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Virulence traits and antibiotic resistance among enterococci isolated from dogs with periodontal disease

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ABSTRACT

Periodontal disease – PD – is one of the most widespread diseases in dogs, but the role of this odontogenic infection in the dissemination of pathogenic bacteria present in the oral mucosa to other animals or pet owners is understudied.

Trying to unveil the putative pathogenicity of enterococci present in the gums of dogs diagnosed with PD, thirty-two animals were investigated during routine visits to a private veterinary clinic. Seventy-one enterococci were recovered and characterized regarding species, genomic variability, virulence traits, antimicrobial resistance and biofilm-forming ability.

Isolates were mainly identified as *Enterococcus faecalis*, with the large majority (95%) being able to produce biofilm. Regarding antibiotic resistance, all dog-enterococci were susceptible to ampicillin, amoxicillin/clavulanate, gentamicin-120, imipenem and vancomycin; while distinct levels of resistance were observed for chloramphenicol (10%), erythromycin (20%), streptomycin-300 (35%) and tetracycline (95%).

For virulence traits incidence levels of 35% were observed for β-hemolysis and 25% for *cylA*, 25% for gelatinase and 35% for *gelE;* 85% harbor *efaAfs* and *ebpABC*; while *ace*, *agg* and *esp* are present respectively in 50, 30 and 10% of the dog-enterococci; *efaAfm* and *acm* were detected in all the *Enterococcus faecium.*

Overall, the widespread prevalence of PD in dogs, associated with the close contact between companion animals, other animals and humans, may act as source for the dissemination of opportunistic pathogenic bacteria. Hence, aforementioned data on virulence and resistance features, emphasizes the need for active surveillance measures, such as the diagnose of PD in companion animals during routine visits to the veterinary clinic.

1. Introduction

Due to the close contact between pets and owners, the emergence of virulent and/or antimicrobial resistant bacteria in companion animals can be considered a major public health concern [1]. The spread of microbial pathogenic variants should not be disregarded; especially considering that improvement in veterinary medical assistance increased the administration of antimicrobials to animals, many times based on human medicine [2]. Additionally, pets interact with other animals, sometimes engaging in fights resulting in dog-bites, which can potentiate the spread of microorganisms. Hence, companion animals constitute important intervenients in the dissemination of pathogenic bacteria [3], justifying the implementation of effective surveillance measures, essential to allow the development of new strategies to confine this increasing problem.

In this context, pathogenicity assessment of bacteria present in pets diagnosed with periodontal disease (PD) plays a major role. Particularly considering that it is one of the most widespread diseases in dogs, with a prevalence of 44–80% [4,5], affecting animals from 2 years old [4], especially small and brachycephalics breeds [5]. PD has a multifactorial etiology, depending on several factors, related with the host and the environment [6]. It requires the formation of a plaque, defined as a microbial biofilm in the oral cavity that leads to the inflammation of tooth supporting structures, progressing from a mild gingivitis to severe periodontitis reaching the periodontal ligament and alveolar bone [4,6].

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Several aerobic and anaerobic microorganisms that colonize dogs' oral cavity have been associated with PD development and progression, including Gram-negative bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus* spp., *Fusobacterium* spp., *Veillonella* spp., *Prevotella* spp., *Porphyromonas* spp., *Capnocytophaga* spp. and *Actinobacillus actinomycetemcomitans*, and Gram-positive bacteria such as streptococci, staphylococci, *Corynebacterium* spp., *Actinomyces* spp., *Bacillus* spp., *Peptostreptococcus* spp., *Eubacterium* spp., *Propionibacterium* spp. and *Lactobacillus* spp. [7]. *Enterococcus* spp., known to be natural inhabitants of the gastrointestinal tract of humans, mammals and birds, are also frequently found in the canine oral environment [8], being increasingly associated with severe diseases in human and veterinary medicine, including bacteremia, meningitis, infective endocarditis, intra-abdominal, wound, urinary tract and healthcare associated infections [9,10]. Enterococci are also known to be intrinsically resistant to several antimicrobials, having the ability to acquire additional antibiotic resistances via genetic mobile elements [10,11], constituting a major challenge for effective antimicrobial therapy. Additionally, several virulence factors, responsible for the establishment and/or severity of enterococci-related infections, have been described over the years [12,13].

The close contact between animals, as well as amongst pets and owners, associated with the scarce available data regarding the role of enterococci in dogs' PD, led the present study. Hence, enterococcal isolates obtained from gum swabs of dogs diagnosed with PD were screened for the presence of antibiotic resistance, virulence factors and biofilm-forming ability.

2. Materials and methods

2.1. Sampling and enterococcal isolation

Samples were collected at a private veterinary hospital located in Cascais, Portugal, from 31 dogs (17 males and 14 females, ages between 7 and 17 years). After the detailed observation of the oral cavity, to allow classifying the stage of PD, swab sampling was performed, including gingivitis and periodontitis lesions.

