BIOFILM FORMING IN *S. PSEUDINTERMEDIUS* ISOLATES FROM ATOPIC DOGS

Ozana Maria HRIŢCU[1], Irina LIPOVAN[1], Mihai MAREŞ[1], Gheorghe SOLCAN[1], Dorina TIMOFTE[2]
[1]UŞAMV, Faculty of Veterinary Medicine, Alley M.Sadoveanu no. 8
[2]University of Liverpool, Leahurst Faculty of Veterinary Medicine
ozy_dulman@yahoo.ro

Abstract
S. pseudintermedius is the main colonizer of skin in dogs, with increased adherence in the ones suffering from atopic dermatitis. It is also the main pathogen involed in pyodermas and has an important zoonotic potential. Biofilm forming was tested using the microtitre plate protocol for 50 S. pseudintermedius sampled from the same number of atopic dogs. Out of these 50 isolates 40 (80%) were methicillin susceptible and 10 (20%) methicillin resistant. S. pseudintermedius isolates sampled from atopic dogs without pyoderma simptoms did not manifest an important biofilm production. Our results show that 60% of all isolates were not biofilm producers, 38% were weak biofilm producers and only one isolate was a moderate biofilm producer. Adherence capability did not show significand differences between MSSPs (methicillin susceptible S. pseudintermedius) and MRSPs (methicillin resistant S. pseudintermedius).
Keywords: *dog, atopic dermatitis, S.pseudintermedius, biofilm*

Introduction

S. pseudintermedius is the main colonizer of skin and mucosal areas in dogs and may also be a pathogen in different types of infections, both in animals and humans. Mucosal sites are considered to be carrier sites from which *S. pseudintermedius* spreads on the skin (Gomez-Sanz, 2014). Its adherence depends on the synthesis of several proteins among which are the clumping factor and the ones involved in biofilm formation and so it may differ from one strain to another (McEwan, 2006). The adherence to canine corneocytes was found to be enhanced in atopic dogs (Latronico, 2014), probably due to structural defects in the epidermis (Marsella, 2011).

Materials and method

The study population included 20 dogs from Romania, and 30 dogs from the UK. The isolates from the atopic dogs were obtained by sampling the gingival mucosa and the perineal area using a sterile swab (sterile sample swab with Amies transport media, FLmedical, Italy) and cultured for *Staphylococcus* spp. The swabs were inoculated onto Columbian Blood Agar (CAB) and Mannitol Salt Agar (MSA; Oxoid, Basingstoke, UK) and incubated at 37^{o}C for 18-24h. Isolates were characterized based on colony morphology, Gram-staining and catalase production. Rabbit plasma agglutination test was performed on all isolates. Species identification was confirmed by performing PCR as previously described for *nuc* gene (*S. pseudintermedius*) (Schmidt, 2014). The mecA gene was identified using protocol and primers from Vannuffel, (1995) and Ishihara (2010). Microtitre plate biofilm forming protocol **(**Stepanovic, 2007):

1. The isolate is transferred from stock-culture on blood agar and incubated at 37^{o}C for 24h.
2. A suspension from 3-4 colonies is made using sterile water reaching a turbidity of 0.5 McFarland – 10^{8} CFU/mL (5 mL).
3. Tissue culture microplates are recommended. In each well we place 180 µL TBS (tryptic soy broth) with 1% glucose and 20 µL vortexed bacterial suspension. For each isolate the test is done in triplicate (3 wells/isolate) and each such test is repeated 3 times. There will be 6 wells for negative controls (200 µL TBS + 1% glucose). In a 96 well microplates we should be able to test 30 isolates.
4. A lid is placed on top and the plates are incubated for 24h at 37^{o}C.
5. After incubation, the supernatant is thrown away and the wells are washed delicately 3 times, with 300 µL PBS each time, using a micropipette. The wells will be emptied by

turning them upside down with a short joggle. The microplates are then left to dry at room temperature.

6. Fixation is done for 60 min at 60ºC.
7. Staining is done using 150 µL cristal violet/well, for 15 min at room temperature. The stain is thrown away and the wells are washed until the water is clean, without stain.
8. The stain fixed in the cellular wall is then diluted using 95% ethanol 150 µL/well. The microplates are covered with a lid and are stored at room temperature for 30 minutes.
9. Optical measuring is done at 570 nm.
   ODc (*cut off value*) is calculated for each microplate = medium OD for negative controls+ (3x SD of negative controls);
   final OD = medium isolate OD– ODc
   0 = no biofilm producer;
   + or 1 = weak biofilm producer;
   ++ or 2 = medium biofilm producer;
   +++ or 3 = strong biofilm producer.

