Biofilm formation of *Staphylococcus*, *Streptococcus*, *Pasteurella* and *Neisseria* strains

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Abstract

The “in vitro” biofilm formation capacity of planktonic bacterial strains with zoonotic potential was tested on *Staphylococcus*, *Streptococcus*, *Neisseria* and *Pasteurella* strains from supragingival sites from dogs with dental disease, different breeds, age, sex, weight and diet. There were isolated and identified 75 strains, 24 belonging to the genus *Pasteurella*, ten to *Staphylococcus* genus, 28 to *Streptococcus* genus and 13 to *Neisseria* genus. After isolation and identification, these strains were tested for the in vitro biofilm formation capacity by microtitre plate assay. Results showed that all *Staphylococcus*, *Streptococcus*, *Pasteurella* and *Neisseria* species had the capacity to form biofilm in vitro, with higher or lower density. It was seen that there are interspecific and intraspecific differences between the ability of biofilm formation of these bacteria. The large frequency of the four bacterial genera from the oral cavity of all studied dogs emphasize the importance of knowing the frequency of isolation from the oral cavity and the presence of these microorganisms in association with dog bite wounds in humans.

Key words: bacteria, dental plaque, planktonic, supragingival, zoonotic

Introduction

The presence of pathogenic microorganisms in the oral cavity is one of the main causes of local and systemic diseases. Thus, the majority of biofilm forming bacteria are able to detach from substrate to which they adhere, to migrate and create favorable conditions for the formation of new microbial groups through the resumption of the initial cycle (J.G. THOMAS & al. [1], M. DECUN & al. [2], J. WIMPENNY [3], J.W. COSTERTON & al. [4]). Supragingival dental plaque (biofilm) is the main cause of subgingival dental plaque accumulation and growth. Accumulation of pathogenic microorganisms to the surface of teeth comprises the steps of dental biofilm formation, which can lead to periodontal diseases and dental caries (C. ZAMBORI & al. [5]). Supragingival dental plaque also provides protection for the bacteria involved in the formation of subgingival dental plaque by decreasing oxygen levels in deep areas of the matrix. Therefore, the primary colonizers of the dental plaque are one of the main
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causes of the outbreak of diseases in the oral cavity, of diseases with systemic evolution and of zoonosis (C. ZAMBORI & al. [6]). The initially attached aerobic bacteria (primary colonizers) consume oxygen, so the potential redox decreases and creates optimal conditions for the growth of anaerobic bacteria. The number of aerobic bacteria does not decrease, but will increase the number of anaerobic bacteria that will change the anaerobic/aerobic bacteria ratio, in favor of the latter (C. GORELL [7], S.L. PERCIVAL & al. [8], D.W. WILLIAMS & al. [9]). What seems to be important is their role as causative agents of disease that can be transmitted from animals to humans. Recently, it was found that microorganisms isolated from the oral cavity (dental plaque) are a key factor in delaying the healing process of wounds. The most common species isolated from human infected dog bite wounds were species of the genus *Pasteurella* (50%), *Streptococcus* (46%), *Staphylococcus*, and *Neisseria* (32%) (D.A. TALAN & al. [10]).

The need of research on the role of microbial biofilm on the evolution of oral cavity diseases is based on the existence of several factors. First of all is that biofilms are very difficult to treat with antimicrobial substances (T.F. MAF & G.A. O’TOOLE [11]). Biofilms increase the capacity of gene transfer between bacteria (bacteria that are resistant to antimicrobial substances can transfer some resistant genes to adjacent sensitive bacteria), thus, by gene transfer, a non-virulent bacteria can become powerful virulent. Biofilm forming bacteria are protected from the natural factors of body defense due to the biofilm structure (P. WILLIAMS [12]). The immune system response occurs only toward the surface of its antigens, and the action of antibodies, other serum and salivary proteins are reduced or absent (K.C. MARSHALL [13]). There are certain bacterial species which communicate between themselves at the level of biofilms, once their density increase, they secrete certain substances with low molecular weight and transmit signals when the bacterial population has reached a certain threshold (D.G. Davie & al. [14]). This process is called *quorum sensing* (QS) and is responsible for the virulence of bacteria (M.E. SHIRTLIFF & al [15], A. COTAR & al. [16], M. SHADABA & M.O. STEVEN, [17]). The biofilm forming bacteria have the ability to express some virulent phenotypes, which were not able to be discovered in the past because the bacteria were grown on nutrient-rich environments, under specific planktonic conditions, while in biofilms, growing conditions are different (nutrients and oxygen are limited). The interest of companion animals owners and their knowledge about the importance of oral cavity hygiene in dogs is still limited, thus lately veterinarians are interested on this topic that has a great importance in veterinary and human medicine. The aim of the present research is to broaden scientific knowledge of veterinary and human pathology, given the fact that it shows the role of microbial biofilm in oral cavity of dogs, an irreplaceable companion animal in human’s life. Thus, we have been pursued to highlight the role of microbial biofilm in oral pathogenesis by the establishment of *in vitro* biofilm formation of planktonic supragingival strains of *Staphylococcus, Streptococcus, Pasteurella* and *Neisseria*.

