Comparison of histological lesions in mink with acute hemorrhagic pneumonia associated with *Pseudomonas aeruginosa* or *Escherichia coli*

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Abstract

Hemorrhagic pneumonia can be a major cause of mortality in farmed mink in the fall. In its classic form, hemorrhagic pneumonia is caused by the bacterium *Pseudomonas aeruginosa.* In recent years, however, outbreaks of this type of pneumonia that are associated with hemolytic *Escherichia coli* have also occurred in farmed mink. The purpose of this study was to compare histological lesions of acute hemorrhagic pneumonia associated with both *P. aeruginosa* and *E. coli* in mink, including a description of tissue distribution of pathogens, in an attempt to differentiate between the 2 disease entities based on histopathology. The study included material submitted for diagnostic investigation to the National Veterinary Institute in Denmark from 2006 to 2009. Altogether, 19 cases of hemorrhagic pneumonia with a pure lung culture of *P. aeruginosa* and 18 cases of hemorrhagic pneumonia with a pure lung culture of *E. coli* were examined. Formalin-fixed paraffin-embedded lung tissue obtained from the mink was examined by histology and fluorescence *in-situ* hybridization (FISH). It was possible to detect a slight histological difference between hemorrhagic pneumonia caused by *P. aeruginosa* and by *E. coli,* as *P. aeruginosa* was most often found surrounding blood vessels and lining the alveoli, while *E. coli* showed a more diffuse distribution in the lung tissue. Furthermore, *P. aeruginosa* often elicited a very hemorrhagic response in the lung, while infection with *E. coli* was associated with a higher frequency of alveolar edema and mild lymphoid cuffing in the lungs.

Résumé

À l'automne, la pneumonie hémorragique peut être une cause majeure de mortalité chez les visons d'élevage. Dans sa forme classique, la pneumonie hémorragique est causée par la bactérie Pseudomonas aeruginosa. *Au cours des dernières années toutefois, des poussées de cas de ce type de pneumonie associées à des isolats hémolytiques d'*Escherichia coli *se sont produits chez des visons d'élevage. Le but de cette étude était de comparer les lésions histologiques de pneumonie hémorragique aiguë associée à* P. aeruginosa *et* E. coli, *incluant une description de la distribution tissulaire des agents pathogènes, dans une tentative de différencier les deux maladies en se basant sur l'histopathologie. L'étude incluait du matériel soumis pour diagnostic à l'Institut National Vétérinaire du Danemark entre 2006 et 2009. Au total, 19 cas de pneumonie hémorragique avec culture pure de* P. aeruginosa *et 18 cas de pneumonie hémorragique avec une culture pure d'*E. coli *ont été examinés. Du tissu pulmonaire provenant des visons fixé à la formaline et enrobé de paraffine a été soumis à un examen histologique et par hybridation avec fluorescence* in-situ *(FISH). Il a été possible de détecter une légère différence histologique entre la pneumonie hémorragique causée par* P. aeruginosa *et* E. coli, P. aeruginosa *étant retrouvé plus fréquemment entourant les vaisseaux sanguins et tapissant les alvéoles, alors qu'*E. coli *montrait une distribution plus diffuse dans le tissu pulmonaire. De plus*, P. aeruginosa *causait souvent une réponse très hémorragique dans le poumon, alors que l'infection par* E. coli *était associée avec une fréquence plus élevée d'œdème alvéolaire et de présence de manchons lymphoïdes dans les poumons.*

(Traduit par Docteur Serge Messier)

Introduction

Outbreaks of fatal hemorrhagic pneumonia caused by *P. aeruginosa* have been described in farmed mink since the disease was first recognized in Denmark in 1953 (1). Hemorrhagic pneumonia due to *P. aeruginosa* spreads rapidly on the farm, with mortalities reaching 75% (2). The disease may also have a milder course, however, with lower mortality (1). In Denmark, hemorrhagic pneumonia occurs almost exclusively from September to December.

Over the last 5 to 10 y, pure cultures of hemolytic *E. coli* have been isolated from outbreaks of acute hemorrhagic pneumonia in Danish mink submitted for diagnostic investigations. Thus, *E. coli* has become an increasingly important differential diagnosis to hemorrhagic pneumonia caused by *P. aeruginosa*. As with *P. aeruginosa*

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pneumonia, pneumonia associated with *E. coli* occurs primarily in the autumn and the 2 disease entities appear to have very similar macroscopic and microscopic pathology.

