

Time-related Pathological Changes in Horses Experimentally Inoculated with Equine Influenza A Virus

Masanori MURANAKA^{1*}, Takashi YAMANAKA¹, Yoshinari KATAYAMA¹,
Hidekazu NIWA¹, Kazuomi OKU², Tomio MATSUMURA¹ and Toshifumi OYAMADA³

¹Epizootic Research Center, Equine Research Institute, the Japan Racing Association, 1400-4 Shiba, Shimotsuke-shi, Tochigi 329-0412, ²Racehorse Clinic, Miho Training Center, the Japan Racing Association, Ibaraki 300-0415, ³Department of Veterinary Pathology, School of Veterinary Medicine, Kitasato University, Aomori 034-8628, Japan

*To investigate the pathology of equine influenza, necropsy of 7 horses experimentally infected with equine influenza A virus (EIV) subtype H3N8 was conducted on post-infection days (PID) 2, 3, 7, and 14. Histopathologically, rhinitis or tracheitis including epithelial degeneration or necrosis with loss of ciliated epithelia and a reduction in goblet cell numbers, was observed in the respiratory tracts on PIDs 2 and 3. Epithelial hyperplasia or squamous metaplasia and suppurative bronchopneumonia with proliferation of type II pneumocytes were observed on PIDs 7 and 14. Viral antigen was detected immunohistochemically in the epithelia of the nasal mucosa, trachea, and bronchi on PIDs 2 and 3. The *sodA* gene of *Streptococcus equi* subsp. *zooepidemicus*, a suspected cause of suppurative bronchopneumonia, was detected in paraffin-embedded lung tissue sections, but only on PIDs 7 and 14. These findings suggest that damage caused to ciliated epithelia and goblet cells by EIV infection results in secondary bacterial bronchopneumonia due to a reduction in mucociliary clearance.*

Key words: equine influenza A virus, equine respiratory disease, experimental infection

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Equine influenza (EI) is one of the most important respiratory diseases of horses and results from infection with H7N7- or H3N8-subtype influenza A viruses [19, 21]. EI outbreaks result in severe economic loss [18, 24]. Horses infected with EI A virus (EIV) develop typical respiratory disorders, including pyrexia, nasal discharge, coughing, and depression [18, 19]. EI is usually curable with symptomatic treatment. However, horses infected with EIV may develop fatal secondary bacterial pneumonia [2, 13, 18, 19]. *Streptococcus equi* subsp. *zooepidemicus* (*S. zooepidemicus*) is one of the most common potential pathogens of secondary bacterial pneumonia following EI [8, 13, 15, 25].

In 2007 in Japan there was an outbreak of EI caused by EIV infection [24]. Few horses had severe clinical manifestations, and the epidemic was contained within a fairly limited area, probably because of mandatory vaccination and/or restriction of horse movements [12, 24]. During the EI outbreak in the Australian horse population in 2007, some deaths of young foals and mature horses occurred

[2, 13]. These fatally affected animals had extensive pulmonary lesions [2, 13]. However, the pathological lesions in these cases consisted mainly of suppurative bronchopneumonia resulting from secondary bacterial infection, which is considered to be only part of the pathological process of EI [19].

Biphasic febrile responses are often observed in horses infected with EIV and are reproducibly observed in horses experimentally inoculated with the virus via an ultrasonic nebulizer in the evaluation of the efficacy of vaccines or antiviral agents [5, 10, 15, 19, 25]. The initial febrile response that occurs 2 or 3 days after inoculation is considered to be related mainly to viral replication in the respiratory epithelial cells. The second febrile response from 7 to 10 days after inoculation is considered to be related mainly to opportunistic bacterial pneumonia [15, 19]. In our previous study [25], we isolated *S. zooepidemicus* from bronchoalveolar lavage fluid 7 days, but not 2 days, after inoculation. Previous studies have approached EI from the perspective of clinical virology or clinical microbiology, but have not been supported by histopathology. Histopathological reports of EI are currently limited to those of cases in which horses have

died from the secondary bacterial pneumonia that follows EIV infection [9, 13]. The gross and histologic lesions of uncomplicated EI have not been adequately described, as horses infected with EIV rarely die in the acute stage [4]. Therefore, to obtain fundamental pathological data useful for the development of prevention and treatment methods for EI, we investigated the pathological changes in the initial (post-infection day [PID] 2 or 3) and second (PID 7) febrile responses, as well as during defervescence (PID 14).

