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Comparative descriptive histopathological study of infection by various strains of Equine Herpesvirus Type 1 (EHV-1) in hamster model

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Summary

Equine Herpesvirus-1 (EHV-1) is a major cause of upper respiratory tract infections in horses, frequently associated with abortion in pregnant mares, neonatal death, and equine herpesvirus myeloencephalopathy (EHM). These clinical outcomes lead to substantial economic losses in the equine industry. Despite the recognized clinical significance of EHV-1, limited information is available regarding the comparative histopathological alterations induced by different viral strains under controlled experimental conditions. The objective of the present study was to investigate the pathogenicity of three distinct EHV-1 strains, namely RacL11, Kentucky D (KyD), and Ab4p, using a Syrian hamster model. A total of 32 specific pathogen-free male Syrian hamsters, three weeks of age, were randomly allocated into four groups (n = 8 per group). Each experimental group was intranasally inoculated with 10^3 plaque-forming units (PFU) of one EHV-1 strain in 50 µL of Eagle's minimum essential medium (MEM), while the control group received MEM alone. All hamsters infected with the KyD and RacL11 strains died on days 4 and 5 post-infection (p.i.), respectively, without exhibiting neurological symptoms. In contrast, animals infected with the Ab4p strain developed neurological signs, with four animals dying on day 5 p.i. and the remaining four on day 6 p.i. Histopathological examination revealed encephalitic changes in all infected groups, characterized by microglial cell infiltration, formation of glial nodules, and perivascular lymphocytic cuffing. The Ab4p group demonstrated the most advanced neuropathological alterations. In addition, prominent viral hepatitis was observed in both the KyD and RacL11 groups. All groups showed a similar moderate degree of interstitial pneumonia. Interestingly, the kidneys of all groups were normal, except those of the KyD group, which showed multiple focal calcified areas with dilatation of Bowman's space and glomerular tuft shrinkage. The spleen of the KyD and RacL11 groups showed a starry-sky appearance with scattered macrophages, and only group KyD exhibited lymphoid follicle depletion. All infected groups exhibited a moderate degree of interstitial pneumonia. The kidneys appeared normal in all groups, except for the KyD group, which showed focal calcification, dilation of Bowman's space, and glomerular tuft shrinkage. The spleens of both the KyD and RacL11 groups exhibited a starry-sky appearance due to scattered macrophages, but lymphoid follicle depletion was noted only in the KyD group. These findings provide novel and detailed insights into the strain-specific histopathological outcomes of EHV-1 infection in a non-equine experimental model. The distinct differences in neurotropism and systemic organ involvement between the strains highlight the complexity of EHV-1 pathogenesis. The data strongly suggest that virulence and tissue tropism are strain-dependent, confirming the need for further investigation into the molecular determinants responsible for pathogenic variability among EHV-1 strains.

