Pyogranulomatous pneumonia in goats caused by an undescribed *Porphyromonas* species: ‘*Porphyromonas katsikii*’.

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Abstract

A yet undescribed bacterial species, tentatively named ‘Porphyromonas katsikii’ has been isolated from diseased individuals with pyogranulomatous pneumonia of a small goat herd during an outbreak of acute respiratory disease. The bacteria isolated grew in the form of black pigmented colonies after 14 days of incubation under anaerobic conditions at 37°C on T-soy - blood agar medium. They were identified as a yet undescribed Porphyromonas species by determination of the nucleotide sequence of the rrs 16S rRNA gene and tentatively named ‘Porphyromonas katsikii’. PCR amplification with specific primers for this yet undescribed species revealed the presence ‘P. katsikii’ in lung tissue of all affected animals while no PCR signals were evidenced from lungs of healthy goats or from goats with pasteurellosis caused by Mannheimia haemolytica. These data indicate ‘P. katsikii’ to be the causative agent of acute respiratory distress. ‘P. katsikii’ is phylogenetically related to Porphyromonas somerae and Porphyromonas levii that cause pathologies in humans and animals respectively. ‘P. katsikii’ could not be detected by PCR from samples of gingival pockets or of faces of healthy goats.
Porphyromonas species belonging to the phylum Bacteroidetes are non-motile, Gram-negative, rod-shaped, anaerobic bacteria that are considered as emerging pathogens in both humans and animals (1, 2). In general, Porphyromonas species, and in particular Porphyromonas gingivalis, the best studied representative of the genus, are known as oral pathogens causing gingivitis, periodontitis, endodontic diseases and apical abscesses in human and animals (3-5). However, several Porphyromonas species are involved in other human and animal pathologies such as metritis, peritonitis, interdigital necrobacillosis and necrotic vulvovaginitis (2, 6-8). Given that phenotypic identification and discrimination of Porphyromonas and Prevotella species has been shown to give inconsistent results (9), genotypic species identification based on 16S rRNA gene sequences was developed and had been used successfully thereafter (10).

Porphyromonas somerae and Porphyromonas levii, two closely related species are regarded as pathogenic species of human and cattle, respectively. Clinical manifestations of P. somerae (P. levii–like) include soft-tissue and bone infections, brain abscesses, and otitis media with mastoiditis (6, 7, 11). In ruminants, P. levii has been isolated from bovine necrobacillosis (8), papillomatous digital dermatitis (12), and acute interdigital phlegmon among cows (13), as well as from an outbreak of bovine necrotic vulvovaginitis (6). However P. levii was also reported as an opportunistic pathogen in the rumen of cattle (2, 14) and was also found in healthy cattle herds (15).
We herein report data on bacteriological isolation and genotypic identification, clinical signs, laboratory results and pathology that are indicative of an outbreak of pyogranulomatous pneumonia associated with acute respiratory disease in goats (Capra aegagrus hircus) caused by a yet unknown Porphyromonas species, which we tentatively name ‘Porphyromonas katsikii’.

Materials and methods

Description of the goat herd

The herd studied that was affected by pyogranulomatous pneumonia consisted of 25 domestic goats (Capra aegagrus hircus) between one and six years. The animals were in the high milking period and not pregnant. They were kept in a closed building with good natural ventilation (permanently open windows), fed with silage and were allowed grazing for 4-5 hours daily during months with moderate climate. Animal traffic was strongly controlled in the area. Twice a year all adult goats were vaccinated against clostridial diseases (Bravoxin10 MSD UK). As a preventive measure against pneumonic and intestinal endoparasites all adult animals were treated with Netobimin (Hapadex 5% MSD UK) once a year. A brief study of the clinical records showed that in 2011 eleven milking goats in this herd showed clinical symptoms of contagious agalactia and were successfully treated with tylosin (Tylan ELANCO).

Gross pathology and histology

A field necropsy was performed 1 h post-mortem from all 6 affected animals of the goat herd after an outbreak of acute respiratory distress. Specimens of the lung
were fixed in 10% neutral buffered formalin, embedded in paraffin wax, and cut in
5 μm thick sections for subsequent hematoxylin, eosin and May-Grünwald Giemsa
staining.