Results and discussion

Our results (Fig. 1, 2) show a low tendency towards biofilm forming for all tested isolates (Table 1).

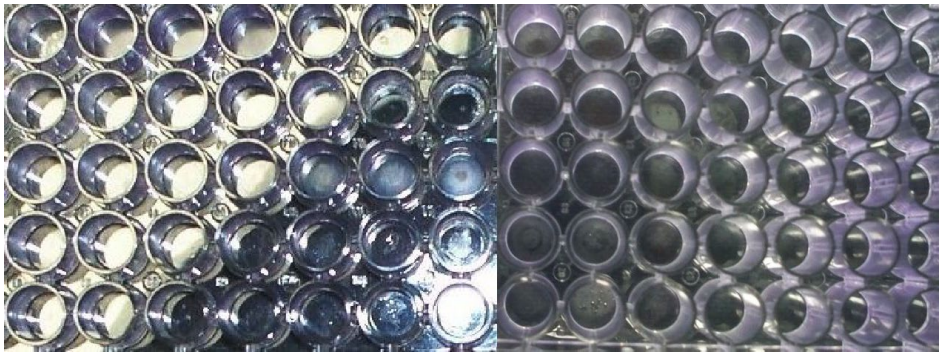

Fig. 1,2 Biofilm on the bottom of the wells from the microtitre plates

Table 1

Results following biofilm forming testing

/	Isolate									
MSSP-RO	1	2	3	4	5	6	7	8	9	10
biofilm producer										
MSSP-RO	11	12	13	14	15	16	17	18	19	20
biofilm producer										
MSSP-UK	21	22	23	24	25	26	27	28	29	30
biofilm producer										
MSSP-UK	31	32	33	34	35	36	37	38	39	40
biofilm producer										
MRSP-UK	41	42	43	44	45	46	47	48	49	50
biofilm producer										

	no biofilm producer
	weak biofilm producer
	moderate biofilm producer
	strong biofilm producer

From the MSSP-RO group we obtained 9 weak biofilm producing isolates (45%); from the MSSP-UK group we obtained 7 weak biofilm producers (35%) and one moderate one (5%). The MRSP-UK group resulted in 3 weak biofilm producing isolates (15%). The rest of the tested isolates did not show biofilm synthesis (60% of all isolates). No strong biofilm producers were found.

DiCicco (2012) tested 20 MRSP to determine biofilm production and found 15%, 35% and 50% as being strong, moderate and weak adherents. Bardiau (2013) determined that from 200 *S. pseudintermedius* isolates, 20 being MRSPs, most of them were strong or moderate biofilm producers. A similar result was reported by Osland (2012) for 23 MRSPs. Pompilio (2015) also found that *S. pseudintermedius* is able to form a well structured biofilm, consisting of multilayered, mushroom-shaped microcolonies embedded in an extracellular polymer substance matrix. Proietti (2015) found, using the same method, 41.6% weak biofilm producers, 35% moderatelly adherent and 16.6% strongly adherent. Only 6.6% isolates were found to be non-adherent. Garbacz (2013) worked on 191 isolates from both healthy and infected dogs and found no isolate with zero adherence, 4.2% and 6.6% weak biofilm producers from healthy/infected dogs; 63.4% and 52.5% moderate producers; 32.4% and 40.8% strong producers. Singh (2013) found 96% of its tested isolates as being strong or moderate biofilm producers, with no important differences between MSSPs and MRSPs.

Although these studies found that the majority of *S.pseudintermedius* isolates did form biofilm, some of them being strong producers, our results showed that more than half of the isolates did not manifest adherents and 38% were only weak biofilm producers. This may be due to the fact that the patients, although atopic dogs, did not manifest at the time of sampling, simptoms of pyoderma.

Biofilm formation may be a factor that enhances the pathogenicity of *S. pseudintermdius*, both MSSPs and MRSPs. Biofilm producing isolates have been found in catheters both in animals and humans, thus representing a threat in view of the zoonotic potential and the increase of antimicrobial resistance.

