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INFECTION DISEASE

Pyogranulomatous Pleuropneumonia and Mediastinitis in Ferrets (Mustela putorius furo) associated with Pseudomonas luteola Infection

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Summary
Between 2008 and 2009, three pet ferrets from different sources presented with acute episode of dyspnoea. Cytological examination of pleural exudates revealed severe purulent inflammation with abundant clusters of rod-shaped microorganisms with a clear surrounding halo. Treatment was ineffective and the ferrets died 2–5 days later. Two ferrets were subjected to necropsy examination, which revealed pyothorax, mediastinal lymphadenopathy and multiple white nodules (1–2 mm) in the lungs. Microscopical examination showed multifocal necrotizing-pyogranulomatous pleuropneumonia and lymphadenitis with aggregates of encapsulated microorganisms, some of which were positively stained by periodic acid–Schiff and alcian blue. In-situ hybridization for Pneumocystis spp., Ziehl–Neelsen staining and immunohistochemistry for distemper, coronavirus and influenza antigen were negative in all cases. Electron microscopically, the bacteria were 2–3 μm long with a thick electron-lucent capsule. Microbiology from one ferret yielded a pure culture of gram-negative bacteria identified phenotypically as Pseudomonas luteola. This speciation was later confirmed by 16S RNA gene amplification.

Keywords: ferret; lymphadenitis; pleuropneumonia; Pseudomonas luteola

Introduction
Pseudomonas (Chryseomonas) luteola is a motile aerobic gram-negative rod with yellow–orange pigmentation of colonies. Its normal habitat is unknown, but it is frequently found in water, soil and other damp environments (Chihab et al., 2004). It contains a polysaccharide capsule and multitrichous polar flagella. The capsule has been associated with adsorption of cadmium and cobalt ions (Hawkins et al., 1991; Kostman et al., 1991; Ozdemir et al., 2005).

P. luteola may cause septicaemia, peritonitis and endocarditis in human patients with underlying disease. The organism may also behave as a nosocomial agent and infect critically ill patients who have undergone surgical procedures and/or had indwelling devices inserted. The infection has also been associated with factors such as immunosuppressive therapy, chronic renal failure and malignancy (Chihab et al., 2004). Recently, this organism has been described as causing an unusual clinical infection mimicking mediastinal malignant lymphoma in a human patient (Goteri et al., 2010). The only report of P. luteola infection in the veterinary literature relates to infection of rainbow trout, which developed septicaemia with haemorrhage in the fins and vent and necrotic foci in the liver and kidney (Altinok et al., 2007).

In recent years there has been an increase in newly-described or emerging infectious diseases in ferrets, particularly systemic coronavirus infection (Garner...
et al., 2008; Martínez et al., 2008) and influenza (Munster et al., 2009). The present report describes a severe respiratory infection characterized by pyogranulomatous pleuropneumonia, lymphadenitis and mediastinitis associated with *P. luteola* in three ferrets (*Mustela putorius furo*). To our knowledge this is the first report of *P. luteola* infection in warm-blooded animals.

**Materials and Methods**

**Animals**

Between 2008 and 2009, three unrelated domestic ferrets (animal numbers 1, 2 and 3) kept as pets by different owners in the metropolitan area of Barcelona, Spain, presented with respiratory disease. All three were males, one was neutered (number 1) and none had contact with other ferrets or animals. Ferrets 1 and 2 were 3 years old and ferret 3 was 2 years old. The animals were not vaccinated against canine distemper virus or rabies. Ferrets 1 and 2 presented with depression, dehydration, anorexia, hyperthermia and acute onset dyspnoea. There was no history of prior medical problems. Complete blood counts and serum biochemical examinations revealed anaemia, severe neutrophilic leucocytosis with toxic neutrophils, hyperglycaemia and hypoalbuminaemia. Ferret 2 also had hyperglobulinaemia. Radiographic findings included a unilateral pleural effusion (right- and left-sided in ferrets 1 and 2, respectively) and a mediastinal mass displacing the trachea dorsally. Ultrasonographically, in ferret 2 there was a large quantity of hypoechoic effusion within the thorax and a heterogeneous ill-defined soft tissue mass was observed in the left mediastinum. Approximately 20 ml of purulent fluid were removed by thoracocentesis. Both ferrets were treated similarly with oxytetracycline, buprenorphine, ranitidine and sucralphate. (enrofloxacin, clindamycin and metronidazole), itraconazole, buprenorphine, ranitidine and sucralfate. The anamnesis revealed anaemia, severe neutrophilic leucocytosis with toxic neutrophils, hyperglycaemia and hypoalbuminaemia. Ferret 2 also had hyperglobulinaemia. Radiographic findings included a unilateral pleural effusion (right- and left-sided in ferrets 1 and 2, respectively) and a mediastinal mass displacing the trachea dorsally. Ultrasonographically, in ferret 2 there was a large quantity of hypoechoic effusion within the thorax and a heterogeneous ill-defined soft tissue mass was observed in the left mediastinum. Approximately 20 ml of purulent fluid were removed by thoracocentesis. Both ferrets were treated similarly with oxygen supplementation, crystalloid fluids, antibiotics (enrofloxacin, clindamycin and metronidazole), itraconazole, buprenorphine, ranitidine and sucralfate. Both animals died within 2–5 days of presentation.