Swabs with Stuart transport medium (Deltalab®) were used to collect samples from the gums of each animal, stored at 4 °C and immediately transported to the Laboratory of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Lisbon, Portugal. After inoculation on Slanetz and Bartley Agar – SBA – (Scharlau, Barcelona, Spain) and incubation under aerobic conditions at 42 °C for 48 h, up to four typical colonies were randomly collected from each plate, transferred individually to new SBA plates and further purified by four steps of sequential streaking. Pure cultures were characterized regarding Gram staining, cell morphology, catalase and oxidase reactions and the ability to grow and hydrolyze esculine in Bile Esculin Azide Agar – BEAA – (Scharlau, Barcelona, Spain). Gram positive, catalase/oxidase negative, esculine hydrolyzers were stored at −80 °C in Brain Heart Infusion – BHI – broth (Scharlau, Barcelona, Spain) containing 20% (v/v) glycerol. For routine use, enterococcal isolates were cultivated on BHI broth or plates, overnight at 37 °C.

2.2. Genus and species allocation

For confirmation of genus and species allocation previously described methodologies were applied [14,15]. Primers and reagents used for PCR amplifications were purchased from NZYTech Lda (Lisbon, Portugal) and positive and negative controls were included at all times.

2.3. Genomic diversity

To assess for the diversity of the enterococcal isolates, bacterial typing was achieved using the primers OPC19 and $(GTG)_5$ in independent reactions, as described elsewhere [16]. The reproducibility level was determined by analyzing a random sample of 10% duplicates.

The BioNumerics software (version 6.6.2, Applied Maths, Kortrijk, Belgium) was used to register fingerprinting patterns, normalize densitometric traces, calculate Pearson product-moment correlation coefficient and perform cluster analysis by the UPGMA algorithm. Analysis of the genomic diversity of the dog-related enterococci was also used to allow the selection of representative strains. The genomic relatedness between representative isolates of the microbial collection was further assessed by *Sma*I-macrorestriction analysis using Pulsed-Field Gel Electrophoresis – PFGE – as previously reported [17].

2.4. Antibiotic susceptibility

Nine antimicrobial agents (Oxoid Limited, Cambrige, United Kingdom) were studied: amoxicillin/clavulanate 30 μg, ampicillin 10 μg, chloramphenicol 30 μg, erythromycin 15 μg, gentamicin 120 μg, imipenem 10 μg, streptomycin 300 μg, tetracycline 30 μg and vancomycin 30 μg. Susceptibility was evaluated by the disk diffusion method, using breakpoints of resistance previously established [18]. Mueller-Hinton medium (BD Difco) was used for antibiotic susceptibility assays, followed by 24 h of growth at 37 °C. For quality control the reference strains *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212 were also included in the study.

2.5. Virulence factors

Production of hemolysin and gelatinase was detected as described by Semedo et al. [19]. Briefly, for gelatinase activity 3% gelatin medium was used, followed by incubation for 48 h at 37 °C, gelatinolytic enterococci were recognized by the development of a transparent halo around the colonies. Hemolysin production was observed after streaking on Columbia agar supplemented with 5% horse blood (BD Difco), followed by incubation at 37 °C for 72 h on anaerobic conditions. Screening for virulence genes performed by PCR amplification using primers and conditions previously reported [20,21], specifically genes coding for aggregation substance—*agg*, *E. faecalis*—*efaAfs* and *Enterococcus faecium* antigen A—*efaAfm*, enterococcal surface protein—*esp*, pili-like—*ebpABC*, gelatinase—*gelE*, adhesin of collagen from *E. faecalis*—*ace* and *E. faecium*—*acm*, cytolysin activator—*cylA*. *E. faecalis* MMH594 was used as positive control for all virulence assays.

2.6. Biofilm-forming ability

Assays were performed using the microtiter biofilm assay already described [22], with minimum modifications. Briefly, flat-bottom polystyrene microtiter plates (Orange Scientific, Belgium) containing 200 μl of bacterial suspensions in Tryptone Soya Broth (TSB) (Scharlau, Spain) supplemented with 5% glucose, at a final concentration of 5×10^5 CFU/ml, were incubated in a humid chamber at 37 °C for 48 h. Biomass quantification was performed after staining with crystal violet 0.1% and evaluation of the corresponding optical density (OD) values at 570 nm using a microplate reader (OPTIMA FLUOstar, BMG Labtech). OD cut-off (ODc) was defined as three standard deviations above the mean OD of the negative control and isolates were classified as non-biofilm producers if $OD \leq ODc$; as weak biofilm producers if $ODc < OD \leq 2 \times ODc$; as moderate biofilm producers if $2 \times ODc < OD \leq 4 \times ODc$; and as