In humans, both *P. aeruginosa* and *E. coli* are a rare cause of acute pneumonia in immune-competent individuals (3–5). *Pseudomonas aeruginosa* is mainly associated with acute lung infections in neutropenic humans or those who require mechanical ventilation (6,7), while community-acquired pneumonia due to *E. coli* is primarily seen in humans with underlying diseases (5). In animals, *P. aeruginosa* is a well-known spontaneous pathogen from chronic otitis externa in dogs (8), but is also found in acute deep pyoderma and ocular infections (9,10). Spontaneous lung infections are not described in healthy animals, however, with the exception of mink. The pathology and histopathology of hemorrhagic pneumonia in mink caused by *P. aeruginosa* have been described in detail (11–13).

Escherichia coli has been described as the cause of spontaneous fatal pneumonia in a few cases among dogs, cats, and a horse (14–16). In a study by Tibbetts et al (17), *E. coli* is associated with hemorrhagic pneumonia in mink, although the pathology or histopathology of the disease is not described. To our knowledge, this study is the first attempt to describe the pathology of *E. coli* associated with outbreaks of fatal hemorrhagic pneumonia in mink.

In this study, we compared the histopathology of hemorrhagic pneumonia in mink caused by *P. aeruginosa* and *E. coli* in an attempt to differentiate between the 2 diseases on the basis of histopathology and to possibly identify disparities in disease pathogenesis. We compared the distribution of *E. coli* and *P. aeruginosa* in the lung using fluorescence *in-situ* hybridization (FISH).

Materials and methods

Samples of lung tissue from 37 mink diagnosed with hemorrhagic pneumonia from 2006 to 2009 and with pure cultures of the bacteria of interest were included in this study. Hemorrhagic pneumonia was diagnosed at the National Veterinary Institute in Denmark based on macroscopic evaluation of the lungs, histology, and bacteriological investigations at the time of submission. Cultures from the lungs of mink included in this study yielded pure growth of either *P. aeruginosa* (*n* = 19) or β-hemolytic *E. coli* (*n* = 18). Samples for bacterial culture were obtained from the lungs at the time of submission by quickly burning the surface of the lung and making an incision in the burned area with a sterile scalpel before introducing a sterile swab, which was streaked onto blood agar and MacConkey agar.

One piece of lung tissue per mink from areas with macroscopic lesions was fixed in 10% buffered formalin for 1 to 3 d and embedded in paraffin as part of a routine diagnostic investigation. The formalin-fixed paraffin-embedded (FFPE) tissue was used in the study.

All mink included in this study were tested for antibodies against Aleutian disease virus using an additive counterimmunoelectrophoresis method (18) with negative results. According to records at the National Veterinary Institute in Denmark, the macroscopic findings in the lungs of the 37 mink included in this study all showed swelling, increased texture, and red discoloration, with tissue distribution reported as most often being diffuse and with some animals displaying diffuse lesions with focal, multifocal, or lobar necrosis.

Table I. Average scores for histopathological lesions. In these cases, vasculitis is regarded as transmural inflammation, while interstitial edema refers to perivascular and peribronchial edema

	E. coli	*P. aeruginosa*
Extent of interstitial edema	1.1	1.3
Extent of alveolar edema	1.9	0.9
Amount of fibrin	1.6	1.6
Number of interstitial leukocytes	1.3	1.2
Number of alveolar leukocytes	1.5	1.9
Extent of necrosis	1.5	1.4
Number of erythrocytes in alveoli	1.6	2.3
Vasculitis	0.3	0.6
Thrombosis	0.1	0.3
Epithelial cell loss in conductive airways	1.6	1.9
Lymphoid cuffing	0.6	0.2

The severity of lesions was graded as 0 — no lesions (0%), 1 — mild lesions (0% to 33% of tissue) or a few cells, 2 — moderate lesions (34% to 66% of tissue) or a moderate number of cells, and 3 — massive/severe lesions (> 66% of tissue) or a massive number of cells.

From the paraffin blocks, 3-μm tissue sections were mounted on glass slides (SuperFrost Plus; Menzel-Gläser, Braunschweig, Germany). Each slide was stained with conventional hematoxylin and eosin (H&E) and examined for severity of lesions. The severity of lesions was graded as follows: 0 — no lesions; 1 — mild lesions (up to 33% of tissue involved) or a few cells, e.g., leukocytes or erythrocytes; 2 — moderate lesions (34% to 66% of tissue involved) or a moderate number of cells; and 3 — severe lesions (more than 66% of tissue involved) or a massive number of cells. The average scores for severity of lesions are given in Table I. The slides were examined twice in a blinded fashion by the same examiner. The entire piece of lung on the slide was included when deciding the histological grades assigned to each lung.