**Strains and culture conditions**

Microbiologic examination of lung samples including aerobic, and anaerobic cultures
for bacteria and mycoplasma were performed according to standard methods (16).
Clinical samples were cultured on T-soy - blood agar base medium (CM0271, Oxoid
LTD Basingstoke Hampshire England) containing 5% sheep erythrocytes at 37°C
under aerobic and anaerobic atmosphere and on Mycoplasma Hayflick medium (17)
under an atmosphere with 5% CO₂ for 14 days with intermittent inspection of the
media plates. To determine growth on antibiotics, T-soy - blood agar base medium
was supplemented with the following antibiotics: Kanamycin 200 µg/ml (Roche
Pharma, Reinach, Switzerland), Vancomycin 5 µg/ml (Sigma-Aldrich, St. Louis, MO,
USA), or Ampicillin 100 µg/ml (Sigma-Aldrich).

**DNA extraction, PCR Amplification and sequence analysis of rrs gene**

DNA from isolates was extracted using the guanidium thiocyanate extraction
method (18) with two subsequent phenol extractions and two ethanol precipitations.

The 16S rRNA genes were amplified from bacterial DNA using FIREPol
Master Mix (Solis BioDyne, Tartu, Estonia) and the universal 16S rRNA gene
primers 16SUNI-L (AGAGTTTGATCATGGCTCAG), 16SUNI-R
(GGTGACGCGGCGGTGATC) with an annealing temperature of 54°C as
described previously (10). These primers amplify a fragment of the 16S rRNA gene
corresponding to nucleotide (nt) positions 8 to 1410 of the reference 16S rRNA gene
of *Escherichia coli* K12 strain MG1655 (GenBank accession nr J01859). To check the quality of the PCR products, they were run on a 1.5 % agarose gel. Ten microliters of each amplicon were purified in order to remove residual deoxynucleotides and primers by adding 2.0 µl of rAPid Alkaline Phosphatase 1 U/µl (Roche Diagnostics, Rotkreuz, Switzerland), 0.4 µl of the corresponding buffer and 0.1 µl of exonuclease I (Exo I; New England Biolabs, Ipswich, MA, USA) and incubation at 37°C for 20 min and subsequently at 80°C for 20 min to inactivate the enzymes. Primers used for PCRs were also applied for sequencing. Internal primers for 16S rRNA were universal primers as described previously (10). Five pmol of the appropriate primer was added to about 20 ng (1.0 µl) of purified PCR product and sequenced with the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) in a thermocycler with 25 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 1 min. After ethanol precipitation of the sequencing products, the samples were run on an ABI Prism 3130 xl genetic analyzer (Applied Biosystems). The sequences were edited using the Sequencher software version 5.0 (Gene Code Corporation, Ann Arbor, MI, USA).

In order to directly detect the newly discovered *Porphyromonas katsikii* in clinical samples, we have designed specific primers based on the *rrs* gene sequence of this novel species and comparison with *rrs* gene sequences of related *Porphyromonas* species, resulting in the following: P_katsikii16S_L: ATAGAGTCGGGCACGTGTG and P_katsikii16S_R: CAGCACCTACATACAGACCC. PCR conditions were the same as described for the universal primers.

Total DNA from 125 mm³ pieces of lung tissues 1 mg feces or swabs taken from sub-gingival pockets of goats was extracted using a guanidium-thiocyanide – magnetic beads method as described earlier (19). The samples were suspended in 1 ml SV lysis buffer (4 M guanindine thiocyanate, 0.01 M Tris-HCl pH 7.5, 1% β-
mercaptoethanol), incubated room temperature for 60 min with gentle shaking, followed by centrifugation at 4’500 x g for 10 min to remove debris. Then 500 μl of the supernatant was mixed with 30 μl Magnesil® RED magnetic bead suspension (Promega, Madison, WI, USA) in a 1.5 ml disposable Eppendorf tube and mixed for 10 min at room temperature with gentle shaking. With the aid of a magnetic separator (scil® Magnetic separator 24, Promega), the supernatant was removed from the magnetic beads with the aid of a magnetic separator (scil® Magnetic separator 24, Promega). The liquid phase was removed again as described above. Magnetic beads were then washed twice with 500 μl of ethanol absolute and beads were air dried for approximately 40 min. Then the DNA retained on the beads was eluted with 50 μl of pyrogen free water by pipetting the beads pellet up and down. The DNA eluate (40 – 42 μl) was removed after 5 minutes incubation at room temperature using the magnetic rack to retain the magnetic beads, and stored at -20°C until further use.

