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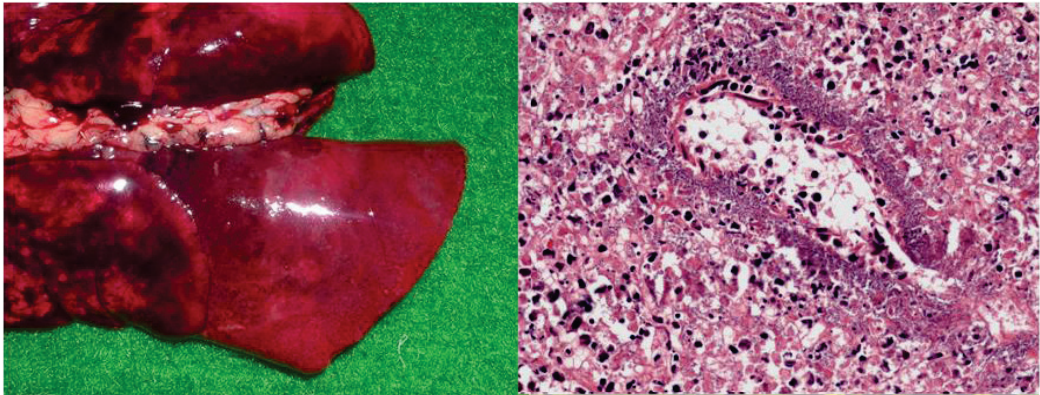
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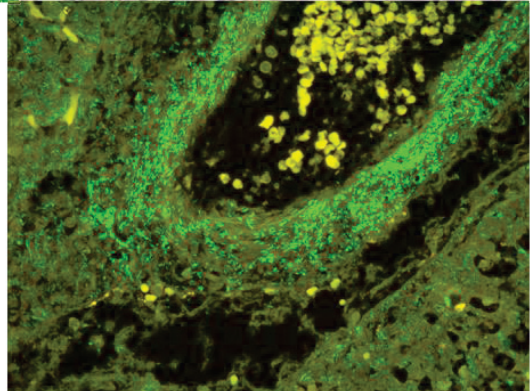
Hemorrhagic pneumonia in mink caused by *Pseudomonas aeruginosa*



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Ph.D. thesis

August 2012



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Table of contents

- Preface and acknowledgements 3
- Abbreviations..... 5
- Summary..... 7
- Sammendrag..... 9
- 1 Introduction..... 11
- 2 Aims and hypotheses..... 12
- 3 Background..... 13
 - 3.1 Mink..... 13
 - 3.2 Hemorrhagic pneumonia in mink 13
 - 3.2.1 Pathology and histopathology..... 14
 - 3.2.2 Pathogenesis..... 18
 - 3.2.3 Epidemiology 19
 - 3.2.4 Hemorrhagic pneumonia associated with *E. coli* 20
 - 3.3 *P. aeruginosa* 21
 - 3.3.1 General 21
 - 3.3.2 Virulence factors..... 22
 - 3.3.3 Antimicrobial drug resistance..... 24
 - 3.3.4 Quorum sensing..... 24
 - 3.3.5 Biofilm..... 24
 - 3.3.6 Population structure and evolution 25
 - 3.3.7 Acute pneumonia caused by *P. aeruginosa* 25
- 4 Materials and methods 27
 - 4.1 Environmental samples 27
 - 4.2 Fluorescence in situ hybridization and histology on tissue from infectious dose experiment 28
 - 4.3 Interview with mink farmers 28
 - 4.4 Typing of isolates from hemorrhagic pneumonia in mink 28
- 5 Results 29
 - 5.1 Environmental samples 29
 - 5.2 FISH and histology on tissue from infectious dose experiment 29

5.3	Interview with mink farmers	34
5.4	Typing of isolates from hemorrhagic pneumonia in mink	34
6	Discussion	35
6.1	Environmental samples	35
6.2	FISH and histology on tissue from infectious dose experiment	35
6.3	Interview with farmers	36
6.4	Typing of isolates from hemorrhagic pneumonia in mink	37
6.5	Overview and discussion of the project	37
7	Conclusion	40
8	Further perspectives.....	41
9	References	43
	Article I: Typing of <i>Pseudomonas aeruginosa</i> from hemorrhagic pneumonia in mink (<i>Neovison vison</i>)	55
	Article II: Comparison of histological lesions in acute hemorrhagic pneumonia in mink associated with <i>Pseudomonas aeruginosa</i> or <i>Escherichia coli</i>	71
	Article III: Effect of infectious dose and season on development of hemorrhagic pneumonia in mink caused by <i>Pseudomonas aeruginosa</i>	85
	Article IV: Investigation of the presence of human or bovine respiratory syncytial virus in the lungs of mink (<i>Neovison vison</i>) with hemorrhagic pneumonia due to <i>Pseudomonas aeruginosa</i>	97

Preface and acknowledgements

This thesis is based upon studies of various aspects of hemorrhagic pneumonia in mink caused by *Pseudomonas aeruginosa*. The studies are presented in four manuscripts accepted for publication, while pilot studies and minor studies are presented in this thesis only. In addition, the thesis includes a review of the literature describing hemorrhagic pneumonia in mink. The intended readers of this thesis are those with a special interest in hemorrhagic pneumonia in mink including veterinarians, mink farmers and researchers working with *P. aeruginosa*.

The work described has primarily been performed at the National Veterinary Institute, Technical University of Denmark (DTU Veterinary) in Aarhus, while some analysis has been conducted at DTU Veterinary in Frederiksberg, at DTU Systems Biology in Lyngby and at Rigshospitalet in Copenhagen.

During my years as a PhD student numerous people have contributed with their support and skills. First of all great thanks is owed to my co-supervisors Professor Niels Høiby at Rigshospitalet and Professor Søren Molin at DTU Systems Biology and my main supervisors, Senior scientist Anne Sofie Hammer and Professor Mette Boye both at DTU Veterinary for fruitful discussions and all kind of help along the way. Anne Sofie was my main supervisor until April 2012 when she left DTU for another position, while Mette Boye was kind enough to guide me through the last three months of work on my PhD. For that I am deeply grateful.

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I would also like to thank the Danish Fur Breeders Association, Nordvacc and DTU Veterinary for financial support of my PhD studies.

Last but not least, great thanks is owed to my lovely family for putting it all into perspective and for their generous supply of all that is important in life.

Charlotte Mark Salomonsen, Aarhus, July 2012.

Abbreviations

ADV: Aleutian disease virus

BRSV: Bovine respiratory syncytial virus

CF: Cystic fibrosis

FISH: Fluorescence *in situ* hybridization

HE: Hematoxylin and eosin

HRSV: Human respiratory syncytial virus

IHC: Immunohistochemistry

MLST: Multilocus sequence typing

PCR: Polymerase chain reaction

PFGE: Pulsed field gel electrophoresis

QTL: Quantitative trait locus

RSV: Respiratory syncytial virus

RT-PCR: Reverse transcriptase polymerase chain reaction

SNP: Single nucleotide polymorphism

Summary

Hemorrhagic pneumonia in mink is an acute and fatal disease caused by *Pseudomonas aeruginosa*. The mink are typically found dead without prior clinical symptoms. The disease can be highly contagious and varying mortalities on the farm level has been reported. Hemorrhagic pneumonia in mink is seasonal with outbreaks almost exclusively occurring from September to November in Denmark. In human medicine, *P. aeruginosa* is regarded as a pathogen for immune compromised individuals but no underlying disease or immune defect has been identified in mink dying of hemorrhagic pneumonia. In fact, little research has been performed in this field and most published work is more than 25 years old.

The studies presented in this thesis aim at elucidating varying aspects of the disease:

Article I investigates the relationships of *P. aeruginosa* isolated from mink hemorrhagic pneumonia using pulsed field gel electrophoresis (PFGE) and a commercial typing system based on single nucleotide polymorphisms (SNP) on chosen strains.

The results presented in this article show that 70% of *P. aeruginosa* isolated from outbreaks of hemorrhagic pneumonia in mink consist of unique strains, while the remaining 30% belongs to either a cluster of closely related strains or unrelated but prevalent strains. This indicates that most outbreaks of hemorrhagic pneumonia are caused by environmental isolates and not by strains specially adapted to mink which spread among mink farms.

Article II compares the histopathological lesions in hemorrhagic pneumonia caused by *P. aeruginosa* and *E. coli* in diagnostic material. The distribution of the two pathogens is visualized using fluorescence *in situ* hybridization (FISH).

Two histological patterns were observed in the work presented in Article II; one was very hemorrhagic with few bacteria while the other was dominated by necrosis, neutrophils and massive amounts of bacteria. The hemorrhagic pattern was predominantly seen in *P. aeruginosa* infected lungs and this bacterium showed a preference for perivascular localization while alveolar edema was more frequently identified in hemorrhagic pneumonia associated with *E. coli* infection. The perivascular localization, tendency for a higher frequency of a very hemorrhagic response and alveolar edema were the only differences noted between hemorrhagic pneumonia caused by *P. aeruginosa* compared to *E. coli*.

Article III describes an infectious dose trial on mink. This experiment was performed in July and in November to elucidate whether the same infectious dose would be able to cause hemorrhagic pneumonia both in and out of the reported season for development of hemorrhagic pneumonia. Furthermore the ability to recover *P. aeruginosa* from the nasal mucosa of experimentally infected mink and from farm mink was investigated.

The results from this study suggest that some mink are predisposed to develop hemorrhagic pneumonia since wide ranges in infectious dose leading to hemorrhagic pneumonia were observed and a tendency towards greater mortality in November as compared to July could be demonstrated. It was possible to culture *P. aeruginosa* from the nasal mucosa up till eight days after experimental infection but no *P. aeruginosa* was ever recovered from the nasal mucosa of farmed mink.

Article IV focuses on the presence of respiratory syncytial virus (RSV) as a possible explanation for the high susceptibility of mink to development of hemorrhagic pneumonia due to *P. aeruginosa*. Polymerase chain reaction (PCR) was used to examine 50 lung tissue samples for the presence of both the bovine and the human type of RSV.

The results of this work show that it was not possible to detect RSV in mink lung samples with hemorrhagic pneumonia caused by *P. aeruginosa*.

Results from investigations not included in articles are also presented. These include interviews with farmers experiencing outbreaks of hemorrhagic pneumonia in their mink, an attempt to culture *P. aeruginosa* from the farm environment and histology of tissue from the experimentally infected mink described in Article III.

It was only possible to culture *P. aeruginosa* from the farm environment in one case on a farm with an outbreak of hemorrhagic pneumonia. The most striking conclusion of the interviews with farmers experiencing outbreaks of hemorrhagic pneumonia among their mink was that the disease always started in the mink kits, never in the adults. Furthermore, 39% reported that most deaths occurred in the male mink.

The results presented in this thesis suggest that factors of the mink make them more prone to develop hemorrhagic pneumonia due to *P. aeruginosa* in the fall. This is based on the discovery that most outbreaks are not due to a special *P. aeruginosa* strain with an increased virulence for mink, but rather local strains. The observed tendency of mink to develop hemorrhagic pneumonia in the autumn irrespective of infectious dose and the difficulty in isolating *P. aeruginosa* in the farm environment also points to something in the mink as being crucial in the development of disease.

Sammendrag

Smitsom lungebetændelse er en akut og fatal hæmorrhagisk lungebetændelse forårsaget af *Pseudomonas aeruginosa* hos mink. Minkene findes ofte døde uden forudgående symptomer. Sygdommen har en meget varierende dødelighed men kan være meget smitsom og forårsage tabsvoldende udbrud. Næsten alle udbrud af smitsom lungebetændelse opstår fra september til november i Danmark. Fra humanmedicin kendes *P. aeruginosa* primært som et opportunistisk patogen, der næsten udelukkende inficerer individer med nedsat immunforsvar. Dette er ikke tilfældet for mink, hvor tilsyneladende raske individer udvikler sygdommen og er i stand til at smitte andre mink. Den publicerede litteratur om emnet er sparsom og de fleste kilder er mere end 25 år gamle.

Denne afhandling er bygget på forskellige undersøgelser med det formål at belyse aspekter af sygdommen smitsom lungebetændelse hos mink:

Artikel I undersøges graden af slægtskab blandt *P. aeruginosa* isoleret fra smitsom lungebetændelse hos mink via pulsed field gel electrophoresis (PFGE) og med et kommercielt typningskit baseret på single nucleotide polymorphisms (SNP) på udvalgte isolater.

Resultaterne viste at 70% af *P. aeruginosa* isoleret fra smitsom lungebetændelse hos mink bestod af unikke stammer, mens 30% tilhørte en gruppe af nært beslægtede stammer eller særligt hyppige stammer. Dette indikerer, at de fleste udbrud af smitsom lungebetændelse skyldes lokale stammer og ikke en speciel virulent stamme, der spredes imellem minkfarmene.

Artikel II sammenligner de histologiske læsioner ved hæmorrhagisk lungebetændelse forårsaget af *P. aeruginosa* og *E. coli*. Fordelingen af bakterierne i vævet blev visualiseret med fluorescens *in situ* hybridisering (FISH).

I denne artikel identificeredes to histologiske mønstre for hæmorrhagisk lungebetændelse forårsaget af *P. aeruginosa* og *E. coli*. Det ene bestod af massiv intraalveolær blødning og få bakterier, mens det andet var præget af nekrose, neutrophile og store mængder bakterier. *P. aeruginosa* var ofte lejret perivaskulært og forårsagede et større antal lungebetændelser med det meget hæmorrhagiske mønster, mens *E. coli* oftere medførte alveolært ødem. Dette var de eneste forskelle observeret for hæmorrhagisk lungebetændelse forårsaget af de to patogener.

Artikel III beskriver et infektionsforsøg med *P. aeruginosa* i mink. Infektionerne blev udført i juli og i november for at belyse om den samme infektiøse dosis ville fremkalde hæmorrhagisk lungebetændelse både i og udenfor sæsonen, hvor smitsom lungebetændelse typisk optræder. Derudover blev det undersøgt om *P. aeruginosa* kunne genfindes på næseslimhinden dels otte dage efter eksperimentel infektion og dels i mink fra farme ved pelsning.

Resultaterne fra dette forsøg indikerer, at nogle mink er prædisponerede for at udvikle smitsom lungebetændelse, eftersom de mink, der udviklede hæmorrhagisk lungebetændelse i forsøget, blev inficeret med vidt forskellige doser. Der kunne påvises en tendens til højere dødelighed i november sammenlignet med juli. Det var muligt at dyrke *P. aeruginosa* fra næseslimhinden af eksperimentelt inficerede symptomfrie mink op til otte dage efter infektionen, mens der ikke kunne findes *P. aeruginosa* på næseslimhinden af raske farmmink.

Artikel IV fokuserer på forekomsten af respiratorisk syncytial virus (RSV) i lungevæv fra mink døde som følge af smitsom lungebetændelse. En underliggende infektion med RSV kunne være forklaringen på minks forøgede modtagelighed for infektion med *P. aeruginosa*. PCR blev brugt til at undersøge 50 prøver af lungevæv for forekomsten af både den humane og den bovine RSV-type.

Der kunne ikke påvises RSV i nogle af de undersøgte lungevævsprøver fra mink med smitsom lungebetændelse forårsaget af *P. aeruginosa*.

Foruden de ovennævnte forsøg, der er beskrevet i artiklerne I-IV præsenteres også enkelte mindre forsøg i denne afhandling. Disse omhandler interviews af minkfarmere, som har oplevet udbrud af smitsom lungebetændelse på deres farm, forsøg på at dyrke *P. aeruginosa* fra miljøet, samt histologi på væv fra de eksperimentelt inficerede dyr beskrevet i artikel III.

Det var kun i ét tilfælde muligt at isolere *P. aeruginosa* i en prøve fra miljøet, dette var på en minkfarm med udbrud af smitsom lungebetændelse. Den mest slående konklusion fra de udførte interviews med minkfarmere var, at de første mink, der døde af smitsom lungebetændelse, i alle tilfælde var hvalpe. Derudover svarede 39% af de adspurgte, at de oplevede en overvægt af hanner blandt de døde mink, mens de øvrige synes fordelingen mellem kønnene var ligelig.

Resultaterne fra dette ph.d. projekt indikerer, at faktorer hos minkene er afgørende for udviklingen af smitsom lungebetændelse. Dette baseres på, at de fleste udbrud forårsages af en lokal *P. aeruginosa*-stamme og altså formodentlig ikke af en stamme med særlige virulens-egenskaber overfor mink. Derudover blev der påvist en tendens til øget mortalitet som følge af infektion med *P. aeruginosa* i november i forhold til i juli uafhængig af den anvendte infektionsdosis. Eftersom det var vanskeligt at dyrke *P. aeruginosa* fra farmmiljøet, må det formodes, at minkene normalt ikke møder store mængder af *P. aeruginosa* i deres nærmiljø, hvilket støtter at minkene er specielt modtagelige for infektion med netop denne bakterie på visse tidspunkter af året.

1 Introduction

Mink is the only animal species known to develop acute, contagious and fatal hemorrhagic pneumonia due to *Pseudomonas aeruginosa* in apparently healthy individuals. Since a vaccine against hemorrhagic pneumonia in mink was developed in the 1980'es only limited research has been carried out on this disease.

Several questions need to be answered in order to understand this disease better:

- 1) Where does the bacterium come from?
 - a. Environment (e.g. cages, water supply, feed, soil), implying that "common" environmental strains are responsible for disease outbreaks.
 - b. Introduction of particularly virulent *P. aeruginosa* strains with certain virulence factors via feed, personnel, animals or water supply.
 - c. Commensal microorganisms living as a natural flora in the mink upper airways and gaining virulence under certain circumstances.

- 2) Why is this bacterium so virulent for mink?
 - a. Is it due to specific virulence factors possessed by the bacterium?
 - b. Is it due to factors of the mink (specific receptors for *P. aeruginosa*, stress, subclinical disease, immune deficiencies, limited space)?
 - c. Underlying viral infections?

- 3) Why is the disease almost exclusively occurring from September to early December?
 - a. Higher numbers and better survival of *P. aeruginosa* in the environment in the autumn months perhaps due to increased humidity, suitable temperature and less sun/UV light.
 - b. Certain factors concerning the mink expressed only at this time of year creating favorable conditions for infection (change of fur, high weight gain, hormonal changes, subclinical diseases e.g. fatty liver)
 - c. Underlying viral diseases which peak at this time of year?

- 4) Why are other bacteria (primarily hemolytic *Escherichia coli*) capable of eliciting similar hemorrhagic response in the mink lung?
 - a. Certain receptors activated by infection with the various pathogens
 - b. Specific virulence factors possessed by the various pathogens

An increased understanding of the pathogenesis of this disease may lead to better disease management through increased protection against transmission and a rational treatment strategy. This would benefit mink welfare and enable lower usage of antimicrobial compounds. Increased understanding of the pathogenesis of *P. aeruginosa* infections might also be of value in human medicine, since the organism is a well known pathogen especially in immune compromised individuals.

2 Aims and hypotheses

This thesis tries to elucidate:

- 1) Are the bacteria responsible for outbreaks of hemorrhagic pneumonia ubiquitous environmental organisms or do they belong to a certain lineage of closely related types circulating in the mink environment?

Hypothesis: If the bacterium originated from the environment, a plethora of different genotypes would be discovered when typing *P. aeruginosa* from outbreaks of hemorrhagic pneumonia. If few types were determined when typing bacterial isolates from succeeding years, specific virulence factors or types of bacteria would be suspected as the disease causing agents.

- 2) Is the susceptibility of infection with *P. aeruginosa* in the mink higher in the autumn?

Hypothesis: If the mink were equally susceptible to developing hemorrhagic pneumonia both in the autumn and out of the season for hemorrhagic pneumonia, it would indicate that the observed seasonality of the outbreaks on farms are due to external factors, e.g. *P. aeruginosa* being present in higher numbers in the autumn, where the disease is normally encountered.

- 3) Are the histopathological lesions observed in hemorrhagic pneumonia associated with *P. aeruginosa* and *E. coli* and the distribution of the pathogens similar?

Hypothesis: If *P. aeruginosa* and *E. coli* are associated with the exact same histopathological lesions and the same distribution in the lung, it would point to a common pathogenesis leading to hemorrhagic pneumonia. This might be in the form of specific receptors or virulence factors common to the pathogens.

- 4) Is respiratory syncytial virus (RSV), which is known to enhance adhesion of *P. aeruginosa* to cells from mice and humans [1-3] present in mink lungs with hemorrhagic pneumonia?

Hypothesis: If RSV was found in lungs from mink dying of hemorrhagic pneumonia, this finding could explain the unexpected susceptibility to *P. aeruginosa*, which is typically not considered a lethal pathogen for healthy individuals.

