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Evaluation of *Staphylococcus pseudintermedius* and incidence of *in vitro* biofilm production in healthy dogs and dogs with pyoderma

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Summary

Staphylococcus (S.) pseudintermedius is a regular component of the skin flora in dogs, while also being a skin pathogen and a common cause of pyoderma in dogs. *S. pseudintermedius* is known to produce biofilms but the frequency of biofilms on healthy and diseased canine skin is not well understood. The aim of this study was to compare the presence of *S. pseudintermedius* on the skin of dogs with pyoderma and on dogs without skin problems and to examine whether *S. pseudintermedius* isolated from dogs with and without skin problems differs in its ability to form biofilms *in vitro*. The skin of 102 healthy dogs without skin problems and 107 bacterial samples from dogs with clinical pyoderma were examined for *S. pseudintermedius*. Thirty samples of *S. pseudintermedius* from each group were tested for the ability to produce biofilms with the Congo red agar test. All (107/107) of the dogs in the pyoderma group were positive for *S. pseudintermedius*, whereas only 29 % (30/102) of the dogs in the healthy group were positive. *S. pseudintermedius* is frequently able to form biofilms: in the pyoderma group, 87% (26/30) of the *S. pseudintermedius* samples were

Zusammenfassung

***Staphylococcus pseudintermedius* und seine Fähigkeit, *in vitro* Biofilm zu produzieren, bei gesunden Hunden und Hunden mit Pyodermie**

Einleitung

Staphylococcus (S.) pseudintermedius ist ein natürlich vorkommender Keim der gesunden Hautflora bei Hunden, aber auch ein Erreger und damit ein häufiger Verursacher von Pyodermien bei Hunden. *S. pseudintermedius* hat die Fähigkeit, einen Biofilm zu bilden, die Häufigkeit des Vorkommens von Biofilmen auf gesunder und kranker Hundehaut ist dabei noch nicht erforscht. Diese Informationen würden in der Entwicklung von neuen Behandlungsmethoden oder wirksamen Mitteln gegen Pyodermie von Nutzen sein. Das Ziel dieser Studie war es somit, das Vorkommen von *S. pseudintermedius* auf der gesunden Hundehaut sowie auf der Haut von Hunden mit Pyodermie zu vergleichen, sowie zu untersuchen, ob dieser *in vitro* in der Lage ist, einen Biofilm zu bilden.

Material und Methode

Es wurden Proben von 102 gesunden Hunden ohne klinische Symptome einer Hauterkrankung und 107 Proben von Hunden mit einer klinischen Pyodermie auf das Vorhandensein von *S. pseudintermedius* untersucht. Von jeweils dreißig Proben aus beiden Gruppen wurde weiters auf Biofilm-Produzenten getestet.

Ergebnisse

Alle Hunde in der Pyodermie-Gruppe (107/107) waren positiv für *S. pseudintermedius*, während nur 29% (30/102) der Hunde in der gesunden Gruppe positiv waren. *S. pseudintermedius* ist häufig in der Lage, Biofilme zu bilden: so zeigten in der Pyodermie-Gruppe 87% (26/30) eine positive Reaktion, während 77% (24/30) der Proben von der gesunden Gruppe Biofilm bildeten.

Schlussfolgerung

Auf der gesunden Hundehaut ist der Keim *S. pseudintermedius* weniger häufig nachweisbar als auf infizierter Haut, aber *S. pseudintermedius* von der Hundehaut – unabhängig davon, ob die Hunde gesund sind oder eine Pyodermie aufweisen – ist häufig in der Lage, einen Biofilm *in vitro* zu bilden.

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biofilm producers, while 77% (24/30) of samples from the healthy group produced biofilms. *S. pseudintermedius* is less often detectable on healthy canine skin than on infected skin but *S. pseudintermedius* isolated from the skin of dogs – irrespective of whether the dogs are healthy or have pyoderma – is frequently able to form a biofilm *in vitro*.

Introduction

Biofilms are structured communities of bacteria in a self-produced polymeric matrix that adheres to an inert or living surface (HØIBY et al., 2015). The development of a biofilm allows microorganisms to multiply in an environment in which they are protected from the host immune system (DONLAN and COSTERTON, 2002). Microbial cells in biofilms are more resistant to antibiotics and the host immune response, which increases the difficulties in clinical treatment. Biofilm formation has been documented in human and veterinary medicine as a complicating virulence factor (FIGUEREDO et al., 2012; MOREIRA et al., 2012; NIVEDITHA et al., 2012; SINGH et al, 2013; SWANSON et al., 2014; KÖNIG et al., 2015).

