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Biofilm formation of *Malassezia pachydermatis* from dogs

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Abstract

Malassezia pachydermatis is a member of the normal skin microbiota which can also cause dermatitis and otitis in dogs. *Malassezia* was shown to form biofilm *in vitro* and *in vivo* which is responsible for colonization of organisms. This study aims in determining the biofilm formation of *M. pachydermatis* isolated from fifteen dogs with dermatitis and ten dogs with otitis. Twenty apparently healthy dogs without any clinical signs of dermatitis were also included in the study as control group. Samples inoculated on Sabouraud's dextrose agar showed cultural, morphological and biochemical properties characteristic of *M. pachydermatis*. Biofilm formation was determined indirectly by crystal violet binding assay and all the isolates in the study showed biofilm formation on 96 well flat bottom plates at various levels. The OD from ELISA reader showed values ranging from 0.075 to 0.56 among the diseased animals and 0.072 to 0.672 among the control group at 620 nm. Statistical analysis showed no significant difference in the biofilm formation assessed between the diseased animals and control group animals. It was observed that the organism irrespective of whether it had caused infection in the animal or has stayed as commensal had the potential to form biofilm at various levels.

Keywords: malassezia, biofilm formation, crystal violet binding

1. Introduction

Malassezia are the commensal organism of skin in most animals, the organism is known to cause diseases only under favourable conditions. *M. pachydermatis* is the most commonly isolated yeast from cases of canine dermatitis and otitis. The infection is most commonly seen in well-fed adult dogs. There are certain properties of *Malassezia* which helps in the pathogenesis and establishment of infection in the hosts. *In vitro* studies have been carried out to understand the mechanism of infection and determinants of virulence such as biofilm formation along with many other factors. Adherence and hydrophobicity was shown to aid in the formation of biofilm and establishment of infection. *Malassezia* biofilm is known to have an impact on antifungal susceptibility and in turn resolution of the disease in dogs treated for the same. It is the prime factor responsible for catheter related fungemia and deaths in neonates supplemented with lipid preparations. The property of *Malassezia* species to form biofilm on animals as well as inanimate objects helps in colonization of the organism and is involved in the pathogenesis of the disease. This study aims to understand the ability of *M. pachydermatis* to form biofilm *in vitro* and to understand the association of the factor in the production of the disease in dogs by comparing it with the isolates from healthy animals.

2. Materials and Methods

Dogs brought to University Veterinary Hospital, Mannuthy and Kokkalai were screened for *Malassezia* infections. Animals were examined for the presence of lesions suggestive of *Malassezia* dermatitis like erythema, hyperpigmentation, greasy exudates, scaling and primary lesions of pustules, papules and macules. Dogs with clinical signs like, excessive discharge from ears, head shaking, scratching of ears and offensive odour were examined for otitis. Both the ears were examined for focal alopecia, redness of ear pinna, scaling of ear flaps, swelling and ectoparasites. Twenty apparently healthy dogs were included in the study as control group. Impression smears were obtained by adhesive tape method where a piece of clear one-sided cellophane adhesive tape 5.5 cm long and 2.5 cm wide was cut from a roll. The adhesive surface of the strip was placed onto the skin surface and was pressed firmly once, for two or three seconds. When the tape strip was removed from the skin, the strip was placed, adhesive side up, on to a clean glass slide (Omodo-Eluk *et al.*, 2003)^[14]. The strip was then stained with Giemsa stain and observed under 1000X.

The samples were obtained from the skin and from the external ear canals of healthy dogs and also from the affected body sites of dogs with confirmed otitis externa and dermatitis by using sterile cotton swabs. The wash fluid, composed of 0.075 M phosphate buffered physiological saline, pH 7.9 containing 0.1 percent Triton X-100 (Bond *et al.*, 1995) [2] was used for processing of the swabs. The samples were inoculated on Sabouraud dextrose agar (SDA) with chloramphenicol (HiMedia, Laboratories, Mumbai, India) for primary isolation (Girao *et al.*, 2006) [6]. The agar was incubated at 37°C, for up to 10 days with daily monitoring. Cultural and morphological characterisation of *M. pachydermatis* was performed. Biochemical tests such as urease test and catalase tests were carried out (Guillot *et al.*, 1996) [11]. Lipid dependency of the organism was evaluated by depriving SDA of any oil supplements.