- 5) Does the bacterium originate from the mink itself?

Hypothesis: If *P. aeruginosa* was found as a commensal bacterium in clinically healthy mink, it would be suspected that mink could be carriers of this organism and that under certain external or physiological circumstances it could become virulent for the mink.

3 Background

3.1 Mink

A short introduction to the mink farming in Denmark is given in this chapter.

The Danish farmed mink (*Neovison vison*) originates from America and has kept its natural seasonal life cycle under farmed conditions. Mating is performed in March, where females are moved to the cages of males. Mink display induced ovulation and delayed implantation of fetuses [4] resulting in a gestation period of 40-60 days [5]. This results in a very short period of giving birth. Almost all mink kits are born in the end of April and the beginning of May. A typical litter size in Danish farmed mink consists of 5-6 viable kits weighing approximately 10 g. In the beginning of July, when the kits are 8 weeks of age, they are separated from the dam and placed in cages of 2-4 mink depending on the cage size. Until the middle of November when pelting begins, the mink quickly gains weight and size reaching a final weight of around 1700 g for females and 3200 g for males. The selection of next year's breeding stock starts in the beginning of November before pelting is initiated.

In October the mink will start to change their fur to winter coat. Several alterations in the endocrine activity happen at the onset of fall related to decreased day length. At the beginning of autumn the level of circulating melatonin increases in parallel with decreasing levels of prolactin [6,7]. Furthermore the onset of testosterone production in the male mink begins in October [8]. Changes in levels of other hormones, like growth hormone and insulin-like growth factor-1 may be speculated to occur but very limited research has been conducted on the function and fluctuations of these hormones in the mink.

Mink are predators adapted to a highly digestible diet primarily consisting of fresh meat. This means that their large intestine is poorly developed and the transit time of feed from mouth to anus only comprises 2-3 hours [9]. In Denmark almost all mink farms are supplied with feed from one of 14 central feed kitchens from where the feed is delivered daily to the farms. The contents of the feed vary according to accessibility of the ingredients but generally consist of fish and fish byproducts as well as blood, fat and other byproducts from slaughtering of poultry, swine and cattle. This is mixed with various cereal products. Some components of the feed are heat treated while others are used raw or acidified. The availability of specific nutrients, vitamins, water content, fibers and pH value is an area of continued research with the aim of optimizing feed intake, health and fur quality and minimizing the production cost of the feed.

The size of a typical Danish mink farm is on average around 2.000 breeding females resulting in a maximum farm size of around 12.000-13.000 animals in the autumn, but the size ranges from below 1.000 to more than 40.000 animals on a single farm in the autumn.

3.2 Hemorrhagic pneumonia in mink

Hemorrhagic pneumonia in mink was first described in 1953 in Denmark [10]. The disease is caused by the bacterium *Pseudomonas aeruginosa* and causes varying mortality on the farm level ranging from below 1% to 75% [10-15]. Hemorrhagic pneumonia has a rapid disease progression in the individual animal. Signs of illness or a drop in feed intake are seldom observed before the animal is found dead, typically with blood around nostrils and mouth. Mink is the only animal species known to develop contagious, acute and fatal

hemorrhagic pneumonia due to *P. aeruginosa*. In humans and other animals, the bacterium is known as an opportunistic pathogen that typically requires the host to be immune compromised or in other ways susceptible to be able to establish an infection [16-18]. Outbreaks of hemorrhagic pneumonia due to *P. aeruginosa* on Danish mink farms are almost exclusively recorded from September to November. Whether this seasonality is related to factors of the mink, which has a highly seasonal life cycle, or it relates to other factors in the environment has not been investigated. Factors in the environment could either be infectious (e.g. viruses) or be related to the composition of the feed at this time of year. Another factor could be the weather conditions of the autumn with high humidity, temperatures typically above freezing but below 15°C and less sunlight and hence less UV radiation than at other times of the year, creating favorable conditions for survival of microorganisms – both bacteria and viruses.

One of the possible predisposing factors of the mink itself could be the fluctuations of hormones experienced by the mink at the onset of fall. A slow rise in testosterone occurs from October [8] and the hormones involved in change of fur and adaption to altered photoperiod also exhibit fluctuations at this time of year. Which hormones are ultimately responsible for molting is debated, but a rise in melatonin and a fall in circulating prolactin are observed in September [6]. It is well established that the immune system and the endocrine system communicate and affect each other [19,20] and that various hormones, including prolactin and melatonin, influence the immune defense [19,21]. Melatonin is believed to be important in regulation of the immune defense by stimulating acute inflammation and attenuating chronic inflammation [19,22]. Females are considered to show a stronger immune response as compared to males [23,24] and the onset of production of testosterone could be one reason for the apparent increased susceptibility of male mink to infection with *P. aeruginosa* [12,15,25]. Since prevalence of hierarchy and territorial fights increase in the autumn, stress hormones (corticosteroids and catecholamines), which are well known suppressors and modulators of the immune system, may also be of importance at this time of year [26,27]. The catecholamines are shown to directly increase the growth of *P. aeruginosa in vitro* [28-31] but it is questionable whether the concentrations of these hormones would be sufficient in the airways to stimulate the growth of *P. aeruginosa*.

3.2.1 Pathology and histopathology

The pathology and histopathology of hemorrhagic pneumonia has been described in details [13,32-36]. Gross lesions typically consist of one or more, dark red, consolidated lung lobes (figure 1) with blood oozing from the cut surface and with dark red froth and hyperemic mucosa in the tracheobronchial tree. The pathologic changes often involve more than 50% of the lungs [10,13]. An overview of the histological changes described in hemorrhagic pneumonia in mink is given in table 3.1.

The timeline of the development of lesions has been described by Long et al. [33], using both electron microscopy and light microscopy. Mink free of antibodies against Aleutian disease and without signs of the heritable disease, Chediak-Higashi syndrome, were inoculated intra-tracheally with 1600 *P. aeruginosa* in total. Eight hours after infection neutrophils and macrophages were present in terminal bronchioles and alveoli and the capillary spaces were widened. Necrosis of alveolar walls and fibrin could be seen after 24 hours along with degenerated neutrophils, perivascular edema and hemorrhage. The inflammation spread in the lungs up till 60 hours after infection, but after 36 hours macrophages increased in numbers and numerous bacteria were seen in necrotic foci, perivascular and along tissue planes. The lesions of animals

dying from the infection were similar to the above described but were more extensive. After 60 hours a perivascular distribution of macrophages were noted along with prominent type II epithelial cells.

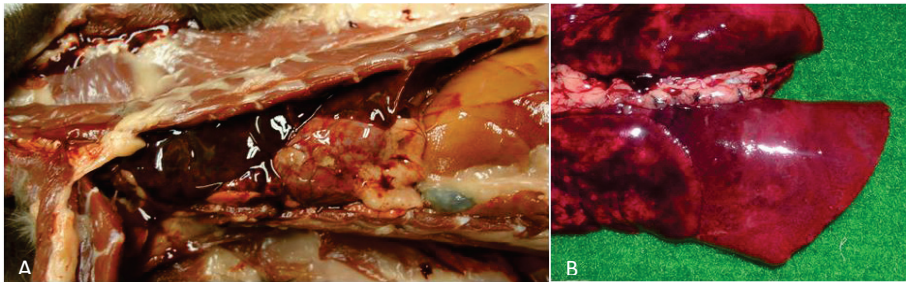


Figure 1: Hemorrhagic pneumonia in mink due to *P. aeruginosa*. A: Dark red, edematous lung lobes with left caudal lung lobe being grayish/brown with increased texture. B: Swollen red/gray left caudal lung lobe with increased texture

Table 3.1: Overview of literature describing histological changes in mink hemorrhagic pneumonia. NS = not stated

Author	Conducting airways	Alveoli	Vessels	Other organs	Lesions in lungs of surviving mink
Homma et al., 1978 [34]	NS	Hemorrhage, leukocytes, fibrin, numerous bacteria and desquamated neutrophils. Perivascular, peribronchiolar and subpleural edema.	Bacteria were especially located in the perivascular space and the vascular walls and thrombi of bacteria were found in capillaries.	In one mink a local purulent interstitial nephritis was noted.	After 13 days: Mild infiltration of leukocytes of the alveolar walls and proliferation of alveolar epithelium along with perivascular cuffing with mononuclear leukocytes.
Long et al., 1980 [33]	Neutrophils, few macrophages, mucosal degeneration in bronchioles.	Necrosis of alveolar walls, exudate, degenerated neutrophils, macrophages and erythrocytes. Numerous bacteria along necrotic septa. Mink dying before 24 hours showed few inflammatory cells but massive hemorrhage.	Perivascular localization of edema and bacteria, occasionally inside vascular walls of small vessels.	No morphologic changes evident in liver and spleen. Unclear if this is microscopically determined.	After 3½ days: Perivascular distribution of macrophages, prominent type II epithelial cells. Occasional necrotic foci. Loss and proliferation of bronchiolar epithelium. After 14 days: Few showed mononuclear vascular cuffs.
Nordstoga, 1968 [32]	Desquamated and necrotic epithelium of bronchioles.	Two histological patterns dominated each of two farms: One, showed few bacteria and massive intra-alveolar hemorrhage. The other was dominated by alveolar neutrophils and numerous bacteria.	Perivascular and mural accumulation of bacteria, but only rarely penetration of the tunica elastica interna was seen and thrombosed vessels were rare.	Congestion, hemorrhages and bacteria were found in other organs, but no inflammatory lesions were observed.	NS
Trautwein et al., 1962 [13]	Exudate, erythrocytes, bacteria and few neutrophils.	Exudate, erythrocytes, bacterial colonies and variable numbers of neutrophils. Sometimes peribronchiolar necrosis.	Hyperemia was marked and small bacterial colonies were found in the capillaries.	NS	After six weeks: No histological lesions.
Elsheikh et al., 1987 [36]	NS	Bacteria, erythrocytes, neutrophils and mononuclear leukocytes. Necrosis, hyperemia and edema.	Perivascular located bacteria.	NS	NS

Shimizu et al., 1976 [35]	NS	Hemorrhage, fibrinous exudate, leukocytes, necrosis of epithelium and bacteria.	Perivascular edema and accumulation of bacteria.	Infrequent granulomas of spleen and lymph nodes, cell foci in liver and fibrinoid necrosis of blood vessels.	After 24 days: Mild thickening of alveolar walls due to leukocytes and proliferation of mesenchymal cells.
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3.2.2 Pathogenesis

Studies have indicated that mink can develop fatal hemorrhagic pneumonia following an intra-tracheal or intra-nasal challenge dose of as low as 10^3 colony forming units (cfu) in total in 1-3 days [11,33,34]. Other animals, like mice and guinea pigs, require substantially higher challenge doses (10^7 - 10^9 cfu in total) to develop fatal disease when infected through the airways [37-39]. In humans, *P. aeruginosa* most often cause acute pneumonias in artificially ventilated patients, in neutropenic individuals or in those with impaired immune defense due to HIV or organ transplants [40].

Male mink kits have been reported to show a higher mortality than female kits and adults [12,15,25]. The incubation period in experimental infections ranged from 0 to 4 days with most reported deaths occurring from 24 hours to 48 hours. An overview of studies including experimental infections is presented in table 3.2. A general concern of the validity of the results presented in the literature, both regarding pathogenesis and pathology, is the general lack of information on the status of the mink regarding infection with Aleutian disease virus (ADV) or occurrence of the Chediak-Higashi syndrome. Both Aleutian disease, which is a chronic parvoviral infection, and the Chediak-Higashi syndrome, which is an inherited autosomal recessive genetic defect related to the "blue" coat color type, result in impaired immune defense [41,42] and hence might lead to higher mortality than would be observed in healthy mink. This has not been documented for hemorrhagic pneumonia, but Long and Gorham [25] reported more serious lesions of hemorrhagic pneumonia in mink with lung changes due to ADV and Honda et al. [12] described three farm outbreaks with the highest mortality occurring on the farm with the highest prevalence of antibodies against ADV.

The pathogenesis of hemorrhagic pneumonia in mink has never been fully elucidated. Earlier studies have suggested that mink are easier to infect via the intra-tracheal route than orally [11,13] and that the transmission route may be airborne [43]. Feed, water troughs, soil, personnel and equipment have been suggested as contamination sources [10,13,44,45] but no investigations have confirmed this. An earlier study has demonstrated *P. aeruginosa* in the oropharynx and rectum of healthy mink at pelting [25] indicating that *P. aeruginosa* may be a commensally living organism in the airways and intestines which only cause disease under certain circumstances.

The bacterium can be isolated in low numbers from liver and spleen 60 hours after intra-tracheal infection [33]. *P. aeruginosa* originating from mink hemorrhagic pneumonia has been isolated as long as 23 days after intra-nasal infection from various organs and with a substantial higher LD₅₀ than *P. aeruginosa* originating from human pus which was not cultured four days after infection [43] indicating that *P. aeruginosa* isolated from disease in mink might be adapted to infect and survive in this species. It was possible to isolate *P. aeruginosa* from mink surviving experimental infections after 13 days in one experiment [34] while others did not find *P. aeruginosa* in survivors after seven days [36,46]. An overview of literature describing experimental infections with *P. aeruginosa* in mink is presented in table 3.2. As can be seen from the table, the results from the various studies are difficult to compare due to the large variations in the age and type of mink used for the experiments and the fact that detailed information is rarely given.

Table 3.2: Overview of publications describing infectious dose experiments. NS = not stated.

Author	Time until death	Total infective dose	Route of infection	Mortality	Bacteria in survivors	Animals
Elsheikh et al., 1987 [36]	0-3 days	10^7 - 10^9 bacteria	Intra-tracheal via mouth	17-88% depending on strain of bacteria	No bacteria after 7-12 days	6-8 months old
Elsheikh et al., 1988 [47]	NS	10^9 - 10^{10} bacteria	Intra-tracheal	100% for non-vaccinated	NS	9-12 months old
Homma et al., 1978 [34]	1-3 days	10^3 - 10^{10} bacteria	Intra-nasal via catheter	LD ₅₀ of 10^3 - 10^4 for non-vaccinated	Yes, in some, after 13 days	Females, sapphire, 3½ months old
Hunter and Prescott, 1982 [46]	16-44 hours	10^6 - 10^8 bacteria	Intra-tracheal	25-100% for non-vaccinated	No bacteria after 7 days	Pastel
Karlsson et al., 1971 [11]	2 days	10^3 - 10^8 bacteria	Intra-nasal via pipette, orally via feed or gavage	Seen from 10^4 bacteria but 100% for doses above 10^7	NS	Young mink
Long et al., 1980 [33]	0-3 days	10^3 - 10^7 bacteria	Intra-tracheal via mouth	15% to 100%	Yes, after 3½ days	Female, pastel, 3 months old. Negative for ADV and CH
Shimizu et al., 1974 [43]	0-3 days	10^4 - 10^9 bacteria	Intra-nasal via vinyl tube	Almost 100% from infectious dose of $>10^5$	Yes after 23 days in few mink	Females, sapphire
Shimizu et al., 1976 [35]	2-4 days	10^8 - 10^{10} bacteria	Intra-nasal via syringe or spray	50% to 100%	No bacteria after 24 days	Females, sapphire, 7 or 17 months old
Trautwein et al., 1962 [13]	1-3 days	NS	Intra-tracheal, in drinking water or in feed	Intra-tracheal infection: 100%. Drinking water infection: 24-33%	NS	Females, pastel, 7 or 12 months old

3.2.3 Epidemiology

The epidemiology of hemorrhagic pneumonia in mink has never been studied in detail. In 2003, Hammer et al. [44] published a study indicating that outbreaks of hemorrhagic pneumonia in Denmark in 1998-2001 were caused either by successful clones, recovered in one year only, or unique *P. aeruginosa* clones causing single outbreaks or small clusters of local outbreaks.

Twenty different serotypes of *P. aeruginosa* have been identified [48]. The only serotypes encountered in Danish outbreaks of hemorrhagic pneumonia in mink are IATS groups 5, 6 and 7/8 (serotyped by Difco™ polyclonal serotyping, Detroit, MI, U.S.A). The records at the National Veterinary Institute in Denmark were used to collect data on outbreaks of hemorrhagic pneumonia in mink. During the last 17 years the distribution of serotypes causing hemorrhagic pneumonia in mink was: serogroup 6; 66%, serogroup 7/8; 18%, and serogroup 5; 11%. It was not possible to serotype 1% and the remaining 4% were not typed at the time of isolation. The seasonality of outbreaks in the last 17 years is shown in Figure 2.

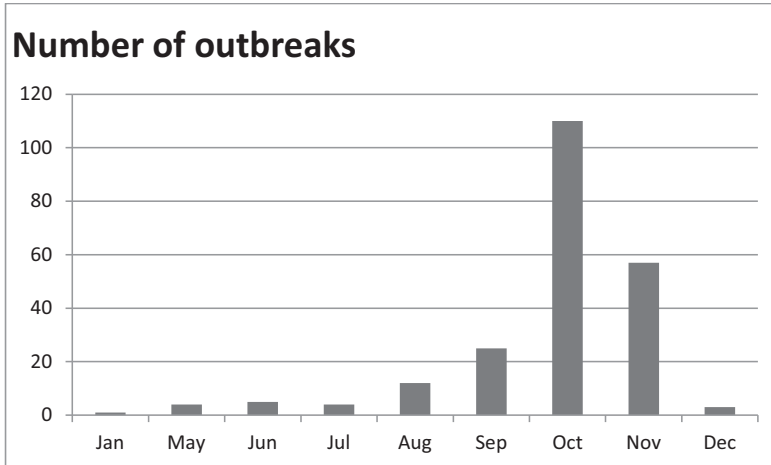


Figure 2: Distribution of outbreaks of hemorrhagic pneumonia from 1999-2011

3.2.4 Hemorrhagic pneumonia associated with *E. coli*

An important differential diagnosis to the classical hemorrhagic pneumonia in mink is pulmonary infection with the Gram-negative rod *Escherichia coli*. The records at the National Veterinary Institute in Denmark show that the disease has been recorded with increased frequency in mink submitted for diagnostic investigations in the last 5-10 years (Figure 3). The peak in outbreaks recorded in 2009 coincided with an outbreak of influenza on Danish mink farms indicating a possible underlying viral etiology of this disease. Hemorrhagic pneumonia associated with *E. coli*, is most often encountered during the autumn months but seldom gives rise to high mortality rates. The macroscopic pathology of the two diseases is indistinguishable making pneumonia caused by *E. coli* the most important differential diagnosis to hemorrhagic pneumonia caused by *P. aeruginosa*. *E. coli* has been described in coli-septicemia in mink [49] in U.S.A. in the period 1994-1999 with the typical lesion being hemorrhagic pneumonia. The pathogenesis of hemorrhagic pneumonia associated with *E. coli* has never been investigated in mink. However, the observed pathologic pattern is lobar involvement of the lungs with no macroscopic changes in other organs, like in *P. aeruginosa* pneumonia, which suggests an aerogen infection route.

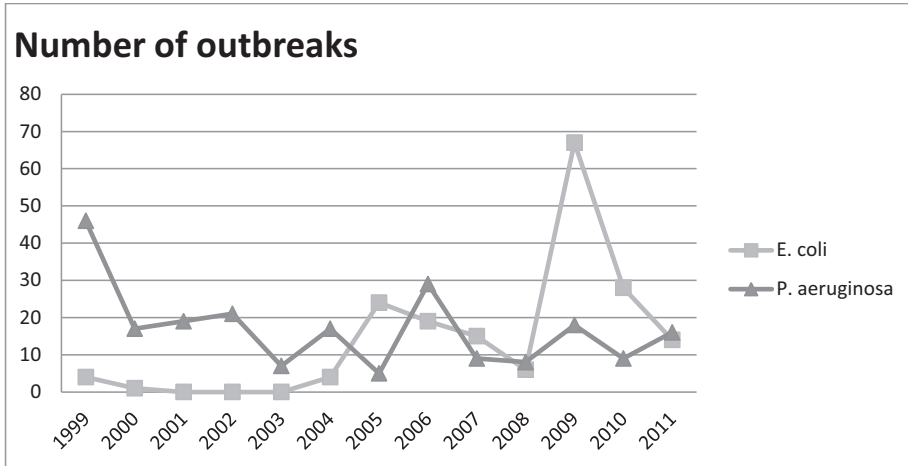


Figure 3: Outbreaks of hemorrhagic pneumonia diagnosed at the National Veterinary Institute in Denmark due to infection with *E. coli* or *P. aeruginosa*

3.3 *P. aeruginosa*

3.3.1 General

P. aeruginosa is a motile Gram-negative rod belonging to the class of gamma-Proteobacteria in the family Pseudomonadaceae. It has a unipolar flagellum, is able to grow up till a temperature of 42°C and can grow under very limited nutritional conditions [50]. Twenty different serotypes of *P. aeruginosa* are identified based on differences in the O-antigen of lipopolysaccharide (LPS) [48].

