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Occurrence and antimicrobial susceptibility of *Porphyromonas* spp. and *Fusobacterium* spp. in dogs with and without periodontitis

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ABSTRACT

The occurrence of *Porphyromonas gulae*, *Porphyromonas macacae*, *Fusobacterium nucleatum* and *Fusobacterium canifelinum* in subgingival plaque from dogs with and without periodontitis as well as their antimicrobial susceptibility were evaluated. From 50 dogs with periodontitis were identified 38 *P. gulae*, 8 *P. macacae*, 26 *F. nucleatum* and 15 *F. canifelinum*, and from 50 dogs without periodontitis were identified 15 *P. gulae*, 12 *F. nucleatum* and 11 *F. canifelinum*. All strains were susceptible to most of the antibiotics tested, however, different resistance rates to clarithromycin, erythromycin and metronidazole among strains were observed. The role of *P. gulae*, *P. macacae*, *F. nucleatum* and *F. canifelinum* in periodontal disease of household pets needs to be defined to a better prevention and treatment of the canine periodontitis.

1. Introduction

In man, periodontal diseases, such as gingivitis and periodontitis, are chronic and multi-factorial diseases affecting the tissues supporting the teeth [1]. Gingivitis and periodontitis lesions in dogs are more closely related to humans than other animals [2]; although, gingivitis did not necessarily progress to periodontitis. The etiologic factors of gingivitis and periodontitis seem to be identical in humans and dogs [2,3]; however, the oral microbiota in animals has been poorly characterized.

It is estimated that approximately 80% of dogs and cats display some degree of periodontal disease around 4 years of age [4]. In animals, different species of *Porphyromonas* and *Fusobacterium* appear to be associated with periodontal disease [5,6], but their pathogenicity is still unclear.

Black-pigmented anaerobic bacteria have been isolated from the periodontal pockets of dogs and cats; however, several differences between human and companion animal *Porphyromonas* strains have been reported [5,7].

Fusobacterium nucleatum is one of the most common bacterial species in gingival crevice of humans and animals and it is associated with periodontal disease. *Fusobacterium canifelinum* is closely related to *F. nucleatum* and it has been recovered from human wounds produced by animal bites, particularly, cat or dog [6,8].

Molecular methods are currently available for typing and subtyping of periodontopathogens, such as *Porphyromonas gingivalis*, but they can vary in efficiency and in amount of required labor [9,10]. Techniques such as bacterial culture are used to identify putative anaerobic pathogens from destructive periodontal disease but can be somewhat cumbersome and expensive [10]. Polymerase chain reaction (PCR) has been used for direct identification of periodontal pathogens from subgingival specimens [11], and for elucidating the role of specific bacteria in the periodontal disease because of ability to accurately detect bacterial species from mixed populations.

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In anaerobic bacteria, the inadequate use of antibiotics has produced an alarming and graduate increase of the resistance to several drugs. Since antimicrobials can produce disequilibrium in oral or intestinal resident microbiota, the choice of a suitable antibiotic therapy is needed, and it must be taken in consideration to the treatment of infectious diseases. Gram-negative anaerobic bacteria, such as *Porphyromonas* spp. and *Fusobacterium* spp. isolated from humans has shown high resistance to several drugs [12]; however, data showing the susceptibility of oral bacteria from animal origin to different antimicrobials are scarce [13].

In this study, the occurrence of *Porphyromonas* spp. and *Fusobacterium* spp. from subgingival biofilm of dogs with and without periodontitis, as well as their antimicrobial susceptibility were evaluated.

2. Materials and methods

2.1. Cohorts

Subgingival plaque samples were taken from 50 dogs with and 50 without periodontitis undergoing routine dental treatment at the Private Dental Veterinary Clinic (Odontovet, São Paulo, SP, Brazil) and at the School of Veterinary Medicine and Zootechny of the University of São Paulo (São Paulo, SP, Brazil). Male and female dogs with (11 different breeds) and without (10 different breeds) periodontitis from 7-months to 10-years of age were selected. All animals with periodontitis were verified by different clinical indices degree of gingival inflammation, amount of supragingival plaque, probing pocket depths ($\geq$5 mm), bleeding on probing, tooth mobility and alveolar bone loss. None of the animals had received antibiotic treatment within the previous three months of the sample collection. The Ethics Committee for Animal Experimentation at the Institute of Biomedical Science/USP (116/CEEA) approved this study.

