Virulence determinants of *Malassezia pachydermatis* isolated from cases of canine dermatitis

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Abstract:

Malassezia is a commensal organism known to cause disease under favourable conditions, and has been isolated from many animals as well as human beings. Malassezia pachydermatis is the most common cause of yeast dermatitis in dogs and there are many determinants involved in the production of disease in the host. This study aims to deterimine the presence of virulent determinants of the organisms isolated from fifteen dogs with dermatitis that may be involved in the pathogenic mechanism of Malassezia. The virulence determinants of M. pachydermatis viz; adherence, cellular surface hydrophobicity and biofilm formation were investigated. All the isolates studied were shown to produce all the virulent factors investigated in vitro which can be compared to the biological system. Adherence of organism on polystyrene plates was evident in all the isolates and the values ranged from 0.14 to 63 per cent with five isolates showing high adherence values. Hydrophobicity was variable and ranged from 1.78 to 69.46 per cent by two phase system with seven isolates showing moderate property. All the isolates were shown to produce biofilm by crystal violet staining technique and the optical density values ranged from 0.075 to 0.56 at 620 nm. No significant correlation was observed between the three virulent determinants examined. The presence of three virulent determinants investigated warrants their consideration in further studies for assessing the pathogenicity of Malassezia dermatitis in dogs.

Keywords: *Malassezia, adherence, cellular surface hydrophobicity, biofilm*

　　　　Malassezia organisms are the normal inhabitants of skin microflora in most of the warm blooded animals; the yeast is known to manifest in the host only under favourable conditions. They are known to cause certain diseases including pityriasis versicolor (PV), folliculitis, seborrheic dermatitis, dandruff, atopic dermatitis in humans and dermatitis in animals. The properties of *Malassezia* which aid in the establishment of infection in the hosts have been studied *in vitro,* to understand the mechanism and pathogenesis of the disease.

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J. Vet. Anim. Sci. 2022. 53 (2) : 201-207

Gagana *et al.*　201

Biofilm formation and several other determinants including adherence, cellular surface hydrophobicity (CSH) have known to cause catheter associated fungaemia in immunocompromised humans. The organism is known to cause life-threatening fungaemia and death in pre-term neonates which makes this organism one of significant public health importance. Since *M. pachydermatis* is the most commonly isolated organism from canine dermatitis, determinants such as adherence, CSH and biofilm formation are being demonstrated in the study. The understanding of virulent factors also paves the way for comprehending the resistance mechanisms exhibited by biofilm forming strains of the organism.

Materials and methods

Dogs brought to University Veterinary Hospital, Mannuthy and Kokkalai were screened for Malassezia dermatitis. Animals were examined for the presence of lesions suggestive of Malassezia infection like erythema, hyperpigmentation, greasy exudates, scaling and primary lesions of pustules, papules and macules.

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 on 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 down, on to a clean glass slide (Omodo-Eluk *et al*., 2003). The strip was then stained with Giemsa stain and observed under 1000X. Cytological quantification with more than two yeast cells per oil immersion objective of the microscope were selected as positive.

The samples were obtained from the skin of dogs which were positive for budding yeast cells on impression smear dermatitis by using sterile cotton swabs. The wash fluid, composed of 0.075 M phosphate buffered physiological saline, pH 7.9 containing 0.1 per cent Triton X-100 (Bond *et al*., 1995) 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 and were incubated at 37°C for a period of 10 days (Girao *et al*., 2006). Cultural and morphological characterisation of *M. pachydermatis* was performed. Biochemical tests such as urease and catalase tests were conducted (Guillot *et al*., 1996). The inoculating medium was deprived of oil supplements to detect lipid dependency of the yeasts.

Adherence, cellular surface hydrophobicity (CSH) and biofilm formation of *M. pachydermatis* were the major virulent determinants that were studied. Adherence and CSH assay were done with some modifications (Angiolella *et al*., 2017) and biofilm formation was determined by crystal violet staining assay (Figueredo *et al*., 2012) with some modifications.