The genome is estimated to an average size of 6.4 Mb [51]. The large size of the genome reflects the extreme versatility of this organism. *P. aeruginosa* inhabits a wide range of environments [52]. The organism has been recovered from soil, sea water, fresh water and distilled water [53-56]. The bacterium is recognized as a pathogen both in plants [57], and in various non-mammalian [58] and mammalian species including humans [59,60].

In humans it is one of the most frequently isolated pathogens in nosocomial infections, especially from urinary tract infections and pneumonia [61]. It is commonly associated with infections with high mortality rates in immune compromised patients and ventilator associated pneumonias [16-18,62], where attributable mortality rate as high as 42% has been reported [63]. *P. aeruginosa* is the most important pathogen for patients suffering from the hereditary disease cystic fibrosis (CF), where the bacterium persists in biofilms in the lungs causing a chronic infection [64-66]. It is also a well known pathogen in burn wound infections, acute keratitis, otitis and folliculitis associated with non-chlorinated spa or swimming pools [16,60]. In healthy humans, *P. aeruginosa* is rarely isolated when examining bacterial samples from oropharynx (0-6.6%) and feces (2.6-24%) [40] and it is very rarely associated with pneumonia in previously healthy individuals [67].

In animals *P. aeruginosa* is a well known pathogen isolated from otitis externa, pyoderma and ocular infections in dogs [68-71]. In horses and farmed blue foxes it has been described as a cause of endometritis [72-74] and in cattle and small ruminants it is associated with mastitis [75,76]. In chinchillas it is the cause of otitis media and interna and healthy chinchillas have been shown to carry *P. aeruginosa* in their oral cavity and rectum [77].

3.3.2 Virulence factors

Many virulence factors have been described in *P. aeruginosa*. Among these are factors associated with adherence, e.g. flagella and pili. The type IV pili are believed to be the major factor responsible for initial adherence to airway epithelial cells [78]. Other factors involved in adherence to mucin and various cell surface receptors have also been described [79].

A major virulence factor is the type III secretion system (TTSS) by which substances are injected directly into cells. This system is recognized as an important virulence factor, especially in acute infections [80]. The proteins secreted by this system include exoenzyme S (exoS), exoenzyme T (exoT), exoenzyme Y (exoY) and exotoxin U (exoU). ExoS and ExoT are closely related bifunctional enzymes that disrupt the actin cytoskeleton, block phagocytosis and cause cell death [81]. ExoU acts as a potent phospholipase with the ability to disrupt host cell membranes thereby leading to cell lysis and it may also be able to cleave surfactant [81]. ExoU is apparently the most virulent effector protein of the TTSS [82,83] followed by ExoS and ExoT while the mechanism and importance of exoY as a virulence factor remains to be established. While almost all *P. aeruginosa* harbors genes for TTSS, genes for the effector proteins ExoU and ExoS rarely occur in the same organism [84]. ExoS or ExoT secretion appears to be more prevalent than ExoU secretion in both clinical and environmental isolates [80,84,85].

P. aeruginosa also secrete virulence factors into the surroundings using four secretion systems. These various systems are responsible for the release of proteins with adverse host effects and include alkaline proteases, elastases, phospholipases, lipases, exotoxin A, iron acquisition proteins and rhamnolipid [86]. Exotoxin A is believed to be a major virulence determinant in *P. aeruginosa* and is responsible for bacterial invasion and necrotic tissue damage by inhibiting protein synthesis [79]. *P. aeruginosa* secretes two elastases, which degrades elastin, a major component of lung tissue and arterial walls. The elastases secreted by *P. aeruginosa* are able to disrupt epithelial cell tight junctions [87], cleave PMN derived elastase [88] and also degrade pulmonary surfactant [89]. One of the secreted phospholipases has potent hemolytic activity and is able to increase vascular permeability and lead to organ damage [79]. *P. aeruginosa* secretes a number of proteases (including elastases) with various functions, ranging from hindering of fibrinogenesis and breakdown of host iron binding proteins and mediators of the immune defense including complement factors, cytokines and immunoglobulin G [79]. Another important virulence factor are the rhamnolipids, which have hemolytic activity and are shown to be important in biofilm growth mode [90] and in the killing of polymorphonuclear leucocytes (PMN) [91]. The characteristic blue-green color of many *P. aeruginosa* strains is due to a phenazine pigment, pyocyanin, which have a great impact on host cells. Among its attributes are the abilities to inhibit host catalase, to induce apoptosis in neutrophils and to inhibit ciliary function and cell respiration [92].

One important non-secreted virulence factor of *P. aeruginosa* and all Gram negative bacteria is lipopolysaccharide (LPS) of the outer membrane. LPS is strongly immunogenic in the host leading to production of several potent inflammatory signal molecules [79].

The impressive number and diversity of virulence factors of *P. aeruginosa* betrays the fact that this organism only very rarely causes disease in healthy human beings. The contributions of the different virulence factors are difficult to assess *in vivo*, since the organism requires some modification of the normal immune defense (e.g. neutropenia, impaired mucociliary escalator in the airways, artificial implants) to establish an infection. The reason for the difficulties experienced by *P. aeruginosa* in causing disease in the immune competent host could be the presence of elastase released by neutrophils, which is capable of degrading exotoxin A [88] and the host anti-proteases (e.g. α_1 -antitrypsin, secretory leukocyte protease inhibitor and α_2 -macroglobulin), which should be capable of neutralizing the proteases secreted by *P. aeruginosa*. Another reason for the lack of virulence in immune competent individuals could lie in the low iron-levels and in the fact that many of the virulence factors are under the control of quorum sensing (described in chapter 3.3.4), which means that they are only secreted when a large number of bacteria are present [16]. However, the single most important host mechanism for avoiding respiratory infections by *P. aeruginosa* appears to be the mucociliary escalator [17,93] which physically removes the bacteria.

3.3.2.1 Importance of virulence factors in mink

The only virulence factors which have been investigated in the mink are alkaline protease, elastase and LPS. The research has been focused on the development of a vaccine and the protective effect of immunizing with elastase, protease or a common protective antigen ("original endotoxin protein", OEP) has been investigated in various Japanese studies. OEP is a cell wall protein complexed with LPS in *P. aeruginosa* and apparently conferring immunity to all serotypes [94,95]. In the various Japanese studies [12,14,15,34] it was shown that immunization with protease toxoid or elastase toxoid alone or in combination with OEP was effective in protecting mink from intra-nasal challenge doses of *P. aeruginosa*. Unfortunately, these studies lack information on the status of the mink regarding infection with ADV, occurrence of the Chediak-Higashi syndrome, or lack controlled challenge trials.

The effect of elastase has been assessed by Elsheikh et al. [36] and was found to be important but not crucial in the mink, since elastase defective mutants were able to cause hemorrhagic pneumonia when instilled in the trachea at high doses. High elastase producing strains, however, caused a significantly higher mortality. The weakness of this study is that 3 of the 5 *P. aeruginosa* isolates used are isolated from clinical outbreaks, meaning that there is no knowledge on other virulence factors possessed by these bacteria. The laboratory strain PAO1 and its elastase defective mutant required high doses to be effective in producing hemorrhagic pneumonia in mink, but PAO1 infected mink showed a higher mortality and a faster disease progress than the mink infected with the elastase defective mutant. Again, the ADV status of the mink used for this experiment was not stated. Long et al. [33] described good protection of a heptavalent LPS vaccine in ADV negative mink suggesting that LPS is either an important virulence factor or that immunizing against five O-antigens renders the mink capable of killing the bacteria before they produce damaging amounts of virulence factors.

3.3.3 Antimicrobial drug resistance

P. aeruginosa has a high level of intrinsic resistance to a wide array of antibiotics. The intrinsic resistance rests on a low permeability of the outer cell membrane, several multi drug efflux pumps and chromosomally encoded β -lactamase conferring resistance to penicillins and cephalosporins [96]. Genetic resistance is also common and results from mutations or acquisition of genetic material (e.g. plasmids, bacteriophages or transposable elements) containing genes leading to resistance. Mutations has been described in target proteins and regulatory genes controlling the expression of certain channels in the outer membrane leading to reduced expression or up regulation of efflux pumps [96]. Other mechanisms of resistance are suspected since altered resistance profiles have been demonstrated, when mutations in genes involved in metabolism and motility occur [97].

The only study carried out on resistance of *P. aeruginosa* isolated from hemorrhagic pneumonia in mink displayed a generally high level of resistance with all isolates being sensitive to only two antibiotics, namely colistin and gentamicin [98]. In human isolates, some report a fairly low sensitivity to gentamicin and colistin [99,100] while others report isolates from CF and biofilms to be sensitive to colistin [101-103].

3.3.4 Quorum sensing

Quorum sensing (QS) is a complex signaling system which enables bacteria to recognize bacterial cell density and coordinate mode of growth and the expression of virulence factors [104,105]. The system comprises positive feedback loops, and uses signal molecules (autoinducers called AI) which are transported or freely diffusible across the bacterial cell membranes [106,107]. When a certain threshold of bacterial density, and hence AI concentration, is reached, the QS system is turned on. The regulation of AI, and the subsequent switching on of QS and transcription of QS controlled genes, is very complex and not yet fully elucidated [108,109]. The two major systems in *P. aeruginosa* consist of the *las* and *rhl* systems with the *las* system classically viewed as regulating the *rhl* system. A third QS system, termed the PQS system, is interspersed between these two systems [107,109]. Other factors like the growth phase of the bacterium and environmental factors are also important in the regulation of QS in *P. aeruginosa* [110]. It is estimated that approximately 10% of the *P. aeruginosa* genome is dedicated to the regulation of this system [111], which in turn controls around 10% of the genome including numerous of the virulence factors [108-110,112] including rhamnolipids and pyocyanin [91,92].

3.3.5 Biofilm

P. aeruginosa is able to grow in biofilms, which are bacterial communities or aggregates. These are often adherent to a surface in the environment or inside a host but can also be non-adherent [113]. The biofilms are organized in microcolonies separated by fluid-filled channels and surrounded by an extracellular matrix composed of exopolysaccharides, proteins and nucleic acids [114,115]. When growing in biofilms, *P. aeruginosa* is very difficult to eradicate with antibiotics, disinfectants or with the components of the immune system [116,117]. This poses great problems in humans, where *P. aeruginosa* can grow as a biofilm on indwelling medical devices and in the lungs of CF patients [118]. Biofilms also easily form in water pipelines and *P. aeruginosa* has been recovered from biofilms in dental equipment chairs [116,119]. The observed resistance of biofilms is believed to be due to different mechanisms existing in the biofilm. These

consist of slow diffusion of antibacterial agents through the biofilm, local changes in pH and altered growth rate and physiological state of the organisms residing inside the biofilm [101,117]. The biofilm under certain conditions harbors hypermutable organisms with increased resistance to antibiotics [102] and a small subpopulation of non-resistant but dormant bacteria (persister cells) which are not killed by antimicrobials [120]. These features generate a highly resistant subpopulation able to survive when other bacteria of the biofilm are eradicated by outside threats.

3.3.6 Population structure and evolution

The population structure of *P. aeruginosa* is considered to be epidemic non-clonal with successful clones and clonal complexes [121,122]. In CF, *P. aeruginosa* is regarded as somewhat epidemic with successful clones residing in a proportion of the chronically infected CF patients [103,123-129]. No specific clones are exclusively isolated from certain diseases but some strains isolated from chronically infected CF patients share common traits [130,131]. Most *P. aeruginosa* possess genes required for infection irrespective of their origin [132-135].

The evolution of *P. aeruginosa* is believed to be based on horizontal gene transfer and genetic recombination, especially in the accessory genome [135-138]. Mutations are also an important mean of evolution at least in the CF lung [129,131].

3.3.7 Acute pneumonia caused by *P. aeruginosa*

Human patients with neutropenia, HIV, transplants and those requiring mechanical ventilation are at risk for developing acute pneumonia due to *P. aeruginosa*, while this type of pneumonia very rarely occurs in the immune competent and healthy human being [17,67].

Pneumonia occurs when a microorganism evades first the mechanical host defenses and secondly overcome the innate and adaptive immune responses. The mechanical host defense in the airways consists of the nasal turbinates, the epithelial cell lining and the mucociliary escalator, while the innate and adaptive immune response supplies various secreted factors to the airway mucus; IgA, IgG, the iron-binding protein lactoferrin, β -defensins, lysozymes, complement and surfactant A and P [17,93,139]. The most important immune cell in acute airways infections with *P. aeruginosa* is the neutrophil granulocytes [39] which are recruited into the lungs in response to various cytokines released from epithelial lining and alveolar macrophages (Figure 4). The importance of the role of the alveolar macrophages and the various types of lymphocytes are debated in acute lung infections [17,93]. The mucociliary escalator is regarded as the single most important host factor in the defense against *P. aeruginosa* airway infections [93]. However, the host-pathogen interaction is incredibly complex with a wide array of cells and signaling pathways and much still remains to be elucidated in this area.

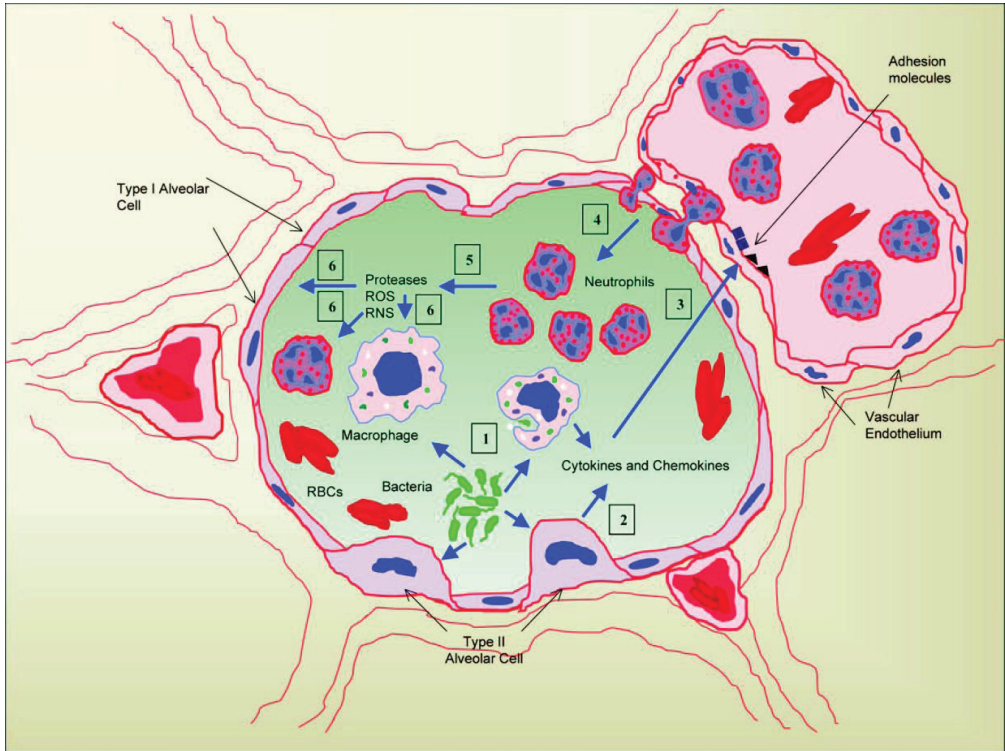


Figure 4: Recruitment of neutrophils into alveoli. From Craig et al., 2009 [140]. (1) Bacteria interact with alveolar epithelial cells and macrophages and (2) induce release of cytokines and chemoattractants, (3) The cytokines upregulate adhesion molecules on capillary endothelia which (4) mediate the migration of neutrophils into alveoli. (5) The neutrophils release proteases, reactive oxygen species (ROS) and reactive nitrogen species (RNS) which (6) cause necrotic cell death in infected cells. RBS = red blood cells.

4 Materials and methods

For a presentation on the materials and methods used in the larger part of this study, the reader is referred to the Articles I-IV. Only minor studies not presented in the articles will be described here.

4.1 Environmental samples

The objective of this small scale study was to isolate *P. aeruginosa* from the environment for comparison with the isolates obtained from hemorrhagic pneumonia in mink. Environmental samples were collected in private gardens, in Aarhus botanical garden, on three mink farms with no history of hemorrhagic pneumonia and on five farms with outbreaks of hemorrhagic pneumonia. Altogether 95 environmental samples were collected. These consisted of 23 samples from the general environment, 6 samples from a mink feed factory, 31 samples from farms without outbreaks of hemorrhagic pneumonia and 35 samples from farms with outbreaks of hemorrhagic pneumonia. The samples were primarily soil samples from areas with increased moisture (small stream in the botanical garden, areas below water supply or muddy areas in the mink farms) and samples from feed on mink cages, drinking water, drinking nipples and manure collecting devices. On one mink farm, with no previous history of hemorrhagic pneumonia, samples from hand washing facilities and a drain tube was also collected. On the mink feed factory samples were taken from fish, poultry, potato and wheat used for manufacturing the feed.

Soil samples were treated as previously described [141] with some modifications. The soil samples (approximately 300 g) were mixed and 50 g were shaken with 100 ml of sterile water for one hour at 300 rpm. From this mixture, 0.5 ml was transferred to liquid broth (brain heart infusion agar or veal infusion broth, Difco™, Detroit, MI, U.S.A.) and allowed to incubate for 24 hours at 37°C. The broth was stirred and 0.1 ml were plated onto CN agar plates for detection of *Pseudomonas* spp. (Thermo Fischer Scientific Inc, Oxoid Microbiology Products, Hampshire, UK) and allowed to incubate at 37°C for 48 hours.

Approximately 100 ml of the water samples were filtered through a sterile 2 µm pore filter (Advantec MFS Inc., Dublin, CA, U.S.A.). The filter was transferred to a CN agar plate and allowed to incubate at 37°C for 48 hours.

Swabs taken from manure collection devices, feed, drinking nipples, hand washing facilities and drains were streaked onto CN agar plates and allowed to incubate at 37°C for 48 hours.

For identification, suspected colonies of *P. aeruginosa* (based on growth and bluish color on CN agar plates) were tested for cytochrome oxidase reaction and when positive, with the API 20 NE identification system (bioMérieux, Marcy l'Etoile, France).

To test whether we would be able to recover *P. aeruginosa* from soil using the above mentioned method, one loop-full of a *P. aeruginosa* isolated from hemorrhagic pneumonia in mink was mixed with approximately 250 g of soil previously tested for presence of *P. aeruginosa*. The soil was left at room temperature for one day and treated as described above for the soil samples.

4.2 Fluorescence in situ hybridization and histology on tissue from infectious dose experiment

Tissue samples were collected from the dose-response experiment described in Article III. Tissue samples from all 72 animals included in this study were obtained from the left caudal lung lobe, liver, spleen, trachea and the nasal turbinates. The tissue samples were fixated in 10% buffered formalin for 24-48 hours before being embedded in paraffin. The nasal turbinates were decalcified in EDTA (14% (w/v) EDTA, 1,5%(w/v) NaOH) for three days before being embedded in paraffin.

Fluorescence *in situ* hybridization (FISH) and hematoxylin and eosin (H.E.) staining were performed as described in Article II.

4.3 Interview with mink farmers

A small interview study was performed on 23 mink farmers experiencing outbreaks of hemorrhagic pneumonia in late 2009 to 2011. The mink farmers were contacted by telephone 3-5 weeks after the National Veterinary Institute had diagnosed hemorrhagic pneumonia due to *P. aeruginosa* on their farm. The farmers answered a questionnaire including questions of the dates for first deaths, mortality, housing types, water supply, treatment and spread on the farm. Information on weather in the week before the first deaths was obtained from the archives of the Danish Meteorological Institute. The weather was defined as either “sunny” or “not sunny”, since air humidity was not recorded and precipitation is not necessarily a feature of humid weather. A week was defined as “not sunny” if days with precipitation amounted to at least 3 days or if the number of hours with sunshine were below 15.

4.4 Typing of isolates from hemorrhagic pneumonia in mink

In the study presented in Article I isolates from 2002-2009 are included. Isolates from 2010-2011 and a few isolates from 2001 were excluded from this article for practical reasons, since the statistical work started before all isolates from 2010 had been obtained. The isolates from 2001 were excluded from the article due to limited ability to revive bacteria from this year, making the proportion of bacteria isolated from 2001 very small. The isolates from 2001 which we chose to PFGE type were isolates of particular interest, since the same farms experienced hemorrhagic pneumonia in later years.

The methods used when performing pulsed field gel electrophoresis (PFGE) on these isolates, were the same as the ones described in Article I. In Article I, PFGE-typing of 164 isolates from 95 outbreaks on 90 mink farms are performed. When including the isolates from 2010-2011 and the two isolates from 2001, in total 189 isolates from 116 outbreaks on 105 mink farms were PFGE-typed.