Keywords: EHV-1, hamsters, histopathology, experimental infection

Equine herpesvirus 1 (EHV-1) is a ubiquitous pathogen affecting equines worldwide. EHV-1 is a member of the genus *Varicellovirus* belonging to the Alphaherpesvirinae subfamily. EHV-1 causes abortion, respiratory disease, neonatal deaths, and fatal myeloencephalopathy in horses. Clearly, the infection by EHV-1 causes serious economic problems in the horse industry worldwide, especially in breeding farms (1, 5, 21, 22, 26, 28). Thromboses in placental and spinal cord vessels are the main causes of abortion and neurological disease in horses. The activation of platelets is a feature of EHV-1 pathogenicity and may be an important factor for thrombosis (31). After EHV-1 infection of the upper respiratory tract epithelium, a highly cell-associated viraemia develops, resulting in infection of peripheral blood mononuclear cells (PBMCs) and spread to endothelial cells lining the blood vessels (16, 36). The virus can reach the vasculature of the uterus and the central nervous system, resulting in tissue damage that can cause abortion or myeloencephalopathy (29, 35, 36). EHV-1 can establish a latent or persistent state of infection, and the efficacy of vaccination against EHV-1 is hindered by the reactivation of latent virus following the appropriate stimuli (8, 10). Therefore, further studies of the pathogenesis and virulence determinants of different EHV-1 strains are required to resolve these problems. The EHV-1 genome has been characterized as a linear double-stranded DNA molecule. The complete DNA sequence of a British isolate, Ab4p, has been determined (34). The genome is 150,223 bp long and contains 80 open reading frames. Since four open reading frames are duplicated, the genome contains at least 76 distinct genes. The large DNA genomes of several herpes viruses have been sequenced, but the function of the majority of putative genes is elusive (6, 34). Field strains of EHV-1 were classified according to restriction site mapping, plaque characteristics in cell cultures, and clinical manifestations in experimentally infected hamsters (12, 32). There are at least two major groups of EHV-1 based on DNA electropherotype patterns, designated type P and type B (2, 17). The virulence of the B and P types of EHV-1 Japanese isolates in hamsters were previously tested and it was found that all EHV-1 B strains were non virulent while EHV-1P strains were virulent, mildly virulent or non-virulent (24). Both RacL11 and Kentucky D are wild-type virulent strains of EHV-1 that were isolated from aborted horse fetuses by newborn hamster inoculation (32). These two strains are parent strains of commercial vaccines. RacL11 is a highly virulent strain isolated from an aborted foal in Poland (19), whereas Kentucky D was isolated in USA, and both of them are P genotype. Ab4p, a field isolate of EHV-1 which shows consistent differences in pathogenicity and has been extensively characterized as a neuropathogenic strain, was originally isolated from a case of a quadriplegic gelding (9). It has been reported that a hamster-adapted strain of equine herpesvirus 1 Kentucky D caused severe hepatic degeneration in both pregnant and non-pregnant hamsters (4). In non-pregnant hamsters, the only consistently affected organ was the liver. Newborn hamsters usually died 1 to 2 days after inoculation. In pregnant hamsters, the virus had a tropism for the placenta as well as the liver. Kentucky D was passaged over 300 times in hamsters and tissue culture (23), whereas RacL11 is a virulent clone of the Rac isolate obtained after 12 passages in porcine embryonic kidney cells (7, 12). Despite these differences, both strains share a common origin: isolation from an aborted horse fetus using newborn hamsters (12, 30). Therefore it is not clear whether the ability to cause hepatitis in hamsters derived from mutations in cell culture passages or the native pathogenicity of both strains. The virulence was also shown by inoculation of newborn hamsters (12, 30). The Kentucky D strain was found to replicate in the liver and lungs and to reach its peak at 4 days post infection following inoculation into hamsters (23). Moreover, Japanese field isolates of EHV-1 and British Ab4p caused encephalitis, but not hepatitis in young hamsters (24). The available literature provides little information on comparative pathological lesions associated with infection by various strains of EHV-1 in a hamster model. Given the above facts, in the present study, clinico-histopathological investigations were performed to evaluate the pathogenicity and virulence of RacL11 and Kentucky D in comparison to Ab4p by experimental infection of 3-week-old (young) hamsters.

Material and methods

Ethical considerations. The study was approved by the Research, Publication and Ethics Committee of the Faculty of Veterinary Medicine, Sohag University, Egypt with approval code number Sohag-2021/04. All experiments were conducted under the approval of our Institutional Animal Ethics Committee.

Viruses and cells. The strains of equine herpesvirus 1 (EHV-1) (Ab4p, RacL11, and Kentucky D) used in this study were a generous gift from Prof. Hideto Fukushi, Gifu University, Japan. Fetal horse kidney cells (FHK) cells were grown in Eagle's Minimum Essential Medium (Eagle's MEM) (Nissui, Tokyo, Japan) supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, and 5% fetal bovine serum (FBS). Viruses were propagated by inoculation in fetal horse kidney cells (FHK) at a low multiplicity of infection (moi) 0.01.

Experimental animal infection. A total of thirty-two specific pathogen-free male Syrian hamsters were used in this study. The 3-week-old hamsters were divided into 4 groups of 8 hamsters. Group 1 was infected with RacL11, group 2 was infected with Kentucky D, group 3 was infected with Ab4p, and group 4 was inoculated with Eagle's MEM. Food and water were freely available during the experiment. The hamsters were observed for 3 days before inoculation. They were inoculated intranasally with 10^3 pfu in 50 µl of MEM per animal. Clinical signs and mortalities were

monitored twice a day. All surviving animals were sacrificed at day 6 post-infection (p.i.) by injection of an excess amount of sodium pentobarbital. The histopathology of the brain, lungs, liver, kidneys, and spleen was done as described previously (13).