2.2. Sample collection and processing

Animals were anesthetized with propofol (2 mg/kg) and diazepam (5.5 mg/kg) by an intramuscular injection, and received isoflurane and oxygen by an endotracheal way. Supragingival biofilm was removed by using sterile gauze and area was isolated by using cotton rolls. Subgingival samples from only one gingival site (healthy dogs) or periodontal pocket (dogs with periodontitis) were collected by using two fine sterile paper points (N°. 30, Tanariman Ind Ltd, AM, Brazil), introduced into the apical region of healthy or periodontal site and allowed to remain for 60 s. Paper points were then placed into VMGA III transport medium [14] and processed within 4 h of collection. All collected samples 10-fold diluted were plated (0.1 mL) onto Brucella blood agar (Difco Laboratories) containing 5% defibrinated horse blood, 0.0005 mg/mL hemin and 0.0001 mg/mL menadione to isolate *Porphyromonas gulae* and *Porphyromonas macacae*, and onto Omata and Disraely agar to isolate *F. nucleatum* and *F. canifelinum*. Plates were incubated in atmosphere of 90% N_2 + 10% CO_2, at 37 °C, for 7 days in anaerobiosis.

2.3. Bacterial identification

Characteristic colonies of each bacterial species were presumptively identified by Gram staining; catalase, H_2S and indol production; and esculin and starch hydrolysis. Further identification by biochemical tests [15] or by RAPID ID 32A kits (bioMérieux) according to the manufacturer's instructions was performed. The resistance to levofloxacin was performed as an additional assay to distinguish *F. nucleatum* from *F. canifelinum* [6].

2.4. Serum resistance and hemolytic activity

Bacterial resistance to human, dog or horse serum was determined [16]. Inactivated sera at 56 °C for 30 min were used as controls. 1 mL (1.5×10^8 cfu/mL) of each bacterial growth was mixed with 1 mL of dog, horse or human serum, and incubated in anaerobiosis at 37 °C for 4 h. 0.1 mL of each mixture was then inoculated onto Brucella blood agar and incubated in anaerobiosis at 37 °C for 72 h. A total inhibition was considered as being susceptible to serum. Hemolysin production was observed onto blood agar and each strain grown in BHI broth was inoculated by using a Steers replicator (*ca.* 10^5 cfu/spot). After incubation as described above, the hemolytic activity was defined as a clear zone around the bacterial growth.

2.5. Hemagglutination (HA) and hemagglutination inhibition (HAI) assays

Each bacterial suspension, in PBS (0.01 M, pH 7.3), was adjusted to *ca.* 1.5×10^8 cfu/mL [17]. Human erythrocytes were resuspended in 1% Alsever's solution. HA was qualitatively assayed by mixing 50 μL of bacterial suspension to 50 μL of erythrocytes suspension in a 96-well microtitre plate. Bacterial two-fold dilutions containing 50 μL/well were performed, and 50 μL of erythrocytes were then added to each dilution. Plate was gently shaken and incubated at 37 °C for 1 h, and after at 4 °C overnight. Hemagglutination titers were expressed as the reciprocal of the highest bacterial dilution showing agglutination.

The ability of D-glucose, D-galactose, D-mannose and D-lactose to inhibit the HA was evaluated. Carbohydrates were prepared at 80 mM in PBS and sterilized by filtration (0.45 micron pore size, Millipore). 0.1 mL of erythrocytes was treated with 9.9 mL of each carbohydrate. The HAI was qualitatively performed by mixing 50 μL of bacteria with 50 μL of erythrocytes treated with each carbohydrate. Two-fold serial dilutions containing 50 μL of bacteria were performed and 50 μL of erythrocytes treated with carbohydrate were added. Plates were gently shaken and incubated at 37 °C for 1 h, and then, at 4 °C overnight. Erythrocytes treated with carbohydrate or mixed with PBS were used as negative controls.