**Materials and methods**

*Isolation and identification of bacterial genus*

The research was conducted on 75 bacterial strains collected from 33 dogs with dental disease, different breeds, age, sex, weight and diet. Samples were taken from 19 different breeds: Metis, Hungarian Vizsla, German Shepherd, Coker Spaniel, Pulli, Cane Corso, Westhighland Terrier, Pekingese, Golden Retriever, Bernese Mountain Dog, Chow-Chow, Bullmastiff, French Bulldog, Boxer, Bichon Havanese, Shar Pei, Fox Terrier, Akita Inu and Dog de Bordeaux. The age of the dogs was between 1-15 years from which 50% females, 50% males with variable weight between 4-60 kg. The nutrition was based on homemade and dog
special diet. The study was performed at the Small Animal Polyclinic at the Budapest “Szént Istvan” University, Faculty of Veterinary Medicine.

Sampling has been carried out from supragingival sites mainly from incisive, canines, premolars and superior molars in dogs under anesthesia with or without intubation. Anesthesia was done according to the animal’s weight with: Dormitum, Calysol, Fentanyl, Propofol. All the owners were informed about the risks of anesthesia and procedures to be performed. The owners received and signed a surgical/anesthetic informed consent form.

Before sampling, in order to highlight recent and most mature dental plaque it was used – Miradent (Mira 2 - Ton, HAGER PHARMA GmbH), (10 ml containing water, sodium benzoate, potassium sorbate, CI 45410, CI 42090), a dental dye which revealed young plaque in pink and mature plaque in dark blue. Teeth were washed with sterile saline water to highlight young and mature plaque and samples were placed in a transport medium.

Subsequently, after harvesting, samples were prepared for microbiological examination. Initially, the collected samples were inoculated on blood agar plates (sheep blood) for Staphylococcus, Pasteurella, Neisseria and on Edwards (OXOID) selective medium for Streptococcus, for the purpose of carrying out cultural and biochemical examinations to confirm bacterial genus. For identification of Staphylococcus and Streptococcus species there were used the catalase and oxidase test. In order to identify the strains of Pasteurella and Neisseria there were used the biochemical tests for catalase, oxidase, urease, nitrate, indole and sulfide hydrogen.

After isolation, the 75 bacterial strains were stored in the freezer in cryotubes in BHI broth (Brain-Heart Infusion Broth, OXOID) and glycerol, at - 50 ºC for further processing.

**Biofilm formation**

The in vitro biofilm formation capacity was done using the microtiter plate technique, described by Djordjecvic et al. (D. DJORDJEVIC & al. [18]). Briefly, the isolated bacterial strains were cultured on BHI agar and were used to make an inoculum that matches 0.5 McFarland standards. This solution was then diluted 1:30 in BHI broth growth medium. After these several dilutions, from each strain 150 µl was added to each well, in 8 wells per strain. The microtiter plates were incubated at 37 °C, for 72 h. After the incubation time the broth was removed from each well and the wells were washed twice with 160 µl 0.9% saline solution, in order to remove planktonic cells. Crystal violet staining was performed adding 160 µl crystal violet 0.1% solution in each well and incubating the plates for 10 minutes at room temperature, then the stain was removed and the wells were washed twice with 170 µl of saline solution 0.9%. Ethanol 96% was added (170 µl) to each well for destaining, for 30 minutes, and then the OD (optical density) was measured at 540 nm using an ELISA reader.

Biofilm growth was carried out separately, in four trials for each bacterial genus, and experiments were repeated twice in order to obtain, the most accurate data.

After the quantification of the densest biofilm formed by the strains of Staphylococcus, Streptococcus, Pasteurella and Neisseria the intraspecific and interspecific differences of the ability of these strains to form biofilm was evaluated.

