
Cultivable Microbiota in Mitis Salivarius Agar from Dental Plaque of Dogs

Fábio Alessandro Pieri^{1,*}, Vitor de Oliveira Silva², Abelardo Silva Junior²,
Maria Aparecida Scatamburlo Moreira²

¹Basic Life Sciences Department, Life Sciences Institute, Federal University of Juiz de Fora, Governador Valadares, Brazil

²Veterinary Department, Health and Biological Sciences Center, Federal University of Viçosa, Viçosa, Brazil

Email address:

fabio.pieri@ufjf.edu.br (F. A. Pieri)

*Corresponding author

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Abstract: Periodontal disease, which affects the support structures and protection of teeth, is the main disease found in dogs, reaching about 85% prevalence in animals over 4 years old. The most important bacteria in the initial colonization of human dental plaque are *Streptococcus*, which produce exopolysaccharides that facilitate fixation of the bacteria to the surfaces. Some studies have sought to identify the dental plaque microbiota in dogs using non-selective culture media, and have reported small amounts of *Streptococcus*, however they did not provide ideal conditions for the growth of this bacteria. The aim of this study was to isolate and identify the initial dental plaque microbiota of dogs cultivable in a selective medium for *Streptococcus*, and verify the presence of this genus. Genotypic identification of the isolates were carried out by partial 16S rDNA sequencing. A total of 179 strains were obtained from the plaque of 10 dogs without periodontal disease. The technique employed allowed 15 different microbial groups to be isolated, including the genus *Streptococcus*. This represented 33 (18.4%) of 179 isolates, thus illustrating the partially selective ability of Mitis Salivarius Agar for samples of dental plaque in dogs.

Keywords: Dogs, Oral Microbiology, Dental Plaque, Microbiota, Bacteria, 16S rDNA

1. Introduction

Periodontal disease, which affects the support structures and protection of teeth, is a disease that is the mostly found in dogs, reaching about 85% prevalence in animals over the age of 4 years [1]. The etiologic agent of this disease is the bacterial plaque which develops on the tooth surface and the of immune system's reaction to these agents. Plaque builds up with the initial colonization of the tooth surface by indigenous bacteria, and promotes the adhesion of other, more virulent bacteria [2].

In humans, the microbial profile of plaque is been defined. It is formed predominantly by Gram-positive, aerobic, non-motile and anaerobic bacteria in the early stages of infection [3, 4], but by Gram negative and mobile bacteria in the later stages of infection and inferred that the same microbial profile occurs in canine plaque [5].

The most important bacteria in the initial colonization of human dental plaque are *Streptococcus* [3], which produce exopolysaccharides that facilitate fixation of the bacteria to the surfaces, especially in places where minor irregularities occur, such as fissures or roughness [5]. Some studies have sought to identify the dental plaque microbiota in dogs using non-selective culture media, and have reported small amounts of *Streptococcus* [6-8]. However, since special conditions are required to isolate this kind of bacteria from samples with a diverse microbiota, its possible that its growth was inhibited by other bacteria. Other work presented low presence of *Streptococcus* spp. in enamel surface of dogs, performing the identification without culture techniques, using pyrosequencing of 16S rDNA, and presented bacteria of *Neisseria*, *Moraxella* and *Bergeyella* species as the main

bacterial groups in this environment [9]. However in human studies is presented the need to use a selective medium such as Mitis Salivarius Agar to obtain higher presence of *Streptococcus* spp., from dental plaque samples [10].

Thus, the importance of the genus *Streptococcus* in the composition of canine dental plaque remains unknown. Such knowledge is essential to determining therapeutic procedures for the treatment or prevention of periodontal disease in dogs. The aim of this study was, therefore, to isolate and identify the initial dental plaque microbiota of dogs cultivable in a selective medium for *Streptococcus* and to verify the presence of this genus in the samples.

2. Materials and Methods

2.1. Animals and Sampling

Bacterial isolates were obtained from the supragingival plaque of 10 mongrel dogs aged between 1 and 2 years, without clinical signs of periodontal disease, periodontal pockets, gingival swelling or redness. All the animals were from the experimental kennels of the Department of Veterinary Medicine or treated at the Veterinary Hospital of the Federal University of Viçosa. All procedures complied with the rules established by the Ethics Committee in the use of animals at the Federal University of Viçosa, and were approved by the same protocol under No. 18/2010.