References

1. Bardiau M., Yamazaki K., Ote I. et al. (2013) - Characterization of methicillin-resistant *Staphylococcus pseudintermedius* isolated from dogs and cats. Microbiol Immunol, 57, 496-501;
2. DiCicco M., Neethirajan S., Singh A., Weese J.S. (2012) – Efficacy of claritromycin on biofilm formation of methicillin-resistant *Staphylococcus pseudintermedius*. BMC Vet Res, 8:225;
3. Garbacz K., Zarnawska S., Piechowicz L., Haras K. (2013) – Pathogenicity potential of *Staphylococcus pseudintermedius* strain isolated from canine carriers and from dogs with infection signs. Virulence, 4:3, 255-259;
4. Gomez Sanz E. (2014) – *Staphylococcus aureus* and *Staphylococcus pseudintermedius* in animals: molecular epidemiology, antimicrobial resistance, virulence and zoonotic potential. doctoral Thesis, Universidad de la Rioja, Spain;
5. Ishihara K., Shimokubo N., Sakagami A., Ueno H., Muramatsu Y., Kadosawa T., Yanagisawa C., Hanaki H., Nakajima C., Suzuki Y., Tamura Y. (2010) – Occurence and Molecular Characteristics of Meticillin-Resistant *Staphylococcus aureus* and Meticillin-Resistant *Staphylococcus pseudintermedius* in an Academic Veterinary Hospital. Applied and Environmental Microbiology, Aug., 5165-55174;
6. Latronico F., Moodley A., Nielsen S.S., Guardabassi L. (2014) – Enhanced adherence of methicillin-resistant *Staphylococcus pseudintermedius* sequence type 71 to canine and human corneocytes. Vet Res, 45:70;
7. Marsella R., Olivry T., Carlotti D.N. (2011) – Current evidence of skin barrier dysfunction in human and canine atopic dermatitis. Vet Dermatol, 22, 239-248;
8. McEwan N.A., Mellor D., Kalna G. (2006) – Adherence by *Staphylococcus intermedius* to canine corneocytes: a preliminary study comparing noninflamed and inflamed atopic canine skin. ECVD and ACVD, Vet Dermatol, 17, 151-154;
9. Pompillio A., DeNicola S., Crocetta V., Guarnieri S., Savini V., Carretto E., Di Bonaventura G. (2015) – New insights in *Staphylococcus pseudintermedius* pathogenicity: antibiotic-resistant biofilm formation by a human wound-associated strain. BMC Microbiol 15:109;

10. Proietti P.C., Stefanetti V., Hyatt D.R., Marenzoni M.L., Copamaccio S., Coletti M., Bietta A., Franciosini M.P., Passamonti F. (2015) – Phenotypic and genotypic characterization of canine pyoderma isolates of *Staphylococcus pseudintermedius* for biofilm formation. The Journal of Veterinary Medical Science;
11. Sasaki T., Kikuchi K., Tanaka Y., Takahashi N., Kamata S., Hiramatsu K. (2007) – Meticillin-Resistant *Staphylococcus pseudintermedius* in a Veterinary Teaching Hospital. Journal of Clinical Bacteriology, Apr., 1118-1125;
12. Sasaki T., Tsubakishita S., Tanaka I., Sakusabe A., Ohtsuka M., Hirotaki S., Kawakami T., Fukata T., Hiramatsu K. (2010) – Multiplex-PCR method for species identification for coagulase positive staphylococci. Journal of Clinical Microbiology, 48, 3, 765-769;
13. Schmidt V.M., Williams N.J., Pinchbeck G. et al. (2014) - Antimicrobial resistance and characterization of staphylococccci isolated from healthy Labrador retrievers in the United Kingdom. BMC Vet Res, 10:17, 1-14;
14. Sigh A., Walker M., Rousseau J., Weese J.S. (2013) – Characterization of the biofilm forming ability of *Staphylococcus pseudintermedius* from dogs. BMC Vet Res 9:93;
15. Stepanovic S., Vukovic D., Hola V., Bonaventura G., Djukic S., Cirkovic I., Ruzicka F. (2007) – Quantification of biofilm in microtitre plates: oveview of testing recommendations for assessment of biofilm production by staphylococci. APMIS, 115, 891-9;
16. Vannuffel P., Gigi J., Ezzedine H., Vandercam B., Delmee M., Wauters G., Gala J.L. (1995) – Specific detection of methicilin-resistant *Staphylococcus* species by multiplex PCR. Journal of clinical Microbiology, 33, 11, 2864-2867.