The 3-μm tissue sections from the paraffin blocks were mounted on SuperFrost Plus glass slides (Menzel-Gläser). A 6-FAM labeled *P. aeruginosa* probe with the sequence 5′-GGTAACCGTCCCCCCTTGC-3′ (19) (DNA Technology, Aarhus, Denmark) and a Texas Red labeled *E. coli* probe with the sequence 5′-GCATAAGCGTCGCTGCCG-3′ (20) (Eurofins MWG Operon, Ebersberg, Germany) were used to detect *P. aeruginosa* and *E. coli* by fluorescence *in-situ* hybridization (FISH) targeting 16S rRNA in a hybridization concentration of 5 ng/μL of hybridization buffer (0.1M Tris pH 7.2, 0.9 M NaCl, 0.1% SDS) and at a hybridization temperature of 45°C. The oligonucleotide probe sequences were found on probeBase (21). It was noted in probeBase that the chosen *E. coli* probe would not bind to 16S rRNA in all *E. coli* strains. In the slides with tissue from lungs showing a pure culture of *E. coli* but without fluorescence when using the *E. coli* probe, the bacteria were visualized with a mixture of Texas Red labeled gammaproteobacterium probes; GAM 42A, 5′-GCCTTCCCACATCGTTT-3′ and 5′-GCCTTCCCACTTCGTTT-3′ (22) (Eurofins MWG Operon).

The slides were washed 3 × 5 min (0.1M Tris pH 7.2, 0.9M NaCl) and mounted with Vectashield Mounting Medium for Fluorescence with DAPI (Vector Laboratories, Burlingame, California, USA).

To verify the specificity of the probes, we initially used a double-staining protocol including a probe targeting the Bacterial Domain EUB338, 5'-GCTGCCTCCCGTAGGAGT-3' (23) (Eurofins MWG Operon).

Results

Records of diagnostic investigations at the National Veterinary Institute in Denmark clearly show that *E. coli* hemorrhagic pneumonia has been detected in mink with increasing frequency over the last 10 y (Figure 1).

Histology

The average scores for the categories of histopathological lesions are shown in Table I. The calculation of averages is not valid for statistical purposes since the data are categorical, but is used in this case only as a way of displaying the differences in scores. The calculations should therefore be interpreted with caution. The agreement between the 2 assessments of each slide was calculated based on each category and ranged from 62% to 100%.

The significance of the difference between the scores for *E. coli* and *P. aeruginosa* was assessed using Fisher's exact test on a 2 × 4 contingency table (2 groups with 4 scores) and a significance level of 0.05. Only the first grading of the slides was included in the statistical evaluation to avoid working with 2 dependent scores, while both gradings were included in the calculation of the average shown in Table I in order to use as much information from the slides as possible. Three types of lesions differed with significance levels below 0.05, namely the extent of alveolar edema, the amount of erythrocytes in alveoli, and lymphoid cuffing.

Based on morphology, the most predominant types of leukocytes were neutrophil granulocytes and macrophages, but lymphocytes were also observed. In *P. aeruginosa* pneumonia, the types of leukocytes consisted predominantly of neutrophils or a mix of neutrophils and macrophages. In *E. coli* pneumonia, the type of inflammation was dominated by a mixture of macrophages and neutrophils. Two main patterns and various mixtures of these patterns were found, with 1 pattern dominated by necrosis of alveolar septa and vascular walls (rarely penetrating the tunica elastic interna), neutrophils, and massive amounts of bacteria (Figure 2) and the other displaying massive intra-alveolar hemorrhage with few bacteria and inflammatory cells (Figure 3). The very hemorrhagic pattern was most often observed in lungs infected with *P. aeruginosa,* while severe alveolar edema (Figure 4) was most often encountered in lungs infected with *E. coli.*

Epithelial cell loss was more prominent in the bronchioles than in the bronchi in all cases of pneumonia. Perivascular accumulation of bacteria around venules and arterioles was noted for pneumonia caused by *P. aeruginosa* (Figure 5), but was noted only in 1 case for pneumonia associated with *E. coli.*

Fluorescence *in-situ* hybridization (FISH)