Phylogenetic analysis

DNA sequences were analyzed for comparison with known sequences by BLAST (20). For the generation of a phylogenetic tree, the 16S rRNA sequence of the novel Porphyromonas species was analyzed by the MEGA 6 software (21) with the following parameters: gap opening penalty 15; gap extension penalty 6.6, DNA weight matrix IUB, transition weight 0.5 using the 16S rRNA gene sequences of the type strains of the most closely related Porphyromonas species: P. levii (GenBank accession number, L16493), P. somerae (NR_043312.1), P. cangengivalis (NR_113080.1), P. cansulci (NR_026137.1), P. gingivalis (NR_119038.1), P. gulae (NR_113088.1) P. endodontalis (NR_042803.1) and related species of other genera, Bacteroides merdae (X83954), Bacteroides fragilis (AB510701) and Prevotella oralis.
The 16S rRNA reference sequence from *Escherichia coli* K12 strain MG1655 (J01859) was used as out-group for the construction of the phylogenetic tree.

Results

An outbreak of acute respiratory disease affecting 6 out of 25 goats occurred in a small farm in the northwest of Greece. The animals that were presented to a private veterinarian showed loss of appetite for 3 days. The clinical examination revealed high fever (41.5°C), lethargy, an empty-appearing abdomen, hyperactive rumen motility, purulent nasal discharge, open-mouth breathing and tachypnea. On auscultation, wheezing sounds were detected. A biochemical blood analysis of a characteristic diseased animal, a 2 year old female goat indicated that globulin (2.9 g/dl, interval: 2.7–4.1 g/dl), creatinine (1.4 mg/dl, ref. interval: 1.0–1.8 mg/dl), glucose (68 mg/dl, ref. interval: 50–75 mg/dl) and total bilirubin (1.1 mg/dl, ref. interval: 0.1–1.7 mg/dl) concentrations were within reference intervals. In addition, hepatic enzyme activities (sorbitol dehydrogenase 17 IU/l, ref. interval: 14–23.6 IU/l; gamma-glutamyl transferase 26 IU/l, ref. interval: 20–56 IU/l) were also within normal ranges. A dipstick screening for proteinuria, hematuria, and ketone bodies in the urine was negative. Additionally microscopic examination of Giemsa-stained, thin and thick blood films did not reveal the presence of any blood parasites (*Anaplasma ovis*, *Babesia ovis* and *Babesia motasi*). The animals were treated intramuscularly with 20 mg/kg of tylosin (Tylan 50, Elanco) twice a day. However, no clinical improvement was achieved and all 6 affected animals died on the 1<sup>st</sup> to 3<sup>rd</sup> day of the
therapy. The remaining animals showed no symptoms. There were no risk factors such as stress, crowding or viral infections recorded for the herd prior to the outbreak.

In the affected animals, gross lesions were confined to the lungs and consisted of generalized interstitial edema with multiple light tan to light yellow branching and anastomosing tracts. The edema were moderately elevating the surface of the whole lung parenchyma. They were consistent with severely ectatic airways frequently distended to form prominent nodular collection of exudate ranging from 1 mm to 4 cm in diameter. Morphologically these lesions are consistent with a sub-acute, severe, and multifocal to coalescing bronchopneumonia with severe bronchiectasis presumably of infectious origin (Fig. 1). Furthermore, microscopic examination of the histologic section of the lung of the affected 2 year old female goat confirmed the macroscopic morphological diagnosis by revealing multifocal to coalescing severe active suppurate bronchopneumonia characterized by bronchial and bronchiolar lumen filled with basophilic amorphous mucoid material admixed with viable and degenerating neutrophils, sloughed degenerated epithelial cells, macrophages and cellular debris. Furthermore the alveolar walls were multifocal expanded by infiltrating lymphocytes, macrophages, plasma cells and neutrophils. Additionally, there was moderate hyperplasia of the bronchus associated lymphoid tissue (BALT) with mild to moderate multifocal hyperplasia of the smooth muscles of the airways, diffuse edema and thickening of the pleura. May-Grünwald Giemsa stained lung tissues revealed the presence of 5 µm long rods indicating the presence of bacteria in affected lung foci.