Histopathological examination. After sacrifice, each animal was necropsied, and organ lesions were described, with special attention focused on the lungs, liver, spleen, kidneys, and brain, which were taken for histopathological examination. Organ samples were fixed in 10% buffered formalin, embedded in paraffin and cut on a microtome in sections 4-5 µm thick and stained with hematoxylin-eosin (33). Depending on the severity of lesions, all changes were semi-quantitatively scored (as severe, moderate, mild, or absent).

Results and discussion

Equine herpesvirus type 1 (EHV-1) is an important cause of respiratory disease, abortion, neonatal death, and neurological disease in horses. Animal models for EHV-1 infection have been developed by using mice and Syrian hamsters. However, few studies have attempted to assess the pathogenesis of EHV-1 infection in hamsters (11, 14, 26). The present study aims to evaluate the pathogenicity of three EHV-1 strains in Syrian hamsters. The results indicate that differences in the pathogenicity of the viral strains were due to the strains themselves, and not to the age of the hamsters.

RacL11, Kentucky D and Ab4p were compared in terms of their pathogenicity and clinical signs by experimental infection of 3-week-old hamsters. From day 3 p.i., hamsters inoculated with Kentucky D became dull and lethargic with prominent weight loss. On day 4 p.i., all hamsters inoculated with Kentucky D died. All hamsters inoculated with RacL11 and 4 out of 8 hamsters inoculated with Ab4p became weak and unable to move as well as lost weight (data not shown). The 8 hamsters inoculated with RacL11 died on day 5 p.i. The hamsters inoculated with Kentucky D or RacL11 did not show any neurological signs until their death. In contrast, the hamsters inoculated with Ab4p showed neurological signs, including salivation, arched back, stiffness, fixed movement of the head, or aimless running, and four animals died on day 5 p.i. On day 6 p.i., the remaining 4 hamsters inoculated with Ab4p died with severe neurological signs.

Histopathological examination revealed that the brain of all groups showed various degrees of viral encephalitis. Hamsters inoculated with Kentucky D group showed hemorrhagic streaks, with microglial cell infiltration forming a glial nodule and a perivascular cuff of lymphocytes. Hamsters inoculated with RacL11, showed only glial nodule formation. Hamsters inoculated with Ab4p showed more advanced changes in the form of focal necrosis with glial nodule formation, multiple perivascular cuffing, neuronal degeneration, as well as petechial and streak hemorrhages. Hamsters inoculated with Ab4p were the only group exhibiting meningitis with dilated blood vessels and infiltration of inflammatory cells in the meninges