2.6. Antimicrobial susceptibility testing

An agar dilution method in Brucella blood agar was used according to CLSI [18]. The antibiotics used were: amoxicillin, ampicillin, clindamycin, erythromycin, and tetracycline (Luper Ind Farm Ltd, SP, Brazil), amoxicillin/clavulanic acid (Smithkline Beecham Ltd, SP, Brazil), penicillin G (Prodoti Lab Farm Ltd, SP, Brazil), metronidazole (Aventis Farm Ltd, SP, Brazil), cefoxitin (Merck, Sharp and Dohme, SP, Brazil), and clarithromycin (Boehringer Ingelheim do Brasil Quim Farm Ltd, SP, Brazil). Breakpoints used for erythromycin and clarithromycin were according to NCCLS [19].

3. Results

3.1. Bacterial isolation and identification

In ten out of eleven dogs with, and six out of ten without periodontitis of different breeds harbored at least one microorganism evaluated (Table 1). From forty-six (92%) out of 50 dogs with periodontitis, 38 *P. gulae,* 8 *P. macacae*, 26 *F. nucleatum* and 15 *F. canifelinum* strains were isolated, and 28 (56%) out of 50 dogs without periodontitis 15 *P. gulae*, 12 *F. nucleatum* and 11 *F. canifelinum* were isolated. No healthy dog harbored *P. macacae*. From each subgingival clinical sample, one to two colonies were picked.

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Table 1
Presence of oral *Porphyromonas* spp. and *Fusobacterium* spp. in dogs with and without periodontitis.

Breed of dogs	Strains			
	P. gulae	*P. macacae*	*F. nucleatum*	*F. canifelinum*
With periodontitis				
Argentine mastiff	5	0	0	0
Australian cattle dog	0	0	0	1
Basset hound	0	0	0	0
Beagle	0	4	3	1
Cocker spaniel	1	0	0	0
Dachshunds	6	0	2	4
Fox terrier	0	0	0	4
Cross breed	9	0	2	0
Poodle	2	4	11	5
West highland white terrier	4	0	4	0
Yorkshire terrier	1	0	4	0
Total	**38**	**8**	**26**	**13**
Without periodontitis				
Cocker spaniel	4	0	0	0
Dachshunds	0	0	5	1
Maltese	1	0	0	4
Cross breed	5	0	0	0
Pinscher	0	0	3	1
Pit bull	1	0	0	0
Rottweiler	0	0	0	0
Schnauzer	0	0	1	0
West highland white terrier	4	0	0	0
Yorkshire terrier	15	0	3	5
Total	**30**	**0**	**12**	**11**

Analysis using Chi-square and Fisher tests showed no statistically significant differences between isolated strains from periodontal and healthy dogs; however, the number of *P. gulae* isolated from dogs with periodontitis was statistically significant ($P = 0.008$). Interestingly, in this study, poodle with periodontitis and yorkshire terrier without periodontitis harbored most of the evaluated bacteria.

The phenotypic characteristics of the four bacterial species are shown in Table 2. All 26 *F. canifelinum* strains were resistant to levofloxacin (MIC > 4). All *P. gulae* strains were β-hemolytic and *P. macacae*, *F. nucleatum* or *F. canifelinum* strains did not produce hemolysis. Most of *P. gulae*, *P. macacae*, *F. nucleatum* and *F. canifelinum* isolated from dogs with or without periodontitis were resistant to human, dog or horse serum.

Table 2
Phenotypic characteristics of *Porphyromonas gulae*, *Porphyromonas macacae*, *Fusobacterium nucleatum*, and *Fusobacterium canifelinum* isolated from dogs with and without periodontitis.

Phenotypic characteristic	% Of positive strains			
	P. gulae ($n = 53$)	*P. macacae* ($n = 8$)	*F. nucleatum* ($n = 38$)	*F. canifelinum* ($n = =26$)
Fermentation				
Glucose	9.4	50	ND	ND
Sucrose	3.8	37.5	0	0
Maltose	3.8	0	0	0
Lactose	1.9	0	0	0
Production				
Catalase	100	100	0	0
Urease	0	100	0	0
α-Galactosidase	0	100	0	0
β-Galactosidase	100	100	0	0
N-Acetyl-β-glucosaminidase	100	100	0	0
Arginine arylamidase	98.1	0	0	0
Alkaline phosphatase	100	100	0	0
NO_3 reduction	0	100	0	0
Levofloxacin resistance	ND	ND	0	100

ND: not determined. No strain fermented xylose or galactose; hydrolyzed esculin or starch; produced H_2S or motility.