Ferret 3 had a history of recurrent and vaguely-defined problems consisting mainly of cough. The animal presented with acute, severe dyspnoea and was humanely destroyed without diagnosis or treatment at the request of the owner.

**Cytological Examination**

Smears of pleural exudates were made from each of the ferrets and these were stained with Diff-Quik.

**Gross Examination**

Two ferrets were subject to necropsy examination. Ferret 1 was submitted to the Pathology Department at the Veterinary School of Barcelona; ferret 3 was examined by the referring clinician and tissue samples were submitted to a private diagnostic pathology laboratory. Tissue samples from mediastinal lymph nodes, lung, heart, liver, kidney, spleen, pancreas, intestine and brain were collected from ferret 1. Samples of lung, mediastinal lymph node, mediastinal mass, trachea and oesophagus were taken from ferret 3. Samples were fixed in 10% neutral buffered formalin and processed routinely.

**Histopathology, Immunohistochemistry and In-situ Hybridization**

Formalin-fixed tissues were embedded in paraffin wax, sectioned (4 μm) and stained with haematoxylin and eosin (HE). Gram, Ziehl–Neelsen (ZN), alcian blue and periodic acid–Schiff (PAS) stains were also performed on serial sections from ferrets 1 and 3. Gram, ZN and PAS staining was performed on the pleural exudate of ferret 2.

Immunohistochemistry (IHC) was used to detect ferret systemic coronavirus (FRSCV) antigen (Custom Monoclonals International, Sacramento, CA, USA; dilution 1 in 400), canine distemper virus (CDV) nucleoprotein (Ingenasa, Madrid, Spain; dilution 1 in 1,000) and influenza A (IA) nucleoprotein (CRESA, Barcelona, Spain; dilution 1 in 100) in lung and mediastinal tissues from ferrets 1 and 3, following the protocols previously described (Domingo et al., 1992; Gooskens et al., 2007; Martínez et al., 2008). Negative control procedures included omission of primary antiserum. Tissue sections known to be negative and positive for expression of FRSCV, CDV and IA were included as additional controls.

For in-situ hybridization (ISH), a 22 base pair (bp) digoxigenin-labelled DNA probe (5’ Dig-TCTCTG AGGTATGGCCGTAACT 3’) was used to detect DNA from *Pneumocystis* spp. in pulmonary and mediastinal tissues from ferrets 1 and 3, as previously described (Rosell et al., 1999). Controls included tissues known to be negative and positive for *Pneumocystis* spp.

**Transmission Electron Microscopy**

Transmission electron microscopy (TEM) was used to examine the pleural exudate obtained from ferret 2 and the formalin-fixed lung and lymph node from ferrets 1 and 3. After evaluating the stained slides, areas of the embedded formalin-fixed tissue where bacterial colonies were most abundant were selected for TEM. Pleural exudate was fixed with 2.5% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde (EM grade, TAAB Laboratories, Berkshire, UK) in 100 mM phosphate buffered saline (PBS, pH 7.4) for 2 h and rinsed four times with 100 mM PBS.

**Pseudomonas luteola in ferrets**
Pellets from pleural exudate, lung and lymph node were then post-fixed in 1% (w/v) osmium tetroxide (TAAB) containing 0.8% (w/v) potassium hexacyanoferrate (III) (Sigma, Madrid, Spain) 2 h and washed with 100 mM PBS. These steps were performed at 4°C. Samples were dehydrated through a graded acetone series, embedded in Epon resin and polymerized for 48 h at 60°C. Ultrathin sections were mounted in copper grids (200 mesh), contrasted with uranyl acetate and lead citrate solutions and evaluated with a transmission electron microscope (Jeol JEM-1400 equipped with a Gatan Ultrascan ES1000 CCD camera).