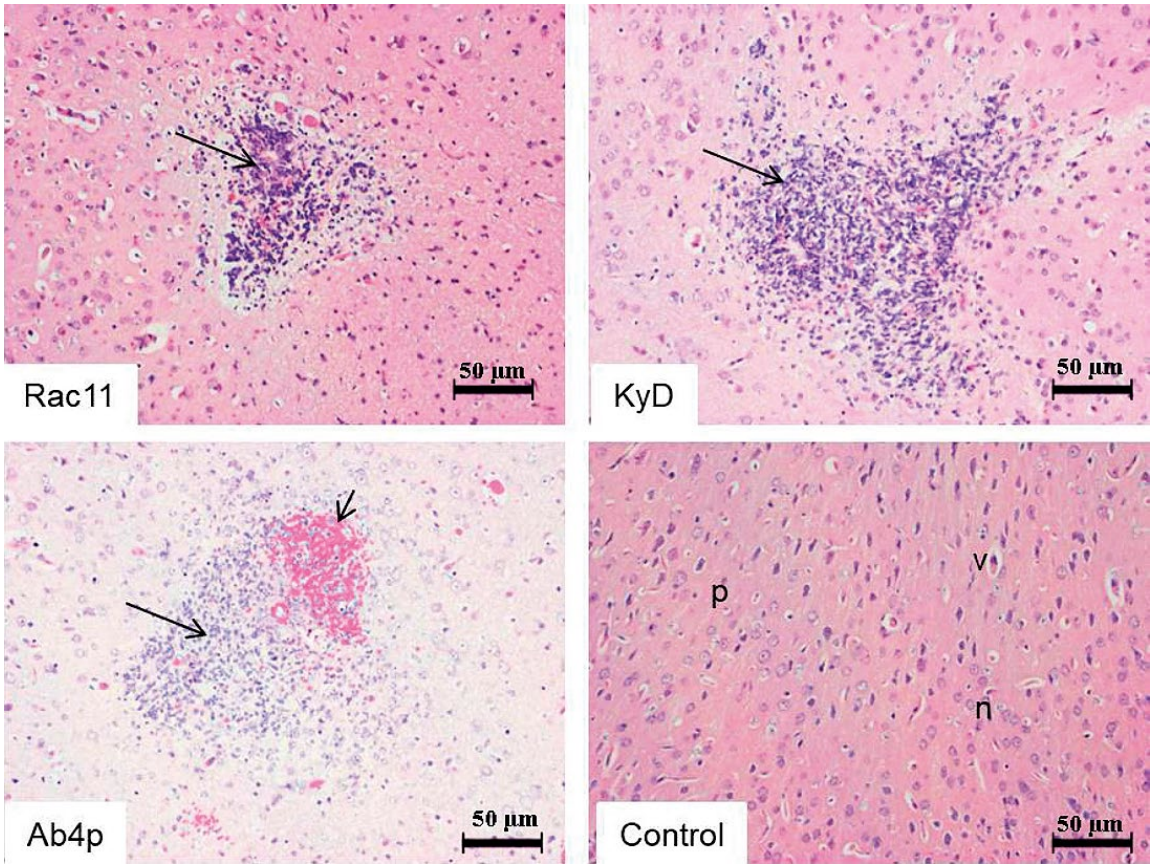

Fig. 1. The brain showed focal necrosis with a collection of microglial cells forming glial nodules (arrows) in the RacL11, KyD, and Ab4p groups. The control group showed normal neuronal cells (N), neuropil (P), and blood vessels (V)

Tab. 1. Summary of lesions observed in the histopathology of hamsters inoculated with RacL11, Kentucky D, and Ab4p

Organ	Lesion	Rac	KyD	Ab4p
Brain	Glial nodule	+ +	++	+++
	Perivascular cuff	–	+	+++
	Neuronal degeneration	–	–	+
	Hemorrhage	–	+	++
Liver	Necrosis	+	++	–
	Inclusion bodies	+++	+++	–
Lungs	Interstitial pneumonia	+	+	+
	Hemorrhage	++	–	+
Kidneys	Focal calcification	–	++	–
	Glomerular dilatation	–	+	–
Spleen	Starry-sky appearance	+	+	–
	Lymphoid follicle depletion	–	+	–

Explanations: Grade of lesions: absent (–), slight (+), moderate (++), severe (+++)

(Fig. 1 and Tab. 1). The liver of hamsters inoculated with KyD and RacL11 showed prominent features of viral hepatitis. The most characteristic microscopic lesion was focal hepatic necrosis, particularly in the peri-portal region. Intranuclear inclusion bodies were prominent, usually in partially degenerated cells. The nucleus was greatly enlarged, and its chromatin was displaced to its peripheral margin, which is described as the margination of chromatin. The inclusion bodies filled the nucleus with distinct outline and usually took a somewhat basophilic tint, which was most obvious in the RacL11 group. Polyhedral eosinophilic material in the center of the nucleus surrounded with a hallo was observed mainly in the KyD group. The liver in the aforementioned groups also showed hemorrhage and sinusoidal dilation as a result of degeneration of the underlined hepatocytes. The liver in the Ab4p group did not show any abnormal changes and resembled that of the control animals (Fig. 2 and Tab. 1). With respect to the kidneys, all groups showed normal kidneys, except hamsters inoculated with KyD, which exhibited multiple focal calcified areas with dilatation of Bowman's space (Fig. 2 and Tab. 1).

As far as the lungs are concerned, all groups showed a similar moderate degree of interstitial pneumonia with edematous and thickened alveolar septa and infiltration of mononuclear inflammatory cells. Hamsters inoculated with RacL11 and Ab4p showed hemorrhage, with RBCs extravasated outside blood vessels and scattered in the alveoli (Fig. 3 and Tab. 1). The spleen of hamsters inoculated with RacL11 and KyD had a starry-sky appearance of macrophages with large and clear cytoplasm scattered within the white pulp. Only the group of hamsters inoculated with KyD exhibited lymphoid follicle depletion, whereas those inoculated with Ab4p did not show any changes (Fig. 3 and Tab. 1).

