocular route may be important in the transmission of many viruses in the laboratory as well as in the field.

In the present study, we describe the use of a suckling hamster model to assess primary infection in the eye, followed by the spread of the virus through neuronal routes to the brain. The suckling hamsters were useful in assessing viral kinetics and pathogenesis because panoramic horizontal views of the head, including the eye, orbital cavity, nasal cavity, cranial nerves, and brain, are very similar to MRI (magnetic resonance imaging) in human radiography. In addition, the eyes were still closed at this age in the suckling hamsters, which enabled the precise delivery of the exact inocula to an enclosed site in the conjunctival sac between the eye and eyelid, which is not available in adult animals with open eyes. Suckling hamsters have been used in the past to elucidate the pathogenesis of various viruses, including the mumps virus, via intracerebral inoculation,[23,33] and recently, the suckling hamster model was used to study the spread of EHV-9 from the nasal cavity to the brain by nasal inoculation.[8]

Materials and Methods

Animals

Nine pregnant Syrian hamsters (*Mesocricetus auratus*) dams at gestational day 10 were purchased from a commercial breeder (SLC Inc., Hamamatsu, Japan). The animals were housed in an isolated biohazard cabinet and were fed a basal pellet diet (Oriental MF, Oriental Yeast Co., Tokyo, Japan) and bottled water ad libitum until they gave birth, after which they were left to rear the pups until the end of the experiment. This experiment was conducted in accordance with the pertinent laws and regulations on the treatment and use of laboratory animals. The experiment protocol was approved by the Animal Experiment Committee for Animal Welfare in the Faculty of Applied Biological Science at Gifu University.

Inoculation of virus. Twenty-nine 6-day-old suckling hamsters were bilaterally inoculated via the conjunctival route with 10 μL (10^6 PFU) of EHV-9 virus solution. The eyelid was grasped, and virus solution was injected into the conjunctival sac between the eyelid and eye (the eyes are still closed in animals at this age). Three each of the inoculated animals were sacrificed at 6, 12, 24, 36, 48, 72, 96, 120, and 144 h postinoculation (PI), respectively, or when they were moribund (one animal after 96 hr and another after 120 hr PI). Five uninfected animals were kept as a control group and were inoculated in the conjunctival sac with a sham inoculum that consisted of sterile cell culture medium (MEM). The animals were checked for clinical signs at least twice daily, in the morning and afternoon. Two inoculated animals died after 6 h PI and their data were excluded from the experiment as we believed that the animals died as a result of the inoculation procedure.

Necropsy, histopathology, and immunohistochemistry. After necropsy, the heads were separated from the bodies and were fixed in 10% neutralized buffered formalin. The fixed heads were sectioned on the horizontal plane at two levels (the first section extending cranially from the supraorbital area passing through the cerebral cortex, the second section extending cranially through the eye and brain; from the second section, ventral [Fig. 1a] and dorsal [Fig. 1b] sections were obtained) to obtain the connection between the eye and brain at the same section and were then dehydrated and embedded in paraffin wax by routine methods. They were then sectioned at 3 μm, stained with hematoxylin and eosin (HE), and examined by light microscopy. The bones, including the skulls, of the suckling hamsters were soft enough to prepare histological slides with good quality without decalcification.

Paraffin-embedded sections of the head, including the brain and eyes, were immunolabeled with EHV-9 rabbit antiserum by the avidin-biotin-complex (ABC) immunoperoxidase method, as described previously,[38] with ABC kits (Vector Laboratories, Burlingame, CA, USA). The primary antibody was EHV-9 antiserum (1:800, 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 hematoxylin counterstain. Tissue sections from confirmed EHV-9–infected hamsters were used as positive control samples, and sera from a nonimmunized rabbit and goat were used as a negative control treatment on positive control specimens.

Results

Clinical Findings

The inoculated hamsters showed various degrees of clinical signs, including depression and uncoordinated movements,

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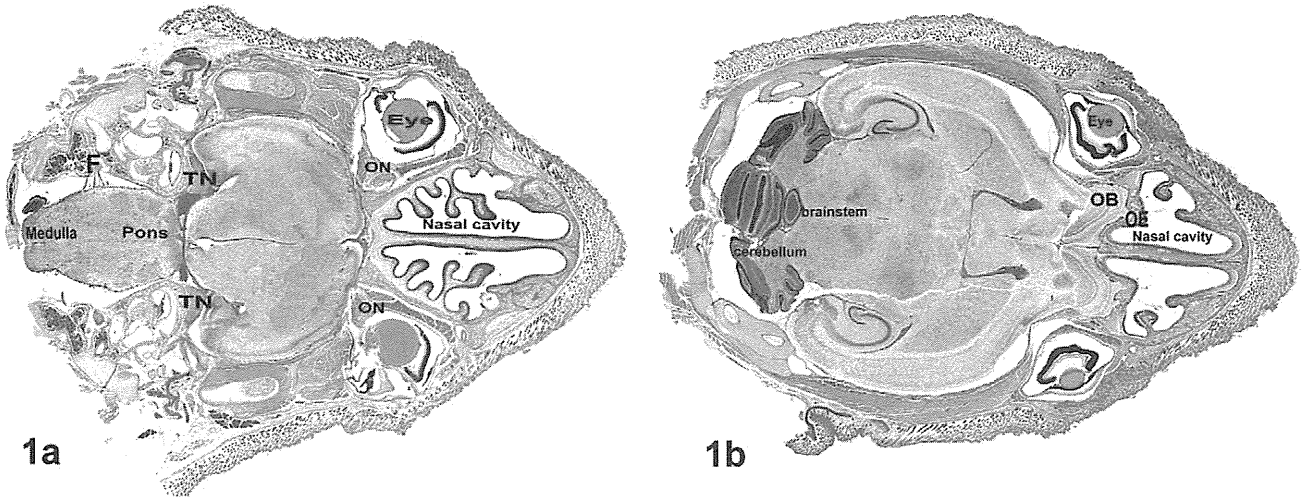

