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Histopathological lesions of *Actinobacillus pleuropneumoniae* serotype 8 in infected pigs

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Abstract

This study aimed to report, for the first time, histopathological lesions caused by an outbreak of acute *Actinobacillus pleuropneumoniae* serotype 8 infections in two farms in Cyprus. Lung tissue samples were collected from two different affected farms (a total of eight samples) for bacterial culture, multiplex polymerase chain reaction (PCR)-based serotyping and histopathological evaluation. Severe respiratory clinical signs, vomiting, anorexia, sudden deaths, a morbidity rate of around 25.00% and a mortality rate of over 60.00% in the fattening stage were reported. Macroscopic lesions included acute to subacute fibrotic, hemorrhagic and necrotizing pneumonia with occasionally encapsulated nodule-like abscesses and fibrous pleuritis. Histopathological evaluation revealed fibrous exudate between alveolar spaces and connective tissue, areas of necrosis mixed with alveolar macrophages, lymphocytes, plasma cells and necrotic leukocytes surrounding colonies of cocci. The bronchial and bronchiolar epithelia were degenerated and replaced by eosinophilic cell debris mixed with inflammatory cells. Several arteries and capillaries were clotted and/or infiltrated by inflammatory cells. In conclusion, these *A. pleuropneumoniae* serotype 8 cases were accompanied by acute illness, death and more pronounced bronchitis and bronchiolitis.

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Introduction

Actinobacillus pleuropneumoniae is a small, Gram-negative anaerobe that was first reported in 1957 in the United Kingdom.[1] Initially, the organism was originally named as *Haemophilus pleuropneucauses* and later classified into the *Pasteurellaceae* family and *A. pleuropneumoniae* genus.[2] It causes severe respiratory disease in pigs (swine pleuropneumonia), having a significant economic impact on affected swine herds worldwide.[3,4] The importance of *A. pleuropneumoniae* infection derives from increased mortality due to pneumonia and chronic or subclinical disease. The chronic or subclinical *A. pleuropneumoniae* disease causes significant economic losses due to decreased farming productivity (daily gain and feed conversion) as well as increased mortality and veterinary costs for antibiotics or vaccines.[3]

Two biovars and 18 serovars of *A. pleuropneumoniae* were reported, performing differences in their capsular polysaccharide composition.[2,5] Generally, serovars 1-12 and 15-18 belong to biovar 1; while serovars 13 and 14 belong to biovar 2. Even if serotyping is routinely performed by serological methods, several cross-reactions are usually reported.[6] Some of these cross-reactions are due to common epitopes being present at the level of long-chain polysaccharides within the lipopolysaccharide (LPS), such as serotypes 1/9/11, 4/7 and 3/6/8/15.[3] Moreover, cross-reactivities between several serotypes have also been reported, including serotypes 1-9, 3-6-8, 1-5 and 4-7.[6] These cross-reactions are likely due to shared species-specific antigens, including LPS or membrane proteins.[7] Early detection and identification of the causative serotype in the field cases are important for the control of swine pleuropneumonia and the design of the appropriate treatment protocols.[7]

This study aimed to report the pathogenesis of *A. pleuropneumoniae* serotype 8, based on the histopathological examination of lung tissue samples from field outbreaks in two farms in Cyprus.

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Materials and Methods

Farms, clinical signs and lesions of infected animals. The swine sector in Cyprus includes 35 farms, with around 25,000 sows. Farms are on the coast and mainland of the island. Since 2017, six swine farms in Cyprus have reported a seasonal increase in mortality in fatteners. The average mortality in these farms in the fattening unit (from 25.00 - 110 kg) was 5.30% from January to September 2016, and the average mortality increased to 7.80% from October to March 2017. Swine owners requested to investigate the possible reasons for this increased mortality in the fattening pigs. The present study focuses on two farms of the same owner, which suffered from common clinical signs of acute *A. pleuropneumoniae* infection, including high temperature/ fever, severe coughing, respiratory distress with dyspnea, bloody foam from the nose and/or at the mouth, vomiting, anorexia and sudden deaths in fattening pigs. The morbidity rate of these farms at the fattening stage was around 25.00%, and the mortality rate of diseased pigs was more than 60.00%. Lung scoring at the slaughterhouse revealed severe lung lesions in infected pigs, located mainly in the dorsal portions of the caudal lung lobes. The macroscopic lesions included acute to subacute fibrinosuppurative, hemorrhagic and necrotizing pneumonia, with occasional encapsulated abscess-like nodules and fibrinous pleuritis.