**Bacterial and Fungal Culture and Molecular Characterization**

The pleural exudate obtained by thoracocentesis from ferret 2 was cultured on 5% sheep blood agar and MacConkey agar. Plates were incubated at 37°C in 5% CO₂ and examined after 24 and 48 h. The isolates were identified using the API-20NE identification system (bioMérieux, Madrid, Spain). For mycological isolation, pleural exudate was cultured on Sabouraud’s glucose agar with chloramphenicol and incubated at 25°C.

The 16S rRNA gene of the bacterial isolate obtained from ferret 2 was amplified using universal primers for bacteria (Calsamiglia et al., 1999) and sequenced using the Big Dye Terminator 3.1 kit (Applied Biosystems, Carlsbad, CA, USA). A fragment of 1399 bp of this gene was analyzed. For sequence identification, Classifier and SeqMatch analysis tools in RDP (http://rdp.cme.msu.edu/) and BLAST at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were used. For phylogenetic reconstruction, the sequence was aligned with 197 type strains of the Pseudomonadales available at RDP. A maximum likelihood (ML) tree was constructed using the Tamura–Nei substitution model and the Nearest Neighbor Interchange (NNI) ML heuristic method and evaluated with 1,000 bootstraps. The tree was rooted using the 16S rRNA gene of *Escherichia coli* K12.

**Results**

**Cytological Examination**

The pleural exudate consisted of neutrophilic inflammation with abundant clusters of rod-shaped to serpentine microorganisms measuring up to 2–3 μm in length, with a clear halo (Fig. 1).

**Gross Examination, Histopathology, Immunohistochemistry and In-situ Hybridization**

At necropsy examination, ferrets 1 and 3 had approximately 5–10 ml of dense and serosanguinous pleural exudate. Ferret 1 showed multiple white and slightly raised nodules (1–2 mm) in the visceral pleura and pulmonary parenchyma. In ferret 1, the mediastinal mass observed radiographically correlated with a severe mediastinal lymphadenopathy (Fig. 2), with abundant purulent exudate on the cut surface. Ferret 3 had a large white mass in the mediastinum which encircled the trachea, oesophagus and regional lymph nodes.

Microscopical examination of the mediastinal lymph node from ferret 1 revealed a severe, multifocal to diffuse, necrotizing and neutrophilic to pyogranulomatous inflammation with variable infiltration of macrophages and lymphoplasmacytic cells. This process extended to the mediastinum. There were abundant colonies of rod-shaped (2–3 μm long) esinophilic and gram-negative microorganisms with a clear and wide halo (capsule). Many of these organisms had capsules that stained positively with...
PAS and alcian blue (Fig. 3). In the lung, the small white nodules observed grossly consisted of multifocal peribronchiolar aggregates of foamy macrophages. Bacterial colonies were not observed associated with these lesions.

Similar lesions were observed in the mediastinal lymph nodes of ferret 3 and these extended to the pleura and oesophagus. This severe and diffuse pyogranulomatous inflammation was also observed in the lungs and was accompanied by thrombosis, lymphoid hyperplasia and mesothelial hypertrophy and hyperplasia. Numerous bacterial colonies, as described above, were observed in the mediastinal lymph nodes, bronchiolar lumen and pleura. Moderate numbers of these organisms had capsules stained by alcian blue, but only a few were PAS positive.

No lesions were observed in the other organs examined and ZN staining, ISH for *Pneumocystis* spp. and IHC for FRSCV, CDV and IA were negative in both ferrets.

**Microbiological Findings and Molecular Characterization**

Pure cultures of yellow-pigmented colonies of gram-negative rods were obtained on both blood and MacConkey agars. The isolate was identified as *P. luteola*. Mycological culture was negative after 14 days of incubation.

Molecular analysis identified the isolate as belonging to the genus *Pseudomonas* with a score of 100% (Confidence interval 80%). Using SeqMatch, the best hit (score 0.972) was *P. luteola* IAM13000, the type strain of this species. Using BLASTn, the best hit was *P. luteola* Marseille, with a nucleotide identity of 99% (1395/1399 bp). This classification inside *P. (Chryseomonas) luteola* was confirmed by phylogenetic reconstruction (Fig. 5).
The present report describes a newly recognized respiratory bacterial infection of ferrets presenting with acute dyspnoea, pyothorax and severe pyogranulomatous pleuropneumonia, lymphadenitis and mediastinitis. Bacterial culture and molecular taxonomy confirmed *P. luteola* as the causative agent in one animal. Although microbiological cultures could not be performed in the other two cases, the intralesional microorganisms were indistinguishable from each other by cytological and histopathological examination as well as by TEM. For this reason, we hypothesize that *P. luteola* could be the cause of this pyogranulomatous pneumonia and mediastinitis in all of these ferrets.