Taken together, there were differences in the clinical picture between RacL11 and KyD and in histopathology between the laboratory strains (RacL11 and KyD) and the field isolates. Previous data obtained from infection of hamsters with Japanese field strains were identical with those obtained from infection of hamsters with Ab4p in the present study. It is well known that EHV-1 causes hepatitis in baby hamsters (23). The data confirmed that RacL11 and Kentucky D can cause hepatitis in young hamsters, as well as in baby hamsters. Like other EHV-1 strains, RacL11 and Kentucky D also caused encephalitis and pneumonia. Our results indicate that, compared to other EHV-1 strains, RacL11 and Kentucky D have an additional ability to cause hepatitis in hamsters. Therefore, these two strains still appear to retain characteristics of wild-type virus strains of EHV-1. However, EHV-1 has a large genomic DNA, and even though various strains have already been isolated, the structure and functions of its genome are not fully understood and need to be further investigated.

Pervious research revealed the whole-genome sequence of attenuated EHV-1 KyA and wild-type RacL11 strains, both of which show several gene alterations compared to the genome of EHV-1 Ab4 (27). The results indicate that genes related to the induction of hepatitis in hamsters are not necessarily the same in the RacL11 and Kentucky D strains. Previous studies utilized DNA RFLP to separate field isolates of EHV-1 into subgroups according to characteristic restriction enzyme site changes and the presence of variable numbers of copies of short sequence repeats (2, 18). Those studies demonstrated a relatively low frequency of genetic polymorphism in EHV-1 and suggested that distinct strains of EHV-1 do exist in the field (1, 3, 15, 17, 20, 25, 32). However, previous studies using RFLP indicated that DNA fingerprints of RacL11 and Kentucky D by *Bam*H1 differed from that of Ab4p (1, 7, 12).

A previous study showed that, according to the nucleotide and deduced amino acid sequences, EHV-1 strain Ab4p would encode a 47,000-Mr US2 protein, whereas wild-type strain RacL11 would encode a 34,000-Mr US2 protein and that the difference in molecular weight might be due to a mutation inducing a frame shift at position 125,540 in strain Ab4p as compared to RacL11 (12, 34). The finding that RacL11 and Kentucky D, unlike other strains of EHV-1, caused hepatitis in young hamsters strongly suggests that hepatitis caused by RacL11 and Kentucky D is due to the tissue tropism and pathogenesis of these strains themselves and not to the age of the hamsters. It has been suggested that the mechanism by which RacL11 and Kentucky D strains cause hepatitis in hamsters is not the same for the two strains. The results indicate that genes related to the induction of hepatitis in hamsters by RacL11 and Kentucky D might be different, and that several genes might be involved in causing hepatitis in these animals. The whole-genome sequencing of RacL11 and Kentucky D strains would