In most *P. aeruginosa*-positive mink lungs, the distribution of bacteria in the lung tissue could be regarded as perivascular with varying amounts of *P. aeruginosa* in alveoli, interstitium, and bronchial epithelium. The bacteria did not usually penetrate into the
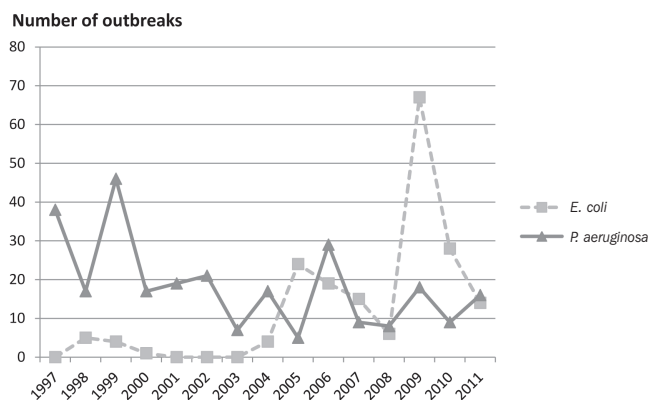
Number of outbreaks

Figure 1. Prevalence of hemorrhagic pneumonia in Denmark caused by *P. aeruginosa* and *E. coli.*

vascular lumen, although this was observed in a few cases in which massive amounts of bacteria were identified surrounding the vessel (Figure 6).

Fewer bacteria were generally found in pneumonia associated with *E. coli* than in pneumonia caused by *P. aeruginosa.* In addition, the distribution of *E. coli* in the tissue was more varied, with bacteria being found mostly in alveoli, bronchioles, and in interstitial spaces surrounding large vessels and bronchi (Figure 7).

Discussion

The average scores for most of the lesions observed in the lung tissue were quite similar for the 2 pathogens. A somewhat similar pattern was expected, since the inclusion criteria for this study were hemorrhagic pneumonia and infection with either *P. aeruginosa* or *E. coli.* Nonetheless, there were significant differences in alveolar edema, the amount of erythrocytes in alveoli, and lymphoid cuffing between the 2 infections. Severe alveolar edema and mild lymphoid cuffing were more often encountered in *E. coli* hemorrhagic pneumonia, while a severely hemorrhagic pattern was most often seen in *P. aeruginosa* hemorrhagic pneumonia. This indicates that *P. aeruginosa* and *E. coli* do not share the same pathological pathway. The animals may die at different time points in the infection, underlying viral infections may be present, or different virulence factors may be employed, all of which could lead to the differences in histological pattern observed between the 2 pathogens. The predominance of neutrophils in *P. aeruginosa* hemorrhagic pneumonia in mink does not agree with a previous study on rats (24), which showed that non-immunized rats developed a response dominated by macrophages and died, while immunized animals cleared an intratracheal challenge dose with *P. aeruginosa* by developing an inflammatory response dominated by neutrophils. The dependence on neutrophils for clearing *P. aeruginosa* infection has been underlined by other studies (25,26), which show that neutrophils are of paramount importance for clearing pulmonary infection with *P. aeruginosa.* In our study, mink displaying a predominantly neutrophil response also died of the infection, which raises the question of whether this dependency is also present in mink. Nevertheless, this cannot be taken as evidence of a lack of dependence on neutrophil leukocytes for clearing of the infection in mink, since no surviving
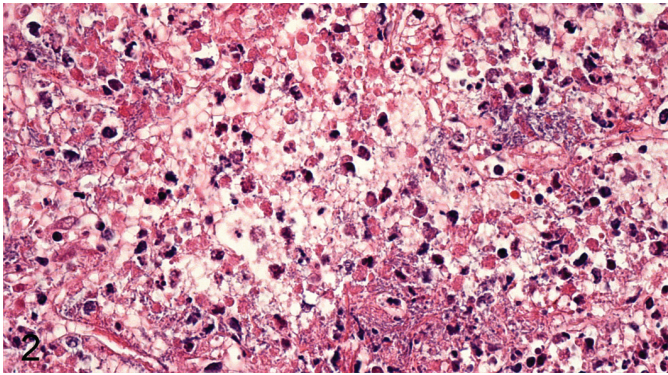
Figure 2. Massive necrosis of alveolar septa, degenerated neutrophils, and myriads of bacteria *(P. aeruginosa)* in mink lung. H&E, 400× magnification.
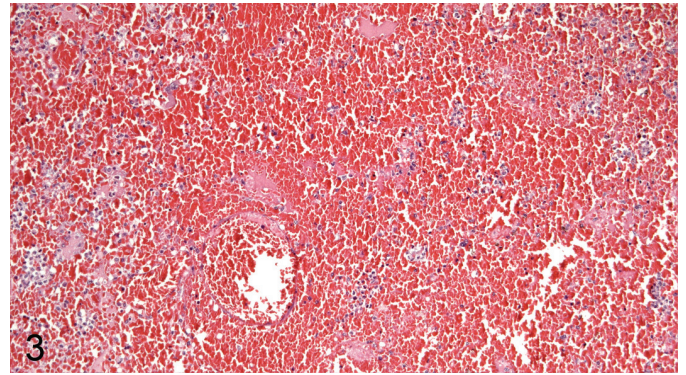