**Results**

Miradent staining showed that all dogs subjected to this procedure had dental plaque of some degree (Fig 1). All the dogs taken into study showed supragingival dental plaque and only 48.48% developed subgingival dental plaque.
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![Figure 1. Dental plaque staining with Miradent dye](https://example.com/image)

After sampling and cultivation, 75 strains were isolated from 33 dogs subjected to this study. From all 75 isolated bacterial strains, 28 were of \textit{Streptococcus} genus (84.85%), 24 of \textit{Pasteurella} genus (72.73%), 13 of \textit{Neisseria} genus (39.39%) and 10 of \textit{Staphylococcus} genus (30.30%) (Table 1).

<table>
<thead>
<tr>
<th>Number of dogs</th>
<th>Bacterial strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pasteurella</td>
</tr>
<tr>
<td>Akita Inu</td>
<td>1</td>
</tr>
<tr>
<td>Bichon Havanese</td>
<td>1</td>
</tr>
<tr>
<td>Vizsla Dog</td>
<td>4</td>
</tr>
<tr>
<td>Pulli</td>
<td>2</td>
</tr>
<tr>
<td>Metis</td>
<td>5</td>
</tr>
<tr>
<td>German Shepherd</td>
<td>2</td>
</tr>
<tr>
<td>Cocker Spaniel</td>
<td>2</td>
</tr>
<tr>
<td>Bernese Dog</td>
<td>1</td>
</tr>
<tr>
<td>French Bulldog</td>
<td>1</td>
</tr>
<tr>
<td>Golden Retriever</td>
<td>2</td>
</tr>
<tr>
<td>Cane Corso</td>
<td>2</td>
</tr>
<tr>
<td>Dog de Bordeaux</td>
<td>1</td>
</tr>
<tr>
<td>Chow-Chow</td>
<td>1</td>
</tr>
<tr>
<td>Bullmastiff</td>
<td>1</td>
</tr>
<tr>
<td>Fox Terrier</td>
<td>1</td>
</tr>
<tr>
<td>Westhighland Terrier</td>
<td>2</td>
</tr>
<tr>
<td>Boxer</td>
<td>1</td>
</tr>
<tr>
<td>Pekingese</td>
<td>2</td>
</tr>
<tr>
<td>Shar Pei</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>33</td>
</tr>
</tbody>
</table>

**Table 1. \textit{Staphylococcus}, \textit{Streptococcus}, \textit{Pasteurella} and \textit{Neisseria} strains isolated from oral cavity of different dog breeds**

Quantification of biofilm formed by the isolated strains

After measuring the optical density (OD) at 540 nm with ELISA reader it was found that all the strains belonging to the genus \textit{Staphylococcus} isolated from the supragingival, have the
ability to form biofilm. It was found that four of the ten isolates, formed the densest biofilm and for three of all ten isolates (C19a, C20a, C32c) the optical density exceeded the value 1.

Although the strains of the genus *Streptococcus* were more frequently isolated from the oral cavity of dogs in the study (28 strains), compared to the strains of the genus *Staphylococcus*, *Streptococcus* strains formed less dense biofilm (Fig 2).

![Figure 2. Biofilm formation of *Streptococcus* and *Staphylococcus* strains, optical density measured at 560 nm](image2.jpg)

*Pasteurella* strength and its ability to form biofilms are intensively studied because of its zoonotic potential or transmission of *pasteurellosis* to humans, by dog bites.

All of the 24 isolates harvested from supragingival sites formed biofilm, of which six developed dense biofilm and for three strains the optical density was above the value 1 (Fig 3).

*Neisseria* are frequently isolated bacteria from the oral cavity and are considered important commensal bacteria of the oral cavity, which can cause serious oral ecosystem imbalance and health problems in dogs and humans. In our study all of the 13 strains, isolated
from the genus *Neisseria* formed biofilm, of which six formed dense biofilm (C7b, C6b, C8b, C11a, C13a, C32b) and only one strain (C7b) had the optical density more than 1 (Fig 3).

In order to assess which of the four bacterial strains of *Staphylococcus*, *Streptococcus*, *Pasteurella* and *Neisseria*, formed the densest biofilm, we have calculated the average growth and average standard deviation for each gender separately (Fig 4).

![Figure 4. Average biofilm forming capacity of Staphylococcus, Streptococcus, Pasteurella and Neisseria strains isolated from dental plaque in dogs](image)

It was found that the strains of the genus *Staphylococcus* formed the densest biofilm, followed by strains of the genus *Neisseria* and the strains of the genus *Pasteurella*, and finally the strains of the genus *Streptococcus*.

**Discussions**

The four bacterial strains were chosen for isolation and identification because of their importance in the transmission of zoonosis from dog bites to humans. D.A. TALAN & al. [10] have shown that aerobic bacteria, found in the oral cavity of dogs, were also isolated from human dog bite wounds.

