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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 13 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.

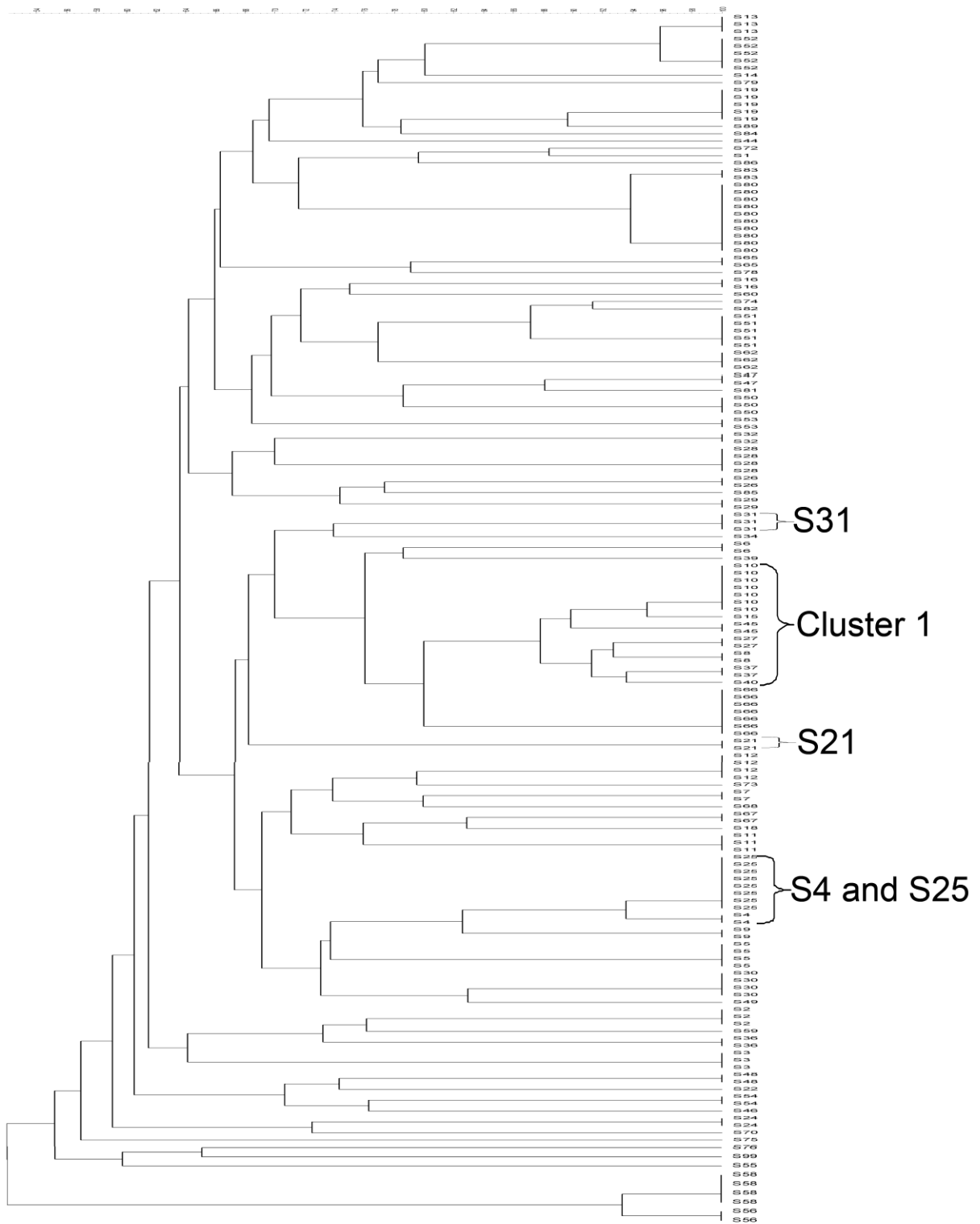


Figure 1: Dendrogram of *P. aeruginosa* isolated from mink hemorrhagic pneumonia. S21, S4, S25 and S31 groups in “Cluster B” based on results from the AT biochip.

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.

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 or three farm outbreaks occurred on farms sharing the same central food kitchen while four strains causing two 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).

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).

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

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	1	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	1	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	1	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.

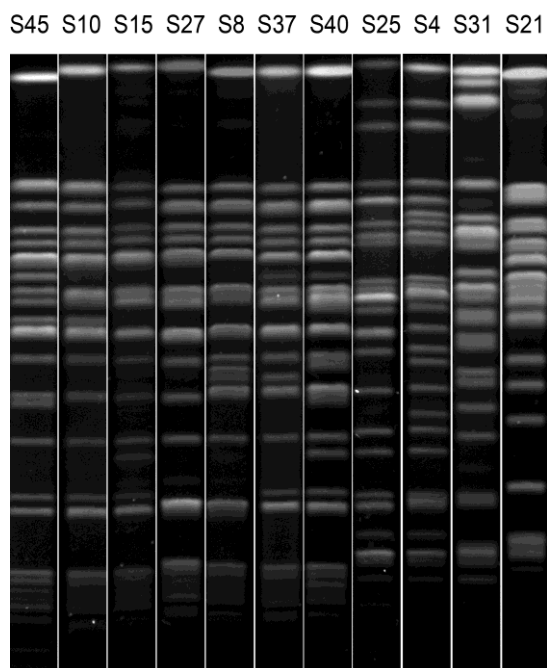


Figure 3: PFGE macrorestriction patterns for Cluster A, B and S37.

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 deduct 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 points 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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