Figure 1. a. Hamster, brain, horizontal section showing the head from the eye to the whole brain from the ventral view; TN, trigeminal nerve; ON, optic nerve; F, facial nerve. Hematoxylin and eosin (HE). b. Hamster, brain, horizontal section showing the head from the eye to the whole brain from the dorsal view at the level of the forebrain, brainstem, and nasal cavity; OE, olfactory epithelium; OB, olfactory bulb. HE.

Table 1. Histopathological Changes Induced in the Eye and Brain in EHV-9 Inoculated Animals.[a]

		Hours Post Inoculation (*n*)[b]								
Tissue	Lesion	6 (3)	12 (3)	24 (3)	36 (3)	48 (3)	72 (3)	96 (3)	120 (3)	144 (3)
Conjunctiva	Necrosis (x)[c]	+ (1)	+ (2)	++ (2)	++ (2)	—	—	—	—	—
	Infiltration of inflammatory cells	+ (2)	++ (3)	+++ (3)	+++ (3)	++ (2)	+ (2)	—	—	—
Lacrimal gland	Infiltration	—	++ (1)	++ (2)	++ (2)	+ (2)	+ (1)	+ (1)	+ (1)	—
Eye muscles	Myositis	—	—	+ (1)	+ (2)	++ (2)	++ (2)	+ (1)	—	—
Brain	Brainstem encephalitis	—	—	—	—	—	—	+ (2)	++ (2)	+++ (3)
	Extended encephalitis	—	—	—	—	—	+ (1)	—	+ (2)	++ (3)

[a]Histopathologic score: —, none; +, slight; ++, moderate; +++, severe.
[b]*n*, number of sacrificed animals at this hour post inoculation.
[c]x, number of animals showed the pathological changes.

starting at 96 h PI. By 120 h PI, all of the animals exhibited severely uncoordinated movement, as well as paralysis, sometimes unilaterally. The animals were unable to crawl normally, were dysphagic, became depressed, and eventually died from the neurologic disorder.

Gross observation and histopathology. Grossly, there were no apparent abnormalities in the organs or tissues of the inoculated animals. Horizontal sectioning of the head in the suckling hamsters made it easy to view the orbital cavity, including the eye with all of its compartments, and most of the cranial nerves connections to the brain and brain itself were visible in a histological section (Figs. 1a, 1b). No microscopic abnormalities were noted in the five uninoculated control mice at any interval of the experiment. Histopathological alterations in the eye and brain in animals inoculated with EHV-9 via the conjunctival route are summarized in Table 1 and as follows:

At 6 h PI: Single cell necrosis in the conjunctival mucosal epithelial cells was observed, along with a few infiltrations predominantly neutrophils, macrophages, and few lymphocytes, in between and associated with the conjunctival mucosal epithelial cells and often free in the conjunctival sac in comparison to control animals.

At 12 h PI: There were variable but increased numbers of neutrophils, macrophages, and lymphocytes in the lamina propria and tarsus of the eyelid, which sometimes extended to the connective tissue of the cutaneous part of the eyelid, along with myocyte necrosis within the eyelid muscles, associated with increased numbers of macrophages and few neutrophils and lymphocytes (Fig. 2). In addition, there were varying increases in macrophages and lymphocytes in the lacrimal glands, as well as a slight focal necrosis and desquamation of the epithelial cells of conjunctival mucosa. In some cases, the inflammatory reaction extended from the subcutis of the eyelid to the skin in the face.

At 24 h PI: There were multifocal necrosis and desquamation of the conjunctival mucosal epithelium (Fig. 3), together with marked increases in neutrophils, macrophages, and lymphocytes in the eyelid, myositis of extraocular muscles characterized by