Biofilm production by single cultures of the isolates of *M. pachydermatis* was determined using a crystal violet staining method (CVS) (Bumroongthai *et al.*, 2016) [3]. Briefly, all isolates were grown in yeast extract peptone dextrose (YEPD) broth for 3 days at 32°C with intermittent shaking. After 3 days incubation, the concentration of inoculum was adjusted at 0.1 optical density at 600 nm using ELISA reader. A total of 150 µl of suspension were added into 96 well flat bottom microtitre plates (Nest Biotech Co., Ltd). Thereafter, the plates were incubated for 24 h at 32°C allowing adherence phase of biofilm. Then, non-adherent cells were gently removed by double washing with 150 µl of phosphate buffered saline solution (PBS, pH7.2). After the rinsing step, a continuous culture was established by adding 200 µl of YEPD broth to each well under the previous conditions. The equal volume of YEPD media were daily replaced for 4 consecutive days. To remove non-adherent yeasts, the microtiter plate wells were gently washed twice with phosphate buffered saline and fixed with 150 µl of 99 percent methanol for 15 minutes then dried at room temperature for 45 minutes. The incubated plates were filled with a 0.5 percent crystal violet solution for 45 minutes and washed with 200 µl of sterile distilled water, and destained with 95 percent ethanol for 200 µl for 45 minutes. A total of 100 µl from each well was transferred to a new microtiter plate. Biofilm production was measured using the crystal violet binding assay, with the quantity of biofilm directly represented by measurement of the OD value at 620 nm in an ELISA microplate reader.

3. Result and Discussion

The lesions and alopecia was mostly noticed on ventral neck, medial thigh and abdomen. Most of the dogs were showing generalised infection with primary and secondary skin lesions (Fig 1). Papule, alopecia, erythema, crusts and excoriations were found to be the predominant lesions noticed on the skin. Dogs with dermatitis showed clinical signs such as pruritus, hyperkeratosis, lichenification, interdigital erythema, scaly lesion, greasy seborrhoea etc. which were similar to the signs described by Gueho *et al.* (1998) and Guillot and Bond (2020) [10]. Yellow to brownish erythematous-ceruminous discharge was seen in cases of *Malassezia* otitis whereas purulent discharge was seen in case of bacterial otitis as observed by Karlapudi (2017) [12] and Guillot and Bond (2020) [10]. Impression smear from adhesive tapes applied on the skin revealed budding yeast cells attached to the corneocytes along with few bacterial cells in case of mixed infection (Fig 2). Adherence of the organism to canine corneocytes was also

observed in the impression smears by Guillot and Bond (1999) [9] and Maynard *et al.* (2011) [13].

Among the dogs screened, 15 samples from dermatitis cases showed colonies characteristic to *Malassezia* species on SDA (without lipid supplementation) after inoculation, 10 isolates could be obtained from otitis and 20 isolates could be obtained from healthy animals.

Macroscopic appearance of colonies showed dull and rounded appearance in SDA medium after incubation for seven days (Fig 3). The colonies were cream to ivory colour with convex to umbonate shape with an average diameter of 5 mm which is in par with Gueho *et al.* (1996) [8]. All the isolates were found to be positive for urease test and catalase test, all the isolates were obtained from SDA without lipid supplementation. *Malassezia* organisms were found to be obligatory lipid dependent species and require lipid supplementation during culture on SDA except *M. pachydermatis* which has been described as lipophilic but lipid independent (Guillot *et al.*, 1996) [11]. All the isolates of *M. pachydermatis* showed growth at 40-42°C whereas 66.67 per cent of the isolates showed accelerated growth which was in par with Ashbee and Evans (2002) [1].

On staining of these colonies, the organisms appeared as small oval budding yeasts with single unipolar blastospores preceded by collarettes, with no germ tubes, hyphae or pseudohyphae characteristic to *M. pachydermatis* (Fig 4).