3.2. HA and HAI assays

Only 21 *P. gulae* and 2 *F. nucleatum* were able to agglutinate erythrocytes with titers from 2 to 64 and 4, respectively. D-glucose, D-galactose, D-mannose and D-lactose were able to inhibit the hemagglutination in most of *P. gulae* and *F. nucleatum* strains. Glucose was not able to inhibit HA in *F. nucleatum*.

3.3. Antimicrobial susceptibility

All strains tested were susceptible to amoxicillin, ampicillin, amoxicillin/clavulanate, cefoxitin, clindamycin, penicillin G, and tetracycline. *P. gulae* and *P. macacae* strains isolated from dogs with periodontitis were respectively resistant to clarithromycin (68.4% and 50%) and erythromycin (34.2% and 12.5%). One (2.6%) metronidazole-resistant *P. gulae* strain was observed (MIC ≥ 512 μg/mL). In addition, *F. canifelinum* and *F. nucleatum* were respectively, 100% and 96% resistant to clarithromycin and erythromycin (Table 3). *P. gulae* strains from dogs without periodontitis were resistant to clarithromycin (86.7%), erythromycin (73.3%) and metronidazole (13.3%). *F. canifelinum* and *F. nucleatum* strains were resistant to clarithromycin (100%) and erythromycin (90.9% and 91.7%, respectively) (Table 4).

4. Discussion

Studies has shown the presence of human periodontitis-related bacterial species, such as *P. gingivalis, Prevotella intermedia, Treponema denticola, T. forsythia, A. actinomycetemcomuitans, C. rectus* and *E. corrodens* from subgingival samples of dogs with and without periodontitis [20,21,24], suggesting that these microorganisms should be regarded as members of the oral microbiota in dogs.

Since the periodontal disease is considered as the most common disease in small animals, previous companion animal studies determining the oral microbiota using biochemical assays have been performed; however, these procedures are limited by the quality of the biochemical databases.

Studies have shown that black-pigmented anaerobic bacteria isolated from animal or human periodontal lesions are phenotipically similar, particularly in oral *Porphyromonas* species [7,22]; however there is little information about the occurrence of oral anaerobic bacteria such as *Porphyromonas* spp. and *Fusobacterium* spp. in animals.

Paper point samples from the crevicular space that are diluted and plated on growth media have proven to be the most reliable method for isolating bacteria from crevicular fluid [23]. Traditional methods of bacterial isolation and identification may limit the bacterial recovery due to the lack or absence of viable cells and growth requirements [5].

In this study, only catalase-producing *P. gulae* and *P. macacae* were observed. This enzyme might allow the bacterial colonization in gingival crevice or periodontal pocket by reducing the superoxide effect [25,26].

The production of hemagglutinins and hemolysis by *P. gingivalis* collaborate with the colonization of diseased sites, and its attachment on erythrocytes might be an important role in the establishment and progression of the infection [27]. Fournier et al. [5] analyzing *P. gulae* and *P. macacae* strains showed that only *P. gulae* strains produced erythrocytes agglutination. On the other hand, the adherence ability of *P. gulae* appears to be lower than *P. gingivalis* and it can be explained because of absence of fimbrillin a protein that influence the adherence [28].

All strains were 100% resistant to dog's serum. The serum bactericidal effect is an important host's defense mechanism

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Table 3
Antimicrobial susceptibility of *P. gulae*, *P. macacae*, *F. nucleatum* and *F. canifelinum* isolated from dogs with periodontitis.