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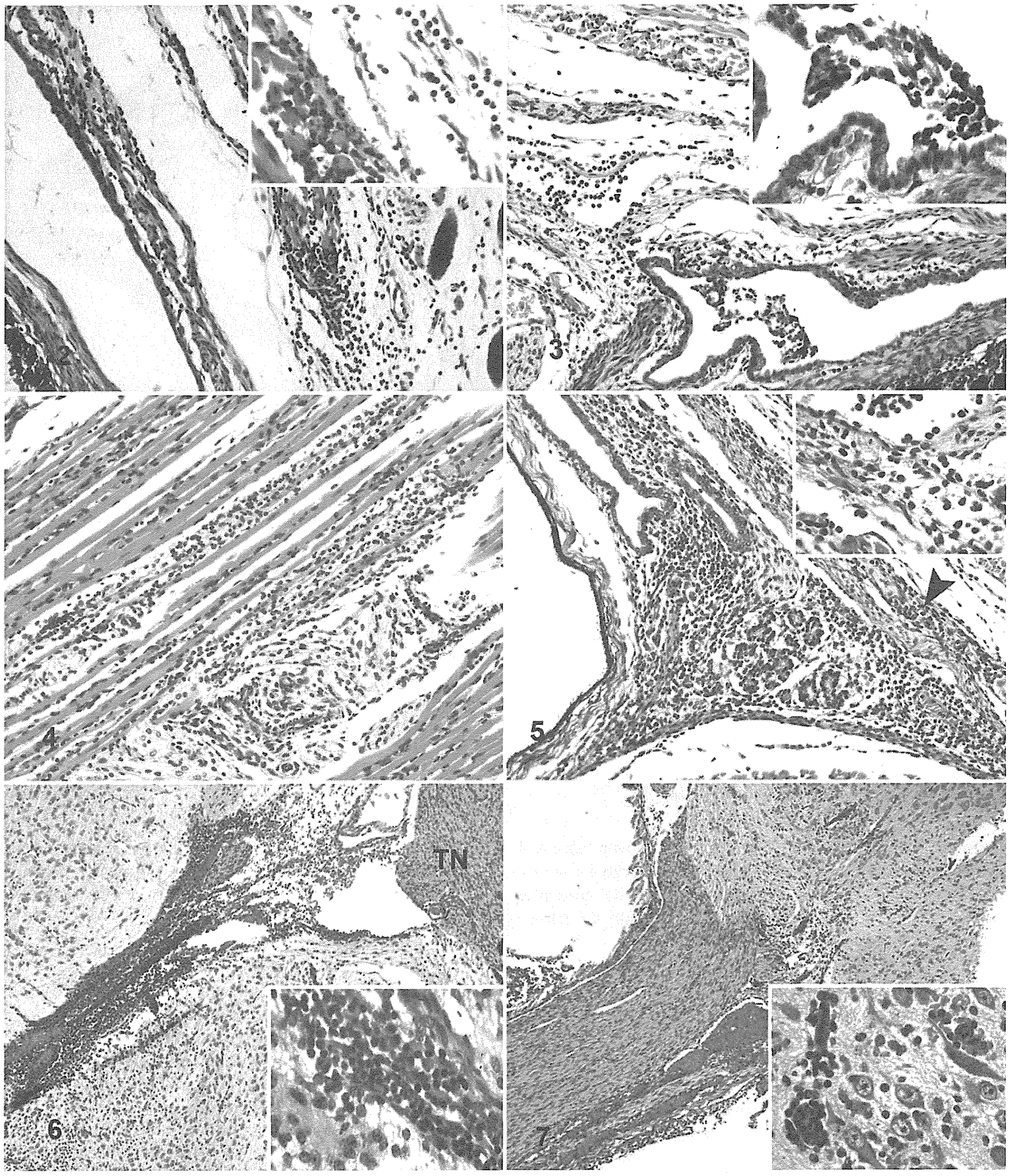

Figure 2. Suckling hamster, eyelid; 12 h post inoculation, there is infiltrations of neutrophils, macrophages, and lymphocytes in the epithelium, lamina propria and muscles of the eyelid, in addition to necrosis of the eyelid muscle. Inset: Higher magnification showing myositis (macrophages and a few lymphocytes and neutrophils) myocyte necrosis and myophagia. Hematoxylin and eosin. **Figure 3.** Suckling hamster, eyelid; 24 h post inoculation, there is conjunctival epithelial cell necrosis and inflammatory infiltrates of neutrophils, macrophages, and lymphocytes, in the lamina propria, tarsus. Inset: Higher magnification showing necrosis and sloughing of the conjunctival epithelial cells. Hematoxylin and eosin.

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moderate increases in macrophages and lymphocytes (Fig. 4). Vasculitis, perivasculitis, and neuritis of the nerves supplying these muscles were observed in few animals and moderate infiltrations of the lacrimal glands with macrophages and lymphocytes were also observed.

At 36h PI: There were marked increases in macrophages, lymphocytes, and few neutrophils in the eyelid (Fig. 5) and within the terminal endings supplying the eyelid, with associated edema of nerve fibers and disruption of nerve axons.

At 48 h PI: There was mild meningitis in the form of mild focal or diffuse lymphocytes infiltration together with macrophages and few neutrophils and hemorrhages at the level of the entrance of the trigeminal sensory nerve to the brainstem, as well as mild neuritis of the trigeminal sensory nerve characterized by few lymphocytic cell infiltrations and vasculitis. The trigeminal and palatine ganglion showed moderate neuronal necrosis and glial reactions consisting of neuronophagia to the ganglion cells. In addition, a few neuroglial cells and lymphocytes had infiltrated the area around the ganglion cells. Very mild increases of macrophages and lymphocytes in the eyelid as well as myositis of the extraocular muscles were observed.

