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.

Introduction

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^{o}C under aerobic and anaerobic atmosphere and on Mycoplasma Hayflick medium (17) under an atmosphere with 5% CO_2 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 (GTGTGACGGGCGGTGTGTAC) 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 mm^3 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. cangingivalis* (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, *Bacteriodes merdae* (X83954), *Bacteriodes fragilis* (AB510701) and *Prevotella oralis*

(L16480). 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^{o}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 1st to 3rd day of the

197 therapy. The remaining animals showed no symptoms. There were no risk factors
198 such as stress, crowding or viral infections recorded for the herd prior to the outbreak.
199 In the affected animals, gross lesions were confined to the lungs and consisted
200 of generalized interstitial edema with multiple light tan to light yellow branching and
201 anastomosing tracts. The edema were moderately elevating the surface of the whole
202 lung parenchyma. They were consistent with severely ectatic airways frequently
203 distended to form prominent nodular collection of exudate ranging from 1 mm to 4
204 cm in diameter. Morphologically these lesions are consistent with a sub-acute, severe,
205 and multifocal to coalescing bronchopneumonia with severe bronchiectasis
206 presumably of infectious origin (Fig. 1). Furthermore, microscopic examination of the
207 histologic section of the lung of the affected 2 year old female goat confirmed the
208 macroscopic morphological diagnosis by revealing multifocal to coalescing severe
209 active suppurate bronchopneumonia characterized by bronchial and bronchiolar
210 lumen filled with basophilic amorphous mucoid material admixed with viable and
211 degenerating neutrophils, sloughed degenerated epithelial cells, macrophages and
212 cellular debris. Furthermore the alveolar walls were multifocal expanded by
213 infiltrating lymphocytes, macrophages, plasma cells and neutrophils. Additionally,
214 there was moderate hyperplasia of the bronchus associated lymphoid tissue (BALT)
215 with mild to moderate multifocal hyperplasia of the smooth muscles of the airways,
216 diffuse edema and thickening of the pleura. May-Grünwald Giemsa stained lung
217 tissues revealed the presence of 5 µm long rods indicating the presence of bacteria in
218 affected lung foci.
219 Microbiological examination of lung samples taken from the multiple ectatic
220 airways lumina revealed no growth on all conditions tested after 96 h. However, after
221 10 – 14 days growth under anaerobic conditions, small black pigmented colonies of

222 rod shaped Gram-negative bacteria were found on blood – agar medium from samples
223 of 3 affected animals. These bacteria also grew on blood – agar medium
224 supplemented with Kanamycin or Ampicillin, but not with Vancomycin. DNA
225 amplification from the black pigmented colonies and subsequent sequence analysis of
226 the amplified *rrs* 16S rRNA gene showed the same 1408 bp sequence for isolates of
227 all 3 samples (Gen Bank accession nr. KM 360064). Comparison of this DNA
228 sequence by BLAST revealed 92% sequence similarity (identical nt) with the *rrs* gene
229 of *Porphyromonas levii* (Gen Bank accession nr. L16493) and *Porphyromonas*
230 *somerae* (Gen Bank accession nr. L16493) as the closest known bacterial species.
231 Both *P. levii* and *P. somerae* are pathogenic *Porphyromonas* species of cattle and
232 human respectively. The inter species variability of the 16S rRNA gene sequence of
233 this yet undescribed *Porphyromonas* sp. from goats compared to the 16S rRNA gene
234 sequence of *P. levii* or *P. somerae* is 8%. This is largely above the 3% difference that
235 is generally recommended to be regarded as a new species (22). We therefore
236 conclude that the *Porphyromonas* strains isolated from the diseased goats represent a
237 novel species that we tentatively name '*Porphyromonas katsikii*' (κατσίκα gr. goat).
238 Figure 2 shows the phylogenetic position of '*Porphyromonas katsikii*' in relation to
239 the closest relatives of *Porphyromonas,* and selected species of *Bacteriodes* and
240 *Prevotella*.

241 In order to determine whether '*Porphyromonas katsikii*' was present as the
242 major bacterial species in the lungs of all diseased animals total DNA extracted from
243 lung tissues of the affected animals was analyzed by PCR with the universal
244 16S rRNA gene primers 16SUNI-L, 16SUNI-R and by sequencing of the PCR
245 amplified fragments. Sequence analysis of the PCR fragments obtained resulted in the
246 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 *rrs* gene amplicons from the lung samples, we conclude that '*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 *rrs* gene of '*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 *Mannheimia haemolytica* used as specificity controls, showed no signal upon PCR amplification with the specific '*Porphyromonas katsikii*' primers. Furthermore sub-gingival samples taken from 6 healthy goats from where *Porphyromonas* species are expected, and feces from healthy goats showed no amplicons when tested with the '*P. katsikii*' – specific PCR, in contrast to PCR with the universal 16S rRNA primers, which was positive in these samples.