In order to examine the adherence capacity to plastic surfaces, the yeasts were grown for 72 h at 37°C in SDB, washed twice with sterile PBS (10 mM phosphate buffer, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4 with 0.5 per cent Tween 20) and then resuspended at 32°C in SDB. Standard inoculum of *Malassezia* was prepared at 7.5 $\times$ 10^2 cells/ml after counting the cells. After incubation for 3 h at 32°C in six-well polystyrene plates (Nest Biotech Co., Ltd) followed by extensive washing with PBS, one ml of SDA was poured into each well and allowed to solidify. After incubation for 72 h at 37°C, colonies were counted, and the results were expressed as a percentage of the inoculum size. The inoculum size for each cell suspension was confirmed by plating aliquots of the culture directly on SDA plates. {Adherence (%) = [Number of colonies in polystyrene plate/ (Number of colonies in control plate) x dilution factor] x 100}

Cellular surface hydrophobicity levels were determined by a two-phase system. The yeast cells were grown in SDB at 37°C for 72 h. Subsequently, cells were washed twice with sterile saline buffer (with 0.5% Tween 20) and resuspended in 0.05 M sodium phosphate buffer (pH 7.2) at a final concentration of 2 $\times$ 10^6 cells/ml. Cell suspension (2 ml), adjusted to an OD of 0.7 at 600nm or OD at 600 of each suspension was recorded (OD600 control).

J. Vet. Anim. Sci. 2022. 53 (2) : 201-207

202 Virulence determinants of *Malassezia pachydermatis* causing canine dermatitis _____

Furthermore, the suspension was transferred to a glass tube containing 500 µl octane (Sigma Aldrich) and shaken for 1 minute using a vortex mixer. After separation of the phases, OD of the aqueous phase was measured at 600nm (OD600 after octane overlay). {Relative CSH was calculated as: [(OD at 600 control – OD at 600 after octane overlay)/OD at 600 control] × 100}

Biofilm production by single cultures of the isolates of *M. pachydermatis* was determined using a crystal violet staining method (CVS). Briefly, all isolates were grown in 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 a spectrophotometer/ELISA reader or approximately 1.0×10^6 cell/ml. The colony forming unit (CFU) of suspension was counted on SDA. 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 YPD 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 per cent methanol for 15 minutes

then dried at room temperature for 45 minutes. The incubated plates were filled with a 0.5 per cent crystal violet solution for 45 minutes and washed with 200 µl of sterile distilled water, and destained with 95 per cent 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.

Results and discussion

The lesions were typically seen on the ventral neck and abdomen with primary and secondary skin lesions (Fig 1). Localised and generalised alopecia along with papules, erythema, crusts and excoriations were observed as the principal findings. Figure 1 shows periorbital alopecia and hyperpigmentation with lesions on the nose. Dogs with dermatitis showed clinical signs such as pruritus, hyperkeratosis, lichinification, interdigital erythema, scaly lesion, greasy seborrhoea which were similar to the signs described by Gueho *et al*. (1998), Bond *et al.* (2010) and Daniel *et al.* (2021).

Impression from cellophane tapes obtained from the skin exposed peanut shaped cells bound to the canine epithelial cells; bacterial cells were seen in pyoderma (Fig. 2). Guillot and Bond (1999) and Maynard *et al*. (2011) observed similar findings with respect to impression smears where they
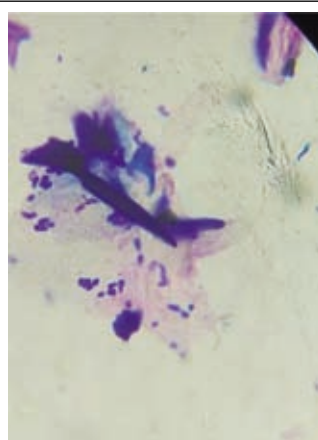