2.2. Sample Processing

The collection of dental plaque was performed using a modified version of the method described by [8]. Plaque was obtained from the fourth premolars of 10 dogs by friction, with the aid of sterile swabs. Following collection, the swabs were transferred to tubes containing a solution of Tris-HCl, pH 7.2. Glass beads were introduced and the tubes were placed in a vortex model MS1 minishaker (IKA Laboratory Equipment, Artur Nogueira, Brazil) at 2200 rpm for 2 minutes.

Suspensions were serially diluted on a decimal scale to 1:10000. All were inoculated on the surface of plates containing 20 ml Mitis Salivarius Agar (Himedia, Mumbai, India) and potassium tellurite 1%, prior to incubation at 37°C for 72 hours in microaerophilic environment, in order to provide optimal conditions for the isolation of *Streptococcus* sp. using BBL GasPak Plus Anaerobic System Envelopes with Palladium Catalyst kit (BD Biosciences, New Jersey, USA).

After incubation, colonies with different morphological characteristics were selected and streaked on Brain Heart Infusion agar (BHI) (Himedia, Mumbai, India), after which they were again incubated, this time at 37°C for 24 hours, in order to obtain pure cultures. Following purification, one colony from each culture was transferred to a tube containing BHI and once again incubated for 24 hours at 37°C, at which point glycerol (20%v/v) was added and the cultures were frozen in liquid nitrogen and maintained at -80°C.

To subsequently activate the cultures, a 10 µL aliquot of each stock was transferred to a BHI broth and incubated at 37°C for 24 hours.

2.3. Phenotypic Determinations

Upon reactivation in BHI broth, the isolates were subjected to Gram staining and tested for catalase production as described by American Society for Microbiology [11, 12].

2.4. Polymerase Chain Reaction – PCR

DNA was extracted using a Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, USA) according to the manufacturer's protocol. DNA extraction was performed using 1.5 mL of the activated culture. The DNA was purified using a Genomic DNA Kit™ GeneJET Purification (Fermentas International Inc., Maryland, USA) according to the manufacturer's protocol, eluted with 70 µL of water and stored at -20°C. DNA from *Streptococcus salivarius* CDC 262 was used as a positive control in the PCR and Nuclease-Free Water (Promega Corporation, Madison, USA) was used as a negative control.

Genotypic identification of the isolates was carried out by partial 16S rDNA sequencing, using the primer pair 616V (5'-AGAGTTTGATYMTGGCTCA-3') and 630R (5'-AAGGAGGTGATCCARCC-3'), binding at positions 11–26 and 1525–1541 of the 16S rDNA, respectively [13], in order to produce an amplicon of approximately 1530 base pairs. The primers were commercially synthesized by Integrated DNA Technologies Inc. (Iowa, USA). Polymerase chain reaction (PCR) was performed using PCR Master Mix (Promega Corporation, Madison, USA) according to the protocol used by Sterr *et al.* [13]. The PCR was conducted in a thermal cycler (Axygen Scientific, California, USA). The amplified fragments were visualized by electrophoresis on 1% agarose gel (Invitrogen) using GelRed™ Nucleic Acid Stain (6X) (Biotium Inc., California, USA) and the UV image capturing system in L-PIX HE (Loccus Biotecnologia, São Paulo, Brazil).

2.5. Sequencing and Data Analysis

Amplified fragments of expected size, together with the positive control, were sent for sequencing by the Macrogen Company (in Korea), which used the method previously described by Sanger *et al.* [14].

The sequences obtained were edited using Sequencher software (version 4.1.4). All isolates were initially identified by performing database searches, comparing 16S rDNA sequences obtained from a single reading with other related sequences in GenBank, using the Basic Local Alignment Software Search Tool (BLAST), available from the National Center for Biotechnology Information - NCBI (<http://www.ncbi.nlm.nih.gov>). Multiple alignments were obtained using Clustal W2 software (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>), from which a phylogenetic tree was also constructed using Molecular Evolutionary Genetics Analysis (MEGA) software (version 4.0 for Windows; www.megasoftware.net) by means of the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Statistical analysis was performed using 2000 bootstrap replicates. We used a sequence of *Sulfolobus* sp. [GenBank: FJ489515.1] 16S rDNA as the outgroup. A

difference of 3% or greater was taken to indicate that the sequences were probably from distinct species [15].