Figure 3. Severe acute pulmonary hemorrhage *(P. aeruginosa)* in mink lung. H&E, 200× magnification.
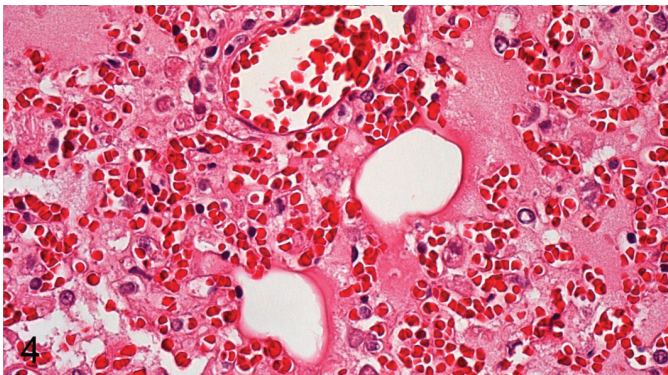

Figure 4. Massive alveolar edema and blood vessel without perivascularly located bacteria *(E. coli)* in mink lung. H&E, 400× magnification.
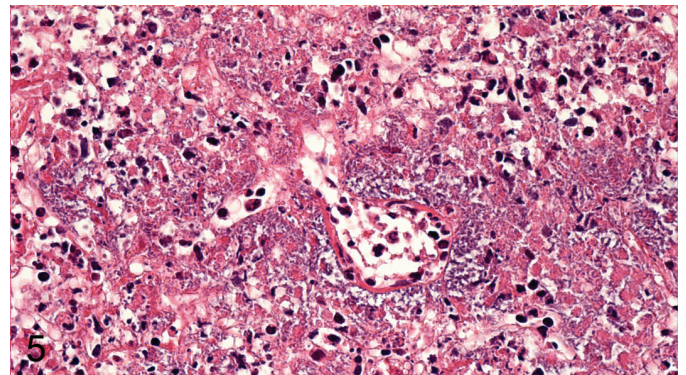

Figure 5. Blood vessel showing massive perivascular accumulation of bacteria, mild vasculitis, and margination of leukocytes along with severe necrosis of the surrounding tissue *(P. aeruginosa)* in mink lung. H&E, 400× magnification.
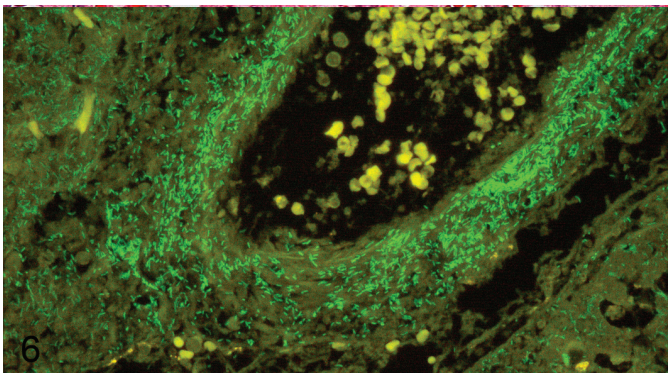