At 72 h PI: One animal with neuronal signs had meningoencephalitis extending from the olfactory bulb to the brainstem, which consisted of neuronal necrosis, perivascular aggregates consisting of lymphocytes, macrophages, and few neutrophils, gliosis, intranuclear inclusion bodies, and diffuse lymphocytic infiltrates together with few macrophages and vasculitis in the meninges. In addition, the olfactory epithelium in this animal showed severe and wide erosion in the mucosa, together with significant purulent inflammation. Trigeminal ganglionitis was also observed. However, the other animals had only meningitis extending to the Virchow-Robin space characterized by infiltration of lymphocytes and neutorphils and haemorrhages (Fig. 6) at the level of the brainstem at the entrance of the trigeminal sensory nerve. Myositis of the extraocular muscles was observed in some cases, in addition to mild neuritis of the nerves supplying those muscles.

At 96 h PI: The brainstem at the level of the pons, especially at the entrance of the sensory branch of the trigeminal nerve to the brain (Fig. 7), exhibited encephalitis consisting of neuronal degeneration and necrosis, perivascular cuffing of lymphocytes, macrophages, and few neutrophils and gliosis, which extended to the trigeminal sensory nucleus.

At 120 h PI: The encephalitis was observed in the pons and medulla oblongata, including the entrance of the sensory branch of the trigeminal nerve, abducent, and the facial nerves.

At 144 h PI: In addition to the pons and medulla oblongata, the encephalitis was also observed in the frontal and temporal lobes of the cerebral cortex in the brain as well as the cervical part of the spinal cord.

Immunohistochemistry. Immunohistochemical staining with EHV-9 antibody was used to determine the viral kinetics of transmission from the eye to the brain. The immunohistochemical detection of EHV-9 in the eye and the brain is summarized as follows:

At 6 to 12 h PI: Occasional nuclear immunoreactivity was observed in the epithelial cells of the conjunctival mucosa (Fig. 8), as well as cytoplasmic and membrane-associated immunoreactivity of most likely infiltrating macrophages and possibly local dendritic cells in the lamina propria and tarsus of the eyelid. Other cells of the eyelid and eyes showed no immunoreactivity.

At 24 h PI: There was frequent immunoreactivity in the macrophages, especially around the fine nerve endings of the eyelid and the macrophages infiltrating the extraocular muscles of the eye as well as those infiltrating the lacrimal glands.

At 36 to 48 h PI: There was frequent and more intense immunoreactivity in the macrophages in the eyelid around the nerve endings (Fig. 9), the macrophages that had infiltrated the lacrimal glands (Fig. 10), the muscles of the eyes, and the area around the nerves supplying muscles before the entrance to the cranial cavity. No immunoreactivity was observed in the brain.

At 72 h PI: In addition to macrophages freely migrating through the eyelid and around the eye, lacrimal glands, and eye muscles, there was cytoplasmic and membrane-associated EHV-9 immunoreactivity in olfactory epithelium and trigeminal ganglia, nuclear and cytoplasmic immunoreactivity in neurons in olfactory bulb, brainstem, and cytoplasmic immunoreactivity in inflammatory cells, and nerve fibers at the trigeminal nerve entrance to the brainstem (Fig. 11) in the case that showed nervous signs. However, no immunoreactivity was observed in the brain in other cases.

At 96 h PI: EHV-9 antigen immunoreactivity was found only in neurons and few inflammatory cells in the brainstem at the level of the pons and cerebellum, close to the entrance of the sensory branch of the trigeminal nerve; however, the olfactory bulb and other areas of the brain showed no immunoreactivity to EHV-9 antigen. In addition, the macrophages present within the eyelid and the eye muscles and lacrimal glands showed immunoreactivity to EHV-9 antigen, but it was less intense than those in

Figure 4 (continued). Figure 4. Suckling hamster, extraocular muscle; 24 h post inoculation, there is myositis characterized by macrophages and lymphocytes infiltration and myocyte necrosis. Hematoxylin and eosin. **Figure 5.** Suckling hamster, eyelid; 36 h post inoculation, there is severe infiltration of inflammatory cells mostly of lymphocytes, as well as neuritis of the terminal nerve endings (arrow) supplying the eyelid. Higher magnification showing neuritis of the terminal nerve endings characterized by infiltration mostly of lymphocytes and disruption of the nerve axon. Hematoxylin and eosin. **Figure 6.** Suckling hamster, brain; 72 h post inoculation, trigeminal sensory nerve (TN) meningoencephalitis and perineuritis, with hemorrhage. Inset: Higher magnification showed infiltration mainly of lymphocytes in the meninges. Hematoxylin and eosin. **Figure 7.** Suckling hamster, brain; 96 h post inoculation, the entrance of the sensory branch of the trigeminal nerve showed mild neuritis and perineuritis. Inset: Higher magnification of the pons showed encephalitis consisting of neuronal degeneration and necrosis, perivascular cuffing, and gliosis. Hematoxylin and eosin.