5 Results

Results not presented in the articles I-IV are presented in this chapter.

5.1 Environmental samples

We were able to culture *P. aeruginosa* from the inoculated soil sample. It was only possible to isolate *P. aeruginosa* in 1 of the 95 tested environmental samples. This sample originated from a farm with an ongoing outbreak of hemorrhagic pneumonia. By PFGE typing this strain was indistinguishable from the strain isolated from diseased mink on the farm.

5.2 FISH and histology on tissue from infectious dose experiment

When examining slides from diseased mink, a perivascular localization of the bacteria was prominent in the lungs (Figure 5). Occasionally *P. aeruginosa* were found inside vessels (Figure 6). It was possible to visualize *P. aeruginosa* in the nasal turbinates of diseased mink using FISH, where *P. aeruginosa* was found in the lumen and along the basement membrane of desquamated epithelium (Figure 7 and 8). Single bacteria were occasionally found in the small vessels of the liver and spleen (Figure 9). No bacteria could be found by FISH in the lungs or other organs of mink not showing symptoms of hemorrhagic pneumonia. The clinically healthy mink were euthanized eight days after infection with *P. aeruginosa*.

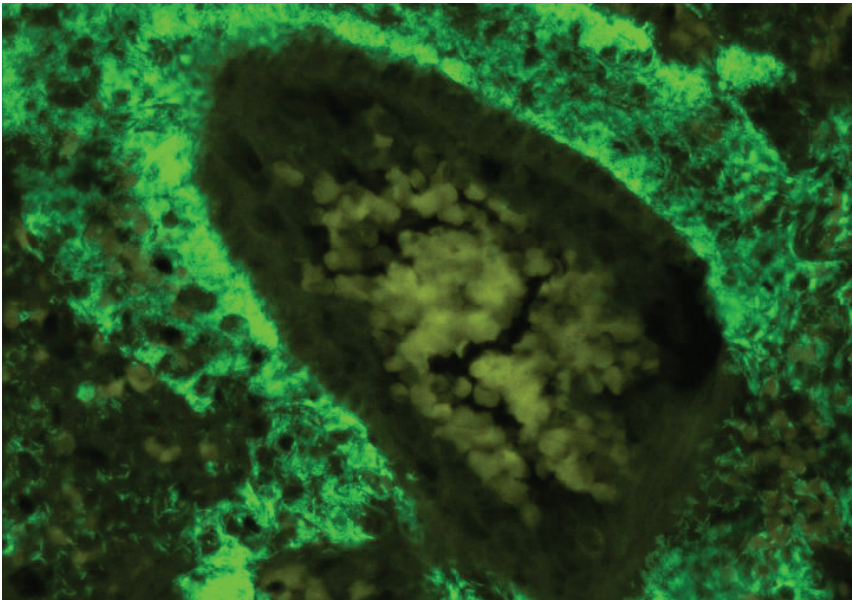


Figure 5: Perivascular localization of *P. aeruginosa* in experimentally infected mink with symptoms of hemorrhagic pneumonia. FISH with 6-FAM labeled *P. aeruginosa* probe. 400x magnified.

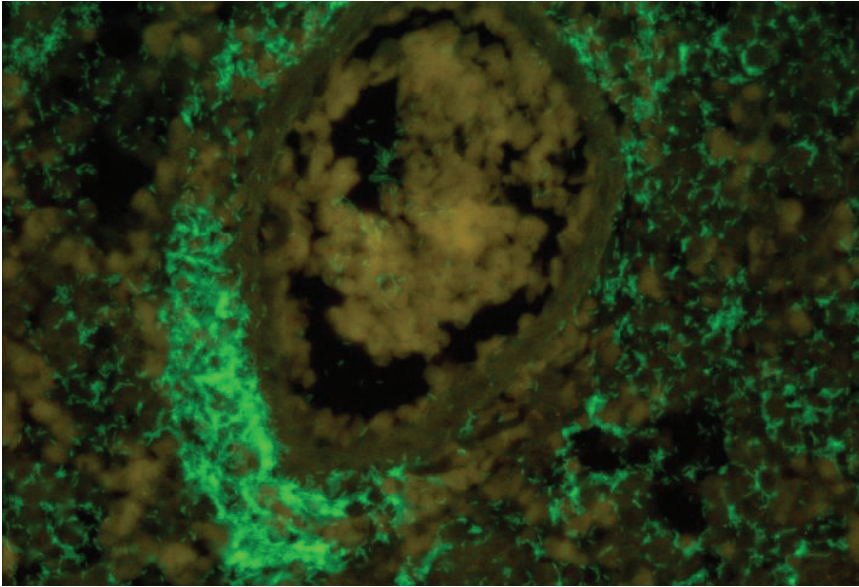


Figure 6: *P. aeruginosa* in and around blood vessel and vessel wall. Experimentally infected mink with symptoms of hemorrhagic pneumonia. FISH with 6-FAM labeled *P. aeruginosa* probe. 400x magnified.

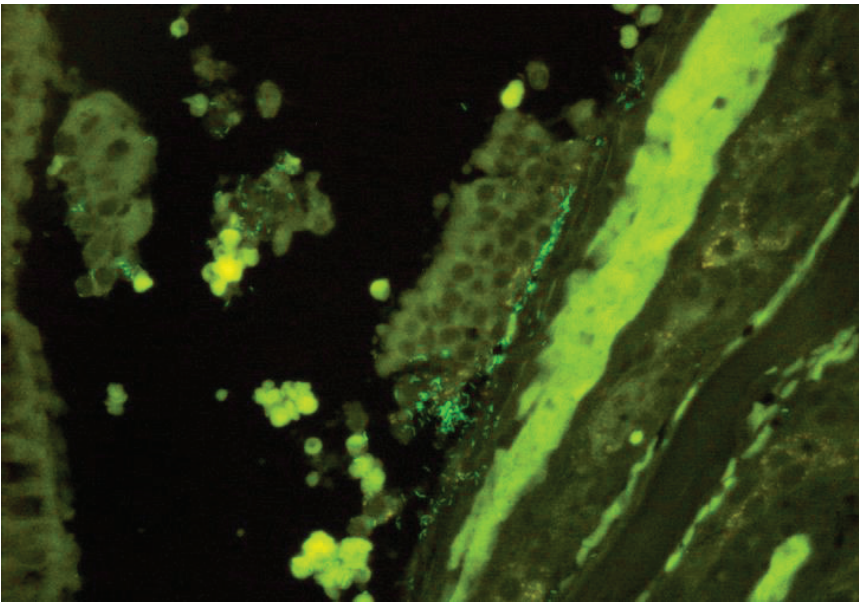


Figure 7: Nasal mucosa with *P. aeruginosa* along basement membrane and loss of epithelium. Experimentally infected mink with symptoms of hemorrhagic pneumonia. FISH with 6-FAM labeled *P. aeruginosa* probe. 400x magnified.

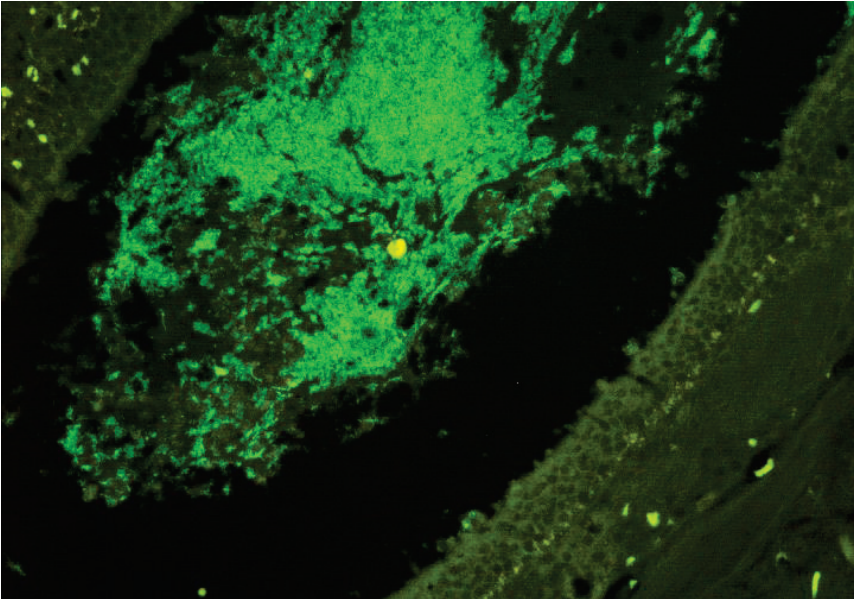


Figure 8: Nasal turbinates from experimentally infected mink with symptoms of hemorrhagic pneumonia. *P. aeruginosa* in lumen, intact epithelium. FISH with 6-FAM labeled *P. aeruginosa* probe. 200x magnified.

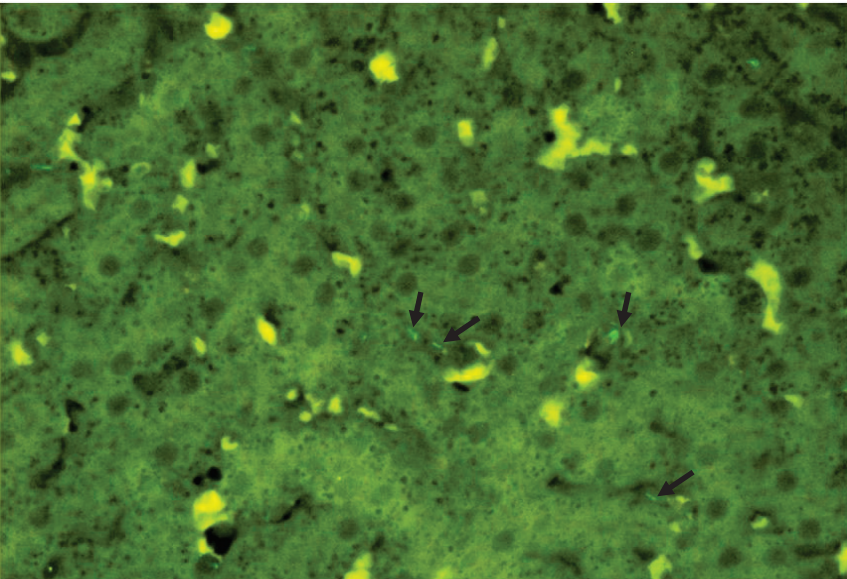


Figure 9: Liver with occasional *P. aeruginosa*(arrowheads) in sinusoids. Experimentally infected mink with symptoms of hemorrhagic pneumonia. FISH with 6-FAM labeled *P. aeruginosa* probe. 400x magnified

The four mink developing hemorrhagic pneumonia showed a diffuse suppurative, hemorrhagic and necrotizing pneumonia on histology along with large numbers of bacteria often with prominent

perivascular localization (Figure 10). Loss of epithelium in the bronchioles was noted, while the greater bronchi and the trachea showed no or only mild lesions. In the spleen moderate numbers of megakaryocytes and several germinal centers were found while the liver showed slight to moderate lipid vacuolization of hepatocytes. The nasal mucosa showed lesions varying from none to erosion of epithelium, penetration of *P. aeruginosa* into the submucosa and accumulations of neutrophils and debris in the lumen and areas with epithelial erosion.

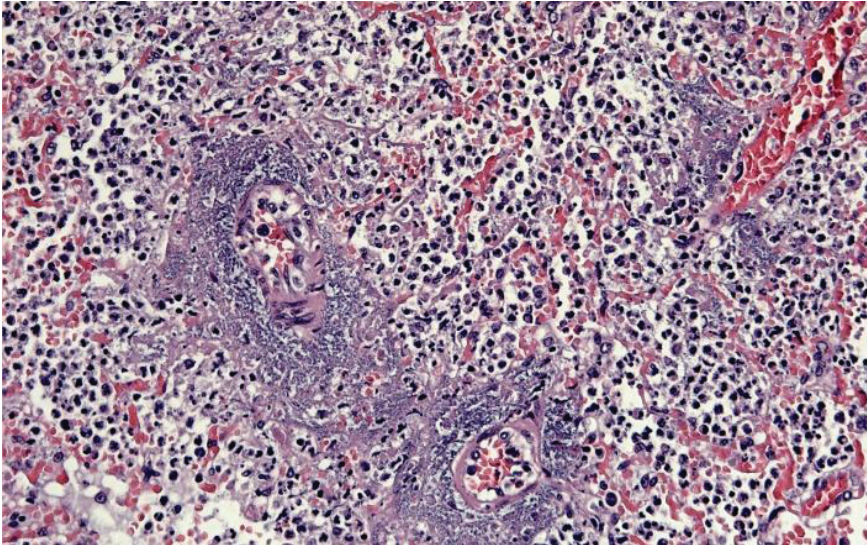


Figure 10: Lungs of experimentally infected mink with symptoms of hemorrhagic pneumonia (November). Perivascular localization of myriads of bacteria, severe necrosis and numerous alveolar neutrophils. HE stain, 200x magnified.

In the lungs of the clinically healthy mink experimentally infected with *P. aeruginosa*, focal or multifocal lesions ranging from slight to moderate perivascular edema, few intra-alveolar free erythrocytes, perivascular and peribronchiolar cuffs of mononuclear leukocytes and hyperplasia of type II pneumocytes were noted (Figure 11 and 12). The spleen typically showed germinal centers and some megakaryocytes, while the livers showed mild to moderate lipid vacuolization of hepatocytes. No lesions were noted in the trachea of any of the symptomless mink. In the experiments conducted in July a larger number of megakaryocytes were present in the spleen, probably reflecting extramedullary hematopoiesis. The lung lesions of the mink infected in July, where none developed hemorrhagic pneumonia, were generally milder, with fewer mink displaying perivascular or peribronchiolar accumulation of mononuclear leukocytes in the lung tissue.

In the control mink, which were not infected with *P. aeruginosa*, mild interstitial edema was seen in the lungs. The livers showed mild lipid vacuolization of hepatocytes while the spleens contained few germinal centers and some megakaryocytes. No lesions were noted in the trachea of the non-infected animals.

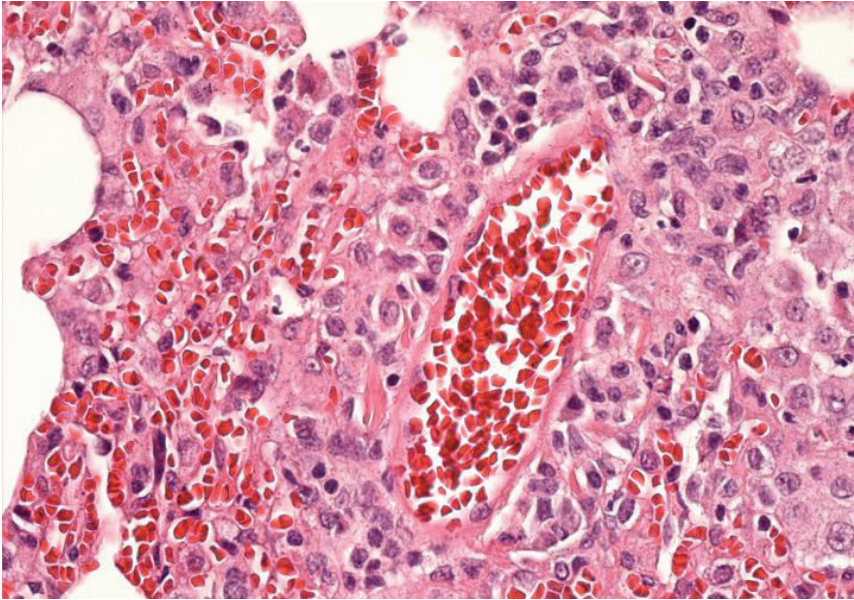


Figure 11: Mononuclear perivascular cuffing in experimentally infected mink without clinical symptoms of disease euthanized eight days after infection. HE stain, 400x magnified.

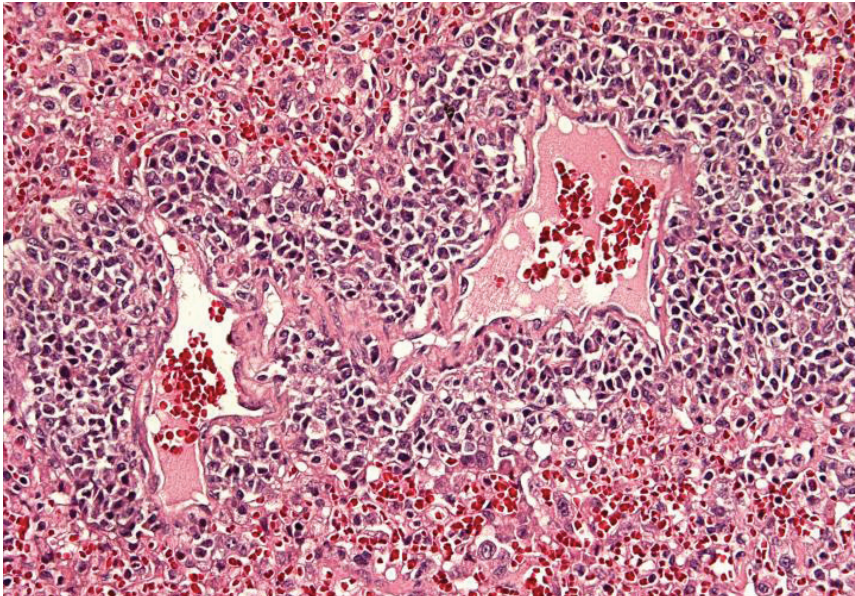


Figure 12: Mononuclear perivascular cuffing in experimentally infected mink without clinical symptoms of disease euthanized eight days after infection. HE stain, 200x magnified.

5.3 Interview with mink farmers

In total, 23 farmers answered the questionnaire. The mortality ranged from below 1% to 56% (Figure 13) and in all farms the first animals to die were kits. Five of the farmers perceived the spread on the farm as sporadic, while 13 recalled the disease as spreading to mink in neighboring cages. Two farmers experienced the disease spreading sporadically within the same shed, while three farmers thought the spread was either a mixture of sporadic and neighboring spread (n=1) or neighboring and within-shed spreading (n=2). In 15 of the outbreaks the weather conditions in one week before the first noticed deaths were “not sunny”. Only four of the farms had their own water supply, the rest were supplied by municipality water. On nine (39%) of the farms the majority of mink dying from hemorrhagic pneumonia were males while the other farmers (n = 14) did not report any difference in mortality between the sexes.

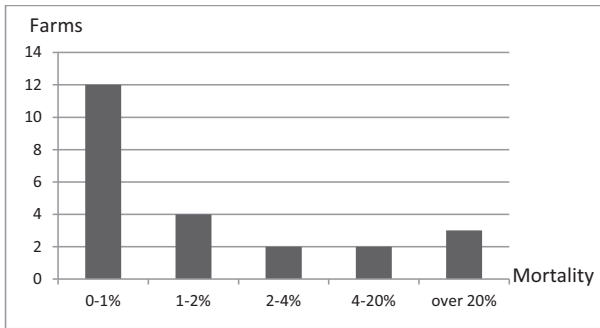


Figure 13: Total mortality on farms.

5.4 Typing of isolates from hemorrhagic pneumonia in mink

We recovered 88 distinct PFGE patterns from the 116 outbreaks on 105 mink farms. Isolates from two or more mink were typed in 50 (43%) of the outbreaks. In 43 (86%) of the outbreaks with two or more isolates typed, the PFGE patterns were indistinguishable. Two strains (S100 and S101) from 2011 were added to “Cluster 1”, which was described in Article I.

On 10 farms repeated outbreaks occurred during the period from 2001-2011. On five of these farms indistinguishable PFGE types were found on the farm level with one to five years between the outbreaks. Little similarity in PFGE-patterns was found between the isolates obtained from the other five farms where the time between the two outbreaks ranged from one to ten years.

6 Discussion

In this section, discussions on the minor studies presented in the sections on material and methods, and results are given. After this, a general overview and sum up on the discussions of all results displayed in this thesis including the results from the Articles I-IV are presented.