Strains (no.)/antibiotic	MIC (μg/mL)			% Resistance
	Range	50%	90%	
***P. gulae* (*n* = 38)**				
Amoxicillin	≤0.25–4	≤0.25	1	0
Ampicillin	≤0.25–2	≤0.25	2	0
Amoxicillin/clavulanate	≤0.25–2	≤0.25	0.5	0
Cefoxitin	≤0.25–2	≤0.25	0.5	0
Clarithromycin[a]	≤0.25–≥512	≥512	≥512	68.4
Clindamycin	≤0.25	≤0.25	≤0.25	0
Erythromycin[a]	≤0.25–32	1	16	34.2
Metronidazole	≤0.25–≥512	≤0.25	0.5	2.6
Penicillin G	≤0.25	≤0.25	≤0.25	0
Tetracycline	≤0.25–0.5	≤0.25	≤0.25	0
***P. macacae* (*n* = 8)**				
Amoxicillin	≤0.25–2	≤0.25	1	0
Ampicillin	≤0.25–0.5	≤0.25	≤0.25	0
Amoxicillin/clavulanate	0.5–2	1	2	0
Cefoxitin	≤0.25–0.5	≤0.25	2	0
Clarithromycin[a]	4–≥512	4	≥512	50
Clindamycin	≤0.25	≤0.25	≤0.25	0
Erythromycin[a]	≤0.25–32	≤0.25	1	12.5
Metronidazole	≤0.25–1	≤0.25	0.5	0
Penicillin G	≤0.25	≤0.25	≤0.25	0
Tetracycline	≤0.25	≤0.25	≤0.25	0
***F. canifelinum* (*n* = 15)**				
Amoxicillin	≤0.25–4	≤0.25	≤0.25	0
Ampicillin	≤0.25–1	≤0.25	≤0.25	6.7
Amoxicillin/clavulanate	≤0.25	≤0.25	≤0.25	0
Cefoxitin	≤0.25	≤0.25	≤0.25	0
Clarithromycin[a]	128–≥512	≥512	≥512	100
Clindamycin	≤0.25–0.5	≤0.25	≤0.25	0
Erythromycin[a]	8–≥512	256	≥512	100
Metronidazole	≤0.25–2	≤0.25	2	0
Penicillin G	≤0.25	≤0.25	≤0.25	0
Tetracycline	≤0.25–0.5	≤0.25	0.5	0
***F. nucleatum* (*n* = 26)**				
Amoxicillin	≤0.25–0.5	≤0.25	≤0.25	0
Ampicillin	≤0.25–4	≤0.25	≤0.25	3.8
Amoxicillin/clavulanate	≤0.25	≤0.25	≤0.25	0
Cefoxitin	≤0.25–4	≤0.25	≤0.25	0
Clarithromycin[a]	≤0.25–≥512	≥512	≥512	96
Clindamycin	≤0.25	≤0.25	≤0.25	0
Erythromycin[a]	2–≥512	256	≥512	96
Metronidazole	≤0.25–2	≤0.25	1	0
Penicillin G	≤0.25	≤0.25	≤0.25	0
Tetracycline	≤0.25–1	≤0.25	1	0

Breakpoints used in according to CLSI (2007): amoxicillin, 8 μg/mL; ampicillin, 1 μg/mL; Amoxicillin/clavulanate, 4 μg/mL; cefoxitin, 32 μg/mL; clindamycin 4 μg/mL; metronidazole, 16 μg/mL; penicillin G, 1 μg/mL; tetracycline, 8 μg/mL.
[a] Breakpoints used for Erythromycin (8 μg/mL) and clarithromycin (8 μg/mL) according to NCCLS (1997).

Table 4
Antimicrobial susceptibility of *P. gulae*, *F. nucleatum* and *F. canifelinum* isolated from dogs without periodontitis.