Microbiological examination of lung samples taken from the multiple ectatic airways lumina revealed no growth on all conditions tested after 96 h. However, after 10 – 14 days growth under anaerobic conditions, small black pigmented colonies of
rod shaped Gram-negative bacteria were found on blood – agar medium from samples of 3 affected animals. These bacteria also grew on blood – agar medium supplemented with Kanamycin or Ampicillin, but not with Vancomycin. DNA amplification from the black pigmented colonies and subsequent sequence analysis of the amplified \textit{rrs} 16S rRNA gene showed the same 1408 bp sequence for isolates of all 3 samples (Gen Bank accession nr. KM 360064). Comparison of this DNA sequence by BLAST revealed 92\% sequence similarity (identical nt) with the \textit{rrs} gene of \textit{Porphyromonas levii} (Gen Bank accession nr. L16493) and \textit{Porphyromonas somerae} (Gen Bank accession nr. L16493) as the closest known bacterial species. Both \textit{P. levii} and \textit{P. somerae} are pathogenic \textit{Porphyromonas} species of cattle and human respectively. The inter species variability of the 16S rRNA gene sequence of this yet undescribed \textit{Porphyromonas} sp. from goats compared to the 16S rRNA gene sequence of \textit{P. levii} or \textit{P. somerae} is 8\%. This is largely above the 3\% difference that is generally recommended to be regarded as a new species (22). We therefore conclude that the \textit{Porphyromonas} strains isolated from the diseased goats represent a novel species that we tentatively name ‘\textit{Porphyromonas katsiki}’ (κατσίκα gr. goat).

Figure 2 shows the phylogenetic position of ‘\textit{Porphyromonas katsiki}’ in relation to the closest relatives of \textit{Porphyromonas}, and selected species of \textit{Bacteriodes} and \textit{Prevotella}.

In order to determine whether ‘\textit{Porphyromonas katsiki}’ was present as the major bacterial species in the lungs of all diseased animals total DNA extracted from lung tissues of the affected animals was analyzed by PCR with the universal 16S rRNA gene primers 16SUNI-L, 16SUNI-R and by sequencing of the PCR amplified fragments. Sequence analysis of the PCR fragments obtained resulted in the same sequence as found for the strains (Gen Bank accession nr. KM 360064). As no
double- or multiple- peaks were found in the chromatograms of the Sanger sequencing of the \textit{rrs} gene amplicons from the lung samples, we conclude that ‘\textit{Porphyromonas katsikii}’ was the predominant or sole bacterial species found in the lungs of diseased animals.

Direct PCR analysis of lung tissues using specific primers designed for the amplification of a specific part of the \textit{rrs} gene of ‘\textit{Porphyromonas katsikii}’ revealed a strong positive PCR signal of 800 bp for all lung samples of the diseased animals. Lung tissue samples from 6 healthy goats taken from slaughter used as negative controls and of 7 goats with pasteurellosis pneumonia caused by \textit{Mannheimia haemolytica} used as specificity controls, showed no signal upon PCR amplification with the specific ‘\textit{Porphyromonas katsikii}’ primers. Furthermore sub-gingival samples taken from 6 healthy goats from where \textit{Porphyromonas} species are expected, and feces from healthy goats showed no amplicons when tested with the ‘\textit{P. katsikii}’ – specific PCR, in contrast to PCR with the universal 16S rRNA primers, which was positive in these samples.