Staphylococcus (S.) pseudintermedius is a normal constituent of the skin flora in dogs, while also being one of the most common causes of skin infections in dogs (BANNOEHR and GUARDABASSI, 2012). However, the frequency of *S. pseudintermedius* on canine skin is not well documented and we know little about the role of this bacterium in biofilm production. We have investigated the occurrence of *S. pseudintermedius* on two sites on the skin of healthy dogs and of dogs with superficial pyoderma. We also evaluated whether the isolated *S. pseudintermedius* is able to form a biofilm *in vitro*.

Materials and Methods

Animals

A total of 209 bacterial samples were evaluated for the presence of *S. pseudintermedius*. Of them, 102 came from clinically healthy dogs (healthy group) with no history of skin problems. They were obtained from the small animal clinic of Dr. Krebitz, Klagenfurt. The remaining 107 samples came from skin swabs from dogs with superficial pyoderma (pyoderma group) and were provided by the laboratory Laboklin.

Sampling

Dry sterile swabs were used for sampling. The swabs were rubbed on the skin, either on the ventral abdomen (n=36) or in the interdigital space (n=66) or on infected skin (n=107), for 10–15 seconds. Swabs in Amies Transport Medium were labelled and stored at room temperature until analysis.

Bacterial isolates

The swabs were directly streaked in three contiguous fractions (streak-plate method) on Columbia blood agar (Becton, Dickinson and Company, Heidelberg, Germany) and CNA agar (Becton, Dickinson and Company, Heidelberg, Germany) and were incubated for 18–24 hours at 36±2 °C. Colonies of *S. pseudintermedius* were isolated and the identification of the bacteria confirmed by MALDI-TOF (Shimadzu, Duisburg, Germany). *S. pseudintermedius* colonies in the first fraction were considered as low amount, those in the second fraction as intermediate amount and those in the third fraction as high amount.

Biofilm growth

Thirty isolates of *S. pseudintermedius* from each group were selected at random and examined for the ability to produce a biofilm *in vitro*, assayed with Congo red agar plates consisting of 37 g/l brain heart infusion broth (Becton, Dickinson and Company, Heidelberg, Germany), 50 g/l sucrose (Merck KGaA, Darmstadt, Germany), 10 g/l agar-agar (AppliChem, Darmstadt, Germany) and 0.8 g/l Congo red (RAL Diagnostics, Martillac, France) according to FREEMAN et al. (1989). Each isolate was inoculated with streaks and dots (5 µl, McFarland 1) on the agar plates for easier interpretation of the colour. *S. aureus* strain ATCC 25923 (biofilm producer) and *S. epidermidis* strain ATCC 12228 (non-biofilm producer) were used as controls. The plates were incubated at 36±2 °C in an aerobic atmosphere. The colour was evaluated after 14, 16, 18 and 20 hours with a final evaluation after 22 hours according to ARCIOLA et al. (2002) and KAISER et al. (2013). Red and very red colonies were considered not to produce biofilms (negative) and brown (weak slime-producing) to dark brown colonies were considered to produce biofilms (positive).

Analysis

The analysis was performed by calculating the percentages and the ratios.

Results

S. pseudintermedius was found in 100% (107/107) of the samples from dogs in the pyoderma group, with 18% (20/107) of the samples in high amounts, 50% (53/107) of them in intermediate amounts, 21% (22/107) in low amounts and 11% (12/107) of the samples only growing following enrichment. However, it was found in only 29% (30/102) of the samples from the healthy dogs. In these dogs *S. pseudintermedius* could be isolated with 0% (0/30) in high amounts, 10% (3/30) in intermediate amounts, 83% (25/30) in low amounts and 7% (2/30) only following enrichment. Within the healthy group, a far greater proportion of dogs had *S. pseudintermedius* in the interdigital space (23/66, or 35% of dogs tested) than in the abdomen (7/36 or 19%).

All of the 102 dogs with healthy skin carried a species-rich flora (not further differentiated), with often only a few colonies of *S. pseudintermedius* found. In contrast, 51% (55/107) of the dogs in the pyoderma group showed a monoinfection with *S. pseudintermedius*. The other 49% (52/107) of the dogs had mixed infections, generally containing one or two, occasionally three, additional species of bacteria, such as *Acinetobacter* spp., *Escherichia coli*, ß-haemolytic streptococci, *Pseudomonas aeruginosa* or *Pantoe agglomerans*.