Materials and Methods

Experimental inoculation with EIV

Four Thoroughbred horses (17–19 months of age), were used. The horses were inoculated by inhalation of EIV A/equine/Ibaraki/1/07 ($10^{8.3}$ 50% egg infectious dose [EID_{50}]/animal) via an ultrasonic nebulizer (SONICLIZER305; ATOM, Tokyo, Japan) for 20 min, as previously described [23]. Rectal temperatures were measured every morning. To detect virus shedding from the nostrils, nasal swabs were taken daily using cotton swabs (JMS menbou, Japan Medical Supply, Tokyo, Japan). Subsequent virus detection and titration were performed as previously described [23]. The swabs were immediately immersed in 2.5 ml of transport medium (PBS supplemented with 0.6 w/v% tryptose phosphate broth, 500 unit/ml penicillin, 500 μ g/ml streptomycin and 1.25 μ g/ml amphotericin B). The swab samples in the transport medium were vortexed for 10 sec and centrifuged at $1,500 \times g$ for 15 min to precipitate debris. Then, 200 μ l of the supernatants that had been diluted at 1:10 (v/v) in transport medium were injected into the allantonic cavities of 10-day-old embryonated hen's eggs (four eggs per sample). The allantonic fluid was harvested after 3 days of incubation at 34.0°C and tested by hemagglutination using 0.5% hen's red blood cells. Virus titers ($\log_{10} EID_{50}/200 \mu$ l) were determined by the same method using further 10-fold dilutions with transport medium for those nasal swab samples that were hemagglutination-positive at 1:10 dilution.

Three horses were necropsied on PID 2 (horse #1) and 3 (horses #2 and #3) (the period of the initial febrile response), and horse #4 was necropsied on PID 7 (the period of the second febrile response). All of the horses were seronegative (titers <1:10) in hemagglutination inhibition tests performed before the study for antibodies to EIV A/equine/Ibaraki/1/07, indicating that they had had no prior H3N8 virus infection or vaccination. Additionally, pathological and bacteriological examinations were conducted on samples collected from horses (#5 to #7) that were experimentally infected with EIV and

necropsied on PID 14 (in the defervescence period) in our previous study [23]; no pathological examination was conducted in that previous study.

This study was conducted in a biosafety level 3 facility, and all animals were studied under the experimental protocol approved by the Animal Care Committee of the Equine Research Institute of the Japan Racing Association.

Pathological examination

Gross examination was performed throughout the whole body, and tissue samples were collected for histopathological examination. These tissue samples were fixed in 20% neutral buffered formalin, embedded in paraffin wax, and cut into 6- μ m-thick sections. All sections were stained with hematoxylin and eosin. Additionally, the lung sections underwent Gram staining.

Immunohistochemical examination

To detect influenza virus antigen, immunohistochemical analysis was performed on sections of the nasal mucosa, trachea, and lung. Tissue samples collected at necropsy were fixed in 4% paraformaldehyde, embedded in paraffin wax, cut into 6- μ m-thick sections, and deparaffinized. For antigen retrieval, the sections were placed in a Pascal programmable pressure cooker (S280033, DAKO, Tokyo, Japan) containing target retrieval solution (pH 6.1; S1699, DAKO); the target temperature was 125°C and the time was set to 30 sec. The sections were then incubated for 5 min with peroxidase blocking reagent (S2023, DAKO), followed by a 10 min incubation with a protein blocking reagent (X0909, DAKO). The sections were exposed overnight to human influenza A (H1, H2, H3) monoclonal antibody (Clone C111, TAKARA BIO, Shiga, Japan) at a dilution of 1:200 at 4°C. Primary antibody was detected with EnVision+ Dual Link System-HRP (K4063, DAKO) and visualized with 3,3'-diaminobenzidine tetrahydrochloride liquid (K3467, DAKO) in accordance with the manufacturer's instructions. The sections were counterstained lightly with hematoxylin. Corresponding sections that had been taken from a healthy horse and stored in the laboratory were used as negative controls.

Bacterial examination

A real-time PCR developed by Båverud *et al.* [1] was performed to detect the *sodA* gene of *S. zooepidemicus* in paraffin-embedded lung tissue sections of all of the horses studied. DNA was extracted from the sections using Takara DEXPAT (TAKARA BIO) in accordance with the manufacturer's instructions.

Bacteria were isolated from post mortem pharyngeal

Table 1. Rectal temperature of each horse experimentally inoculated with equine influenza virus

Horse #	Post-infection day							
	0	1	2	3	4	5	6	7
1	37.8	39.8	37.6	ND ^{a)}	ND	ND	ND	ND
2	37.7	37.8	39.5	37.8	ND	ND	ND	ND
3	38.2	38.0	40.1	38.8	ND	ND	ND	ND
4	37.7	38.2	39.6	38.4	38.2	38.5	38.0	40.2

^{a)} Not Done: Horses were already necropsied.