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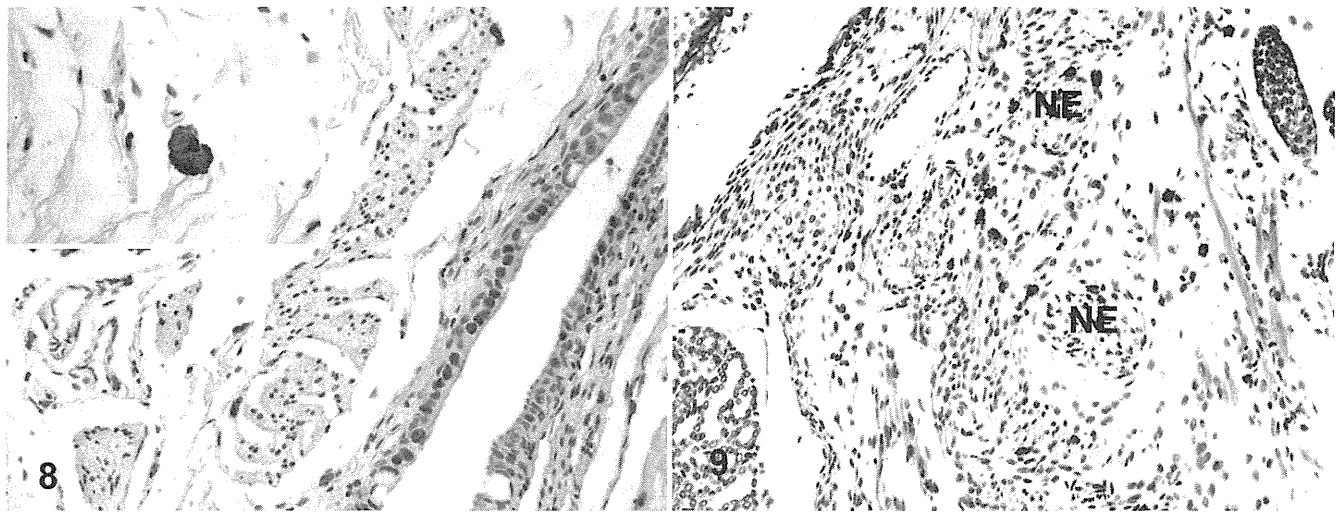

Figure 8. Suckling hamster, eye; immunolabelling with EHV-9 antibody, the conjunctival epithelial cells exhibited a positive immunoractivity. Inset: Higher magnification showed immunoreactive macrophage. ABC method, counterstained with Mayer's hematoxylin. **Figure 9.** Suckling hamster, eye; immunolabelling with EHV-9 antibody. At 36 to 48 h post inoculation, positive reaction was observed in most of the macrophages circulating around the nerve endings (NE). ABC method, counterstained with Mayer's hematoxylin. **Figure 10.** Suckling hamster, eye; immunolabelling with EHV-9 antibody. At 36 to 48 h post inoculation, positive reaction was observed in some of the macrophages infiltrating the lacrimal gland in the eyelid. ABC method, counterstained with Mayer's hematoxylin. **Figure 11.** Suckling hamster, brain; immunolabelling with EHV-9 antibody. At 72 h post inoculation, viral antigen was present in some of the inflammatory cells of the trigeminal sensory nerve root. ABC method, counterstained with Mayer's hematoxylin.

previous cases. The neurons of trigeminal ganglia showed weak immunoreactivity in their cytoplasm.

At 120 to 144 h PI: There was immunoreactivity in the neurons and some inflammatory cells in the medulla oblongata, with specific intensity in the midline and close to the entrance of the abducent nerve, as well as the pons and cerebellum. In addition, the viral antigen immunoreactivity was detected in some neurons in the frontal and temporal lobes of the cerebral cortex. EHV-9 was detected in the cervical part of the spinal cord, in the neurons and inflammatory cells in areas showing myelitis.

The distributions of the viral antigen immunoreactivity in the horizontal sections of the brain after 96 h and 144 h PI are depicted in Figures 12a and 12b, respectively. At 96 h, EHV-9 antigen immunoreactivity was detected only in the brainstem at the level of the pons close to the entrance of the sensory branch of the trigeminal nerve; however, at 144 h PI EHV-9 antigen immunoreactivity was detected in the medulla oblongata, close to the entrance of the abducent nerve, as well as the pons and extended to frontal and temporal lobes of the cerebral cortex.

Discussion

EHV-9, the newly emerging neurotropic herpesvirus, has been studied with respect to pathogenesis and route of brain infection including the nasal, ocular, peritoneal, oral, and