The large majority (26/30, 87%) of *S. pseudintermedius* isolated from dogs in the pyoderma group was able to produce biofilms. Most of the isolates (23/30, 77%) formed dark brown colonies (strong slime production), while 10% (3/30) produced brown colonies (weakly slime producing). A similarly high proportion (23/30, 77%) of *S. pseudintermedius* isolated from the healthy group produced biofilms: of these, 7/7 were in the abdomen group and 16/23 in the paw group. 57% (17/30) gave dark brown colonies and 20% (6/30) produced brown colonies.

Discussion

Bacterial pyoderma is very common in dogs and superficial pyoderma is generally caused by *S. pseudintermedius*, the main pathogen of the canine skin (BANNOEHR and GUARDABASSI, 2012). The bacterium is normally found on the nose, oropharynx, oral mucosa and anus but can spread to other parts of the body. In most cases of pyoderma, *Staphylococcus* infects the ventral, sparsely haired abdomen (IHRKE, 1987; LLOYD et al., 1991). For this reason we initially elected to sample the ventral abdomen in healthy dogs. Because we only rarely found *S. pseudintermedius* on the abdomen, we started taking samples from the paws. We found more *S. pseudintermedius* on the paws (in the interdigital space) than on the abdomen in healthy dogs. The difference possibly relates to the greater humidity of the skin on the paws. *S. pseudintermedius* was more frequent in samples from infected dogs than on normal healthy dog skin. We could detect *S. pseudintermedius* in all swab samples (n=107) from dogs with clinical pyoderma. Monoinfections were often found and *S. pseudintermedius* was isolated in higher amounts than found in samples from healthy dogs.

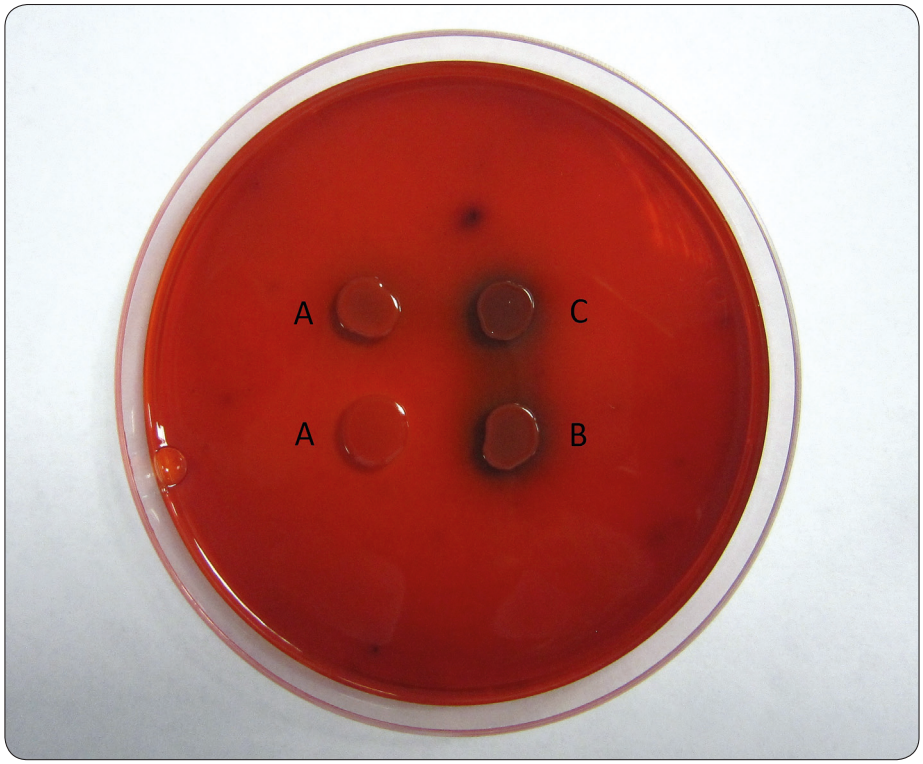

Fig. 1: *Staphylococcus pseudintermedius* from dog skin, biofilm production (Congo red agar, aerobic incubation at 36±2 °C for 20 hours): (A) red colony, no biofilm production; (B) brown colony, weak biofilm production; (C) dark brown colony, biofilm production / Biofilmproduktion von *Staphylococcus pseudintermedius* von Hundehaut (Kongo-Rot-Agar; Inkubation bei 36 ± 2 °C; aerob; nach 20 Stunden): (A) rote Kolonien, nicht- Biofilm-Produzent; (B) braune Kolonie, schwache Biofilmbildung; (C) dunkelbraune Kolonie, Biofilmbildung