Table 2. Virus detection by egg culture and titers ($\log_{10}EID_{50}/200 \mu l$) of nasal swabs collected from each horse experimentally inoculated with equine influenza virus (H3N8)

Horse #	Post-infection day							
	0	1	2	3	4	5	6	7
1	- ^{a)}	-	2.3	ND ^{b)}	ND	ND	ND	ND
2	-	-	3.5	2.0	ND	ND	ND	ND
3	-	-	4.0	3.0	ND	ND	ND	ND
4	-	≤ 0.7	3.5	2.3	1.5	2.8	1.7	-

^{a)} <0.7 (no virus was isolated from four eggs inoculated with nasal swab specimens diluted 1:10).

^{b)} Not Done: Horses were already necropsied.

and tracheal swabs and the lung tissue of horse #4 using Columbia agar plates supplemented with 5% horse blood. The isolated bacteria were identified using the colony morphology, Gram stain, and a commercial identification kit (Api 20E, Api 20NE, Api 20 Strep: SYSMEX bioMérieux., Tokyo, Japan).

Results

Pyrexia

Table 1 shows the rectal temperature of each horse (horses #1 to #4) experimentally inoculated with EIV. All horses developed pyrexia (above 39.0°C) at PID 1 or 2. In horse #4, a second increase in rectal temperature was observed on PID 7.

Virus shedding

Table 2 shows the virus shedding results for each horse inoculated with EIV.

Virus shedding was detected on PID 1 (horse #4) and PID 2 (horses #1 to #3). Horse #4 showed virus shedding until PID 6.

Necropsy

Gross morphological changes were confined to the respiratory organs and/or adjacent lymph nodes. In horse #1 (PID 2), the nasal mucosa was slightly hyperemic. The retropharyngeal and pulmonary lymph nodes were mildly swollen. In horses #2 and #3 (PID 3), the nasal

mucosa was hyperemic, and serous secretions were seen in both nasal cavities. Petechial hemorrhages and mucopurulent exudate were observed in the bronchi (Fig. 1). The retropharyngeal and pulmonary lymph nodes were mildly swollen. In horse #4 (PID 7), red-brown lesions and interstitial edema in the cranial lobe and cranial part of the caudal lobe of the lung, together with petechial hemorrhages and mucopurulent exudate in the trachea and bronchi, were observed. The retropharyngeal and pulmonary lymph nodes were moderately swollen. In horses #5 to #7 (PID 14), hepatization and mild interstitial edema were observed in the cranial lobe of the lung (Fig. 2). The retropharyngeal and pulmonary lymph nodes were moderately or severely swollen.

Histopathology

The histological changes in all the horses experimentally inoculated with EIV were also confined to the respiratory organs and adjacent lymph nodes. Table 3 summarizes the histopathological lesions in the respiratory organs of all horses. In horse #1 (PID 2), rhinitis, including diffuse epithelial degeneration (cloudy swelling or vacuolar degeneration) or necrosis, sometimes with loss of ciliated epithelium, reduction in goblet cell numbers, and moderate lymphocytic infiltration of the lamina propria, as well as mild tracheitis (Fig. 3a), was observed. In horses #2 and #3 (PID 3), moderate to severe tracheitis (Fig. 3b) and bronchitis, including diffuse epithelial degeneration or necrosis (often with loss of

Table 3. Distribution of histological lesions in respiratory organs from horses experimentally inoculated with equine influenza virus (H3N8)

	Horse #						
	(post-infection day)						
	1	2	3	4	5	6	7
	(2)	(3)	(3)	(7)	(14)	(14)	(14)
Nasal mucosa							
loss of ciliated epithelium	+	+	+	+++	++	+++	+++
epithelial degeneration or necrosis	++	+	+	++	+	++	++
epithelial hyperplasia or squamous metaplasia	-	++	+	++	++	++	++
reduction in goblet cell numbers	+	+	+	+++	+	+	++
lymphocytic infiltration	++	++	++	+++	++	+	++
Trachea							
loss of ciliated epithelium	-	+++	+	+++	+++	+++	+++
epithelial degeneration or necrosis	+	+	++	++	++	++	+
epithelial hyperplasia or squamous metaplasia	-	+	+	++	+++	+++	+++
reduction in goblet cell numbers	-	+++	++	+++	+	+	++
lymphocytic infiltration	+	+	+	++	++	+	++
Bronchus							
loss of ciliated epithelium	-	++	++	+++	+++	+++	+++
epithelial degeneration or necrosis	-	+	++	++	++	++	+
epithelial hyperplasia or squamous metaplasia	-	+	+	++	+++	+++	+++
reduction in goblet cell numbers	-	+++	+	+++	+	-	-
lymphocytic infiltration	-	+	+	+++	+	+	+
neutrophilic infiltration	-	-	-	+++	++	+++	++
Bronchiole							
epithelial degeneration or necrosis	-	+	+	++	++	+	+
epithelial hyperplasia or squamous metaplasia	-	-	-	++	+++	+	++
lymphocytic infiltration	-	+	+	+	-	-	-
neutrophilic infiltration	-	-	-	+++	++	+++	++
hemorrhage	-	-	+	+	-	-	-
Alveoli							
pulmonary edema	-	++	+	+++	+++	++	++
neutrophilic infiltration	-	-	-	+++	+++	++	++
alveolar macrophage proliferation	-	-	-	+	+++	+++	+++
proliferation of type II pneumocytes	-	-	-	+	+++	+++	+++
hemorrhage	-	-	-	++	+	+	++