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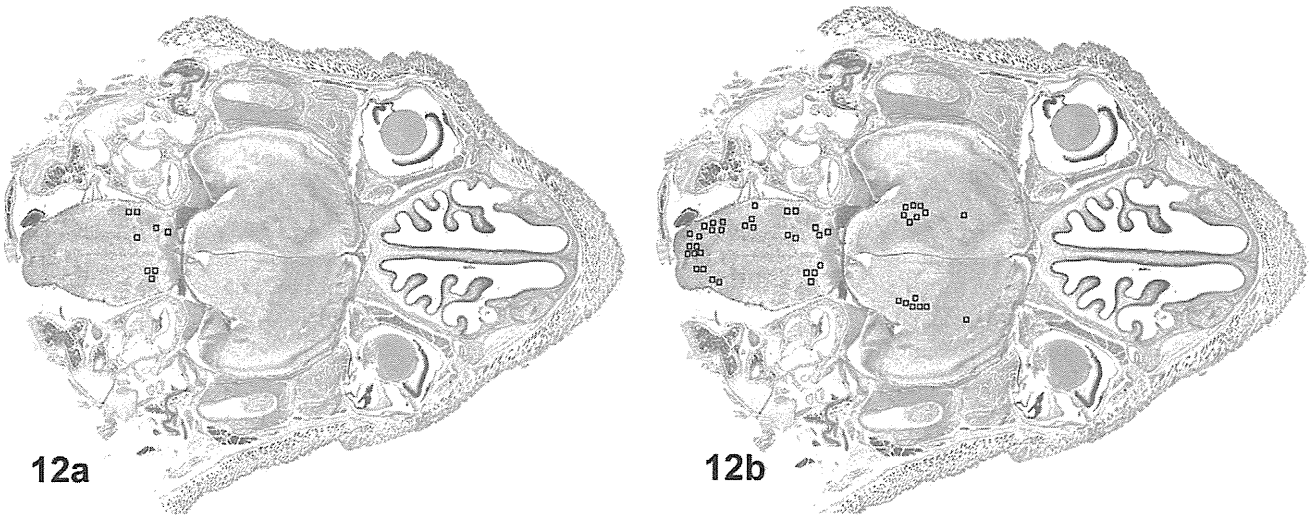

Figure 12. a. Suckling hamster, brain, distribution of EHV-9 antigen immunoractivity. At 96 h post inoculation (PI), viral antigen was detected in the pons. b. Suckling hamster, brain, distribution of EHV-9 antigen immunoractivity. At 144 h PI, viral antigen was found in the pons, cerebellum, medulla oblongata, and the frontal and temporal lobes of the cerebral cortex.

intravenous routes. The nasal and ocular routes show higher incidences of infection (100% and 75% of animals became infected, respectively) in comparison to the other routes.[9] The ocular route for the transmission of neuropathogenic viruses is gaining in importance in the laboratory as well as in the field. Fatal infections with Cercopithecine herpes virus 1 (B-virus) via ocular splashes with biological fluid from macaque monkeys without percutaneous injury have been reported in humans,[4,5] and in this case, conjunctivitis developed 10 days later after exposure, and the victim then died from B-virus–induced encephalomyelitis 6 weeks later. Possible infection in the CNS following ocular inoculation was reported with respect to many viruses, such as EHV-9 in hamsters,[9] poliomyelitis virus,[10,22] rabies virus,[29] vesicular stomatitis virus,[30] fox encephalitis virus,[15] and equine encephalomyelitis.[20] However, the detailed kinetics and pathogenesis in terms of access to the CNS are still unclear because of difficulties in tracing the entrance of the cranial nerves to the brain through the cranial bone. The suckling hamster model following conjunctival inoculation with EHV-9 was developed to assess the kinetics and pathogenesis of EHV-9 using a panoramic view that includes the eye, olfactory epithelium, olfactory nerve, and optic nerve as well as other cranial nerves and the brain by using horizontal sections. Horizontal sections at two levels were prepared in the present study to assure easy tracking from the viral inoculation site of the eye to the main affected site of the brain. Based on histology and immunohistochemistry, conjunctival inoculation with EHV-9 first results in conjunctivitis characterized by conjunctival epithelial cell necrosis, mononuclear cell infiltrations in the lamina propria of the eyelid, as well as myositis of the eyelid muscles and infiltration of lacrimal glands, which may be an essential stage for propagation of the virus in the entry site for further travel to the CNS. At the early stage of infection (6 hr PI), the conjunctival epithelial cell as well as macrophages exhibited immunoreactivity to EHV-9 antigen, especially around the fine nerve endings, and this extended later to the eyelid muscles, lacrimal and extraocular muscles, as well as the nerves supplying those muscles. At the initial stage of infection, the macrophages migrating from surrounding tissue may play a very important role in the propagation and transmission of EHV-9 to the nerves of the eyes. Previously, the importance of macrophages in the pathogenesis of virus infections was reviewed by Mires.[24] Blood clearance studies suggested that most viruses are taken up by macrophages in the same way as other colloidal materials.[3,24] Since macrophages move freely through tissues, it has also been suggested that they play a primary role in the dissemination of viruses.[11] Therefore, the susceptibility or resistance to viral diseases could depend in large part on the macrophage-virus interaction. In an in vitro study on macrophages in suckling mice, herpesvirus was shown to have spread to adjacent cells of various types by macrophages.[17] The migrating macrophages may pick up the EHV-9 and transport it to the nerve endings supplying the eyelid and eye, including the ophthalmic branch of the trigeminal nerve and the abducent, oculomotor, and facial nerves. EHV-9 may then travel by way of these nerves to the brainstem causing encephalitis at the level of the pons and cerebellum. CNS lesions in these animals were restricted to the pons, medulla oblongata, and cerebellum, suggesting viral transport through the trigeminal, abducent, and facial nerves. A similar transneural passage has been described in porcine Pseudorabies virus (PRV) infection where virus gained access to the brain via the trigeminal and oculomotor nerves.[32] In cases of herpes simplex virus infection in rabbits, the inoculated virus was passed serially onto the scarified cornea, producing fatal encephalitis, where the virus did not enter the eye after inoculation onto the cornea, but passed to the conjunctiva and thereafter to the brain via the