2.6. Nucleotide Sequence Accession Numbers

The nucleotide sequences determined in this study have been deposited in GenBank [GenBank: HQ717175 to HQ717353].

3. Results

A total of 179 strains were obtained from the plaque of 10

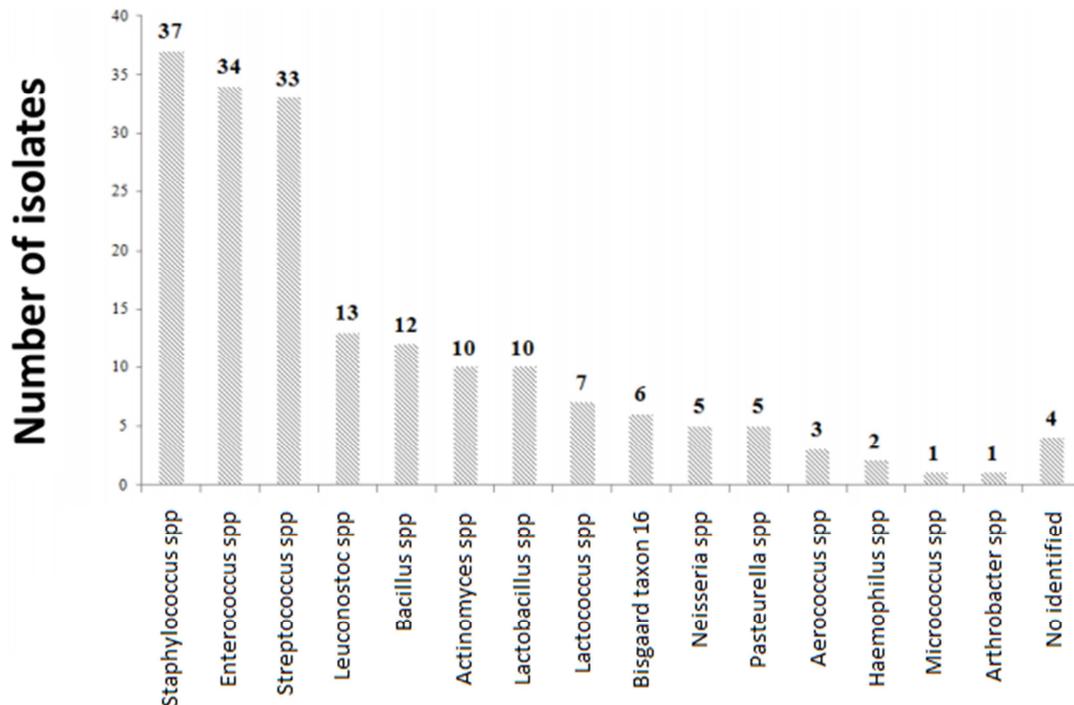


Figure 1. Identification to genera level of the 179 isolates obtained from the dental plaque of dogs based on sequencing of the 16S rDNA fragment.

Despite the targeting of the isolation technique for the genus *Streptococcus*, the bacterial diversity found in this study indicated a total of 14 genera belonging to three phyla. The genera *Aerococcus*, *Bacillus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Enterococcus*, *Staphylococcus* and *Streptococcus* represented the phylum Firmicutes (low G+C, Gram-positive), *Actinomyces*, *Arthrobacter* and *Micrococcus* represented the phylum Actinobacteria (high G+C, Gram-positive), and *Neisseria*, *Haemophilus* and *Pasteurella* represented the phylum Proteobacteria (Gram-negative). A total of six isolates of the bacteria known as Bisgaard taxon 16 [16] were also found in two dogs.

The bacterial profile constructed from the dental plaque isolates obtained from the dogs is shown as a phylogenetic tree (Figure 2). *Streptococcus* sp., 33/179 (18.4%), *Staphylococcus* sp. 37/179 (20.7%) and *Enterococcus* sp., 34/179 (19%) were the prevalent genera in the dental plaque stages (Figure 1). These genera were found in 9/10 (90%), 8/10 (80%) and 5/10 (50%) of the dogs respectively, representing 105/179 (58.6%) of the total number of isolates.

dogs without periodontal disease (Figure 1). As expected, Gram staining indicated 90% positive cocci and rods, representing 164 isolates. These were 66% catalase negative. The 179 isolates had fragments of 16S rDNA amplified and sequenced. The sequences obtained were deposited in GenBank (Supplementary Material 1 and 2) and identified by comparison with other gene sequences from the same database. Of the 179 isolates sequenced, 46 were identified to species level, while the other 133 were not (data not shown).