Lesions were classified as follow; -:negative, +:mildly positive, ++: moderately positive, +++: strongly positive.

ciliated epithelium), reduction in goblet cell numbers, and lymphocytic infiltration of the lamina propria, accompanied the same rhinitis as was seen in horse #1. The tracheitis tended to be more severe at the bottom of the trachea. In horse #4 (PID 7), extensive moderate epithelial hyperplasia or squamous metaplasia was observed from the nasal mucosa to the bronchi (Fig. 3c). Lymphocytic infiltration of the nasal, tracheal, and bronchial lamina propria was more severe than in horses #2 and #3. The bronchial and bronchiolar lumina were filled with degenerated neutrophils and necrotic epithelial cells. Occasionally, large numbers of neutrophils were seen to have infiltrated the peripheral alveoli from damaged terminal bronchioles (Fig. 4). The peribronchiolar alveolar spaces were collapsed, with large numbers of neutrophils and variable numbers of macrophages, sometimes containing

Gram-positive cocci (Fig. 5). Pulmonary edema, with fibrin and neutrophilic infiltration, was observed in the alveoli (Fig. 6). In horses #5 to #7 (PID 14), severe tracheitis (Fig. 3d) and bronchitis (Fig. 7) with epithelial hyperplasia or squamous metaplasia was noted. The tracheal and bronchial epithelia sometimes had short cilia or small goblet cells. Neutrophilic infiltration of the lamina propria and epithelium was observed in the bronchial and bronchiolar epithelia. The bronchial and bronchiolar lumina were filled with large numbers of neutrophils and variable numbers of necrotic epithelial cells. The bronchopneumonia included extensive neutrophilic infiltration and alveolar macrophage proliferation, with pink proteinaceous material in the alveoli (Fig. 8), fibrin exudates, pulmonary edema, and often marked proliferation of type II pneumocytes (Fig. 9).

Table 4. The results of immunohistochemical staining with influenza A virus antigen of the respiratory organs

Horse # (post-infection day)	Nasal mucosa	Trachea	Lung		
			Bronchus	Bronchiole	Alveoli
1 (2)	+++ ^{a)}	+++ ^{a)}	++ ^{a)}	+ ^{a)}	–
2 (3)	++ ^{a)}	+++ ^{a)} + ^{b)}	+ ^{a)}	–	–
3 (3)	+ ^{a)}	+++ ^{a)} + ^{b)}	++ ^{a)}	–	–
4 (7)	+ ^{a)} + ^{b)}	+ ^{a)} + ^{b)}	+ ^{b)}	–	–
5 (14)	–	–	–	–	–
6 (14)	–	+ ^{b)}	–	–	–
7 (14)	+ ^{b)}	+ ^{b)}	–	–	–

The assessment for the immunohistochemical staining was determined according to the number of positive cells as follows: –, negative, +; a small number, positive, ++; a moderate number, positive, +++; a large number, positive.

^{a)} epithelium.

^{b)} macrophages in the lamina propria.

Reactive lymphoid hyperplasia was observed in the mandibular, retropharyngeal, and pulmonary lymph nodes of all horses, with the exception of the retropharyngeal lymph nodes of horse #2. This feature did not differ among the different stages of the infection at which necropsy was done.