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sensory branches of the trigeminal nerve.[14] Keeble et al. described lesions of natural B-virus infection in monkeys where CNS lesions were minimal and consisted of localized involvement of the pons and medulla oblongata.[19] In addition there were glial and lymphocytic infiltrations around the nerve roots of the trigeminal and facial nerves, in the nucleus and tract of the descending branch of the trigeminal nerve and in the solitary tract.[19] Encephalitis restricted to the brainstem was reported previously in cases of human herpes simplex virus 1 infection[16,25] and herpes simplex virus 2 infection.[6,28]

At the final stage EHV-9 virus expanded rostrally to the cerebrum as well as caudally to the spinal cord. There were some discrepancies in severity and distribution of the lesions. In some cases, the encephalitis was restricted to one-half of the brain, or there was bilateral involvement. The ocular EHV-9 suckling hamster model may be useful in pathogenesis studies of neurotropic virus such as B-Virus, especially those concerned with how virus gains access to the brain.

The optic nerve is considered to be part of the central nervous system, which is completely encased by three layers of meninges and is protected from contact with macrophages carrying the virus. With some viruses, such as the lymphocytic choriomeningitis virus (LCM), multiplication of the virus in the eyes does not induce ocular pathological changes following intraocular inoculation except mild transitory hyperemia;[27] however, in the present study marked conjunctivitis was noted. In our previous work with EHV-9 after nasal inoculation, we found that the virus traveled through the olfactory nerve after initial propagation in the olfactory receptor neurons, and that area of the brain that was affected earliest and to the greatest degree was the olfactory bulb;[8] however, in the present study, the pons and cerebellum were affected earliest.

There is still a possibility of other routes of transmission of EHV-9 after conjunctival inoculation, via the oral or nasal routes, because the eyes and the oral and nasal cavities are connected by means of the lacrimal (tear) duct. After conjunctival inoculation, (1) that virus may secondarily infect the olfactory nerve via the infection of the lacrimal gland, and (2) virus may also spread to the nasopharynx with the tear film via the nasolacrimal duct, providing an alternate method of infecting the olfactory nerve and accounting for the rhinitis noted in one animal with possibly dominant and earliest lesions in the olfactory bulb.

The suckling hamster model, using horizontal sections of the head following conjunctival inoculation, is very useful in elucidating the mechanism of EHV-9 transmission from the eye to the brain. Following conjunctival inoculation, EHV-9 appears to result in local conjunctivitis and infection of local macrophages, which transport the virus to local fine nerve endings; because of this, EHV-9 is able to invade the brain via the trigeminal, abducent, oculomotor, and facial nerves, inducing meningitis, encephalitis, and myelitis.

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Declaration of Conflicting Interests

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the contents of the paper.

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References

1. Banfield BW, Yap GS, Knapp AC, et al. A chicken embryo eye model for the analysis of alphaherpesvirus neuronal spread and virulence. *J Virol.* 1998;**72:**4580–4588.
2. Borchers K, Wiik H, Frolich K, et al. Antibodies against equine herpesviruses and equine arteritis virus in Burchell's zebra (*Equus Burchelli*) from the Serengeti ecosystem. *J Wildl Dis.* 2005;**41:** 80–86.
3. Brunner KT, Hurez D, McCluskey RT, et al. Blood clearance of P32-1abelled vesicular stomatitis and Newcastle disease viruses by the reticuloendothelial system in mice. *J Immunol.* 1960;**85:** 99–105.
4. Centers for Disease Control and Prevention. Fatal Cercopithecine herpesvirus 1 (B virus) infection following a mucocutaneous exposure and interim recommendations for worker protection. *Morb Mortal Wkly Rep.* 1998;**47:**1073–1076, 1083.
5. Centers for Disease Control and Prevention. *Hazard ID 5-Cercopithecine Herpesvirus 1 (B Virus) Infection Resulting From Ocular Exposure.* Atlanta, GA: National Institute for Occupational Safety and Health;1999.
6. Chu K, Kang D-W, Lee J-J, et al. Atypical brainstem encephalitis caused by herpes simplex virus 2. *Arch Virol.* 2002;**59:**460–463.
7. Donovan TA, Schrenzel MD, Tucker T, et al. Meningoencephalitis in a polar bear caused by equine herpesvirus 9 (EHV-9). *Vet Pathol.* 2009;**46:**1138–1143.
8. El-Habashi N, El-Nahass El-S, Fukushi H, et al. Experimental intranasal infection of equine herpesvirus 9 (EHV-9) in suckling hamsters: kinetics of viral transmission and inflammation in the nasal cavity and brain. *J Neuro Virol.* 2010;**16:**242–248.
9. El-Habashi N, Murakami M, El-Nahass E, et al. Study on the infectivity of equine herpesvirus 9 (EHV 9) by different routes of inoculation in hamsters. *Vet Pathol.* 2011;**48:**558–564.
10. Flexner S, Amoss HL. Localization of the virus and pathogenesis of epidemic poliomyelitis. *J Exp Med.* 1914;**20:**249–268.
11. Florman AL, Enders JF. The effect of homologous antiserum and complement on the multiplication of vaccinia virus in roller-tube cultures of blood mononuclear cells. *J Immunol.* 1942;**43:** 159–174.
12. Fukushi H, Taniguchi A, Yasuda K, et al. A hamster model of equine herpesvirus 9 induced encephalitis. *J Neuro Virol.* 2000; **6:**314–319.
13. Fukushi H, Tomita T, Taniguchi A, et al. Gazelle herpesvirus 1: a new neurotropic herpesvirus immunologically related to equine herpesvirus 1. *Virology.* 1997;**227:**34–44.