6.1 Environmental samples

In this study, we examined 95 environmental samples for presence of *P. aeruginosa*. It was only possible to isolate *P. aeruginosa* in one soil sample from a farm experiencing an outbreak of hemorrhagic pneumonia. The retrieval of *P. aeruginosa* from inoculated soil and from one farm with an outbreak of *P. aeruginosa* pneumonia shows that the applied method should be able to detect *P. aeruginosa* in soil. Perhaps the numbers of *P. aeruginosa* in the soil samples and in the various other samples collected have been below detection limit. On the other hand, it is well known that *P. aeruginosa* and other bacteria can enter a non-culturable but viable state [142-144], which might have been the case for *P. aeruginosa* in samples from a hostile environment like soil. Likewise the biofilm-mode of growth, which is believed to be the mode of growth of more than 99% of the world's population of bacteria, might have yielded fewer bacteria especially in the samples from soil, water and surfaces on the farm since the bacteria would have been difficult to free from the biofilm or could be in a non-culturable state [143,145,146]. The lack of retrieval of *P. aeruginosa* from water sources on the farms is not surprising, since bacteria would be expected to grow in biofilms inside the pipelines and water containers and hence be difficult to obtain in a free-living culturable form [143,145]. The free-living or planktonic bacteria are not expected to comprise more than 5% of the bacterial population of a drinking water system [147]. *P. aeruginosa* are, however, encountered in biofilms inside drinking water distribution systems and different materials of the distribution system are shown to have an effect on the number of *P. aeruginosa* in the biofilms [148]. The number of culturable *P. aeruginosa* in biofilm and water of a biofilm model system has been shown to be orders of magnitude lower than the number of *P. aeruginosa* as identified by FISH [148].

Detection of biofilms containing *P. aeruginosa* in the farms' water system would require physical disruption of the biofilms inside the water distribution systems or the analysis of large volumes of water. Filters, insides of pipelines and water containers could be physically scraped and the resulting material be analyzed for the presence of *P. aeruginosa* by FISH or PCR, since these methods are more sensitive than culturing in the detection of bacteria growing in biofilms [148,149]. Attempts of resuscitation of the non-culturable bacteria could be tried with non-selective medium and long incubation times perhaps supplemented with hormones, known to stimulate growth of *P. aeruginosa* like catecholamines [29,148].

6.2 FISH and histology on tissue from infectious dose experiment

The finding of severe histological changes and massive amounts of bacteria in the lungs of mink developing hemorrhagic pneumonia corresponds to previous findings of acute hemorrhagic pneumonia in mink [13,32-36]. *P. aeruginosa* could be cultured both from the nasal cavities, lungs and livers of these mink but the histological lesions were only pronounced in the lungs, which show that the septicemia most likely occurred

very late in the progress of disease. This is in accordance with previous reports also reporting no or only rare lesions in other organs than the lungs of mink with hemorrhagic pneumonia [32,34,35].

The histological lesions in the infected mink, which did not develop hemorrhagic pneumonia, indicate that the bacteria have reached the lungs and elicited a reaction, but that the mink were capable of clearing themselves from the infection. This is also in agreement with previously published observations [13,33-35].

The hemorrhagic pneumonia observed in mink does not present different histological lesions than what is observed in rats receiving high doses (5×10^8 cfu) of *P. aeruginosa* intra-tracheally [150]. In mice given lower doses (6×10^5 cfu) of *P. aeruginosa* intra-tracheally the histological lesions are comparable but milder and with less hemorrhage [151].

6.3 Interview with farmers

The purpose of the questionnaire was to investigate possible common factors shared among farms experiencing outbreaks of hemorrhagic pneumonia caused by *P. aeruginosa*. The most striking finding was the report from all farmers that the disease always started in the kits and that in 9 (39%) of the outbreaks almost exclusively males were affected. Since only 23 farmers were interviewed, no firm conclusions can be drawn from this study, but the tendency of male kits to be more susceptible in clinical outbreaks has been reported in a few previous studies [12,15,25] and this tendency is supported by the answers in these interviews.

The weather conditions in 15 (63%) of the outbreaks were “not sunny” which could indicate that moist weather conditions could lead to outbreaks of hemorrhagic pneumonia. However, a larger dataset and measurements of air humidity are needed to further investigate this. On 19 of the farms the water supply was municipality water, which should not be contaminated with *P. aeruginosa*. However on the farms, the water is held in underground containers and distributed into pipelines leading into the sheds and individual cages. Biofilms of *P. aeruginosa* could easily grow in these containers and in the pumps and pipelines.

More than half of the farmers (n=13) perceived the progression of the outbreak on the farm to occur by spread of disease between neighbors while the rest experienced sporadic spread or within shed spread or combinations of the above. This is interesting in view of the common practice of vaccinating animals around the diseased animals (“ring vaccination”). Sporadic spread was defined as the finding of diseased mink in various sheds on the farm with apparently no diseased mink between them. This type of spread was recorded by six farmers (including the one reporting a mixture of spreading methods), meaning that in one fourth of the farm outbreaks of hemorrhagic pneumonia ring vaccination would probably not protect the farm from spread of the disease. The spread on the mink farm to neighboring mink has been described in earlier studies [10,43], while the sporadic spread has never been reported in the literature. Only 5 farmers experienced sporadic spread as the only type of spread (i.e. without spread of the disease to neighbors or as a within shed spread), and in 4 out of these 5 outbreaks the mortality did not exceed 2%. With so low mortalities probably only few animals on the farm were susceptible to infection. This could be due to low virulence of the *P. aeruginosa* strain or perhaps because of factors of the mink itself.

The farmers' memory of the events can be very subjective, but most often number of deaths is recorded in a farm diary as this is mandatory in the Danish regulation. Often the cages having contained dead animals are marked, making it possible to assess the spread on the farm in retrospect.

6.4 Typing of isolates from hemorrhagic pneumonia in mink

When including isolates from 2001, 2010 and 2011, two strains isolated from the Northern part of Jutland in 2011 were added to cluster 1. The other strains belonging to this cluster all occurred from 2002-2006. The geographical distribution of these isolates was widespread and the isolates from 2011 differed by 1 to 6 bands from the other isolates in cluster 1. It is peculiar to find related strains so widely separated in time and space, but apparently genetic drift has been low in this particular strain.

About 10% of the farms included in this study witnessed more than one outbreak in the study period. Of these, half of the farms (n = 5) were infected with a *P. aeruginosa* with a PFGE pattern identical to the one causing the first outbreak and the time between these outbreaks ranged from 1 to 5 years. This indicates that *P. aeruginosa* is able to survive on the farm or in a contaminating source and that the genotype of *P. aeruginosa* able to induce hemorrhagic pneumonia is quite stable, not impairing the fitness of the bacterium.

6.5 Overview and discussion of the project

The results from PFGE typing of 189 bacterial isolates obtained from 105 mink farms experiencing in total 116 outbreaks from 2001 to 2011 clearly show that the majority of outbreaks are caused by unrelated strains of *P. aeruginosa*. However, approximately 30% of the outbreaks can be attributed to either related strains or particularly prevalent strains. This is in agreement with the view of the general population of *P. aeruginosa* as being epidemic non-clonal [121,122] meaning that the population is regarded as consisting of many unique strains and a number of related epidemic strains. The observation of a strain in cluster 1 occurring in 2011 differing by 1 to 6 bands from the other members of this cluster is somewhat peculiar, since members of this cluster was otherwise only recovered in 2002-2006. The reason for the low number of band differences on the PFGE patterns may be attributed to mutations or genetic material loss or that acquisition of genetic material has either not happened or has happened in an extent that is difficult to recognize on the PFGE pattern (i.e. point mutations not leading to new restriction sites or small pieces of DNA not altering the size of the bands to a visible degree).

The finding of a related subpopulation of *P. aeruginosa* strains causing hemorrhagic pneumonia in mink might imply that these strains are somehow genetically adapted to be virulent for mink or they may be more prevalent in the environment. As described in Article I, the 18 strains analyzed by AT biochip were not present in a large dataset [122], which supports the idea that these strains are not highly prevalent in the environment or in other habitats. However, 18 strains of the 88 recovered PFGE patterns are not a lot and it would be interesting to test all the isolates by the AT biochip, both to establish connections to previously isolated organisms from human clinical isolates or the environment and to reveal clusters of genetically related strains which are not identified by PFGE. The AT biochip could also be used to conduct phylogenetic analysis on the bacteria.

The recovery of strains with indistinguishable PFGE patterns from outbreaks of hemorrhagic pneumonia on the same farm as much as five years apart, shows that *P. aeruginosa* is able to survive in the farm environment or in a contaminating source without losing virulence and without major changes in the genome for at least five years. This finding and the isolation of a strain belonging to cluster 1 five years after the last isolation of members of this cluster, implies that if a certain genotype is required to cause hemorrhagic pneumonia in mink, and these strains possess this feature, the genotype probably does not influence fitness of the bacterium negatively. Since indistinguishable strains were found in 50% of the cases with more than one outbreak on a farm in the study period, it would be suspected that a source on the farm was suitable for the survival and propagation of *P. aeruginosa*. This could very well be biofilms inside drinking water distribution systems, as *P. aeruginosa* is able to survive in fresh water [152] and has been detected in biofilms in water distribution systems as discussed in Chapter 6.1. Distribution of *P. aeruginosa* from central food kitchens has been proposed [44] but the results in Article I, where almost half of the farms with outbreaks caused by indistinguishable PFGE types did not share food kitchen, indicates that this is probably not the case.

In 43% of the outbreaks more than one isolate was PFGE typed. Of these 86% displayed indistinguishable PFGE types. However, as explained in article I, it is not wise to deduct that the mortality in the majority of outbreaks are caused by the same strain. If two strains of *P. aeruginosa* were causing an outbreak with the same frequency of disease attributed to each strain, one would need to type at least five mink to be 95% certain of detecting both strains (page 37-38 in [153]). No more than four isolates were ever typed from the same outbreak. The finding of more than one PFGE type in 14% of the outbreaks with more than one isolate PFGE typed supports the idea that more than one *P. aeruginosa* strain can be involved in a farm outbreak. This again indicates that factors of the mink make them vulnerable to developing disease, since one must assume that factors of the mink are more important than factors of the bacterium, if more than one bacterial strain can cause an outbreak. The idea is supported by the fact that even though related or more prevalent strains were isolated in approximately 30% of the outbreaks, distinct strains were encountered in 70% of the outbreaks. These strains most likely originate from the local environment surrounding the mink and hence should not be specially adapted to the lungs of mink.

It was not possible to isolate *P. aeruginosa* in the nasal cavities of clinically healthy mink at pelting, which reveals that mink are not normally carrying this organism on their nasal mucosa. This is rather surprising, as an American study demonstrated *P. aeruginosa* in the nasal cavity or rectum of 39 out of 100 tested mink at pelting [25]. In retrospect, it would have been preferable to test the Danish mink in this study for rectal presence of *P. aeruginosa* as well. However, since the results presented in Article III reveal that mink are able to carry *P. aeruginosa* on their nasal mucosa for at least eight days, it would be expected that at least some of the mink would be carriers of this organism in their nasal cavity, if they were housing this bacterium in their intestinal microbiota. Since the prevalence of a possible carrier state is not known, the sample size of 20 animals per farm might have been too small to detect any carriers, but relying on the prevalence reported by Long and Gorham, 1981, this should not be the case. On the other hand, the mink investigated in the study by Long and Gorham, 1981, lived on farms with a prevalence of antibodies to ADV of 13%-97% which may have made these mink more prone to developing a carrier state for *P. aeruginosa* making the expected prevalence of a carrier state too high for Danish conditions. The sample size of 20 animals per farm should be able to detect at least one mink with *P. aeruginosa* in the nasal cavity if more

than 14% of the mink on the farm were carriers, or in other words, we are 95% sure that the prevalence of mink carrying *P. aeruginosa* on their nasal mucosa at pelting is below 14% (page 36 in [153]).

It is noteworthy that Danish mink are able to carry *P. aeruginosa* on their nasal mucosa for at least eight days without developing hemorrhagic pneumonia when inoculated intra-nasally, as demonstrated in Article III. Whether this niche is important for colonization of the lungs, as has been described for human CF patients [154], remains uncertain. The results of histological examination of tissue from the experiment described in Article III strongly suggest that mink infected intra-nasally with *P. aeruginosa* are able to clear themselves of the infection, since lesions are present in the lungs of mink without clinical symptoms. These results furthermore reveal that *P. aeruginosa* reaches the lungs also in the mink not developing clinical signs of hemorrhagic pneumonia.

The observed histological lesions both in tissue from the experimentally infected mink and in tissue from farm outbreaks are in good accordance with what has previously been described [32-34,155]. The perivascular localization of *P. aeruginosa* was a prominent feature in many of the lungs from farm outbreaks and in all of the cases of experimentally induced hemorrhagic pneumonia. Two patterns were observed, with one being very hemorrhagic with almost no bacteria or neutrophils and another being dominated by massive amounts of neutrophils, bacteria and necrosis. This has also been reported previously [32]. *E. coli* did not show perivascular localization in the lung tissue of mink with hemorrhagic pneumonia as described in Article II. Otherwise a similar histological pattern as the one described for *P. aeruginosa* hemorrhagic pneumonia with massive amounts of neutrophils, necrosis and bacteria were encountered. The very hemorrhagic pattern observed in some *P. aeruginosa* pneumonias were rarely found for *E. coli* pneumonia. This demonstrates that even though the two bacteria elicit a hemorrhagic pneumonia in the lungs of mink, the pathogenesis is probably not shared. The preference of *P. aeruginosa* for perivascular localization could be due to higher amounts of specific nutrients in this area or due to the presence of specific receptors. Receptors for adherence to specific carbohydrates have been recognized in the epithelium and capillaries of mink pancreas and lungs [156]. If this is part of the reason why mink are so vulnerable to infection with *P. aeruginosa* remains to be elucidated.

It is peculiar why otherwise clinically healthy mink develop hemorrhagic pneumonia due to *P. aeruginosa*. A total infectious dose resulting in hemorrhagic pneumonia as low as 10^3 cfu has been reported both in Article III in this thesis and in the literature [11,33,34]. In the experiment described in Article III, 4 out of 30 mink developed hemorrhagic pneumonia in November, while 0 out of 30 mink developed hemorrhagic pneumonia when infected in July. Even though the difference was not significant (p -value=0.1), it indicates that mink infected with the same dose of bacteria more easily develop hemorrhagic pneumonia in November as compared to in July. This suggests that factors which are present in some mink in November but not in July, perhaps due to the highly seasonal life cycle of the mink, are important in the development of hemorrhagic pneumonia. This is underlined by the fact that some of the mink in the infectious dose experiment were infected with doses as high as 10^8 and 10^9 cfu in total in July and in November but did not develop clinical signs of disease. It is odd that the mink are able to survive being infected with such large numbers of bacteria, while it was very difficult to isolate this organism in samples from the mink's environment. This strongly indicates that some mink are more prone to develop hemorrhagic pneumonia when exposed for *P. aeruginosa* than others. The possibility of importance of certain virulence factors in *P. aeruginosa* for the initiation and progress of disease cannot be ruled out, but since 70% of the strains

isolated from outbreaks of hemorrhagic pneumonia in mink are more or less unique and hence most probably local, the presence of such a virulence factor or set of virulence factors most likely exists in many distinct *P. aeruginosa* strains. Co-infection with respiratory viruses may make the mink more vulnerable for *P. aeruginosa* infection. Such viruses have not been identified and our attempt to demonstrate RSV (and subsequently influenza virus, since it was part of the commercial kit used for RSV detection) in the lungs of mink dying of hemorrhagic pneumonia caused by *P. aeruginosa*, was unsuccessful.

The genetics of the mink might also influence the ability to withstand an attack of *P. aeruginosa*. From studies on humans, it has been shown that mortality due to infection is influenced by genetics [157-159] and a high selection pressure on certain desirable coat colors or types could disseminate a particularly vulnerable genotype on a mink farm. Since no specific color types are associated with hemorrhagic pneumonia in mink, this is, however, not likely.

The prevalence of the Chediak-Higashi syndrome is not known for Danish mink, making defect neutrophils a possible explanation for the heightened susceptibility of this species. However, as stated above, no particular fur coat color is associated with hemorrhagic pneumonia making this hypothesis unlikely. The prevalence of Aleutian disease on Danish mink farms is closely monitored and outbreaks occur on both antibody-positive and negative farms. Whether outbreaks on ADV antibody positive farms are more severe remains to be elucidated. The results from the questionnaire presented in Chapter 4.3. identify four farms with ADV antibodies in the mink. The outbreaks on these farms were, in two cases, quite severe but since these farms were infected with the same strain of *P. aeruginosa*, nothing can be concluded from this observation.

7 Conclusion

Few differences were observed when comparing the histological lesions present in *P. aeruginosa* and *E. coli* infected lungs, indicating that these two pathogens do not share the exact same pathological pathways. Based on the results presented in this thesis it is proposed that factors of the mink may be the most important factor in the development of hemorrhagic pneumonia in mink. These results include the fact that in 70% of outbreaks of hemorrhagic pneumonia distinct (and hence most probably local) strains of *P. aeruginosa* are isolated, the tendency for mink to develop hemorrhagic pneumonia more easily in the fall irrespective of infection dose of *P. aeruginosa* and the indication that *P. aeruginosa* is very difficult to isolate from the mink farm environment, probably making it difficult to reach a high infectious dose. The results of interviewing farmers experiencing outbreaks of hemorrhagic pneumonia in their mink furthermore identifies a subpopulation of mink (male kits), which are more prone to develop hemorrhagic pneumonia emphasizing that something in the mink must be important for the development of this disease. The possibility of this factor being infection with RSV or influenza virus is unlikely based on the findings presented in Article IV.

8 Further perspectives

Now, what are the factors that make mink susceptible to lethal hemorrhagic pneumonia due to *P. aeruginosa*? This is a very open question and as previously stated the apparent increased susceptibility of this species could be related to various factors including shedding, high weight gain, hormonal fluctuations including those related to stress, immune deficiencies, limited space, subclinical diseases like fatty liver or specific receptors for *P. aeruginosa* perhaps only expressed in a subpopulation of animals at a given time.

It should be possible to investigate the importance of some of these factors while others are very difficult to assess *in vivo*. The effect of various hormones on the infection with *P. aeruginosa* in the mink is problematic to study, since handling and restraint of the animal for blood sampling will have an impact on the levels of especially the stress hormones (catecholamines and glucocorticoids) but probably also on the sex hormones and prolactin levels [160,161]. Indications as to whether specific hormones were important in the pathogenesis could come from animal experiments where the hormone of interest was removed either by surgical removing the hormone-producing organ or by chemically inhibiting the production or function of the hormone. However, experiments like this will yield very limited information on the actual importance of the hormone on infection with *P. aeruginosa* in the mink. The physiological level of the hormone would still be unknown and the possibility of the removal or inactivation of one hormone might influence other hormones or components of the immune system. Even though a possible effect of a hormone on the pathogenesis of hemorrhagic pneumonia most likely would be related to interplay with the immune defense, the induced defect in the immune defense might not be mediated through this hormonal interaction in real life.

In the future, it could be interesting to include genetic markers in the research, and compare the genetics of mink succumbing to hemorrhagic pneumonia to that of surviving mink. It might be possible to use the results from the newly started “FurMap” project, which investigates the relationship between genes and fur quality using quantitative trait locus (QTL) analysis.

Presence of viral infections could be studied using PCR techniques or electron microscopy of lung tissue from mink dying of hemorrhagic pneumonia. Other subclinical diseases, like fatty liver, would be troublesome to assess, since mink develop fatty liver very fast in response to limited feed intake and is almost always found on mink dying from disease including those dying from hemorrhagic pneumonia. Furthermore, this condition is very common in the autumn when the mink in many farms are fed ad libitum to obtain optimal growth rates before pelting. One of possible effects of a subclinical fatty liver could be decreased blood levels of α_2 -macroglobulin, since this protein is produced by the liver, leading to a higher susceptibility to the proteases secreted by *P. aeruginosa*.

The presence of carbohydrate-receptors for *P. aeruginosa* in the mink lung has already been observed [156]. It would be of interest to use the experimental work described in this publication to compare a possible difference in distribution of these receptors among animals dying of hemorrhagic pneumonia and those infected with *P. aeruginosa*, but not developing disease.

Furthermore, the presence of a reservoir of *P. aeruginosa* on the mink farms remains to be identified. A possible approach could be detection by PCR techniques and subsequent culturing of samples obtained from sites showing presence of *P. aeruginosa*.

Another aspect of these infections that would be valuable to study are the virulence factors of the particularly prevalent *P. aeruginosa* strains identified by PFGE and the AT biochip (Article I). These could be assessed in *in vitro* settings and compared to virulence factors of *P. aeruginosa* identified in only one outbreak or with *P. aeruginosa* not related to disease in mink. The value of such studies is limited by the fact that 70% of the recorded outbreaks were caused by distinct strains. However, since only three serotypes are encountered in disease from mink as opposed to what is known from human infections [85,162], some factor in the bacterium are expected to be crucial for the development of hemorrhagic pneumonia. Whether this relates directly to the O-antigen or some serotypes more frequently possess important virulence factors has not been investigated, but some studies has shown a differential occurrence of virulence factors in the proteins secreted by the type III secretion system related to the O-antigen of LPS [85,162].