Immunohistochemistry

Table 4 summarizes the results of immunohistochemical staining of the respiratory organs for EIV antigen. Viral antigen was diffusely present in the nasal mucosa (horses #1 to #4) and the tracheal (horses #1 to #4) (Fig. 10a, b, c) and bronchial (horses #1 to #3) epithelia. Particularly positive signals were clearly observed in the intact ciliated epithelia of the trachea (Fig. 10a, b) and bronchi from horses #1 to #3. Viral antigen was also detected in macrophages in the lamina propria of the nasal mucosa (horses #4 and #7), trachea (horses #2 to #4, #6 and #7) (Fig. 10d), and bronchus (horse #4). Viral antigen, however, was not detected in the pneumocytes or alveolar macrophages. No immunohistochemical reaction was observed in negative control sections from the healthy horse.

Bacterial isolation and real-time PCR for *S. zooepidemicus*

Real-time PCR for the *sodA* gene of *S. zooepidemicus* detected the gene in lung samples from horses #4 to #7, but not in those from horses #1 to #3.

Bacterial isolation from the pharyngeal swab of horse #4 yielded several bacteria, namely *S. zooepidemicus*, *Pasteurella caballi*, *Actinobacillus equuli*, *Streptococcus* sp., and Gram-positive facultative anaerobic bacilli. However, only *S. zooepidemicus* was isolated from the tracheal swab and lung tissue.

Discussion

The purpose of this study was to investigate time-related pathological changes in horses inoculated with EIV. Our findings show that the lesions at the time of the initial febrile response (PIDs 2 and 3) were characterized by rhinitis or tracheitis, including epithelial degeneration or necrosis with loss of ciliated epithelium and a reduction in goblet cell numbers. In the bronchioles and alveoli only a few pathological changes were observed. In contrast, the lesions at the time of the second febrile response (PID 7), or later (PID 14), had expanded to include the bronchioles and alveoli, where epithelial hyperplasia or squamous metaplasia and suppurative bronchopneumonia with proliferation of type II pneumocytes were observed. Furthermore, Gram-positive cocci were observed inside the alveolar macrophages on PID 7. Begg *et al.* [2] reported on the pathology of pneumonia associated with natural EIV infection in unvaccinated horses. They found that the essence of the pathological lesions in these cases was proliferative tracheitis, ranging from epithelial

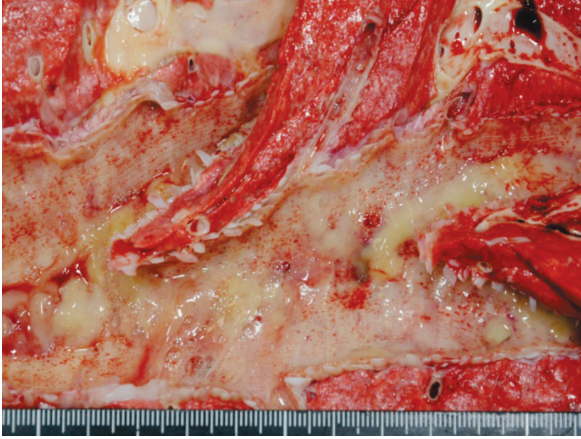


Fig. 1. Macroscopic findings on the cut surface of the lung (horse #3, PID 3) showing petechial hemorrhages and mucopurulent exudate in the bronchus.



Fig. 2. Macroscopic findings in both cranial lung lobes (horse #6, PID 14) showing hepatization and mild interstitial edema.

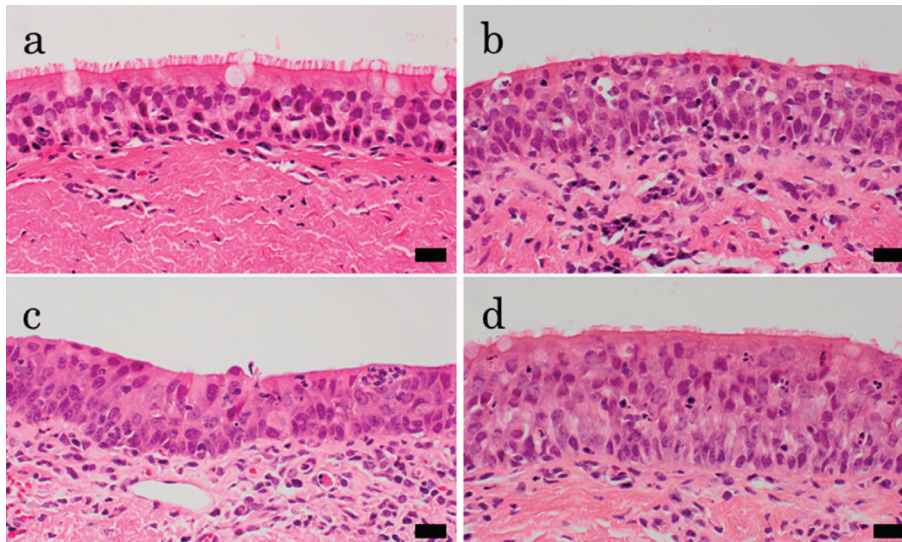


Fig. 3. Histopathological findings in the trachea.