Bacteria within a biofilm can be more resistant to antimicrobial therapy (HØIBY et al., 2015). Understanding whether *S. pseudintermedius* isolates from healthy and infected skin are able to form biofilms is important when developing new treatment regimes. However, our knowledge of the pathogenesis of biofilms of *S. pseudintermedius* is very limited. We thus compared the ability to form biofilms of bacteria from healthy dogs with those from dogs with skin infections. We found that *S. pseudintermedius* strains from dogs with and without skin lesions are able to form biofilms *in vitro*. Our results are consistent with two previous studies, which showed no significant difference in the ability to produce biofilms between *S. pseudintermedius* isolates from healthy and infected dogs (GARBACZ et al., 2013; SINGH et al., 2013). In contrast to the present report, previous studies included samples from different locations and diseases (GARBACZ et al., 2013; SINGH et al., 2013). In general, the vast majority of *S. pseudintermedius* isolates (95–96%) were able to produce biofilms (SINGH et al., 2013; CASAGRANDE PROIETTI et al., 2015). We could confirm that biofilm production by canine pyoderma isolates is common, although we find it to be less frequent than claimed by SINGH et al. (2013) and CASAGRANDE PROIETTI et al. (2015). The difference could relate to the different tests used to assess the ability to form biofilms.

Antibiotics alone are in most cases insufficient to treat biofilm infections (HØIBY et al., 2015; WU et al., 2015). Pyoderma within biofilms can be up to 1000 times more resistant to antibiotics than when it is outside biofilms (O`NEILL et al., 2014). An improved understanding of biofilm production is required to develop better treatment regimes. There are no guidelines for the therapy of dogs with biofilm-induced skin infections. The goal of treatment should be the reduction of biofilm load and the avoidance of the rebuilding of a biofilm in diseased patients. In the treatment of wounds, the physiological removal of biofilms through debridement or strong wiping seems the most effective method. Some products for wound cleaning may also help to destroy the biofilm (e.g. polyhexanide; PHILIPPS et al., 2010).

Our study is not without problems. The test method represents a major concern. There are many tests for the ability to form biofilms, including phenotypic and genotypic examination. We elected to use the Congo red method as OLIVERIA et al. (2010) described it as easier and faster to perform than other phenotypic tests.

CASAGRANDE PROIETTI et al. (2015) and ARCIOLA et al. (2002) found good agreement between the results of PCR and Congo red staining, although OLIVERIA et al. (2010) reported the method to be less sensitive than the use of PCR for genes involved in biofilm production, giving a sensitivity of 89% and a specificity of 100% compared with PCR.

We could not apply the colorimetric scale of ARCIOLA et al. (2002) with six colour nuances. The minimal differences between the colours makes interpretation difficult and we feel that there is a risk of differences in interpretation by individual investigators. In our study the colour was classified as very red or red (not producing a biofilm), brown (weak biofilm production) or dark brown (biofilm production), as depicted in Fig. 1.

Our results highlight the frequency of *S. pseudintermedius* and the potential role of biofilm production on canine skin. Further work is needed to clarify the effect of biofilms on healthy dog skin and their role in skin diseases. Additional investigation is also needed to study the impact of biofilm formation on clinical treatment failure.

Fazit für die Praxis:
Im Rahmen dieser Studie wurde das Vorkommen von *S. pseudintermedius* auf der gesunden Hundehaut sowie auf der Haut von Hunden mit Pyodermie verglichen. Desweiteren wurde untersucht, ob dieser Keim *in vitro* in der Lage ist, einen Biofilm zu bilden. Gerade im Hinblick auf die Problematik von MRSP (Methicillin resistenter *S. pseudintermedius*) in der Veterinärmedizin kann die Identifizierung von Biofilmproduzenten ein wichtiger Therapie-Ansatz sein. Die hier vorgestellte Methode kann helfen, die Therapiemaßnahmen gezielt abzustimmen und einzuleiten.

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