Sampling. Lung tissue samples were collected from dead pigs, showing common clinical signs of acute *A. pleuropneumoniae* infection. During the study, four lung tissue samples were collected from two different affected farms (a total of eight samples) for laboratory examinations including bacterial culture and polymerase chain reaction (PCR). Afterwards, the lung tissue samples were sent to Ridgeway Biologicals Ltd. Laboratory (Newbury, UK) to be microbiologically and molecularly assayed as described below. In addition, four lung tissue samples (a total of eight samples) were collected from the same animals for histopathological examination.

Bacterial culture. The lung tissue samples were chopped and suspended in phosphate-buffered saline solution before being homogenized using a stomacher. The homogenized samples (1.00 µL) were inoculated with a loop on blood agar plates (Thermo Fisher, Hennigsdorf, Germany) with a *Staphylococcus aureus* feed streak. Following primary isolation, chocolate agar with added 50.00 µg mL $^{-1}$ nicotinamide adenine dinucleotide (NAD; Columbia agar base, Thermo Fisher), with sheep blood added at 80.00 ˚C and NAD added at 50.00 ˚C was used for culture. The plates were incubated at 37.00 ˚C for 24 hr in an aerobic environment with the addition of 5.00% CO_2.

DNA preparation and PCR assays. A loopful of overnight bacterial colonies was suspended in 200 µL of sterile water. The suspension was vortexed, placed on ice for 5 min and heated for 5 min at 98.00 ˚C. This step was repeated once. Following cell lysis, the suspension was centrifuged at 13,000 *g* for 2 min. The bacterial DNA supernatant was removed and stored at – 20.00˚C pending PCR analysis. The PCR typing system based on *Apx* gene patterns that have been developed by Gram *et al*., for *A. pleuropneumoniae* isolates was used for serotype group identification.[8] The primers used have been developed by Gram *et al*., for the detection of the structural A gene of the Apx toxins and by Frey *et al*., for the detection of *BDI* and *BDIII* genes.[8,9] The PCR reaction was prepared in a final PCR mix volume of 50.00 µL and the PCR thermal profile was performed as described by Gram *et al*.[8] A multiplex PCR amplifying fragments of the *A. pleuropneumoniae*-specific *ApxIV* gene and serovars 1 to 3, 5 to 8, 10 and 12 specific sequences derived from the capsule loci, was used to confirm and further distinguish the serovars implicated.[10] The primers used for the multiplex PCR, the reaction mixture preparation in a final volume of 50.00 µL, and the PCR cycling conditions were performed as Bossé *et al*., described formerly.[10] In-house serotype-positive controls were used in each PCR run. The PCR-grade water was used as a negative control. Amplification products were subjected to electrophoresis in 1.50% agarose gel stained with ethidium bromide (0.50 µg mL^{-1}) in 1X Tris-Borate-EDTA buffer and visualized under ultraviolet light. The PCR reaction was performed using an automated DNA thermal cycler (G-Storm GS4822 thermal cycler, Somerton Biotechnology Centre, Somerton, UK). Serotype confirmation was performed at the Innovative Veterinary Diagnostics Laboratory (Seelze, Germany) and *A. pleuropneumoniae* Reference Laboratory (Animal and Plant Health Agency, Weybridge, UK).

Histopathological examinations. The samples were fixed in neutral buffers of 10.00% formalin, processed routinely and stained with Hematoxylin and Eosin for histopathological evaluation.

Results

PCR results. The PCR typing systems used in this study showed that the isolated field strains had the same gene pattern. Based on Apx toxins, the isolates corresponded to serotypes 2, 6 and 8.[8] Based on the serovar-specific multiplex PCR derived from the capsule loci, the field strains belonged to serovar 8, being also confirmed by the *A. pleuropneumoniae* Reference Laboratory results.[10]

Histopathological results. The histopathological evaluation of the examined lung samples revealed diffuse lesions in both lobes. Fibrinoid exudate between alveolar spaces and connective tissue, areas of necrosis admixed with alveolar macrophages, lymphocytes, plasma cells and necrotic leukocytes surrounding small colonies of cocco-

bacilli were detected. Bronchial and bronchiolar epithelia were degenerated and replaced by eosinophilic cellular debris admixed with inflammatory cells (lymphocytes, plasma cells and neutrophils). Additionally, the lumen of the above lung structures contained exudate composed of cellular and inflammatory debris. Several arteries and capillaries were thrombosed and/or infiltrated by neutrophils, lymphocytes and plasma cells (Fig. 1).