- a) Horse #1 (PID 2): mild epithelial degeneration or necrosis. H & E. Bar=20 μ m.
- b) Horse #2 (PID 3): moderate epithelial degeneration or necrosis with loss of ciliated epithelium and reduction in goblet cell numbers. H & E. Bar=20 μ m.
- c) Horse #4 (PID 7): epithelial degeneration or necrosis with loss of ciliated epithelium and squamous metaplasia. H & E. Bar=20 μ m.
- d) Horse #6 (PID 14): severe epithelial hyperplasia with short cilia or small goblet cells. H & E. Bar=20 μ m.

stunting with loss of cilia to hyperplasia and often squamous metaplasia, as well as secondary bacterial bronchopneumonia with many neutrophils and some macrophages filling the bronchioles and infiltrating into the adjacent lung parenchyma. Their findings are essentially consistent with our pathological findings on PID 7 and 14.

The hemagglutinin of influenza virus binds specifically to host-cell-surface sialyloligosaccharides that act

as receptors when the virus infects the target cells [7]. Although all influenza viruses recognize oligosaccharides containing a terminal sialic acid (SA), the specificity of the hemagglutinin toward these molecules differs [7]. From the nasal mucosa to the bronchioles, the equine respiratory tract has SA α 2,3Galactose linked receptors (SA α 2,3Gal) [6, 11, 17]. EIV preferentially binds to SA α 2,3Gal [17, 26]. Our immunohistochemical findings

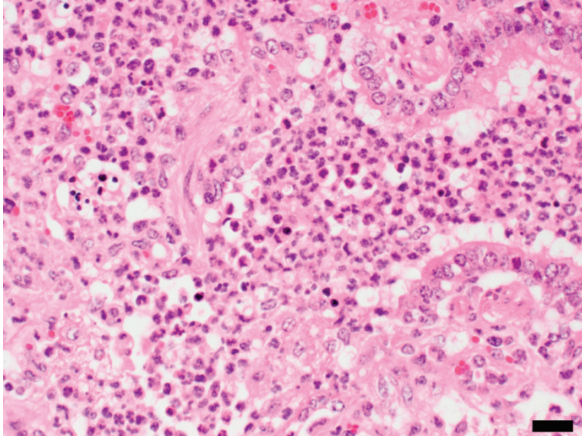


Fig. 4. Histopathological findings in a terminal bronchiole (horse #4, PID 7) showing large numbers of neutrophils infiltrating the peripheral alveoli from the damaged terminal bronchiole. H & E. Bar=20 μ m.

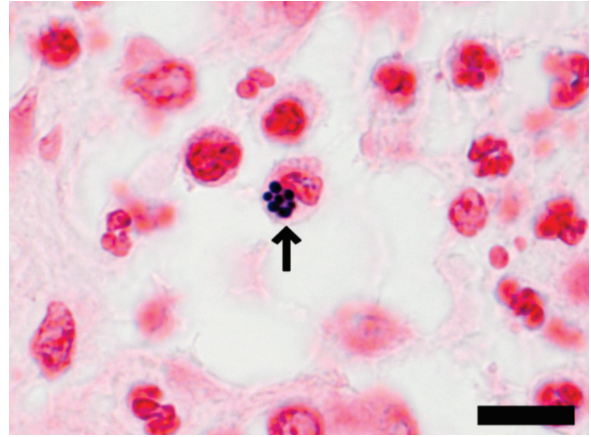


Fig. 5. Histopathological findings in the alveoli (horse #4, PID 7) showing suppurative bronchopneumonia with Gram-positive cocci in a macrophage (arrow). Gram stain. Bar=10 μ m.

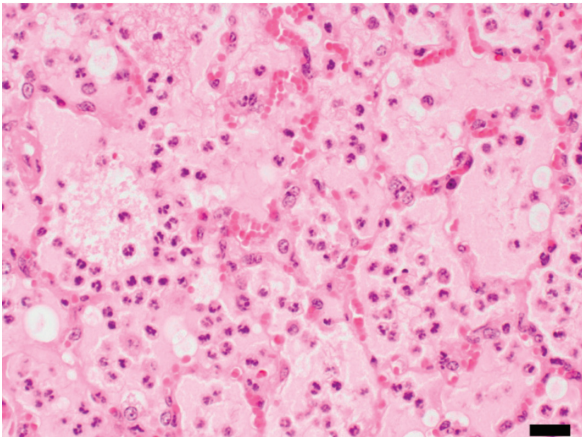


Fig. 6. Histopathological findings in the alveoli (horse #4, PID 7) showing pulmonary edema with fibrin and neutrophilic infiltration of the alveolar spaces. H & E. Bar=20 μ m.