9 References

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Article I

Typing of *Pseudomonas aeruginosa* from hemorrhagic pneumonia in mink (*Neovison vison*)

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Abstract

Hemorrhagic pneumonia in mink (*Neovison vison*) is caused by *Pseudomonas aeruginosa* and is an acute and fatal disease in farmed mink. Earlier work has demonstrated that some outbreaks of hemorrhagic pneumonia are caused by pathogenic strains while most outbreaks are caused by local strains. The objective of this study was to determine the genetic and geographical relationship among outbreaks of hemorrhagic pneumonia by pulsed field gel electrophoresis typing of *P. aeruginosa* isolates. Furthermore, chosen isolates were typed by a commercial genotyping method based on single nucleotide polymorphisms (SNPs) and compared to a larger dataset of human and environmental origin. The bacterial isolates were obtained from diagnostic samples from 2002-2009 and contained 164 isolates from 95 outbreaks on 90 farms. Our results show that most outbreaks of hemorrhagic pneumonia in mink are caused by distinct strains of *P. aeruginosa*. We also identified related *P. aeruginosa* strains which, together with two prevalent but unrelated clones, caused one third of the outbreaks of hemorrhagic pneumonia supporting the sparse literature on this subject. None of the SNP typed strains were identified in a large dataset of human and environmental origin.

Keywords: *Pseudomonas aeruginosa*, mink, hemorrhagic pneumonia, pulsed-field gel electrophoresis, single nucleotide polymorphism

Introduction

Pseudomonas aeruginosa has been described as a cause of hemorrhagic pneumonia in mink since 1953 (Knox, 1953). The disease is almost exclusively seen from September to early December in Denmark and can cause an epizootic on the mink farm with mortalities ranging from 1% to 50% (Knox, 1953; Honda et al., 1977). It is acute and characterized by sudden deaths among the mink which are often found dead with blood around nostrils and mouth. No known underlying cause has been identified preceding *P. aeruginosa* pneumonia in mink, which make mink the only species known to be susceptible to contagious, acute and fatal lung infections with *P. aeruginosa*.

Only descriptive epidemiological research has been published on *P. aeruginosa* pneumonia in mink (Knox, 1953; Shimizu et al., 1974; Long and Gorham, 1981; Hammer et al., 2003). *P. aeruginosa* is widespread in both environment and various disease habitats. Earlier work has demonstrated that specific clones only rarely are associated with certain habitats and that most *P. aeruginosa* possess the genes required for establishing an infection (Römling et al., 1994; Alonso et al., 1999; Wolfgang et al., 2003; Morales et al., 2004; Stewart et al., 2011)

To identify possible successful clones and explore the relationship of *P. aeruginosa* included in this study, bacterial isolates were typed by pulsed-field gel electrophoresis (PFGE) which is considered the "gold standard" for discriminative typing of *P. aeruginosa* (Grundmann et al., 1995; Tenover et al., 1997; Johnson et al., 2007). To uncover the relationship of mink isolates with a larger dataset of human and environmental isolates 18 PFGE-typed isolates were further characterized by an array hybridization kit; the AT biochip (*P. aeruginosa* Genotyping Kit, Clondiag Chip Technologies, Germany) as previously described (Morales et al.,

2004; Wiehlmann et al., 2007). While PFGE is based on mutations in restriction sites dispersed in the chromosome, the AT biochip recognizes SNPs in the core genome and genetic markers for specific gene islets and islands in the accessory genome.

Materials and methods

Materials

One hundred and sixty-four isolates representing 95 outbreaks of hemorrhagic pneumonia on 90 mink farms were typed by PFGE. Only *P. aeruginosa* isolated from lungs of mink dying of hemorrhagic pneumonia were included in the study. The mink were submitted for diagnostic investigations from Danish mink farms during the period 2002-2009 and formed 85% of the recorded outbreaks of hemorrhagic pneumonia in this period. *P. aeruginosa* were diagnosed on the basis of characteristic colony morphology on blood agar and MacConkey agar, smell, Gram-stain and positive cytochrome oxidase reaction. Serotyping was performed using polyclonal antisera (Difco™ polyclonal serotyping, Detroit, MI, U.S.A).

The isolates were freeze-dried at the time of isolation and stored at room temperature before they were revived by incubation in veal infusion broth (Difco™) for 24 hours at 37°C and plated on blood agar plates.

Eighteen isolates were furthermore typed using the AT Biochip. The AT biochip recognizes 16 highly conserved genetic regions and several regions in the accessory genome including genetic islands and islets. The isolates selected for genotyping either belonged to a cluster which showed only few band differences on the PFGE profile, or were identified as particularly prevalent or as originating from repeated outbreaks on the same farm. In addition, two isolates were chosen only on behalf of serotype to represent the serotypes 5 and 7/8 since the rest of the chosen isolates belonged to serotype 6. The 18 PFGE types were responsible for 36 of the 95 outbreaks.

Methods

The PFGE procedure has been described elsewhere (Nauerby et al., 2000) and was followed with some modifications. The agarose plugs were digested with 0.1 mg/ml proteinase K in lysis buffer (1M Tris pH=8.0, 0.5M EDTA pH=8.0, 10 % N-lauroyl sarcosine) for 2 hours in a shaking water bath (56°C, 200 rpm). Thin slices cut from the plugs were digested with *SpeI* (BioLab, Ipswich, MA, USA) for 4 hours at 37°C. The restriction fragments were separated as previously described (Nauerby et al., 2000). Lambda Ladder PFG markers (BioLab) were run with the samples on the gels. The gels were stained with ethidium bromide (2 µg/ml) for 7 min., washed in distilled water for 15 min and photographed under UV-light by GelDoc-It Imaging System (AH Diagnostics, Aarhus, Denmark) with the software VisionWorks LS (UVP, Upland, CA, USA).

The resulting band profiles were analyzed using Bionumerics (Applied Maths, ver. 4.50) with Dice band based comparison and a position tolerance of 1.7 % as suggested by Carriço et al. (2005)

The isolates were defined as belonging to the same strain if the isolates had indistinguishable PFGE profiles. If the isolates differed by 1 to 5 bands (corresponding to similarities above 85 %) they were regarded as belonging to a cluster of closely related strains.

Band profiles were exported from Bionumerics as binary data into Arlequin v.3.5.1.2 (Excoffier and Lischer, 2010). Pairwise measures of genetic distance (F_{ST}) were calculated using Nei's average number of pairwise differences (Nei and Li, 1979). Geographical distances between farms were calculated in ArcGIS (Redlands, CA, U.S.A.) based on their geographical coordinates. A Mantel test was used for fitting regression models between the two matrices, consisting of the genetic distance (response matrix) and the Euclidean geographic distance (explanatory matrix) (Mantel, 1967). The Mantel test was performed using Arlequin.

The manufacturer's protocol for the AT-Biochip was followed closely and has been described elsewhere (Jelsbak et al., 2007; Wiehlmann et al., 2007).

Results

We determined the PFGE profiles of 164 isolates sampled from 95 outbreaks of hemorrhagic pneumonia on 90 mink farms, and found 72 distinct PFGE patterns (Figure 1). Seventy-two per cent of the outbreaks were caused by serotype 6 while serotype 5 was recovered in 22% of the outbreaks and serotype 7/8 in the final six per cent. Isolates with similarities of their PFGE profiles of more than 80% invariably displayed the same serotype.

Isolates from two or more mink were typed in 47 (50%) of the outbreaks. In 41 (87%) of these outbreaks the isolates showed indistinguishable PFGE profiles when recovered from the same outbreak while two apparently unrelated PFGE profiles were discovered in six outbreaks represented by two or more isolates. Six farms experienced hemorrhagic pneumonia outbreaks twice in the study period with one to six years between the outbreaks. In three of these farms the outbreak was caused by a *P. aeruginosa* strain with indistinguishable PFGE profile from the one causing the previous outbreak (S3, S12, S19). On the other three farms, the PFGE profiles between the isolates causing outbreak one and two showed similarities ranging from 33-52%. The serotypes of *P. aeruginosa* causing these outbreaks belonged to the same serotype (6) in two out of three cases while the last farm was infected with serotype 6 in the first outbreak and serotype 5 in the next.

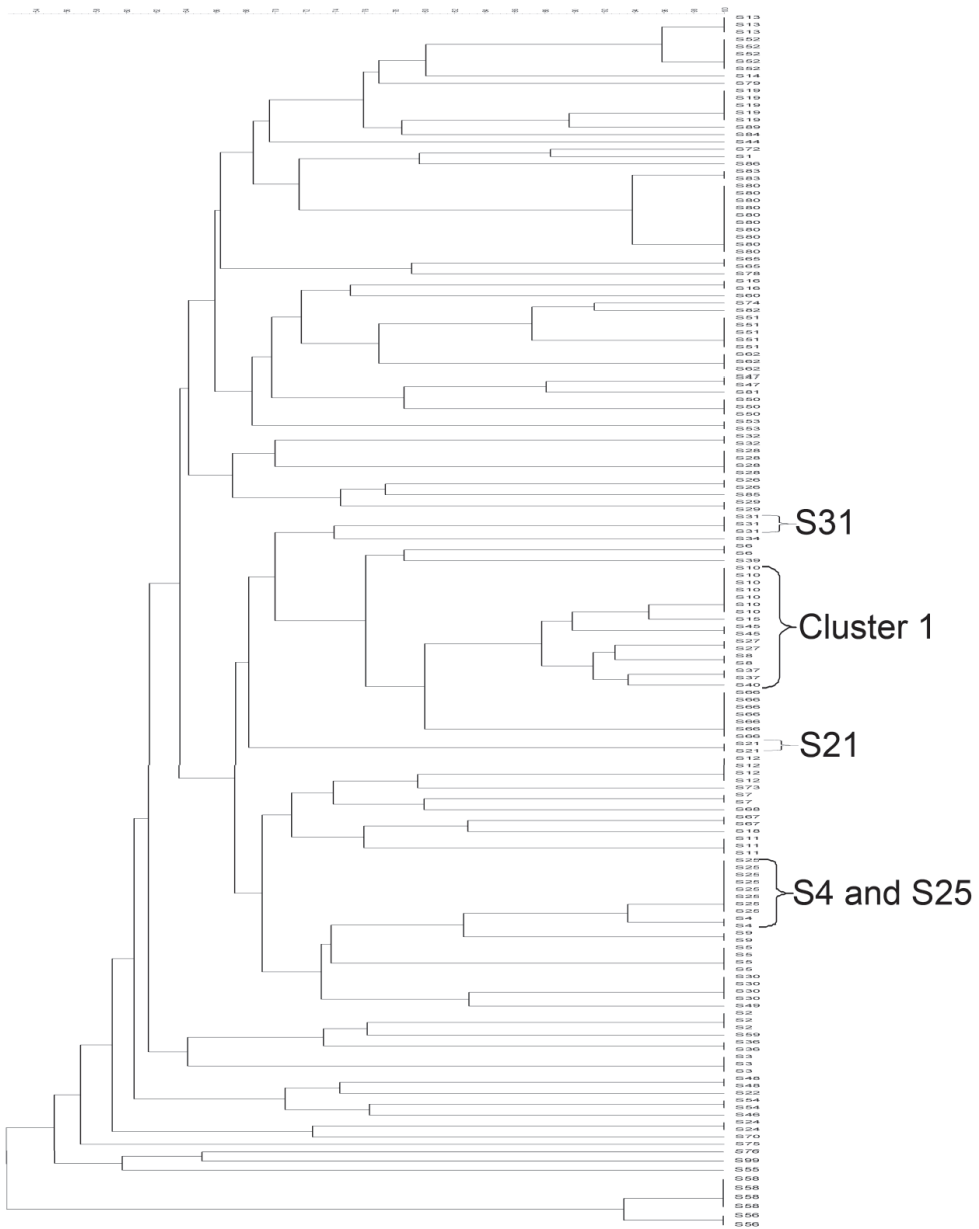


Figure 1: Dendrogram of *P. aeruginosa* isolated from mink hemorrhagic pneumonia. S21, S4, S25 and S31 groups in “Cluster B” based on results from the AT biochip.

Eleven strains were each recovered from two outbreaks on different farms, while four strains were each recovered from three or more outbreaks on different farms. The outbreaks caused by the same strains were sometimes located in the same geographic areas but also widely apart. Eight strains responsible for two outbreaks occurred on farms sharing the same central food kitchen while four strains causing two or three outbreaks did not share food kitchen. The remaining three strains caused outbreaks on several farms of which some shared food kitchen and others did not (Table 1).

Table 1: PFGE-types causing more than one outbreak. The approximate distance between farms and sharing of food kitchens between farms are listed.

PFGE type	No. outbreaks	Year	Approximate distance between farms	Shared food kitchen
S2	2	2002	1 km	Yes
S5	2	2002	1 km	Yes
S10	2	2002 and 2003	170 km	No
S28	2	2004	8 km	Yes
S30	2	2004	1 km	Yes
S37	2	2004 and 2005	350 km	No
S51	2	2006	7 km	No
S52	2	2006	1 km	Yes
S53	2	2006	18 km	Yes
S62	2	2006	1 km	Yes
S83	2	2009	10 km	Yes
S31	3	2004	From 170 to 350 km	No
S25	4	2004	From 1 to 7 km, 1 farm 300 km	Some
S66	4	2006	From 25 to 170 km	Some
S80	8	2009	From 0 to 25 km	Some

Seven strains (S10, S15, S45, S27, S8, S37 and S40) grouped in a cluster with similarities of above 85% and PFGE profiles with 5 band differences or less among each other. This group was called “Cluster 1” (Figure 1). The members of this cluster caused nine outbreaks; they were geographically widespread and occurred from 2002 to 2006.

The Mantel test between geographical distance and genetic difference (F_{st}) was not significant ($p=0.59$), meaning there was no correlation between the outbreak locations and relationship among the isolates. The dendrogram also revealed a large amount of differentiation among the PFGE types (Figure 1). A larger version of the dendrogram can be viewed in supplementary material.

Eighteen isolates were further typed using the AT biochip. The isolates either belonged to Cluster 1 (S10, S15, S45, S27, S8, S37 and S40), were particularly prevalent (S25, S31, S66 and S80), showed similarities of above 85% to one of the above mentioned strains (S4 to S25 and S83 to S80) or occurred on a farm with previous outbreaks of hemorrhagic pneumonia (S73 and S84, together with S66, S21, S25 and S37, which also belonged to Cluster 1 or were particularly prevalent). Two isolates represented the serotypes 5 and 7/8 (S72 and S79).

The isolates in Cluster 1 showed a high degree of relationship on the AT biochip except from isolate S37 (Figure 2) and was named Cluster A. S37 differed from the others in Cluster 1 by three to four bands on the PFGE profile but did not group together with the other strains in Cluster A on the AT biochip. In fact, this isolate showed a remarkably dissimilar SNP-profile to the other isolates in Cluster A but a somewhat similar profile on the gene islands markers in the accessory genome (outline of the entire information obtained by the AT biochip can be viewed in Table S1 in supplementary material).

	S37	S66	S79	S72	S84	S73	S10	S15	S8	S27	S40	S45	S4	S25	S21	S31	S80	S83
oriC	1	0	1	0	0	0	1	1	1	1	0	1	1	1	1	1	0	0
oprL	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	1	1
fliC a	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
alkB2	0	1	1	1	0	1	0	0	0	0	0	0	1	1	1	1	0	0
citS-1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
citS-2	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
oprI	0	0	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1
ampC_1	0	0	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1
ampC_3	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	1
ampC_4	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1
ampC_5	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ampC_6	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ampC_7	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1
fliC a	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1
fliC b	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
exoS	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
exoU	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	882E	682E	F64A	7458	4992	699A	8E9A	8E9A	8E9A	8E9A	8E9A	8E9A	AE9A	AE9A	AE9A	AE9A	4F9A	4F9A

Figure 2: Outline of AT-chip. 0 = wild-type (PAO1 type), 1 = differing from wild-type. Cluster A (S10, S15, S8, S27, S40, S45) and cluster B (S4, S25, S21, S31) are shown. S25, S31, S66 and S80 were recovered from three or more outbreaks and S4 and S83 showed only few band differences to S25 and S80, respectively. Three farms with previous outbreaks of hemorrhagic pneumonia due to distinct PFGE-types included the strains S37 and S25, S21 and S84, and S73 and S66. S72 represented serotype 5 and S79 represented serotype 7/8.

Cluster A showed a high degree of relationship with isolates S4, S25, S21 and S31 on the AT Biochip. These four PFGE-types had the same SNP-type and were named “Cluster B”. The only difference between these two clusters on the AT biochip was a polymorphism in *alkB*. Both the relationship among the strains in Cluster B and the relation to Cluster A were not obvious from their PFGE-profiles (Figure 3). The presence or absence of gene islands were somewhat similar in the two groups but showed some variation especially in Cluster B. This was also the case for S80 and S83 which showed PFGE patterns with 86% similarity. These types were almost identical on the AT biochip with the exception of few gene islands in the accessory genome.

S45 S10 S15 S27 S8 S37 S40 S25 S4 S31 S21

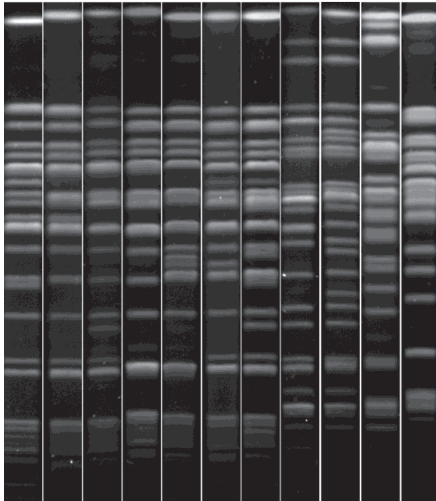


Figure 3: PFGE macrorestriction patterns for Cluster A, B and S37.

Discussion

When isolates from two or more mink were obtained from the same farm, the PFGE profiles were indistinguishable in 87% of the outbreaks. However, it is not possible to deduce that the mortality in the majority of outbreaks are caused by the same strain since isolates from only two mink were typed in 30 of the 41 outbreaks with indistinguishable PFGE profiles occurring on the same farm. No more than four animals were ever examined from the same farm. If two strains of *P. aeruginosa* were causing an outbreak with the same frequency of disease attributed to each strain, one would need to type at least 5 mink to be 95% certain of detecting both strains. The number of animals to be examined is even higher if the prevalence of a different PFGE type is lower (Martin et al., 1987, pp. 37-38). Unfortunately typing of more isolates from each farm outbreak was not possible using the present dataset.

Six farms experienced repeated outbreaks in the study period. On three of these farms indistinguishable PFGE-types were found on the farm with one to three years between the outbreaks which supports previous work (Hammer et al., 2003). This demonstrates that *P. aeruginosa* is able to survive in the environment for at least three years and remain infectious for mink. No relationship was found between the isolates found on the other three farms with two outbreaks where the time between the two outbreaks ranged from one to six years. Since some of the farms probably vaccinate against the disease after having experienced an outbreak, only little can be said of the ability of *P. aeruginosa* to re-infect farms in succeeding years and how well it thrives in the environment on the mink farm.

Differences in relationship among the strains were observed when comparing the results from PFGE and the AT biochip. While the prevalent strain S80 was related to S83 both by PFGE typing and especially by the AT biochip, S25, S31 and S21 (isolates from Cluster B) showed a relationship only when typed by the AT

biochip, while their PFGE profiles were quite dissimilar (Figure 3). Similarly the strain S37 was included in Cluster 1 based on the results from PFGE analysis but was unrelated to the other members of this cluster when examined by the AT biochip. The reason for the observed difference might lie in the fact that PFGE is considered more discriminative than the AT-biochip and in some settings it may be too discriminating, making it hard to identify related isolates (Morales et al., 2004; Johnson et al., 2007; Maatallah et al., 2011). Changes in the accessory genome may lead to differences in the macro-restriction pattern, while the core genome is unchanged and shared among related strains. This is more easily recognized by a SNP-based typing system like the AT biochip or conventional multilocus sequence typing (MLST) (Kidd et al., 2011; Waters et al., 2012). Since horizontal transfer and genetic recombination of the accessory genome are believed to be major factors of evolution in *P. aeruginosa* (Römling et al., 1997; Kiewitz and Tümmler, 2000; Larbig et al., 2002; Morales et al., 2004) few genetic events can cause significant changes in the PFGE pattern if the bacterium gains or loses large pieces of genetic material. The observed differences in the accessory genome might be the reason for the differences in PFGE pattern recovered in Cluster 1 and in the isolates belonging to Cluster B and the types S80 and S83. Most of the isolates carried genes for type A flagellin and ExoS while all isolates contained the PAGI-1 gene island which carries regulatory genes and genes required for detoxification of reactive oxygen species (Kung et al., 2010). ExoS is an effector of the type III secretion system. While the type III secretion system is recognized as an important virulence factor, ExoU is regarded as being the most virulent effector of this system (Shaver and Hauser, 2004). The biological significance of the presence or absence of certain genes in the accessory genome is difficult to assess since the AT biochip was developed purely for typing and does not reveal the level of expression of these genes. Furthermore nothing can be concluded on the presence of these genes in the larger population of *P. aeruginosa* originating from hemorrhagic pneumonia in mink since the isolates typed by the AT biochip in this dataset were not randomly chosen.