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14. Goodpasture EW, Teague O. Transmission of the virus of herpes febrilis along nerves in experimentally infected rabbits. *J Med Res.* 1923;**44:**139–184.
15. Green RG, Stulberg CS. Distemperoid virus interference in canine distemper. *Science.* 1946;**103:**497–498.
16. Jereb M, Lainscak M, Marin J, et al. Herpes simplex virus infection limited to the brainstem. *Wiener Klinische Wochenschrift.* 2005;**117:**13–14, 495–499.
17. Johnson RT. The pathogenesis of herpes virus encephalitis II. A cellular basis of the development of resistance with age. *J Exp Med.* 1964;**120:**359–374.
18. Kasem S, Yamada S, Kiupel M, et al. Equine herpesvirus type 9 in giraffe with encephalitis. *Emerg Infect Dis.* 2008;**14:**1948–1949.
19. Keeble SA, Christofinis GJ, Wood W. Natural virus-B infection in rhesus monkeys. *J Pathol Bacteriol.* 1958;**76:**189–199.
20. King LS. Experimental encephalitis: some factors affecting infection with certain neurotropic viruses. *J Exp Med.* 1940;**72:** 573–593.
21. Kodama A, Yanai T, Yomemaru K, et al. Acute neuropathogenicity with experimental infection of equine herpesvirus 9 in common marmosets (*Callithrix jacchus*). *J Med Primatol.* 2007;**36:** 335–342.
22. Landsteiner K, Levaditi C. La paralysie infantile experimentale (Deuxieme note). *Compt Rend Soc Biol.* 1909;**67:**787–789.
23. Lawrence K, Overman JR. Natural pathogenicity of mumps virus for suckling hamsters on intracerebral inoculation. *J Immunol.* 1953;**70:**147–151.
24. Mires CA. Aspects of the pathogenesis of virus diseases. *Bacteriol Rev.* 1964;**28:**30–71.
25. Miura S, Kurita T, Noda K, et al. Symmetrical brainstem encephalitis caused by herpes simplex virus. *J Clin Neurosci.* 2009;**16:** 589–590.
26. Narita M, Uchimura A, Kimura K, et al. Brain lesions and transmission of experimental equine herpesvirus type 9 in pigs. *Vet Pathol.* 2000;**37:**476–479.
27. Overman JR, William F, Friedewald MD. Multiplication of certain neurotropic viruses in the rabbit eye following intraocular inoculation. *J Exp Med.* 1950;**91:**39–51.
28. Pelligra G, Lynch N, Miller SP, et al. Brainstem involvement in neonatal herpes simplex virus type 2 encephalitis. *Pediatrics.* 2007;**120:**e442–e446.
29. Remlinger P. Contribution ~ t'gtude de la toxine rabique. Faits cliniques. *CR Soc Biol.* 1904;**5b6:**349.
30. Sabin AB, Olitsky PK. Influence of host factors on neuroinvasiveness of vesicular stomatitis virus. II. Effect of age on the invasion of the peripheral and central nervous systems by virus injected into the leg muscles or the eye. *J Exp Med.* 1937;**66:**35–57.
31. Schrenzel MD, Tucker TA, Donovan TA, et al. New hosts for equine herpesvirus 9. *Emerg Infect Dis.* 2008;**14:**1616–1619.
32. Takahashi H, Kai C, Yoshikawa Y, et al. Immunohistochemical analysis of pseudorabies virus spread through neurons innervating the eyeball. *Comp Immunol Microbiol Infect Dis.* 1995;**18:**275–281.
33. Takano T, Ohno M, Yamano T, et al. Experimental Hydrocephalus in suckling hamster induced by Myxovirus infection: pathogenesis of Hydrocephalus caused by Mumps virus. *Cong Anom.* 1991;**31:**115–128.
34. Taniguchi A, Fukushi H, Matsumura T, et al. Pathogenicity of a new neurotropic equine herpesvirus 9 (Gazelle herpesvirus 1) in horses. *J Vet Med Sci.* 2000;**62:**215–218.
35. Taniguchi A, Fukushi H, Yanai T, et al. Equine herpesvirus 9 induced lethal encephalitis in experimentally infected goats. *Arch Virol.* 2000;**145:**2619–2627.
36. Yanai T, Sakai T, Fukushi H, et al. Neuropathological study of gazelle herpesvirus 1 (equine herpesvirus 9) infection in Thomson's gazelles (*Gazella thomsoni*). *J Comp Pathol.* 1998;**119:**159–168.
37. Yanai T, Fujishima N, Fukushi H, et al. Experimental infection of equine herpesvirus 9 in dogs. *Vet Pathol.* 2003;**40:**263–267.
38. Yanai T, Tsujioka S, Sakai H, et al. Experimental infection with equine herpesvirus 9 (EHV-9) in cats. *J Comp Pathol.* 2003;**128:** 113–118.

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