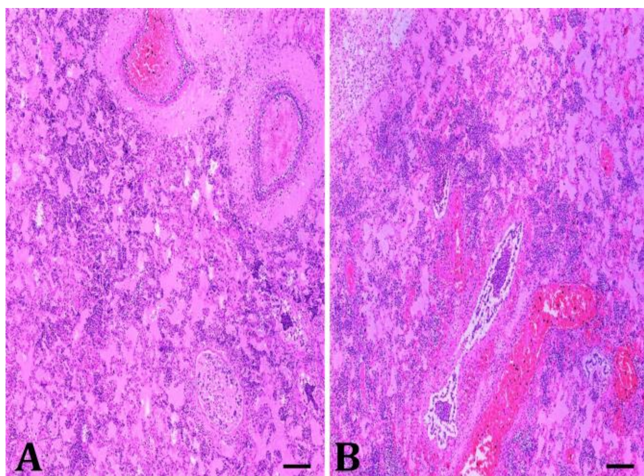

Fig. 1. Fibrinosuppurative bronchopneumonia and necrotizing hemorrhagic pneumonia. **A)** Fibrinoid necrosis and intra-luminal thrombosis of vessels (vasculitis); **B)** Bronchi infiltrated by inflammatory cells and filled with fibrinosuppurative exudate (Hematoxylin and Eosin staining, bar = 250 μm).

Discussion

The prevalence and virulence distribution of isolated serotypes from field cases varies significantly worldwide.[3] For example, serotypes 2, 9 and 11 are more prevalent in Europe; while, serotypes 1 and 5 are most prevalent in North America.[3,11] However, serotypes 9 and 11 are absent in North America.[3] Our study reported for the first-time serotype 8 in pig farms in Cyprus. Previous studies have reported that serotype 8 is identified only sporadically in European countries (Czech Republic, Spain, Norway and Denmark).[11-13] Moreover, serological studies of the last decade have reported frequent detection of serotype 8 in Germany and the most common and predominant presence of serotype 8 in the United Kingdom.[14,15] Except in Europe, serotype 8 was also reported to be prevalent in Argentina.[16]

Generally, antibiotics are efficacious for the recovery of acute *A. pleuropneumoniae*-affected pigs and are used in control strategies of an acute outbreak.[17] In addition, vaccines against *A. pleuropneumoniae* are used in preventive programs under field conditions. Several commercial vaccines are available, based mainly on serotypes 1, 2, 9 and 11, differing in their composition being appointed into one of three categories including killed *A. pleuropneumoniae* whole-cell components only (bacterins), subunit vaccines containing ApxI-III toxins only and combination of these. Antibodies against APXI-III are responsible for serovar-independent protection against lung lesions. Due to the limited cross-protection between the serovars, bacterin vaccines have a lack of efficacy compared to ApxI-III combined bacterin vaccines.[18] Furthermore, vaccines of the combined category are quite heterogeneous, with a wide variety in composition, containing the ApxI-III and only one of the cell wall outer membrane proteins or whole-cell components of two serotypes or whole-cell components of several serotypes together with some exotoxins.[18] Consequently, the findings of this study can be helpful in the future development of more effective vaccines against *A. pleuropneumoniae*, including the homologous antigens to the most prevalent serotypes *per* each specific geographical area. For this reason, serotyping of *A. pleuropneumoniae* from field cases is important for epidemiology and vaccine-development purposes, as vaccination is an effective preventive tool in cases of high risk for introducing the disease in a farm or if the *A. pleuropneumoniae* outbreak is severe.

The histopathological results of this study are in agreement with previous studies reporting fibrinous pleuritis and fibrinohemorrhagic necrotizing pneumonia with focal pulmonary vascular thrombosis as well as pulmonary lesions characterized by severe edema, inflammation, hemorrhage and necrosis.[19] In addition, our findings indicated tissue damage and activation of neutrophils and macrophages being attributed to produced toxins by *A. pleuropneumoniae*, as previous researchers reported.[20] Furthermore, it was noticed that bronchi and bronchioles were also affected in diseased pigs.

In conclusion, this study reported for the first time the detection of serotype 8 from field *A. pleuropneumoniae* cases in commercial pig farms in Cyprus, accompanied by acute respiratory illness and increased mortality.

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Conflict of interest

There is no conflict of interest.

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