*P. luteola* infection has been described in rainbow trout (Altinok *et al.*, 2007), but no cases have been found in animals, birds or reptiles. In mink, *P. aeruginosa* causes a fatal and acute haemorrhagic suppurative pneumonia, but no predisposing factors have been conclusively identified (Trautwein *et al.*, 1962; Long and Gorham, 1982). Previous studies in man showed that *P. luteola* may cause septicemia, peritonitis, meningitis, dermatitis and endocarditis in patients with different underlying diseases or with indwelling devices (Chihab *et al.*, 2004). The ferrets of this report probably acquired the infection by the respiratory route, as other organs were not affected. However, the origin of the infection and the incubation period remain unknown. Humans (owners and clinicians) that came in contact with these animals did not show clinical signs of illness at any time, including before and after the animals were clinically ill. However, a clinical infection in a human patient mimicking mediastinal lymphoma caused by *P. luteola* has been described recently, and in that case there were similar histopathological lesions and microorganism morphology (Goteri *et al.*, 2010) as described for the ferrets of this report. These results may suggest a predilection of this infection for the lower respiratory tract and the mediastinal lymph nodes of man and ferrets. Thus, this infection should be included in the differential diagnosis of pyogranulomatous pleuropneumonia and mediastinitis in the ferret and further research is needed to elucidate the zoonotic potential of ferrets in the transmission of the infection.

The unusual morphology of the microorganisms initially suggested that they may be fungal in nature (e.g. *Sporothrix* spp.). However, TEM confirmed that the agent was a bacterium. TEM revealed numerous viable and dividing bacteria with surfaces frequently contained multiple vesicular blebs of cell membranes, a change consistent with endotoxin release. Moreover, viable leucocytes were present, but no phagocytosis was observed, suggesting that the inflammatory response in these animals was unable effectively to fight the infection. Based on this observation, we offer two hypotheses. The first is based on the possibility that bacteria were able to elude the inflammatory response; the second hypothesis speculates an ineffective immune reaction due to immune suppression or concomitant infectious and non-infectious diseases.

In support of the first hypothesis, the literature shows evidence that the genus *Pseudomonas* has a polysaccharide capsule and flagella. The polysaccharide capsule has been associated with an anti-inflammatory effect in *P. aeruginosa* infection in human patients with cystic fibrosis. In contrast, flagella
are related to the stimulation of innate host defence (Cobb et al., 2004). In this case, the abundant bacterial colonies with a thick polysaccharide capsule, coupled with the absence of flagella and phagocytosis observed by TEM, could have triggered a similar evasion mechanism of the inflammatory reaction in ferrets. On the other hand, bacterial capsules were positively stained by PAS and alcian blue, with more abundant positive colonies in ferret 1 than 3. This could suggest the presence of different clones of the same bacteria or, possibly, chemical changes in bacterial capsule during the course of infection, for which the significance is unknown.

With regard to the second hypothesis, the diagnostic techniques used showed no predisposing, concomitant, infectious or non-infectious disease. No previous immunosuppressive therapy was reported, no histological evidence of predisposing viral or toxic damage was observed and IHC was negative for some of the most common ferret viral pathogens, including AI,
CDV and FRSCV. Other microorganisms that could produce similar pyogranulomatous lesions (e.g. *Mycobacterium, Nocardia, Actinomyces, Pneumocystis* or fungi) were discarded by microbiological culture, ISH and special stains. However, stress periods or other intrinsic immune defects that may induce immune suppression could not be ruled out.

*P. luteola* has been considered to be a secondary pathogen in human patients. However, a primary pathogenic role remains possible in the ferret. As these animals are becoming very popular domestic pets, gaining a greater understanding of the origin and dynamics of infection in ferrets may improve our knowledge of possible transmission to man, especially in those patients who are immune compromised or affected by cystic fibrosis.

**Conflict of Interest**

None of the authors has any financial and personal relationships with other people or organisations that could have inappropriately influenced this work.

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