When using PFGE as the typing method, the strains in Cluster 1 caused nine outbreaks of hemorrhagic pneumonia which accounted for 9% of the recorded outbreaks. When using the AT biochip as the typing method the strains in Cluster A and B caused 16 of the recorded outbreaks (17%). Furthermore 12 outbreaks (13%) were caused by two unrelated but frequently prevalent strains (S80 and S66). This means that two thirds of the recorded outbreaks of hemorrhagic pneumonia in mink were caused by unique genotypes. This number may have been lower if a slightly less discriminating typing method like MLST had been used since MLST is considered more likely to reveal clonal relatedness (Kidd et al., 2011; Waters et al., 2012). Nonetheless, a large proportion of outbreaks of hemorrhagic pneumonia are caused by distinct genotypes, which are probably transmitted to the mink from the farm environment. Feed, water troughs, equipment, personnel, air and feed manufacturers have been suggested as contamination sources (Knox, 1953; Shimizu et al., 1974; Gierløff, 1980; Hammer et al., 2003). Since many outbreaks caused by indistinguishable *P. aeruginosa* strains occurred on closely situated farms, it was not surprising that some of these farms also shared food kitchen. However, in almost half of the outbreaks caused by indistinguishable PFGE-types all farms infected with a particular strain did not share food kitchen. This point to local environmental factors as being more important as contamination sources than the central food kitchens. Investigations including genetic typing of *P. aeruginosa* in both animals and suspected sources have never been carried out and hence no confirmed contaminating source has ever been identified.

Roughly one third of the outbreaks could be attributed to infection with genotypes that for some reason were more prevalent in the population of *P. aeruginosa* isolated from hemorrhagic pneumonia in mink.

This might be an underestimation due to the highly discriminating typing technique used in this study. Having identified these more prevalent genotypes, we now have an opportunity to further investigate their genomes and their virulence. The isolates in Cluster A and B might share common traits making them more virulent to mink or they might be overrepresented in the environment. When comparing the SNP-types of the 18 isolates typed by the AT biochip with 240 isolates from a previously published dataset (Wiehlmann et al., 2007), *P. aeruginosa* from mink grouped with *P. aeruginosa* isolated from various infections (bacteremia, intensive care units, cystic fibrosis) and even from water but the specific types as identified by their hexadecimal codes were not present. This might imply that the mink strains are not overrepresented in the general environment.

The relatively high numbers of distinct *P. aeruginosa* strains causing hemorrhagic pneumonia in mink supports previous work in this field (Hammer et al., 2003). The results are to some degree comparable to what has been found in human *P. aeruginosa* infections, where multiple distinct and a number of highly clonal isolates are identified as the cause of various infections (Curran et al., 2004; Scott and Pitt, 2004; Jelsbak et al., 2007; Tramper-Stranders et al., 2008). In contrast to what has been found in human infections, the four most frequently isolated strains (S25, S31, S66 and S80) (Table 1) appear to cause disease in one year only and hence cannot be considered as “widespread successful clones”. This has been demonstrated for isolates from mink hemorrhagic pneumonia before (Hammer et al., 2003). The relationship between genetic relatedness and geographical distance was not significant implying that most often no particular successful strain is circulating between mink farms in a geographical region. However, no records of the farms’ relationship with each other are available and the investigation spans many years, so it is possible that some *P. aeruginosa* strains have been spread vast distances by vehicles, people or trading of mink, which would bias the test towards no relationship between geographic region and strain.

Conclusion

Clusters of highly related isolates were recognized using PFGE and the AT biochip. The related clusters, together with two unrelated but frequently isolated strains, caused almost one third of the outbreaks indicating that some clones have a higher virulence for mink or are more prevalent in the environment. A comparison of the biochip typed mink isolates to 240 *P. aeruginosa* from various sources showed that none of the mink isolates had been identified in this large dataset. Outbreaks caused by the same strain of *P. aeruginosa* most often occurred in the same year with both local and widespread geographical distribution. In almost half of these outbreaks the farms did not share food kitchen. Two thirds of the recorded outbreaks could be attributed to distinct strains emphasizing that environmental strains are likely the most frequent cause of hemorrhagic pneumonia in mink.

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Conflict of interest statement

The authors do not have any financial or personal conflicts of interest regarding the work presented in this communication.

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Article II

Comparison of histological lesions in acute hemorrhagic pneumonia in mink 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. Hemorrhagic pneumonia in its classic form is caused by the bacterium *Pseudomonas aeruginosa*. However, in recent years, we have experienced outbreaks of this type of pneumonia in farmed mink associated with hemolytic *Escherichia coli*. The purpose of this study was to compare histological lesions of acute hemorrhagic pneumonia in mink associated with *P. aeruginosa* and *E. coli* including a description of tissue distribution of pathogens in an attempt to differentiate the two disease entities on histopathology. The study included material from 2006-2009 submitted for diagnostic investigation at the National Veterinary Institute in Denmark. 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 were examined by histology and fluorescence in situ hybridization. It was possible to detect a slight histological difference between hemorrhagic pneumonia caused by *P. aeruginosa* and *E. coli*, as *P. aeruginosa* was found mainly 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* were associated with a higher frequency of lungs displaying alveolar edema and mild lymphoid cuffing.

Keywords: *Escherichia coli*, fluorescence in situ hybridization, histology, mink, pneumonia, *Pseudomonas aeruginosa*

Introduction

Outbreaks of fatal hemorrhagic pneumonia caused by *Pseudomonas aeruginosa* have been described in farmed mink since it was first recognized in Denmark in 1953 [1]. Hemorrhagic pneumonia due to *P. aeruginosa* has a rapid disease spread on the farm with mortalities able to reach 75% [2]. However, the disease may also have a milder course with lower mortality [1]. Hemorrhagic pneumonia is almost exclusively seen from September to December in Denmark.

During the last 5-10 years 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*. Pneumonia associated with *E. coli* primarily occurs in the autumn, like *P. aeruginosa* pneumonia, and the two 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]. *P. 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 in healthy

animals are however not described for other animals than mink. The pathology and histopathology of hemorrhagic pneumonia in mink caused by *P. aeruginosa* has been described in detail [11-13].

E. coli has been described as the cause of spontaneous fatal pneumonia in few cases among dogs, cats and in a horse [14-16] and in one report, is associated with hemorrhagic pneumonia in mink [17]. In the publication by Tibbetts et al., no attempt to describe the pathology or histopathology of the disease is made. To our knowledge, this is the first report which describes 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 the two diseases on 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

Materials

Samples of lung tissue from 37 mink diagnosed with hemorrhagic pneumonia during 2006-2009 and with pure cultures of the bacteria of interest were included in this study. Diagnosis of hemorrhagic pneumonia was done at the National Veterinary Institute in Denmark and was based upon 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 shortly 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 pr. mink from areas with macroscopic lesions was fixed in 10% buffered formalin for 1-3 days and embedded in paraffin as part of routine diagnostic investigation. The formalin fixed paraffin embedded (FFPE) tissue was utilized for the study described below.

All mink included in this study were tested for antibodies against Aleutian disease virus using an additive counter-immunoelectrophoresis method [18] with negative results.

The records at the National Veterinary Institute in Denmark described the macroscopic findings in the lungs of the 37 mink included in this study as all displaying 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.

Methods

From the paraffin blocks, tissue sections of 3 μ m were mounted on glass slides (Superfrost, Menzel-Gläser, Braunschweig, Germany). Each slide was stained with conventional hematoxylin and eosin (HE) and examined for severity of lesions in different categories (Table 1). The severity was graded as follows: 0; describing no lesions, 1; describing mild lesions (up till 33% of tissue involved) or few cells (e.g. leukocytes

or erythrocytes), 2; describing moderate lesions (34-66% of tissue involved) or a moderate number of cells and 3; describing severe lesions (above 66% of tissue involved) or a massive number of cells. The slides were examined twice in a blinded fashion by the same examiner. The entire piece of lung present on the slide was included when deciding the histological grades appointed to each lung.

3 µm tissue sections from paraffin blocks were mounted on Superfrost® Plus glass slides (Menzel-Gläser). A 6-FAM labeled *P. aeruginosa* probe with the sequence 5'-GGTAACCGTCCCCCTTGC-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 for detection of *P. aeruginosa* and *E. coli* by fluorescence in situ hybridization (FISH) targeting 16S rRNA in a hybridization concentration of 5 ng/µl hybridization buffer (0.1M Tris pH 7.2, 0.9 M NaCl, 0.1% SDS) and 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 gamma-proteo-bacterium probes; GAM 42A, 5'-GCCTCCACATCGTTT -3' and 5'-GCCTCCCACTTCGTTT -3'[22] (Eurofins MWG Operon).

The slides were washed 3 x 5 min (0.1M Tris pH 7.2, 0.9M NaCl) and mounted with Vectashield Mounting Medium for Fluorescence with DAPI (Vector Laboratories, Burlingame, CA). For verification of 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 in mink has been detected with increasing frequency over the last 10 years (Figure 1).

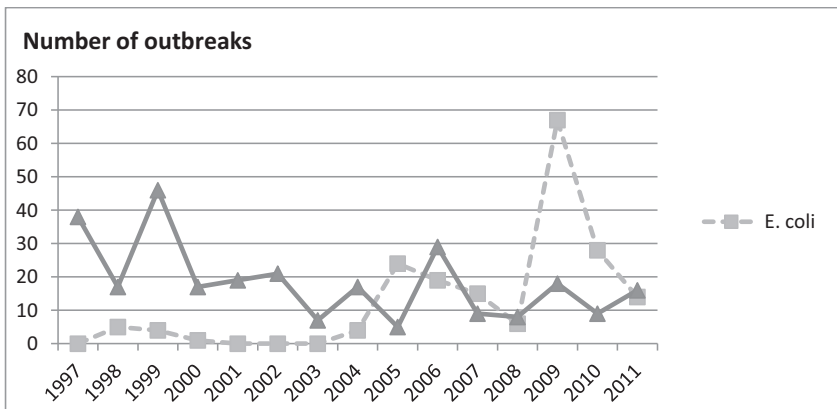


Figure 1: Prevalence of hemorrhagic pneumonia in Denmark caused by *P. aeruginosa* and *E. coli*

Histology

The average of the histological grades assigned to the categories of pathological lesions can be seen in Table 1. The calculation of averages is not valid for statistical purposes since the data are categorical but is in this case only used as a way of displaying the differences in scores and should be interpreted with caution.

The agreement between the two assessments of each slide was calculated based on each category and ranged from 62-100%.

Table 1: Average scores for histopathological lesions. Vasculitis is in these cases regarded as transmural inflammation while interstitial edema refers to perivascular and peribronchial edema. 0 = none (0%), 1 = mild lesions involving 1-33% of tissue or few cells, 2 = moderate lesions involving 34-66% or a moderate number of cells and 3 = massive/severe lesions involving >66% of tissue or a massive number of cells.

	<i>E. coli</i>	<i>P. aeruginosa</i>
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	1.4	1.4
Lymphoid cuffing	0.6	0.2

The significance of the difference between the scores for *E. coli* and *P. aeruginosa* was assessed using Fisher's exact test on a 2 x 4 contingency table (2 groups with 4 scores) and a significance level of 0.05. Only the first grading of the slides were included in the statistical evaluation to avoid working with two dependent scores, while both gradings were included in the calculation of the average displayed in table 1 to utilize as much information from the slides as possible. Three lesion types differed with significance

levels below 0.05, namely extent of alveolar edema, amount of erythrocytes in alveoli and lymphoid cuffing.

The predominant leukocyte types based on morphology were neutrophil granulocytes and macrophages but lymphocytes were also observed. In *P. aeruginosa* pneumonia the leukocyte types predominantly consisted of neutrophils or a mix of neutrophils and macrophages. In *E. coli* pneumonia the inflammation type was dominated by a mixture of macrophages and neutrophils. Two main patterns and various mixtures of these patterns were found, with one being 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 *P. aeruginosa* infected lungs while severe alveolar edema (figure 4) was most often encountered in *E. coli* infected lungs.

Epithelial cell loss was most prominent in the bronchioles compared to the bronchi in all cases of pneumonia. Perivascular accumulations of bacteria around venules and arterioles were noted for pneumonias caused by *P. aeruginosa* (Figure 5) but only in one case for pneumonias 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 most often did not penetrate into the vascular lumen although it was observed in few cases where massive amounts of bacteria were identified surrounding the vessel (Figure 6).

In pneumonia associated with *E. coli*, fewer bacteria were generally found 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).

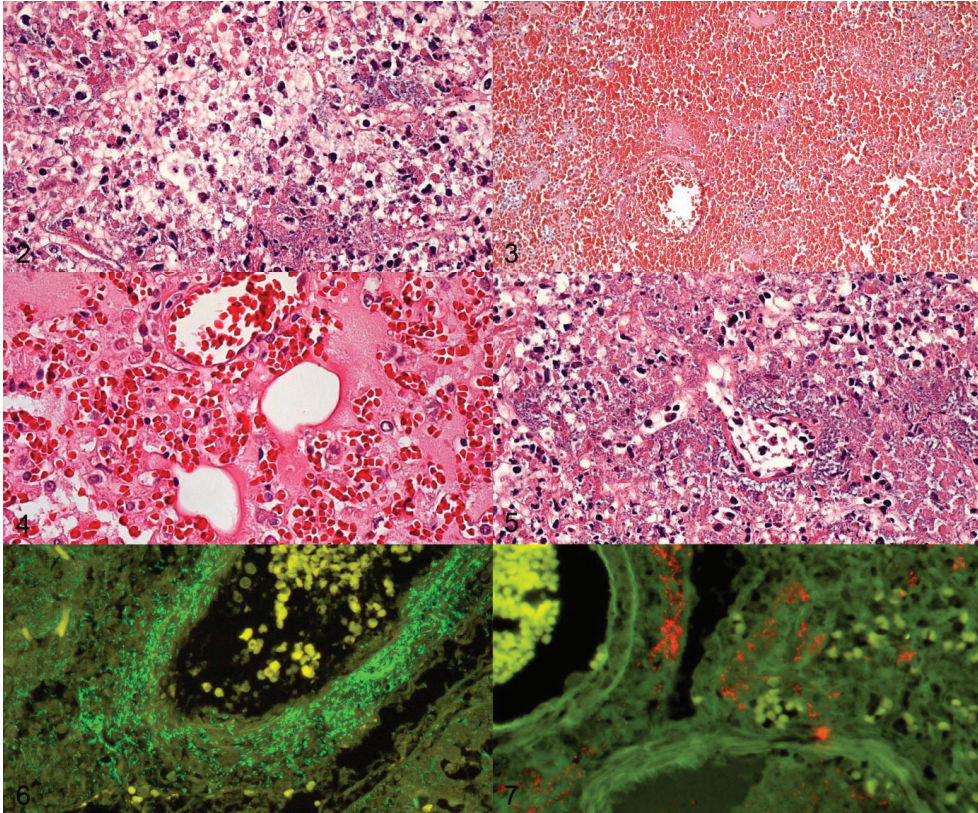


Figure 2: Lung, mink. Massive necrosis of alveolar septa, degenerated neutrophils and myriads of bacteria (*P. aeruginosa*). HE, 400x magnification

Figure 3: Lung, mink. Severe acute pulmonary hemorrhage (*P. aeruginosa*). HE, 200x magnification.

Figure 4: Lung, mink. Massive alveolar edema and blood vessel without perivascular located bacteria (*E. coli*). HE, 400x magnification.

Figure 5: Lung, mink. Blood vessel showing massive perivascular accumulation of bacteria, mild vasculitis and margination of leukocytes along with severe necrosis of the surrounding tissue (*P. aeruginosa*). HE, 400x magnification.

Figure 6: Lung, mink. *P. aeruginosa* around and in blood vessel wall. FISH with 6-FAM labeled *P. aeruginosa* probe. 400x magnification.

Figure 7: Lung, mink. *E. coli* between blood vessel (left upper corner) and bronchiole (right lower corner). FISH with Texas Red labeled *E. coli* probe. 400x magnification.

Discussion

The average scores for most of the lesions observed in the lung tissue were quite similar for the two pathogens. A somewhat similar pattern was expected since the inclusion criteria for this study was hemorrhagic pneumonia and infection with either *P. aeruginosa* or *E. coli*. Nonetheless, alveolar edema, amount of erythrocytes in alveoli and lymphoid cuffing displayed significant differences between the two 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 observed differences in histological pattern between the two 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 clearance of *P. aeruginosa* infection has been underlined by others [25,26] showing neutrophils to be of paramount importance for clearance of pulmonary infection with *P. aeruginosa*. In our study mink displaying a predominantly neutrophil response also died of the infection, questioning 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 animals were included in the study which makes it very difficult 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 were highly dependent on the activity of neutrophils, this coat color type should be more prone to develop hemorrhagic pneumonia. No coat color types were particularly prevalent in this study, which however represents too few mink to further explore this theory.

In 1968 Nordstoga described two histopathological patterns in *P. aeruginosa* infected mink lungs [13]. One pattern, which was found in mink from one farm, showed a hemorrhagic necrosis without inflammation and with only 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 not penetrating the tunica elastica interna and hence only rarely seen in the vessel lumen. This report 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]. Why *P. aeruginosa* shows a predominantly perivascular localization has not been established but it could be speculated that the amount of certain nutrients are 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 be part of the reason 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 are consistent with the sparse literature on the subject [14,15]. The perivascular distribution, as was observed in pneumonias caused by *P. aeruginosa*, was seen only once in our study, while a higher tendency for provoking alveolar edema was observed. If this reflects a difference in receptors or a tendency for evoking different responses by the immune system is unclear and further investigations including experimental studies are necessary to investigate the pathogenesis of hemorrhagic pneumonia in mink.

The use of diagnostic material is not optimal for this type of study but is, at present, the only accessible source of tissue from this disease. The macroscopic lesions described in the materials and methods section

are based upon records used for diagnostic purposes which are subject to variations due to various personnel and handling procedures and should be cautiously assessed.

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 pathogens leading 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. *P. aeruginosa* was also recognized as more often evoking a predominantly hemorrhagic pattern in the mink lungs while *E. coli* more often was associated with higher amounts of alveolar edema and mild lymphoid cuffing than *P. aeruginosa*. Whether these differences reflect aspects of pathogenesis of hemorrhagic pneumonia in mink caused by the two pathogens remain to be elucidated.

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Article III

Effect of infectious dose and season on development of hemorrhagic pneumonia in mink caused by *Pseudomonas aeruginosa*

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Abstract

Hemorrhagic pneumonia is an acute and fatal disease of farmed mink caused by *Pseudomonas aeruginosa*. The pathogenesis of this disease has not yet been resolved. Mink are the only animals known to be susceptible to acute, contagious and fatal lung infections caused by *P. aeruginosa*. To elucidate the pathogenesis of the disease, an infectious dose-response trial was performed on adult mink and mink kits both in the season for hemorrhagic pneumonia (November) as well as out of season (July). It proved difficult to infect mink via the intra-nasal route. Only 4 out of 60 infected mink developed clinical disease and were euthanized, all of them in November, indicating that predisposing factors in the mink itself might be crucial for disease development. We were able to culture *P. aeruginosa* from the nasal cavity of the clinically healthy experimental mink eight days after inoculation showing that the mink can carry *P. aeruginosa* on their nasal mucosa without developing disease. It was, however, not possible to culture *P. aeruginosa* from the nasal cavity of clinically healthy mink obtained from farms in November indicating that the organism is not a normal part of the nasal mucosal flora of mink.

Keywords: Mink, hemorrhagic pneumonia, *Pseudomonas aeruginosa*

Introduction

Pseudomonas aeruginosa was first described as the cause of hemorrhagic pneumonia in farmed mink in 1953 [1]. This disease can cause high mortality among farmed mink [2,3] and is regarded as highly seasonal since it is almost exclusively diagnosed in the autumn period from September to November. The disease has an acute onset and the mink are often found dead with blood around nostrils and mouth without prior clinical signs.