Fig. 1. Malassezia hyperpigmentation

Fig. 2. Adhesive tape impression showing yeast cells on corneocytes

J. Vet. Anim. Sci. 2022. 53 (2) : 201-207

Gagana *et al.* 203

Fig. 3. Colonies appeared as ivory colored convex structures
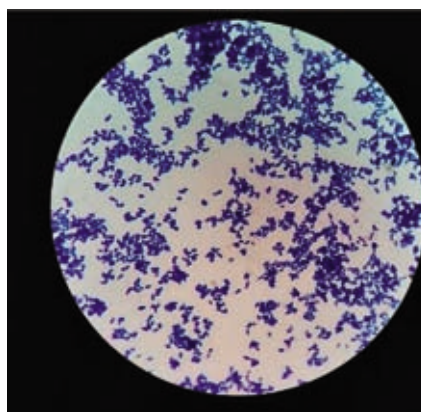
Fig. 4. Budding yeast cells X1000, Giemsa stain

Fig. 5. Colonies on polystyrene plate

Fig. 6. Network of budding yeast cells on CVS assay X400, crystal violet stain
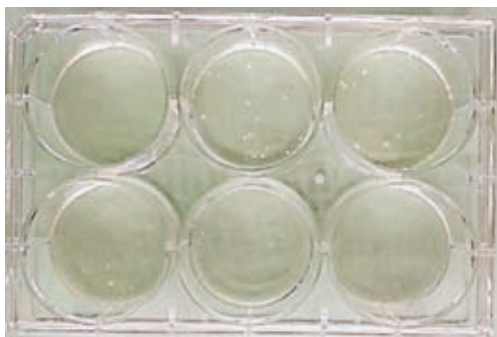
observed yeast cells attached to corneocyes. As the epidemiological studies and clinical signs in dogs with respect to bacterial and malassezia dermatitis intersect, it is important to differentiate both based on microbiological examinations (Shyma and Vijayakumar, 2012).

Among 45 cases of canine dermatitis with evidence of budding yeast cells on impression smear, 15 samples showed colonies typical of *Malassezia* species on SDA (without lipid supplementation).

Macroscopic appearance of colonies showed dull and rounded appearance in SDA medium after incubation for seven days (Fig.3). On microscopic examination, peanut shaped, unipolar budding yeast cells without any hyphae or pseudo hyphae were noticed (Fig. 4). The colonies were ivory coloured and convex with mean diameter of around 5 mm, positive for urease and catalase activity which was in par with Gueho *et al*. (1996). All the isolates of in the study showed growth
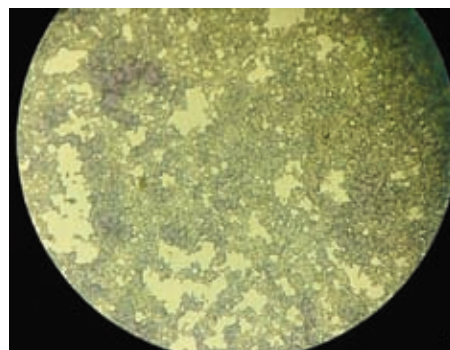
at 37°C and 40°C as described by Ashbee and Evans (2002). Cultural, biochemical and morphological qualities were characteristic of *M. pachydermatis*.

The detergent action of Triton X-100 was found to remove the adherent yeast cells on the epidermal cell layer especially the corneocytes. Bond *et al*. (1995) observed similar findings while quantifying the yeast cells.
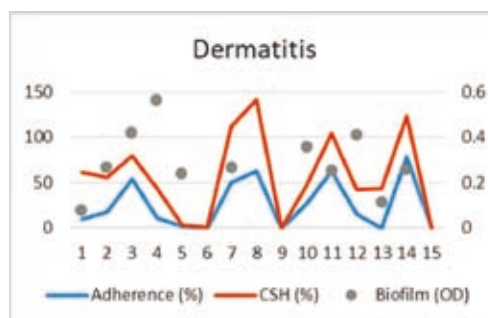

Fig. 7. Determinants in dermatitis group

J. Vet. Anim. Sci. 2022. 53 (2) : 201-207

204 Virulence determinants of *Malassezia pachydermatitis* causing canine dermatitis _____

All the isolates showed adherence property on polystyrene plate (Fig. 5) with five isolates showing high adherence values ranging from 50 to 78 per cent. The adherence of isolates on plastic surface was evaluated on six welled polystyrene plates and the percentage of adherence values varied from 0.14 to 78 per cent.