4. Discussion

The technique employed in this study not only enabled the isolation of bacteria of the genus *Streptococcus*, but also other bacteria (Figure 1), including rods and Gram-negative, demonstrating the partially selective capability of the Mitis Salivarius Agar. Among the main genera isolated, *Streptococcus* was the third most common, surpassed by both *Staphylococcus* and *Enterococcus* (Figure 1).

Elliot *et al.* [6] identified the microbiota in the subgingival plaque of 10 dogs, with *Streptococcus* sp. representing only 1% of the total. Those investigations, however, utilized plates of a different nature and different culture media from those employed in the present study. From a total of 32 isolates obtained from dogs without gum disease using non-selective culture media, Riggio *et al.* [8] identified only one isolate of *Streptococcus minor*, which was also identified in this study, as well as in other studies examining plaque from dogs and cats [6, 17, 18].

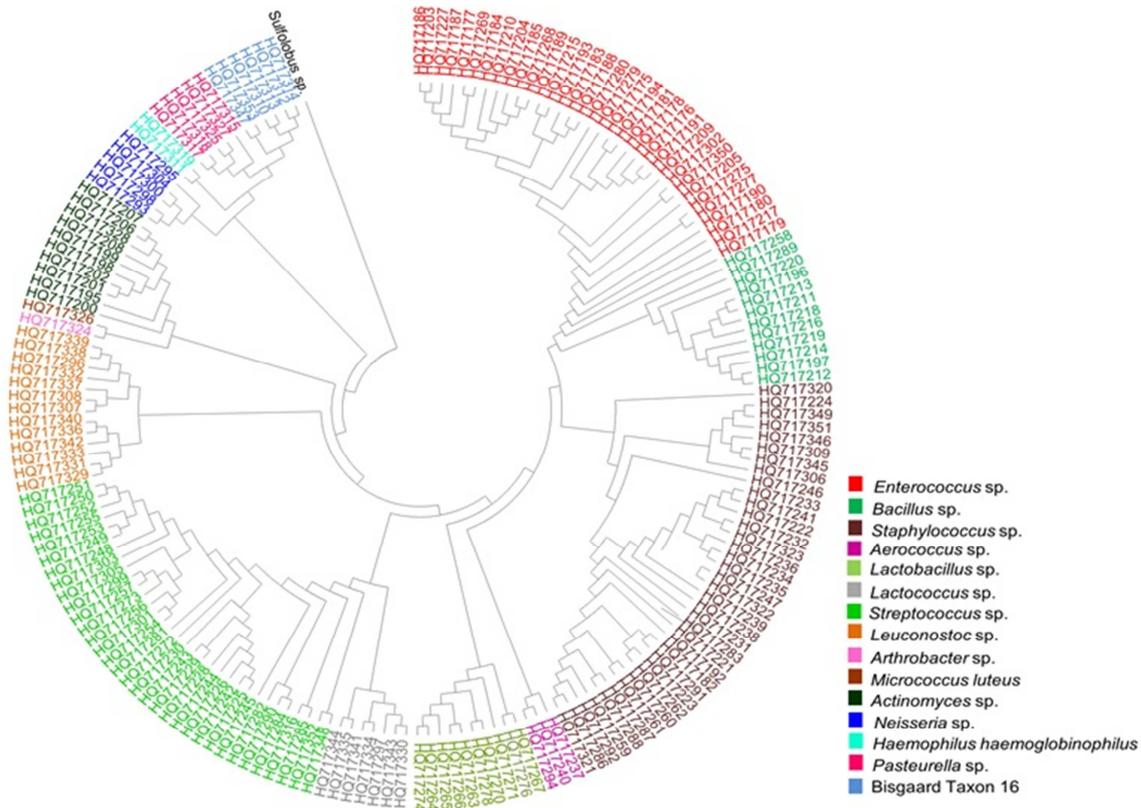


Figure 2. Phylogenetic Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrogram showing all isolates for which sequences were obtained from ten dogs aged between one and two years identified by 16S rDNA sequencing. Sequences are identified by their access numbers in GenBank. 179 sequences are included in the tree.