Strains (no.)/antibiotic	MIC (μg/mL)			% Resistance
	Range	50%	90%	
***P. gulae* (*n* = 15)**				
Amoxicillin	≤0.25	≤0.25	≤0.25	0
Ampicillin	≤0.25	≤0.25	≤0.25	0
Amoxicillin/clavulanate	≤0.25	≤0.25	≤0.25	0
Cefoxitin	≤0.25 to 0.5	0.5	0.5	0
Clarithromycin[a]	≤0.25 to ≥512	≥512	≥512	86.7
Clindamycin	≤0.25	≤0.25	≤0.25	0
Erythromycin[a]	≤0.25 to 64	8	64	73.3
Metronidazole	≤0.25 to ≥512	1	8	13.3
Penicillin G	≤0.25	≤0.25	≤0.25	0
Tetracycline	≤0.25	≤0.25	≤0.25	0
***F. canifelinum* (*n* = 11)**				
Amoxicillin	≤0.25 to 1	≤0.25	1	0
Ampicillin	≤0.25 to 4	≤0.25	≤0.25	9
Amoxicillin/clavulanate	≤0.25 to 2	≤0.25	≤0.25	0
Cefoxitin	≤0.25 to 16	≤0.25	≤0.25	0
Clarithromycin[a]	32 to ≥512	256	≥512	100
Clindamycin	≤0.25	≤0.25	≤0.25	0
Erythromycin[a]	0.5 to ≥512	≥512	≥512	90.9
Metronidazole	≤0.25 to 1	≤0.25	0.5	0
Penicillin G	≤0.25	≤0.25	≤0.25	0
Tetracycline	≤0.25 to 1	≤0.25	≤0.25	0
***F. nucleatum* (*n* = 12)**				
Amoxicillin	≤0.25	≤0.25	≤0.25	0
Ampicillin	≤0.25	≤0.25	≤0.25	0
Amoxicillin/clavulanate	≤0.25	≤0.25	≤0.25	0
Cefoxitin	≤0.25 to 1	≤0.25	≤0.25	0
Clarithromycin[a]	128 to ≥512	≥512	≥512	100
Clindamycin	≤0.25	≤0.25	≤0.25	0
Erythromycin[a]	1 to ≥512	≥512	≥512	91.7
Metronidazole	≤0.25 to 8	0.5	2	0
Penicillin G	≤0.25	≤0.25	≤0.25	0
Tetracycline	≤0.25 to 2	≤0.25	≤0.25	0

Breakpoints according to CLSI (2007): amoxicillin, 8 μg/mL; ampicillin, 1 μg/mL; Amoxicillin/clavulanate, 4 μg/mL; cefoxitin, 32 μg/mL; clindamycin, 4 μg/mL; metronidazole, 16 μg/mL; penicillin G, 1 μg/mL; tetracycline, 8 μg/mL.
[a] Breakpoints used for erythromycin (8 μg/mL) and clarithromycin (8 μg/mL) according to NCCLS (1997).

against bacterial infections [29]. The resistance of gram-negative anaerobic bacteria to serum appears to be a multi-factorial process and LPS or proteolytic enzymes produced by *P. gingivalis* may suppress the serum bactericidal activity [30]. In addition, the presence of serum-resistant *P. gulae* strains may represent a selection of virulent and non-virulent strains and it could explain its pathogenesis in canine periodontitis.

The presence of *F. nucleatum* is often observed in human periodontal sites, although, this organism was also observed in dogs with (16%) and without (12%) periodontitis. These results suggest that this microorganism plays an important role in canine periodontitis, in accordance with Kornman et al. [31], Nishiyama et al. [21] and Syed et al. [8].

In the last years, the resistance of anaerobic bacteria to multiple drugs has increased, and the indiscriminate use of antibiotics, alone or as adjuncts to conventional periodontal therapy, may be responsible for the bacterial resistance, and it has been a target of several studies [32]. All tested strains were susceptible to amoxicillin, ampicillin, amoxicillin/clavulanate, cefoxitin, clindamycin, penicillin G and tetracycline. Studies have shown the presence of bacterial strains isolated from small animals, such as cats and dogs, as highly susceptible to several antimicrobials [33].

In addition, high MIC values to clarithromycin and erythromycin of *P. gulae*, *F. canifelinum* and *F. nucleatum* were observed, as well as, *P. macacae* resistant to clarithromycin. The bacterial resistance to macrolides has increased, particularly in those considered as resident microbiota in humans and animals. Clarithromycin and erythromycin act on the bacterial protein synthesis, however, their MIC values for anaerobic bacteria have not been yet established [18]. The resistance to macrolides observed in *P. gulae*, *F. canifelinum* and *F. nucleatum* strains suggests the presence of resistance-encoding genes, but it was not determined. Moreover, further studies might be needed to determine the real role that *P. gulae*, *P. macacae*, *F. canifelinum* and *F. nucleatum* could play in dogs' periodontal disease.

5. Conclusions

The presence of oral *P. gulae*, *P. macacae*, *F. canifelinum* and *F. nucleatum* was determined in oral cavity from dogs of different breeds, and it could play an important role in dogs' periodontal disease. All strains were susceptible to most of the antibiotics tested.

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Conflict of interest statement

Authors declare no conflict of interest.

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