The property of *Malassezia* species such as adherence and cellular surface hydrophobicity helps in the formation of biofilm in living as well as inanimate objects. Attachment of *Malassezia* yeast cells on biological surface or inanimate object serves as a basis for colonisation of the organism and delay in the resolution of disease if caused. The adherence values obtained in the study are similar to the values from Angiolella *et al*. (2017) for *M. furfur* and Angiolella *et al*. (2020) for *M. sympodialis*, *M. globosa*, and *M. slooffiae*.

Results of CSH obtained by two-phase system for the isolates showed values ranging from 1.78 to 69.46 per cent. It was observed that seven isolates showed medium hydrophobicity with values 40.95 to 69.46 per cent while rest of the isolates showed low hydrophobicity values. The CSH values obtained in the study are similar to the values from Angiolella *et al*. (2017) for *M. furfur* and Angiolella *et al*. (2020) for *M. sympodialis*, *M. globosa*, and *M. slooffiae,* none of the isolates showed high CSH property. Studies have been carried out by Sivasankar *et al*. (2015) targeting the hydrophobic property of *Malassezia* thereby indirectly aiming at the treatment of *Malassezia* by decreasing the colonisation.

Biofilm formation was estimated by crystal violet staining technique, which is an indirect assessment, indicated by OD values in an ELISA microplate reader. The OD values from ELISA reader ranged from 0.075 to 0.56 at 620 nm. All the 15 isolates from dermatitis cases produced biofilm in different concentrations. In this study, all the isolates produced biofilm at various levels similar to Bumroongthai *et al*. (2016).

On ELISA plate, the organisms were seen as network of cells with clumps of budding yeasts, proving the ability of *Malassezia* to

Table 1. Pearson's correlation coefficient for the virulent determinants

Virulent determinants	Pearson's correlation coefficient
Adherence & CSH	0.358
CSH & Biofilm	-0.217
Biofilm & Adherence	0.036

p>0.05=not significant (ns)

adhere and form biofilm (Fig. 6). The networking of cells was more in samples with high OD and vice-versa which was in par with Cannizzo *et al*. (2007).

The relationship between adherence, hydrophobicity and biofilm values were assessed group using the Pearson correlation. There was no statistically significant correlation between these virulent determinants tested. Pearson correlation coefficients between the virulence determinants are given in Table 1 for different groups. The values of these determinants are plotted against each other from the same sample (Fig. 7). Angiolella *et al*. (2017) had tried to correlate between CSH, adherence and biofilm formation and no statistically significant correlation was obtained which agrees with the findings of this. Regardless of this, the presence of other virulence determinants of *Malassezia* such as pigment production, lipid metabolism (Mayser and Hort, 2011), genes encoding for azelaic acid, arachidonic acid and various enzymes such as chondroitin-sulphatase, esterase, lipase, phospholipase etc. may also contribute to the pathogenicity of the disease.

Conclusion

The dogs with typical signs of Malassezia lesions were examined for the presence of virulent determinants. The *M. pachydermatis* isolates were characterised by cultural, morphological and biochemical properties. All the isolates included in the study displayed properties of adherence, hydrophobicity and biofilm formation at various intensities. Further study may be required to correlate the levels of the determinants and the disease status of the animal. No statistically significant correlation has been observed between the virulence factors tested. Biofilm helps in forming of colonies on the skin surface as well as intravenous catheters and hence

J. Vet. Anim. Sci. 2022. 53 (2) : 201-207

Gagana *et al*. 205

considered as the most important virulent factor among the three. Thorough study comprising the above factors and other dependent factors may be required to understand the pathogenesis of the organism.

Conflict of interest

The authors declare that they have no conflict of interest.

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J. Vet. Anim. Sci. 2022. 53 (2) : 201-207

Gagana *et al.* 207