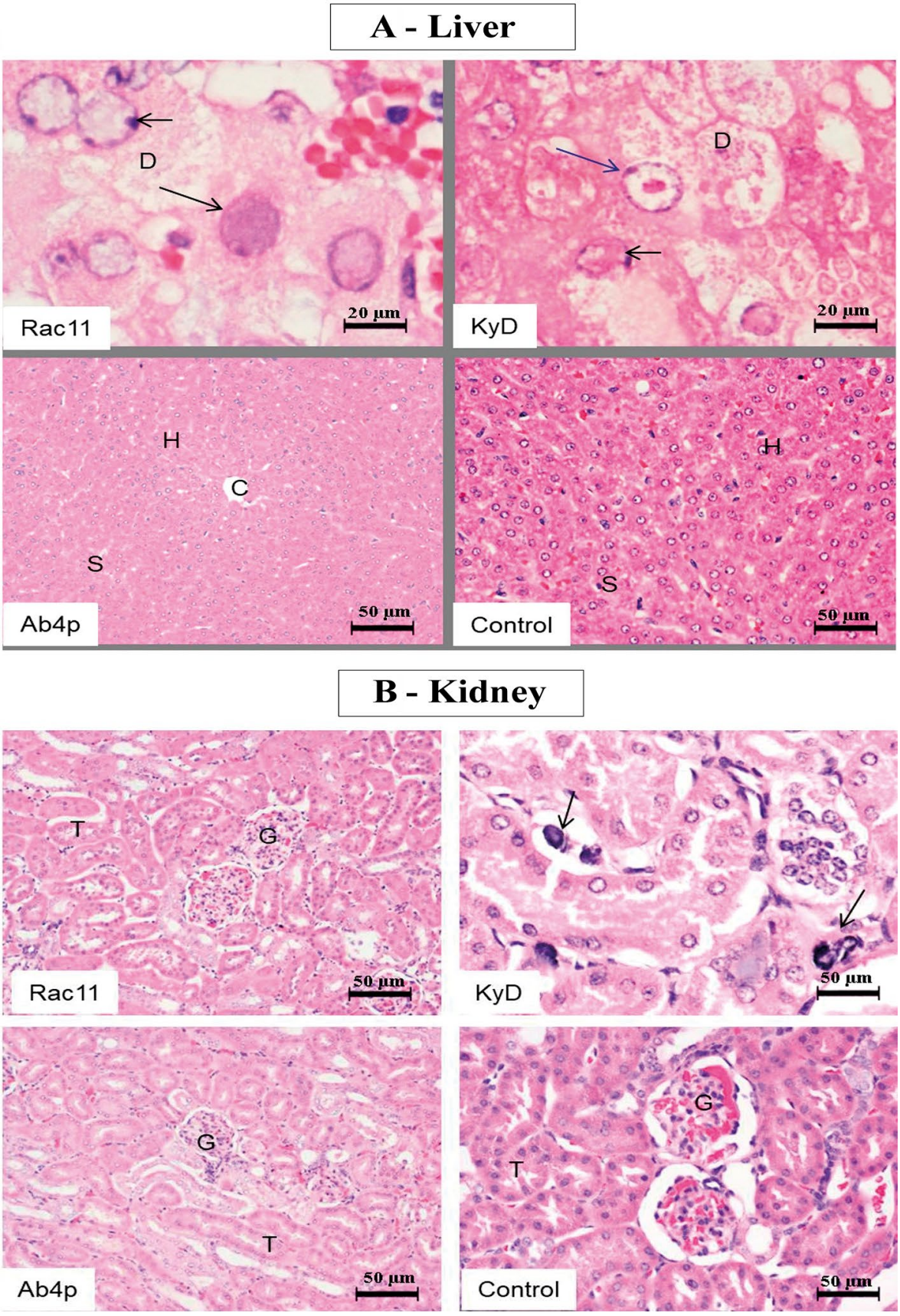

Fig. 2. Hepatic tissues (upper panel): Both the RacL11 and KyD groups exhibited signs of viral hepatitis. Degenerated hepatocytes (D) contained greatly enlarged nuclei with peripheral chromatin margination (small arrows). In RacL11, basophilic intranuclear inclusion bodies filled the nuclei with a distinct outline (black arrow), whereas KyD showed polyhedral eosinophilic bodies (blue arrow) centered in the nucleus and surrounded by a halo. The Ab4p group displayed a normal hepatic pattern similar to the control, with intact central vein (C), hepatocytes (H), and sinusoids (S). Kidneys (lower panel): Multiple focal calcified patches (arrows) were observed in the renal tubules of KyD animals, while the RacL11 and Ab4p groups showed a normal structure like the control, with preserved glomeruli (G) and tubules (T)