Figure 6. *Pseudomonas aeruginosa* around and in blood vessel wall in mink lung. Fluorescence *in-situ* hybridization with 6-FAM labeled *P. aeruginosa* probe. 400× magnification.
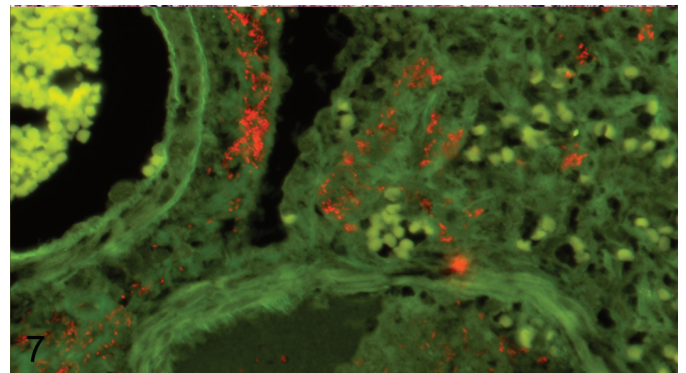

Figure 7. *Escherichia coli* between blood vessel (left upper corner) and bronchiole (right lower corner) in mink lung. Fluorescence *in-situ* hybridization with Texas Red labeled *E. coli* probe. 400× magnification.

animals were included in the study. This makes it impossible to reveal a difference in leukocyte response between survivors and non-survivors. Defect neutrophils are present in the Chediak-Higashi syndrome recognized in mink with the blue coat color type (27–29). If development of hemorrhagic pneumonia in mink was highly dependent on the activity of neutrophils, this type of mink should be more prone to develop hemorrhagic pneumonia. No coat color types were particularly prevalent in this study, although too few mink were represented to further explore this theory.

In 1968, Nordstoga described 2 histopathological patterns in mink lungs infected with *P. aeruginosa* (13). One pattern, which was found in mink from 1 farm, showed a hemorrhagic necrosis without inflammation and with only a few bacteria present. Mink from another farm showed a severe inflammation dominated by neutrophils. In these mink, high numbers of bacteria were often found in the perivascular space and inside the vessel wall but did not penetrate the tunica elastica interna and therefore were rarely seen in the vessel lumen. This study (13) is in good agreement with our finding of

P. aeruginosa in the perivascular space and with our assessment of the slides as showing an either predominantly hemorrhagic picture or a pattern dominated by necrosis of the alveolar septa and vascular walls and massive numbers of neutrophils. The finding of a perivascular localization of *P. aeruginosa* is also in agreement with previously described septic lesions in rats (30). It has not been determined why *P. aeruginosa* shows a predominantly perivascular localization, but it could be speculated that the amount of certain nutrients is higher in this area or that *P. aeruginosa* targets specific receptors. Receptors for specific adherence to some carbohydrates have been identified in the capillaries and epithelium of mink lungs and pancreas (31). This might partially explain why mink are more easily infected with *P. aeruginosa* than other species.

The localization of *E. coli* primarily to the bronchioles, alveoli, and interstitial spaces around larger vessels and bronchi is consistent with the sparse literature on the subject (14,15). The perivascular distribution, as was observed in pneumonia caused by *P. aeruginosa*, was seen only once in our study, while a greater tendency for provoking alveolar edema was observed. It is not clear whether this reflects a difference in receptors or a tendency for evoking different responses by the immune system. Further investigations including experimental studies are necessary to investigate the pathogenesis of hemorrhagic pneumonia in mink.

Diagnostic material is not optimal for use in this type of study but, at present, is the only accessible source of tissue from this disease. The macroscopic lesions described in the materials and methods section are based on records used for diagnostic purposes. These records are subject to variations due to various personnel and handling procedures and should be assessed with caution.

Studies involving experimental infections have previously been conducted with *P. aeruginosa* in mink with the purpose of revealing pathogenesis (11,12,32–34) but this has never been attempted with *E. coli* in mink. It is well-known that both *E. coli* and *P. aeruginosa* possess a plethora of different virulence factors and a further study of the expression of these in bacterial isolates from mink could lead to a better understanding of the pathogenesis of this disease entity. Studies of recruitment of specific cell types and cytokines could also be relevant, since these factors apparently play an important role in the progression of *P. aeruginosa* pneumonia in humans (7,35).

This study revealed a difference in distribution of the pathogens that lead to hemorrhagic pneumonia in mink; *P. aeruginosa* showed a preference for perivascular localization compared to the more diffuse distribution of *E. coli* in the lungs. It was also recognized that *P. aeruginosa* more often evokes a predominantly hemorrhagic pattern in mink lungs, while *E. coli* was more often associated with higher amounts of alveolar edema and mild lymphoid cuffing. It remains to be determined whether these differences reflect aspects of pathogenesis of hemorrhagic pneumonia in mink caused by the 2 pathogens.

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