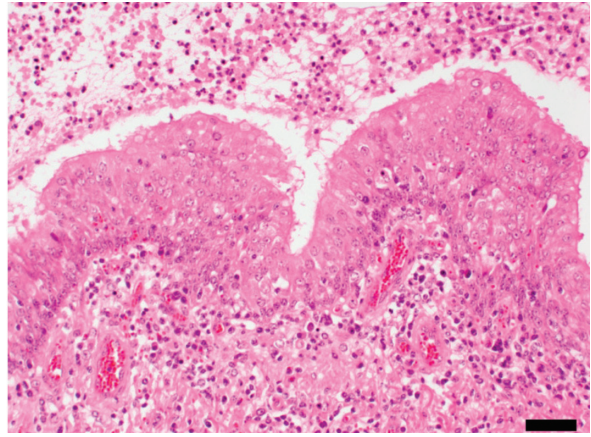


Fig. 7. Histopathological findings in a bronchus (horse #6, PID 14) showing epithelial hyperplasia with necrosis in the bronchus. H & E. Bar=50 μ m.

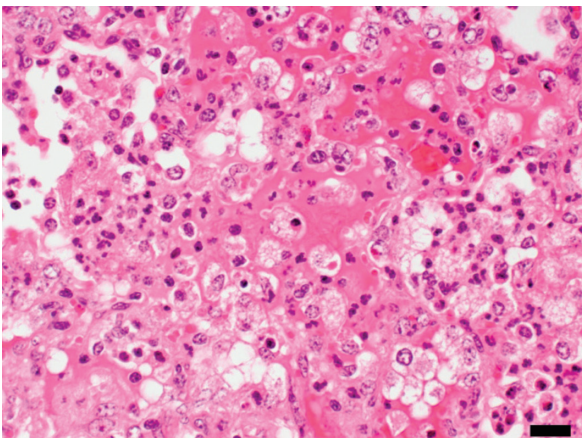


Fig. 8. Histopathological findings in the alveoli (horse #5, PID 14) showing alveolar spaces filled with neutrophils and macrophages with pink proteinaceous material. H & E. Bar=20 μ m.

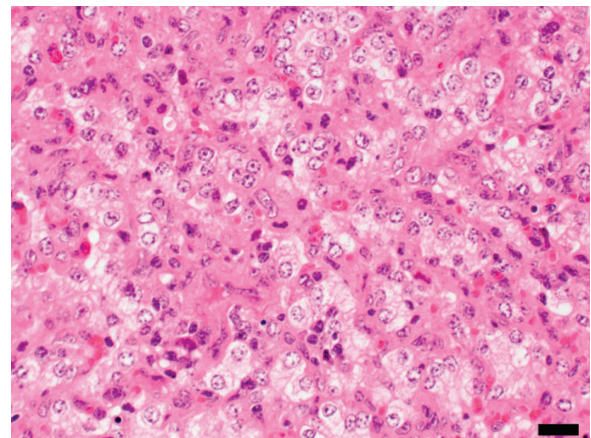


Fig. 9. Histopathological findings in the alveoli (horse #5, PID 14) showing alveolar spaces are narrowed by proliferation of type II pneumocytes. H & E. Bar=20 μ m.

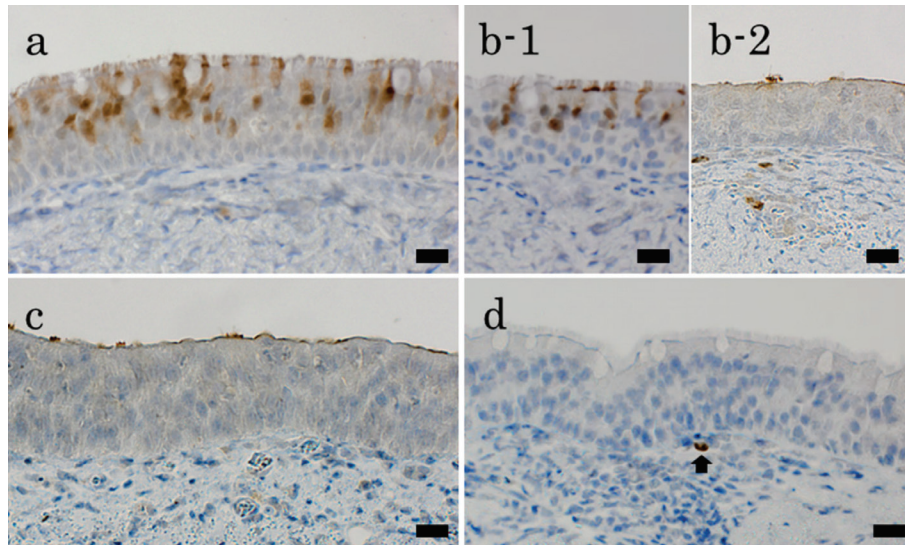


Fig. 10. Immunohistochemistry of the trachea for the influenza A virus antigen.