Takada *et al.* [10] were able to isolate *Streptococcus* using Mitis Salivarius Agar with supplements, with a recovery rate of 21.2% of all isolates. They also reported that the medium removed 89.7% of total bacteria grown in BHI from the same blood sample. The main species identified by Takada *et al.* [10] were *S. mitis* and *S. sanguis*, demonstrating the bias of this medium towards the growth of *Streptococcus*.

It is noteworthy that none of the above studies was able to isolate *S. mutans*, which is considered the most important bacteria in human plaque formation. This corroborates the observation of Dent and Marsh [19] that there is a correlation between the presence of *S. mutans* and diets rich in sucrose, which is not the case with dogs. Takada *et al.* [10] suggest that the different pH of a dog's oral cavity, compared to that of a human, may be a factor responsible for the absence of this type bacteria in these animals.

Studies on the microbiota of supragingival plaque from beagle dogs, assessed using DNA–DNA hybridization, identified several *Streptococcus* species, including some associated with dental plaque microbiota in humans, such as *S. mitis*, *S. constellatus*, *S. oralis* and *S. sanguis* [20]. These species were not isolated in the current study, since the sequences of *Streptococcus* sp. obtained showed no similarity with the examples of these species deposited in GenBank. Controversially, Holcombe *et al.* [9] did not present significant presence of *Streptococcus* spp., in enamel dental plaques of 12 dogs with initial dental plaque formation

between 24 and 48 hours after dental professional cleaning, which could suggest that the presence of this bacterial group could be increased just after later events in the development of dental plaque in animals.

The difficulty in identifying bacteria to the species level, especially when dealing with the genera *Staphylococcus*, *Streptococcus* and *Enterococcus*, can be because closely related species may have virtually identical 16S rDNA gene sequences. Some species of *Streptococcus* group D, for example, share a 99% similarity in their 16S rDNA sequence [21].

Bacteria of the genera *Actinomyces*, *Porphyromonas*, *Fusobacterium*, *Neisseria*, *Staphylococcus*, *Streptococcus* and *Veillonella* were isolated in most samples analyzed by Dent and Marsh [19], Kim *et al.* [22], Zambori *et al.* [23] and Jeussete *et al.* [24], suggesting that representatives of these genera can be considered as characteristic or indigenous members of the microbial community in dental plaque. This was confirmed in the present study, which verified the presence of the genera *Staphylococcus*, *Streptococcus*, *Actinomyces* and *Neisseria* (as shown in Figure 1). As expected, the genera *Porphyromonas*, *Fusobacterium* and *Veillonella*, found in other works [19, 22, 23, 24] were not isolated as result of the methodology applied and the nutritional and strict anaerobic needs of these bacteria.

Study by Nowroozilarki *et al.* [25], presented *Helicobacter* spp. and *Wolinella* spp., in oral cavities of toy breed dogs,

different of our findings. However, the dog population studied differ from that used in our work. In that work the aim was to study dogs older from six years with periodontal disease, meanwhile in the present study were used young dogs without oral diseases, suggesting that the inclusion of that genera [25] occur in a new environment provided in oral dental plaque of older dogs by the evolution of that specific oral disease.

It is suggested that Bisgaard taxon 16 [16], that has not yet formally referred in the bacterial taxonomy, it is part of oral microflora of dogs, since the few reports of them were obtained from this site [6, 16] and in this study, it was obtained from two different dogs.

The sequence analyses and literature review indicated that 59/179 (33%) of the bacteria isolated from dogs in this study are not normally found in human oral cavities [26, 27]. Only 5/15 (33.3%) of the identified groups (14 genera plus Bisgaard taxon 16), namely *Actinomyces*, *Neisseria*, *Haemophilus*, *Streptococcus* and *Lactobacillus*, are considered members of human dental supragingival plaque [22, 23].

Recent works [23, 28-30] presented the sensitivity profile of some isolates presented in this work when challenged by natural compounds from vegetable and other bacteria, with important findings in the study of dental plaque control in dogs. These studies have to be conducted to confirm these alternative therapeutic drugs to replace chlorhexidine use, that presents a set of side effects on its long-term use [5].

5. Conclusions

It follows that the technique employed in this study allowed 15 different microbial groups to be isolated, including the genus *Streptococcus*, which represented 18.4% of 179 isolates. This demonstrates the partially selective ability of Mitis Salivarius Agar when analyzing samples of dental plaque from dogs. In addition the results indicated that the genus *Streptococcus* spp. bacteria can be present in higher levels in dogs dental plaque environment, particularly those included in group D streptococci.

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