After sampling it was found that all dogs taken in the present study had dental plaque, the main cause of gingivitis, periodontitis, calculus and tooth decay, thereby it was confirmed the theory supported by S.S. SOCRANSKY & A.D. HAFFAJEE. [19], according to which dental plaque is the main cause for the imbalance between the normal microbial and pathogenic microflora that favors the transition from health to disease (W.J. LOESCHE [20] S.S. SOCRANSKY & A.D. HAFFAJEE [19] C.K. HOPE & M. WILSON [21]).

The presence of dental plaque in all dogs suggests its importance in the formation of microbial biofilm and, thereby, the researches in the last decades had contributed to the recognition of dental plaque as a biofilm, a well organized community, attached to the tooth surface (R. SHANTIPRIYA & al. [22], M.R. WIRTHLIN & al [23]).

Cuesta & al. (A.I. CUESTA & al. [24]) have studied the role of the genus *Staphylococcus* species to form biofilm in the oral cavity, and have found that most of them are resistant to a large number of antimicrobials, because of the ability of resistant genes transfer against the activity of antimicrobial agents.

The results of previous research that show the resistance of bacterial strains of the genus *Staphylococcus*, from the oral cavity of pet carnivores, along with data obtained from other experiments underline two important conclusions. First conclusion is that strains of the genus *Staphylococcus* isolated from the oral cavity are able to form biofilms. The second conclusion
is that the resistance of microorganisms to antimicrobials action increases with creating optimal conditions for the development of pathological processes.

Most of the researches in the last years have suggested the role of streptococci in the initiation of primary stages of biofilm formation in oral cavity. *Streptococcus* species are considered primary colonizers of dental biofilm which contribute, together with other bacterial species to the formation of mature biofilm (C.K. HOPE & M. WILSON [21]). Some results reported by other researchers in the scientific literature state that the strength of *Streptococcus* species from the oral cavity is smaller compared to that of *Staphylococcus* species due to carnivore food that is less fermentable and their salivary's pH is more alkaline (C. STOIAN [25]), which confirmed as well our results.

The above results confirm that *Pasteurella* species are able to form biofilm in vitro. This can be associated with the isolation frequency of the *Pasteurella* species in the oral cavity (20 % in dental plaque), their resistance to the various niches of the oral cavity and the frequency of isolation in wounds after dog bites, which due to some authors can reach 50 % (D.A. TALAN & al. [10]).

In dogs, the researches on pathogenicity of bacteria of the genus *Neisseria* are in constant change. D.R. ELIOTT & al. [26] have highlighted that the frequency of isolation of *Neisseria* species from dental plaque was 10 % of the total strains analyzed and the source of infection in humans was the dog bite. The authors of the above-mentioned confirm the importance of *Neisseria* in the pathogenesis of diseases of the oral cavity and of dental biofilm formation as primary colonizers (B. HOLMES & al. [27]). In humans the common wound infection is with *Neisseria* species after dog bites. The most pathogenic *Neisseria* species isolated from the oral cavity of dogs are *Neisseria animaloris* and *Neisseria zoodegmatis*. These bacteria have been identified by J.B. EUZEBY & F.V. GUÉRIN [28] in 75-80 % of the cases investigated in saliva, gums and nasal passages.

In the present study, despite of the low frequency of isolation of *Neisseria* species in oral cavity of dogs, it was observed that all species formed a dense biofilm, which was much densest than the biofilm formed by *Streptococcus* and *Pasteurella* strains.

**Conclusions**

The research carried out within the framework of this experiment confirmed that there are differences in the ability of the isolated strains from the tooth surfaces to form biofilm in vitro. All isolates had the capacity to form biofilm in vitro with higher or lower density depending on the type of the studied strains.

Although the frequency of isolation of strains belonging to the *Staphylococcus* genus was much smaller than that of the strains of *Streptococcus* genera, they formed the densest biofilm. Despite this the strains of *Streptococcus* genus were the most frequently isolated from the oral cavity of dogs taken into study.

The density of biofilm was higher for the strains of the genus *Neisseria*, followed by those from *Pasteurella* genera. For all species of the genera *Staphylococcus*, *Streptococcus*, *Pasteurella* and *Neisseria* it was highlighted interspecific and intraspecific differences in terms of their ability to form biofilm.

Throughout this study it was noticed that may be a strong relationship between the pathogenic role of microbial biofilm in oral veterinary pathology and direct implications in the evolution of microbial zoonosis regarding the possible maturation and resistance of microbial biofilms under favorable conditions in the oral cavity and the transmission of these resistant microorganisms by dog bites to humans.
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