The pathogenesis of the disease has not yet been fully elucidated. Earlier studies have suggested that the transmission route may be airborne [4] and that mink are easier to infect via the intra-tracheal route than orally [5,6]. Feed, water troughs, personnel and equipment have been suggested as sources of *P. aeruginosa* but no investigations have confirmed this [1,7,8]. Earlier studies have indicated that mink develop fatal hemorrhagic pneumonia following an intra-tracheal or intra-nasal challenge dose of 10^6 cfu/ml after 18-66 hours [4]. Species like guinea pigs and mice typically require higher challenge doses to show any mortality when infected through the airways [9,10]. *Pseudomonas aeruginosa* has been isolated from the oropharynx and rectum of healthy mink at pelting [11] indicating that *P. aeruginosa* may be a commensal organism which only causes disease in certain circumstances.

In humans, *P. aeruginosa* is primarily regarded as a pathogen for immune-compromised patients and some studies have proposed aerogen transmission for lung infections [12-14]. Mink are, to our knowledge, the only species known to develop acute, fatal and contagious pneumonia caused by *P. aeruginosa* in otherwise apparently healthy individuals.

The purpose of the study was to investigate the correlation between dose-response and season of infection and to clarify whether Danish mink are carriers of *P. aeruginosa* on their nasal mucosa in the season for hemorrhagic pneumonia.

Materials and Methods

Animal experiments

Four animal experiments were conducted each including 18 brown mink obtained from the same Danish mink farm. All mink had been tested for antibodies against Aleutian Disease virus with negative results. In both 2010 and 2011 two experiments were performed; one in July and one in November. In 2010 barren females were used for the study while in 2011 we used female mink kits. The mink were assigned to individual cages separated with metal plates and with separate water supply. All animals were anesthetized with an intramuscular injection of ketamine/xylazine (Ketaminol®/Rompun®) mixture and were assigned to six infectious dose groups of three mink. The mink were infected intra-nasally with *P. aeruginosa* suspended in 0.5ml 0.9% saline which was instilled into the nasal cavity using a pipette (Figure 1A). The infectious doses ranged from 10^3 - 10^9 cfu/ml in the four conducted experiments and the concentration of the bacterial solution was determined using the plate count method. In 2010 the mink were infected with dose ranges from 10^3 - 10^7 cfu/ml. In July 2011 the doses ranged from 10^5 - 10^9 cfu/ml and in November 2011 from 10^4 - 10^8 cfu/ml. The mink were placed with their noses tilted slightly upward (10 - 20°) during recovery from the anesthesia. The *P. aeruginosa* isolate used for infection was recovered from an outbreak of hemorrhagic pneumonia in mink on the Danish island Mors in 2008 (Difco serotype O6, laboratory journal number at the National Veterinary Institute: 2008-52-937). One group of mink in each experiment served as controls and received 0.9% saline intra-nasally.

The mink were observed every six hours in the period from 18-66 hours after infection and in the rest of the period at least three times daily. Eight days after infection the mink were anesthetized with a ketamine/xylazine mixture injected intramuscularly and euthanized with an injection of T-61® (Intervet, NL) in the liver. Necropsies were performed immediately after the animals were euthanized. Mink showing signs of illness (lethargy, labored respiration) were euthanized and necropsied immediately or cooled and necropsied no later than 16 hours after death. At necropsy of all mink, swabs were taken from the nasal cavity, while bacterial samples from the lungs and livers were collected after shortly burning the surface of the organs and making a stab incision with a sterile scalpel before the swab was introduced into the organ. The swabs were streaked on blood agar and grown at 37°C for 24 hours. The procedure for taking swabs from the nasal cavity was changed from 2010 to 2011. In 2010, the swabs were collected by cutting the nose transversely with an oscillating saw midway between the eyes and the tip of the nose and introducing a sterile swab into the caudal part of meatus nasi dorsalis and ventralis (Figure 1B). In 2011 the nose was cut in the dorsal plane right above the hard palate and transversely at the level of the eyes so a large piece of the nasal cavity could be separated from the head. The nasal turbinates were removed with sterile forceps and the swab was streaked on the inner sides of the nasal cavity and inserted into a fold in the nasal epithelium laterally in the nasal cavity at the level of the eyes (Figure 1C and D). *Pseudomonas aeruginosa* isolated from lungs or nasal cavities were typed by pulsed field gel electrophoresis (PFGE) essentially as has

previously been described [15] to verify that the same PFGE type was isolated from the mink as the one used for infection.

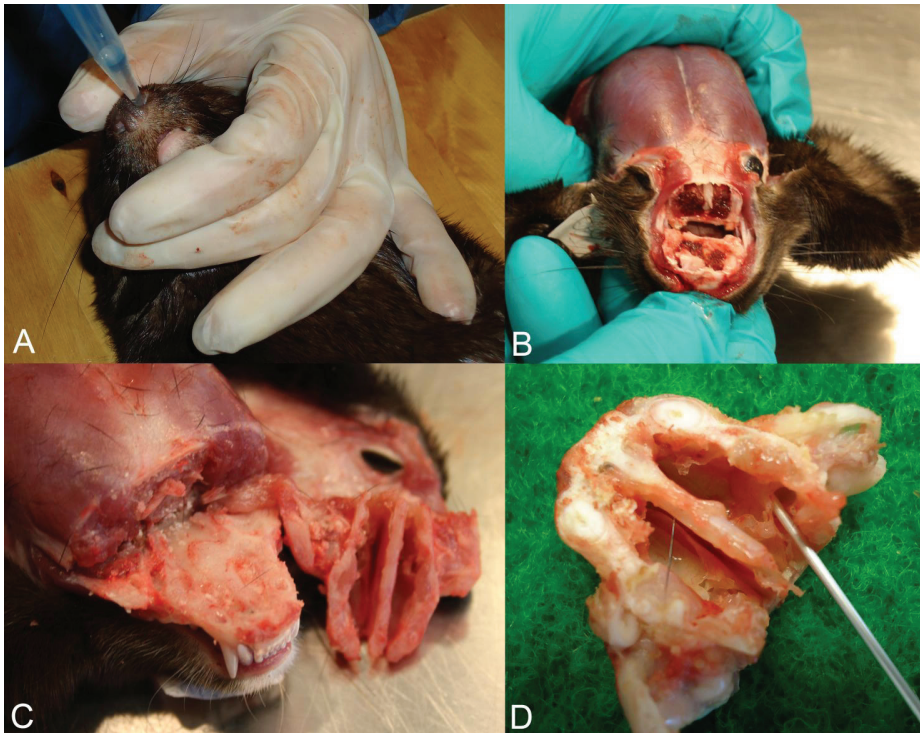


Figure 1: A: Intra-nasal infection of anesthetized mink. B: Method for taking nasal swabs in 2010: A sterile swab was inserted caudally into meatus nasi dorsalis and ventralis. C and D: Method for taking nasal swabs in 2011: The swab was inserted into a lateral fold in the nasal cavity after removal of the nasal turbinates.

Farm mink

Heads from ten female mink kits and ten male mink kits were collected from freshly euthanized animals on four farms at pelting in November 2011. All four farms were negative for antibodies against Aleutian Disease virus. Outbreaks of hemorrhagic pneumonia had occurred on two of these farms in October in the same year as the investigation. On one of the two farms, the animals had been vaccinated against *P. aeruginosa* and treated with antibiotics after the diagnosis of hemorrhagic pneumonia while the mink in the outbreak on the other farm were treated with antibiotics only. The mink heads were opened and swabbed as previously described.

Results

Experiments conducted in July

No mink showed clinical signs of hemorrhagic pneumonia in July 2010 while one mink showed slight depression and reduced feed uptake for three days in July 2011 but fully recovered. In 2010 *P. aeruginosa* was found in the nasal cavity of one mink while the same number for July 2011 was nine, including the mink showing mild disease signs.

Experiments conducted in November

In 2010 and 2011 two mink showed severe lethargy and labored breathing between 24-52 hours after infection. These mink were euthanized and on necropsy the lungs were found to be swollen and dark red. The two mink developing disease in 2010 were infected with 3.7×10^3 and 3.7×10^7 cfu/ml, respectively. The two diseased mink in 2011 were infected with 4.2×10^6 and 4.2×10^7 cfu/ml, respectively. *Pseudomonas aeruginosa* could be found in the nasal cavity of all four mink, as well as in three clinically healthy mink from 2010 and in seven clinically healthy mink from 2011. The ability to find *P. aeruginosa* in the nasal cavity of the mink appeared to be dose related (Figure 2) with a high, but not statistically significant, correlation coefficient ($R^2 = 0.93$). *Pseudomonas aeruginosa* was recovered from the lungs and livers of mink with clinical signs of hemorrhagic pneumonia while it was not possible to culture the bacterium from these organs in mink without clinical disease.

All *P. aeruginosa* cultured from the lungs and nasal cavities of the mink in this experiment had PFGE types indistinguishable from the isolate used for intra-nasal infection.



Figure 2: Proportion of mink with *P. aeruginosa* on nasal mucosa in relation to dose. Time from intra-nasal infection to necropsy and culturing was eight days for clinically healthy mink and 24-52 hours for mink with clinical signs of disease. Vertical bars represent 95% confidence interval.

Samples from farm mink

It was not possible to culture *P. aeruginosa* from the nasal cavity of the farm mink obtained at pelting. From 90% of the mink a mixed bacterial flora with occasional fungi were found, while the remaining 10% showed no growth.

Discussion

In this study it was proven difficult to induce hemorrhagic pneumonia in mink. This might be due to the infection route which could be considered as “milder” compared to earlier used infection routes where the bacteria were instilled directly into the trachea or deep into the nasal cavity using a small catheter [4,16-19]. The method used in this experiment was selected to mimic the suspected natural infection route and was previously used with good results [5]. In a study from Japan [2] it was shown that males and kits developed hemorrhagic pneumonia more easily than females and adults. The decision to use females for this study might have influenced the morbidity causing fewer diseased animals than expected. In this experiment no effect of age was found.

The difference in mortality between July and November was not significant when using Fishers exact test with a confidence level of 0.05 (two-tailed p-value=0.11), but showed a tendency toward higher mortality in November. This indicates that predisposing factors in the mink may influence the development of disease, since the same amount of bacteria was used for infection. Predisposing factors could be related to change of fur in the autumn, or subclinical metabolic conditions associated with high growth rate (e.g. fatty liver) or a higher amount of stress and pressure of infection due to smaller free space in the cages as the mink grow.

Previous studies have shown a correlation between high infectious dose and development of disease [4,5,16-19]. This study cannot support this view since the mink developed hemorrhagic pneumonia irrespective of the infectious dose. Unfortunately, the results were not conclusive due to the low number of diseased animals.

A strong correlation was found between infectious dose and isolation of *P. aeruginosa* in the nasal cavity of the mink. The correlation coefficient was not significant, due to the low number of mink in the infectious dose groups. However this study shows that mink are capable of carrying *P. aeruginosa* on the nasal mucosa for at least eight days without developing hemorrhagic pneumonia.

The method for taking nasal swabs was changed from 2010 to 2011 and *P. aeruginosa* was found in more cases in 2011 as compared to 2010. This difference was proved significant using Fisher’s exact test (two-tailed p-value=0.001). The method of sampling was altered due to description of the paranasal sinuses as a reservoir for human lung infections in patients with cystic fibrosis [20] and the epithelial fold found deep in the nasal cavity was regarded as approximately equivalent to the paranasal sinus in mink. The results from this study show that these folds may also serve as a niche for *P. aeruginosa* in the mink. No significant difference between the numbers of animals carrying *P. aeruginosa* on their nasal mucosa was found when

comparing the trials in July and November showing that this ability apparently is not dependent upon season.

Pseudomonas aeruginosa was not cultured from the nasal cavity of 80 clinically healthy mink, collected at mink farms in the autumn. This is not in agreement with a previous study [11] which found *P. aeruginosa* in the pharynx or rectum of 39% of the examined mink. The difference might be due to a true disparity between the countries (U.S.A. versus Denmark), differences in sampling procedure or that the previously studied mink may have been infected with Aleutian Disease virus making them more prone to general infections. Half of the mink included in this study had been treated with antibiotics three weeks before collection of the heads due to outbreaks of hemorrhagic pneumonia. This treatment could possibly limit the ability to culture *P. aeruginosa* and could have eradicated the bacterium from healthy carriers of this organism.

Conclusion

The results of this experiment show that *P. aeruginosa* is not part of a normal flora of the nasal mucosa in mink in November and that mink are able to carry viable *P. aeruginosa* in their nasal cavity for at least eight days without developing hemorrhagic pneumonia. There is a tendency toward higher morbidity when infected with *P. aeruginosa* in November as compared to July, even when using the same bacterial doses. These results indicate that it is not the dose of bacteria that are responsible for whether the mink develops hemorrhagic pneumonia or not, but perhaps predisposing factors in the mink itself.

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Article IV

Investigation of the presence of human or bovine respiratory syncytial virus in the lungs of mink (*Neovison vison*) with hemorrhagic pneumonia due to *Pseudomonas aeruginosa*

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Abstract

Background: Hemorrhagic pneumonia is a disease of farmed mink (*Neovison vison*) caused by *Pseudomonas aeruginosa*. The disease is highly seasonal in Danish mink with outbreaks occurring almost exclusively in the autumn. Human respiratory syncytial virus (RSV) has been shown to augment infection with *P. aeruginosa* in mice and to promote adhesion of *P. aeruginosa* to human respiratory cells.

Findings: We tested 50 lung specimens from mink with hemorrhagic pneumonia for bovine RSV by reverse transcriptase polymerase chain reaction (PCR) and for human RSV by a commercial real-time PCR. RSV was not found.

Conclusions: This study indicates that human and bovine RSV is not a major co-factor for development of hemorrhagic pneumonia in Danish mink.

Keywords: Hemorrhagic pneumonia, mink, *Pseudomonas aeruginosa*, respiratory syncytial virus

Findings

Pseudomonas aeruginosa was identified as the cause of hemorrhagic pneumonia in farmed mink (*Neovison vison*) in 1953 [1]. This disease can cause mortalities as high as 50% on the farm level [2, 3] but can also follow a milder course [1]. Hemorrhagic pneumonia is characterized by a rapid disease progression in the individual mink and it is highly seasonal in Denmark with farm outbreaks occurring almost exclusively from September to November. Whether the seasonal pattern is related to the seasonal life cycle of farmed mink or if it relates to co-infection with other pathogens has not been investigated. Thus, no infectious co-factors have been identified but the highly seasonal appearance of disease may indicate the presence of a viral co-factor. In human cystic fibrosis (CF) patients, a seasonal pattern for acute and chronic *P. aeruginosa* infection has been identified [4] and the authors proposed respiratory viruses as the underlying cause for this pattern. Respiratory viruses have a seasonal infection pattern coinciding with that of *P. aeruginosa* infection in CF patients and respiratory viruses are known to precede bacterial infections [5, 6].

Respiratory syncytial virus (RSV) is an enveloped single-stranded negative sense RNA virus belonging to the genus *Pneumovirus* in the family Paramyxoviridae. Variants of the virus infect various species including sheep, goats, mice, cattle and humans. A number of animal species can be experimentally infected with human RSV (HRSV) [7, 8]. The occurrence of HRSV infections in humans is known to be highly seasonal with the majority of cases occurring in the winter or spring [9]. HRSV has been shown to promote adhesion of *P. aeruginosa* to cells in the respiratory tract of mice [10, 11] and to directly mediate binding of *P. aeruginosa* to human epithelial cells *in vitro* [12]. HRSV has also been shown to replicate in mink lung cells *in vitro* [13] and in the lungs of infant ferrets [14]. RSV has never been described as naturally occurring in mink and experimental studies on RSV infections in mink has not been published. However mink may be exposed to HRSV by their human caretakers and to bovine RSV (BRSV) from the feed which often include slaughter offal from cattle.

The aim of the present study was to investigate whether HRSV or BRSV could be found in lung tissue from mink succumbing to hemorrhagic pneumonia caused by *P. aeruginosa* and hence possibly be part of the pathogenesis of this disease.

Fifty samples of lung tissue from Danish mink were selected of which 40 originated from mink submitted from 10 fur farms for routine diagnostic examination and found to have hemorrhagic pneumonia associated with culturing of *P. aeruginosa* in pure culture from the lungs. Twenty of these mink originated from three farms with high mortality due to hemorrhagic pneumonia (above 15%) while 15 mink were submitted from five farms with low mortality (below 2%). Data on mortality was not available for the last five mink submitted for routine diagnostic investigation. Ten samples originated from normal wildmink experimentally infected intra-nasally with 10^3 - 10^9 viable *P. aeruginosa* bacteria. Two of the ten experimentally infected mink developed clinical symptoms of hemorrhagic pneumonia (lethargy, labored breathing) and were euthanized two days after inoculation and necropsied immediately. The remaining eight clinically normal mink were euthanized eight days after inoculation and necropsied immediately. These eight mink served as negative controls. The animal experiments were approved by the Danish Animal Experiments Inspectorate. From the control mink, specimens of the left caudal lobe was obtained, while lung tissue with gross lesions of pneumonia was chosen from the experimentally infected clinically diseased mink and the mink submitted for routine diagnostic. The samples were collected during 2011 and stored at -20 °C until further testing.

Total RNA was purified from the lung tissue using the RNeasy Mini Kit (Qiagen, Düsseldorf, Germany) on a QIAcube (Qiagen) according to manufacturer's instructions. A BRSV positive cell culture was used for positive control while nuclease-free water (Amresco, Solon, OH, USA) was used as a negative control of the purification step. The recovered RNA was stored at -80 °C until further analysis.

The presence of BRSV was tested with a modification of a previously published conventional reverse transcriptase polymerase chain reaction (RT-PCR) assay targeting the F-gene with the forward primer sequence 5'-AACCGCTTCCTCAGTAGAGC-3' and the reverse primer sequence 5'-CAATACCACCCACGATCTGTCC-3' [15]. The RT-PCR was performed on a Biometra T3 Thermocycler (Biometra, Goettingen, Germany) in a total volume of 25 µl using Qiagen OneStep RT-PCR kit (Qiagen) and 3 µl of extracted RNA, 400µM dNTP mix and 500 nM of each primer. The amplification temperature profile was 30 min at 50 °C for reverse transcription, 15 min at 95 °C and 35 cycles of 30 s at 95 °C, 30 s at 55 °C and 1min at 72 °C, followed by 10 min at 72 °C. In each PCR reaction a known BRSV positive sample and a non-template control was run with the samples as controls. PCR products with the expected size of 730 bp were visualized on E-gel® 2% General Purpose Agarose (Invitrogen, Carlsbad, CA, USA).

For detection of HRSV a commercially available real-time PCR (Prodesse® ProFlu™+, Gen-probe, San Diego, CA, USA) was employed. The primers used for this detection were not available due to the commercial nature of the PCR. The amplification and detection were performed on a MX3005p (Stratagene, La Jolla, CA, USA). The manufacturer's protocol was followed in detail.

The 50 mink lung samples were negative when tested in the BRSV and HRSV specific assays. In 5 samples tested with the BRSV specific primers, weak PCR products of the predicted size were recognized but sequence analysis showed no resemblance to the F gene of BRSV or to other known genes for these products. Therefore, they probably represent unspecific annealing to mink specific nucleic acids. No PCR products were obtained using the HRSV specific assay.

Evidence of concurrent infection with HRSV and BRSV and *P. aeruginosa* in cases of mink hemorrhagic pneumonia was not found. Whether this was due to breakdown of the virus, e.g. due to severe inflammation, collection of samples after clearance of the virus, or because HRSV or BRSV simply was not present in the lung samples remains uncertain.

Histological examinations of lungs from mink suffering from hemorrhagic pneumonia have not revealed syncytial cells [16, 17] but due to the severe lung lesions seen in this type of infection, syncytial cells may be difficult to recognize or may be necrotic at the time of histological examination. RSV can experimentally infect a number of species [8] but is apparently quite host-specific in eliciting disease even though the various RSVs are closely related [7]. If a specific mink RSV is existing and is associated with hemorrhagic pneumonia in mink, the primers used for detection of BRSV and HRSV in this study may not be able to detect this virus. To further elucidate whether a novel RSV-like virus is present in mink, other primer-sets may be generated based on highly conserved parts of genomes belonging to the genus *Pneumovirus*. Electron microscopy, immunohistochemistry of lungs and nasal epithelium or *de novo* sequencing of tissue from animals showing acute respiratory symptoms may also be a valuable tool for identifying unrecognized respiratory viruses that may act as co-factors to *P. aeruginosa* hemorrhagic pneumonia.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CMS participated in the design of the study, identified and collected suitable material, coordinated the study and drafted the manuscript. SØB participated in the design of the study, supervised the BRSV testing, interpreted the sequencing of the weak PCR products and revised the manuscript. LEL contributed to the study design and revised the manuscript. JJ planned the HRSV testing and performed the practical experiment. NH contributed to the study design. ASH participated in the design of the study and the initial identification of tissue used in the study. All authors read and approved the final manuscript.

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