Discussion

To the authors' knowledge, this is the first report of a *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 *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 *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 vuvlovaginitis 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

297 in goats reared in backyard farms, could be the primary site of infection prior to
298 dissemination of the bacterium to the lung. However, no ova or larvae of nematodes
299 were evidenced in the affected animals. '*Porphyromonas katsikii'* is an anaerobic
300 bacterium, although slight growth of this bacterium in 5% CO_2 atmosphere on
301 mycoplasma medium was observed. Hence, one can speculate that airways filled with
302 exudate could have served as anaerobic conditions in order to initiate the infection.
303 The absence of '*P. katsikii*' in healthy goats as revealed by analyzing sub-gingival
304 samples where *Porphyromonas* species most likely are expected to have their
305 ecological niche, and from feces, suggests that the infectious agent may not be a
306 commensal of these animals.

307 *Porphyromonas* infections are most probably underdiagnosed in veterinary
308 and also human medicine because appropriate media and growth conditions and in
309 particular long incubation times up to two weeks are not implemented in the
310 procedures of routine diagnostic laboratories. However, in cases of disease outbreaks
311 with undetermined etiology, we suggest to implement procedures with extended
312 incubation times and the use of various growth conditions in order to isolate and
313 identify rare bacterial pathogens such as *Porphyromonas* species and other slow
314 growing *Bacteroidetes* as potential infectious agents. Furthermore amplification of
315 16S rRNA genes using universal primes and subsequent DNA sequence analysis was
316 shown to be a valuable rapid diagnostic tool. The current report indicates that the
317 newly identified bacterium '*Porphyromonas katsikii'* should be considered as a
318 possible etiological agent of lung infections in goats and related animal species.

319

320 **Conflict of interest**

321 The authors declare no conflict of interest.

Acknowledgments

We are grateful to Paola Pilo and Francesco Origgi, Institute of Veterinary Bacteriology, Bern, for their expert help in editing this manuscript and to Amandine Ruffieux for excellent technical support. This project was financed by the internal research fund of the Institute of Veterinary Bacteriology, University of Bern.

329 Figure Legends

330

331 Fig.1. Diaphragmatic lobe of the goat. Generalized interstitial edema and multiple
332 ectatic airways elevating the lung surface forming branching and anastomosing tracts.
333 Morphologically these lesions are consisted with a subacute, severe, multifocal to
334 coalescing, bronchopneumonia with bronchoectasia.