- a) Horse #1 (PID 2): ciliated epithelia positive for the antigen. Bar=20 μ m.
 b-1) Horse #2 (PID 3): intact ciliated epithelia positive for the antigen at a site of mild lesion. Bar=20 μ m.
 b-2) Horse #2 (PID 3): remaining cilia or epithelial surfaces and macrophages in the lamina propria positive for the antigen at a site of severe lesion. Bar=20 μ m.
 c) Horse #4 (PID 7): cilia or epithelial surfaces positive for the antigen. Bar=20 μ m.
 d) Horse #6 (PID 14): macrophage in the lamina propria for the antigen (arrow). Bar=20 μ m.

indicates that the epithelia from the nasal mucosa to the bronchioles were infected with EIV in horses necropsied on PID 7 or before. This coincides well with the period of virus shedding recorded here and in our previous study [23], in which virus shedding was recorded for 5 or 6 days from PID 2 in horses #5 to #7. These EIV-infected epithelia were not observed in horses necropsied on PID 14. Viral replication in epithelia with SA α 2,3Gal on their surfaces likely leads to epithelial degeneration or necrosis and loss of ciliated epithelium. Similarly, in pony foals experimentally infected with EIV, Sutton *et al.* [16] detected EIV antigens on PIDs 3 to 9 in epithelial cells collected from the nasopharynx, trachea, and bronchus by using cytology brushes. However, Sutton *et al.* [16] also found that viral antigens were retained in alveolar macrophage-like cells collected by bronchoalveolar lavage on PIDs 7 and 21. In contrast, no viral antigens were detected in pneumocytes or alveolar macrophages throughout the course of our study. Although it is not clear why the results of these studies differ in this regard, individual differences of the horses (i.e., age, sex, and breed), and/or of the EIV strains used in the studies might have affected the outcomes. In other species viral antigen was detected in alveolar macrophages in the lungs of dogs

from 1 to 3 days after inoculation with canine influenza virus H3N8 [3], and in type II pneumocytes in the lungs of cats at 7 days after inoculation with highly pathogenic avian influenza viruses H7N7 [20]. Therefore, more study is needed to evaluate the distribution of viral antigens in the lungs of horse infected with EIV.

Real-time PCR for the *sodA* gene of *S. zooepidemicus* in paraffin-embedded lung samples from all horses did not detect the gene in any of the horses during the initial febrile response (PIDs 2 and 3), but the gene was detected in horses necropsied at the time of the second febrile response (PID 7) or later (PID 14). Therefore, the second febrile response was probably caused by secondary bacterial infections, and in fact *S. zooepidemicus* was isolated from the tracheal swab and lung samples of horse #4 necropsied on PID 7. Pittet *et al.* [14] reported that secondary bacterial pneumonia following influenza virus infection is associated with decreased mucociliary clearance induced in the mouse trachea by the virus. In horses infected with EIV, tracheal clearance rates decrease after EIV infection [22]. Here, we found diffuse epithelial degeneration or necrosis, with loss of ciliated epithelium and a dramatic reduction in goblet cell numbers in the trachea on PID 2 and 3. Thereafter, the moderate suppura-

tive bronchopneumonia observed on PID 7 and 14 would have developed after secondary infection with *S. zooepidemicus*. It is therefore likely that secondary bacterial pneumonia following EI is the result of EIV-related damage to the ciliated epithelial cells and goblet cells, which play important roles in mucociliary clearance in the respiratory tract.

To our knowledge, this is the first report to demonstrate the time-related morphological changes caused by EIV infection in horses. Our findings show that EIV infection and viral replication lead primarily to cell damage in the respiratory tract. This suggests that vaccination and early administration of a specific antiviral agent are important in EI for the reduction of primary cell damage. Furthermore, we showed that the decrease in mucociliary clearance in the trachea leads to secondary bacterial pneumonia. Our results suggest that specific antibiotic treatment should be effective in the treatment of secondary bacterial pneumonia in EIV-infected horses with secondary febrile responses.

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