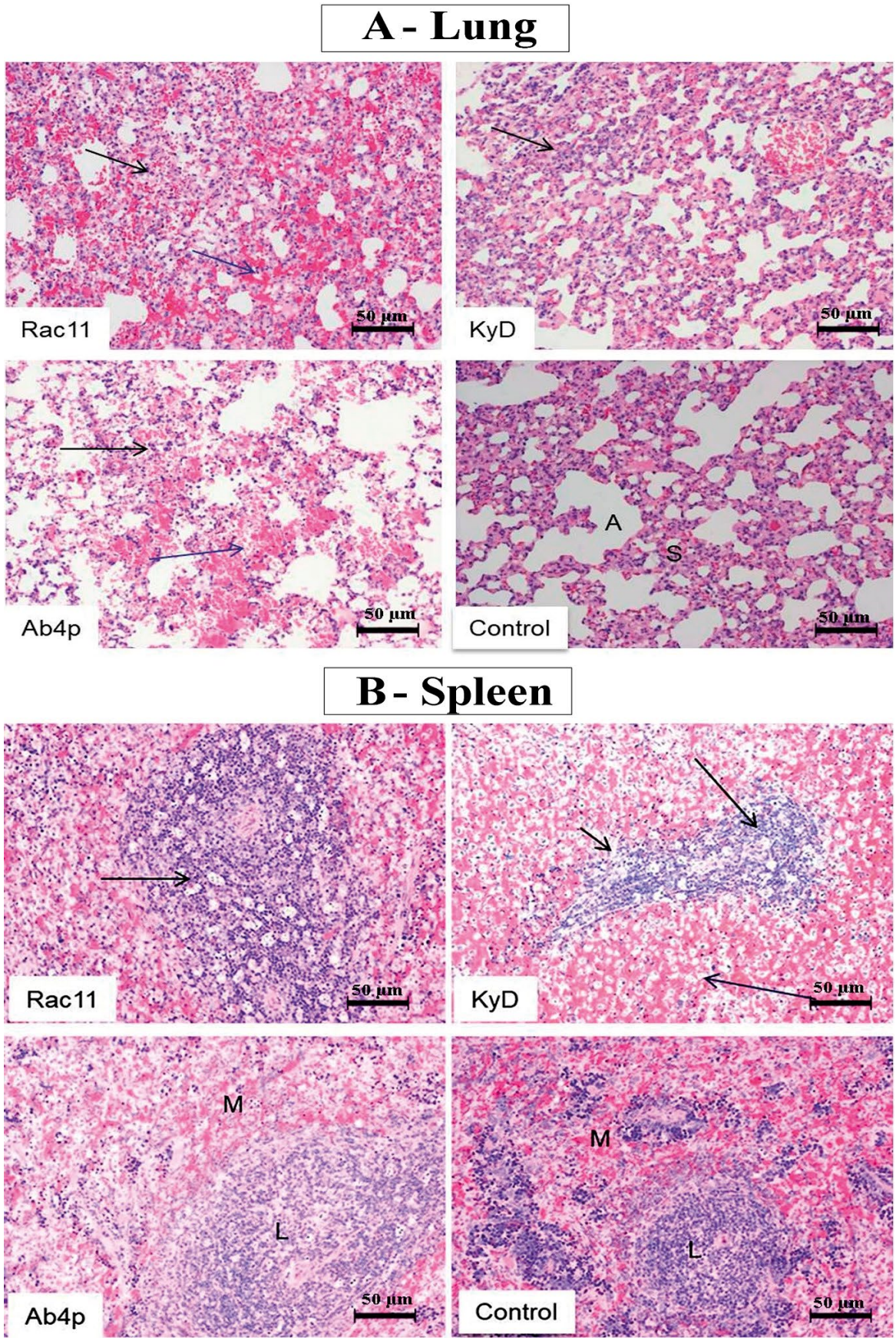

Fig. 3. Lungs (upper panel): Moderate interstitial pneumonia was observed in the RacL11, KyD, and Ab4p groups, with thickened alveolar septa infiltrated by mononuclear inflammatory cells (black arrows). Hemorrhagic areas (blue arrows) were detected in RacL11 and KyD only. Control lungs showed normal alveoli (A) and interalveolar septa (S). Spleen (lower panel): In the RacL11 and KyD groups, scattered macrophages with large, clear cytoplasm produced a starry-sky appearance within the white pulp (black arrows). The KyD group also showed lymphoid follicle depletion (small arrow) and hemorrhages in the medulla (blue arrow). Ab4p spleens appeared normal, similar to the control, with intact lymphoid follicles (L) and medulla (M)

probably help resolve the question, but it is hard to perform. Little information is available on the correlation between genomic structures and expression of specific pathogenicity in various EHV-1 strains. The determinants of the character of pathogenicity in the hamster model for Ab4p and wild-type virulent strains, such as RacL11 and Kentucky D, remain unknown and need to be investigated.

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Informed Consent Statement: Not applicable.

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