Fig 1: Dog with malassezia dermatitis

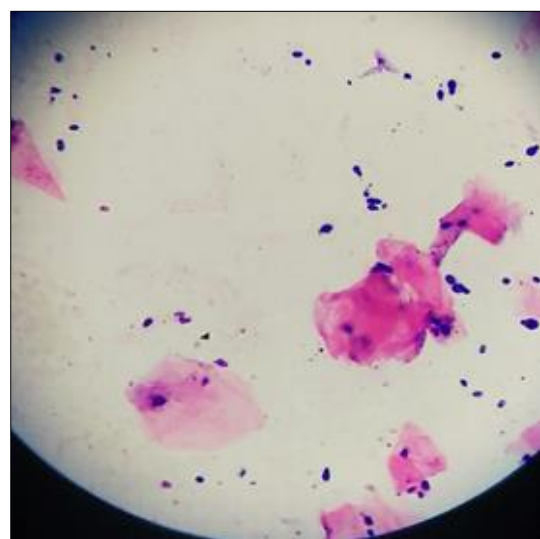


Fig 2: Adhesive tape impression showing yeast cells



Fig 3: Colonies appeared as ivory colored convex structure

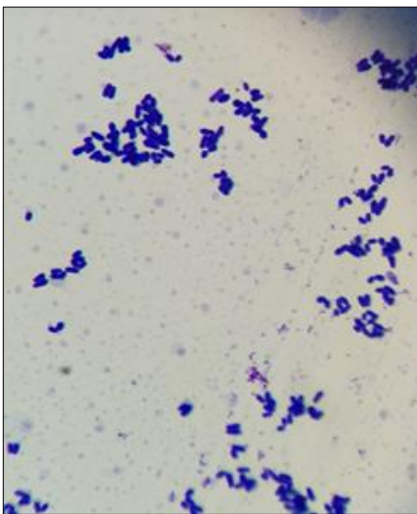


Fig 4: Budding yeast cells X1000, Giemsa stain

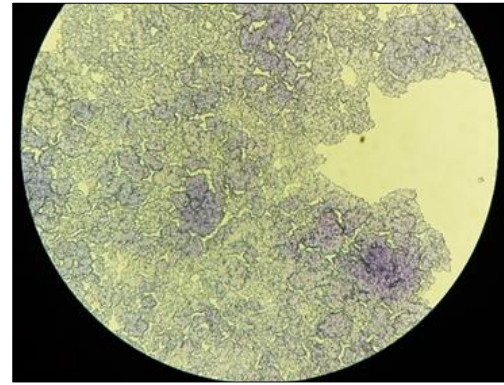


Fig 3: Network of budding yeast cells on CVS assay X400, crystal violet stain

In this study, the assay for biofilm formation was an indirect estimation of the binding ability of the crystal violet to the already formed yeast biofilm. All the isolates in the study produced biofilm at various levels irrespective of whether they have been isolated from either a healthy or a diseased dog. These findings were in agreement to Cannizzo *et al.* (2007) [4], Figueredo *et al.* (2012) [5] and Bumroongthai *et al.* (2016) [3]. Figueredo *et al.* (2012) [5] opined that the biofilm formation of *M. pachydermatis* might depend on the strain of the yeast and not the origin of the isolate. The estimation of biofilm by CVS assay in terms of OD values was said to be the direct representation of the amount of biofilm visualized under scanning electron microscope Cannizzo *et al.* (2007) [4]. Cannizzo *et al.* (2007) [4] has studied the biofilm formation on catheter surface recreating the biological transmission of organisms in neonatal feeding tubes and has found profuse formation of biofilm in the catheters used. Biofilm formed by the organism was found to have role in the establishment of infection along with other factors such as adherence, hydrophobicity (Cannizzo *et al.*, 2007) [4]. Detailed study involving the above factors is required to understand the pathogenesis and mechanism of biofilm formation by the organism.

4. Conclusion

The dogs included in the study showed typical signs of *Malassezia* lesions. Lipid independent *Malassezia* could be isolated from dogs with dermatitis, otitis as well as apparently healthy animals. The *M. pachydermatis* isolates were characterized by cultural, morphological and biochemical properties. All the isolates obtained from dogs with dermatitis, otitis as well as animals without any clinical signs of *Malassezia* infection was shown to produce biofilm at various levels by CVS assay. All the isolates included in the study including those obtained from apparently healthy dogs were shown to be equally potent to form biofilm *in vitro* similar to the isolates from diseased animals.

5. References

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Biofilm production was measured using the crystal violet binding assay, with the quantity of biofilm directly represented by measurement of the OD value at 620 nm in an ELISA microplate reader. All the isolates included in the study showed biofilm formation at various levels. The OD values ranged from 0.075 to 0.56 among the diseased animals and 0.072 to 0.672 among the control group at 620 nm. The biofilm formation of the diseased animals and control group were compared using one way ANOVA, it showed no statistical difference between the groups ($p=0.057$). The mean OD values for biofilm from dogs with dermatitis, otitis and healthy animals are given in Table 1.

Table 1: The mean OD values of biofilm and standard error

Groups	N	Mean±SE
Dermatitis	15	0.259500±0.0360858
Otitis	10	0.262700±0.0410439
Control	20	0.160400±0.0299066
Total	45	0.216167±0.0210455

Observation of the biofilm formed by CVS assay under inverted microscope showed network of yeast cells with clumping of organisms at some areas, also the yeasts were found adhered to the walls of the polystyrene plate. The networking of cells could be readily correlated with the OD values obtained from CVS assay (Fig 3).

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