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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 to 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.

1. 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"

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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.

2. Materials and methods

2.1. 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, USA).

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 h 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.

2.2. 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 (1 M Tris pH = 8.0, 0.5 M EDTA pH = 8.0, 10% N-lauroyl sarcosine) for 2 h 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 h 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 1–5 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, USA) 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).

3. 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 (Fig. 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% to 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