\section*{Discussion}

To the authors’ knowledge, this is the first report of a \textit{Porphyromonas} lung infection in goats. As bacteriological diagnostics using culture methods often result in overgrowth by secondary much faster growing bacteria resulting from contaminations of the dead carcasses, the impact of \textit{Porphyromonas} species in specimens of diseased animals is not easy to pinpoint and the primary pathogen quite often remains undetected due to its slow growth properties. In the current case \textit{Porphyromonas} only grew after 10 days or more and could only be identified in 3 animals. However, we
were able to detect ‘Porphyromonas katsikii’ in lung samples of all affected animals by PCR both with universal 16S rRNA gene primers followed by DNA sequencing, where it revealed to be the only species recognized. It has to be underlined that universal bacterial 16S rRNA gene primers used for PCR resulted uniquely in detectable 16S rRNA gene fragments corresponding to ‘Porphyromonas katsikii’, hence indicating that other bacteria were comparatively at very low abundance or absent in the affected lung tissue. Furthermore, ‘P. katsikii’ was also detected in the affected lung tissues by the specific PCR primers. In contrast, tissues from lungs of healthy goats or from goats with other pneumonic infections were devoid of the specific PCR signal. Hence we postulate that the new Porphyromonas species, tentatively named ‘Porphyromonas katsikii’, was the causative agent of lung diseases in the affected goats of the outbreak. The closest relatives to ‘P. katsikii’ are P. levii and P. somerae that cause various infections in animals and humans respectively such as vulvovaginitis, chronic skin and soft tissue infections and bone infections (6, 7, 15).

Local infections due to Porphyromonas species are generally caused by bacterial strains present in tissues prior to the development of the clinical infection (1). In a previous study (6), cases of necrotic vulvovaginitis were associated with P. levii in cows. In this study, lesions of the genital tract and immunosuppressive stressors as calving, age and primiparity of the cows were considered as risk factors of that outbreak. In our current case, the primary site of infection was not determined. ‘P. katsikii’ could not be detected by specific PCR from gingival pockets of healthy goats where the habitat of Porphyromonas species is expected, nor from feces of healthy goats. Furthermore, there was no evidence of immune suppression of the goat. It could be speculated that lungworm which is very common among goats, especially...
in goats reared in backyard farms, could be the primary site of infection prior to
dissemination of the bacterium to the lung. However, no ova or larvae of nematodes
were evidenced in the affected animals. ‘Porphyromonas katsikii’ is an anaerobic
bacterium, although slight growth of this bacterium in 5% CO₂ atmosphere on
mycoplasma medium was observed. Hence, one can speculate that airways filled with
exudate could have served as anaerobic conditions in order to initiate the infection.
The absence of ‘P. katsikii’ in healthy goats as revealed by analyzing sub-gingival
samples where Porphyromonas species most likely are expected to have their
ecological niche, and from feces, suggests that the infectious agent may not be a
commensal of these animals.

Porphyromonas infections are most probably underdiagnosed in veterinary
and also human medicine because appropriate media and growth conditions and in
particular long incubation times up to two weeks are not implemented in the
procedures of routine diagnostic laboratories. However, in cases of disease outbreaks
with undetermined etiology, we suggest to implement procedures with extended
incubation times and the use of various growth conditions in order to isolate and
identify rare bacterial pathogens such as Porphyromonas species and other slow
growing Bacteroidetes as potential infectious agents. Furthermore amplification of
16S rRNA genes using universal primes and subsequent DNA sequence analysis was
shown to be a valuable rapid diagnostic tool. The current report indicates that the
newly identified bacterium ‘Porphyromonas katsikii’ should be considered as a
possible etiological agent of lung infections in goats and related animal species.

Conflict of interest

The authors declare no conflict of interest.
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Fig. 1. Diaphragmatic lobe of the goat. Generalized interstitial edema and multiple ectatic airways elevating the lung surface forming branching and anastomosing tracts. Morphologically these lesions are consisted with a subacute, severe, multifocal to coalescing, bronchopneumonia with bronchoectasia.

Fig. 2. Phylogenetic comparison of the newly discovered ‘Porphyromonas katsikii’ in relation to the most closely related Porphyromonas species and to selected species of Bacteriodes and Prevotella, based on the 16S rRNA gene sequences using UPGMA: Escherichia coli K12 was used as out-group. Botstrap values of 1000 simulations are shown at the branches. The scale represents sequence divergence.


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