335

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

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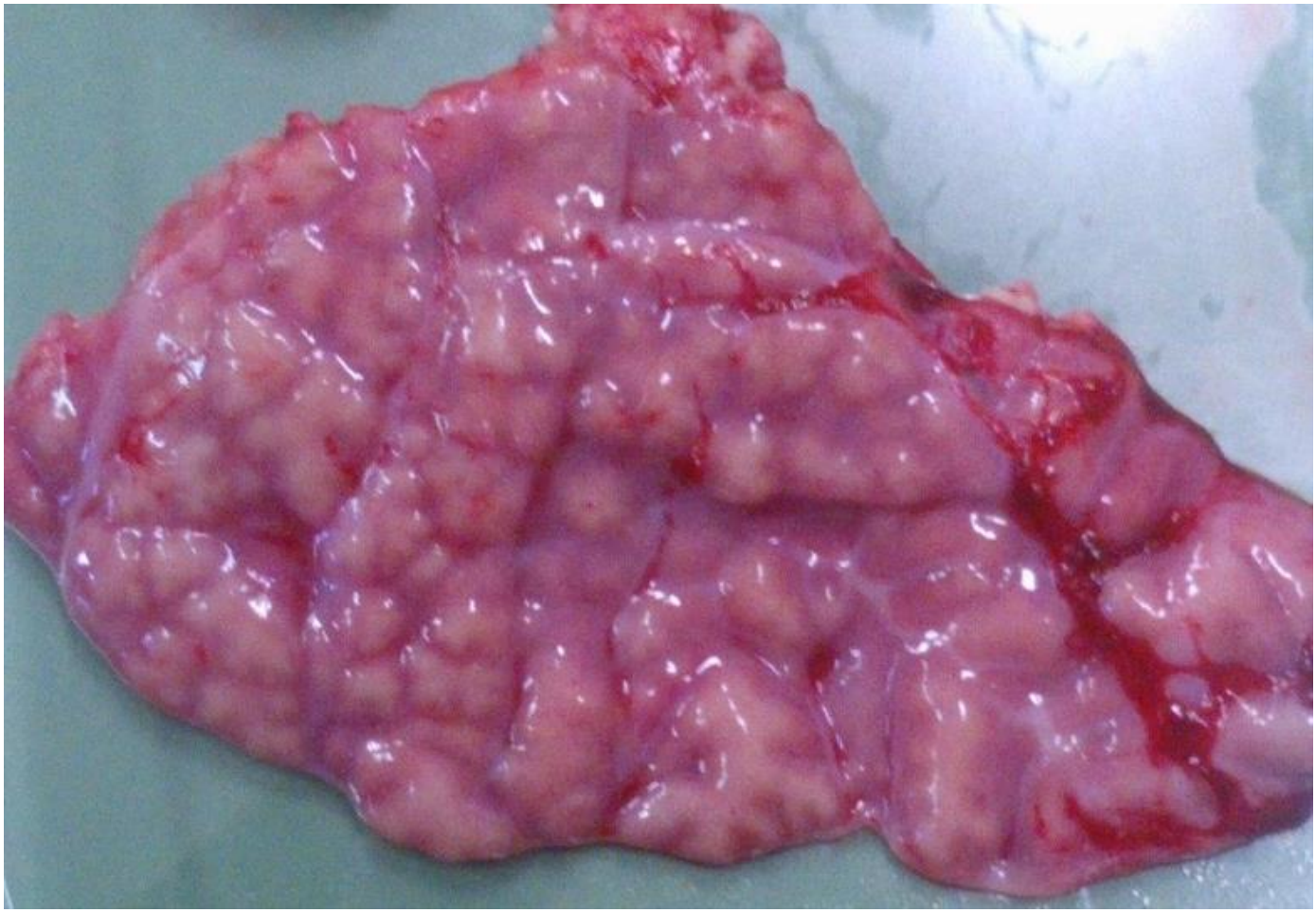
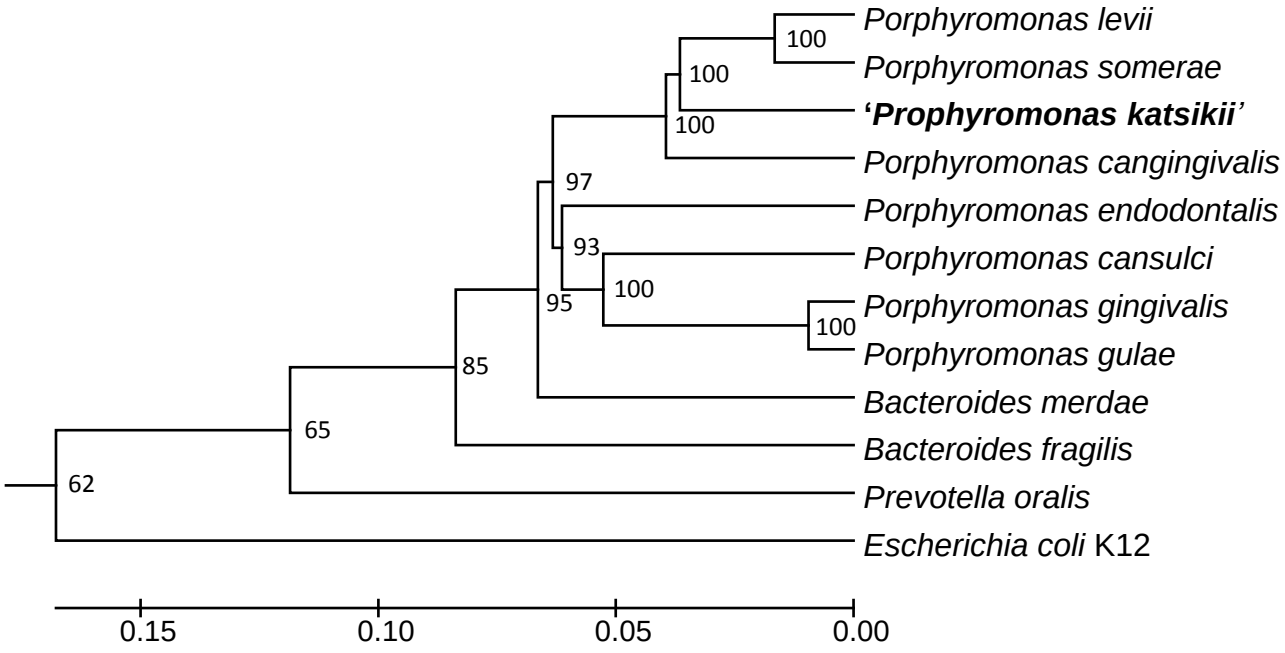


Fig. 1


Porphyromonas levii
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'Prophyromonas katsikii'
Porphyromonas cangingivalis
Porphyromonas endodontalis
Porphyromonas cansulci
Porphyromonas gingivalis
Porphyromonas gulae
Bacteroides merdae
Bacteroides fragilis
Prevotella oralis
Escherichia coli K12
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Fig. 4