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Isolation and molecular characterisation of *Malassezia pachydermatis* from dogs in Kerala

S. Abhijith[1]*, Chintu Ravishankar[1], R. Rajasekhar[1], K. Sumod[1], P. Vinu David[2], P. Habeeb Biju[2], C. Harish[3], A. Javed Jameel[4], K. Daniel Anju[2], Suprativ Sarma[1], P. Vishnu[1] and K. Sandhiya[1]

[1]Department of Veterinary Microbiology, [2]Department of Veterinary Clinical Medicine, Ethics and Jurisprudence, College of Veterinary and Animal Sciences, Pookode, Wayanad- 673 576, Kerala Veterinary and Animal Sciences University, Kerala, India, [3]Clinical Laboratory, District Veterinary Centre, Thiruvananthapuram, Animal Husbandry Department, Government of Kerala, [4]Assistant Professor, University Veterinary Hospital, Kokkalai, Thrissur, Kerala

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Abstract

Malassezia spp. *are commensals of the normal cutaneous microbiota of animals that may become opportunistic pathogens under certain conditions and cause dermatitis and otitis externa in dogs. In the present study, a total of 47 dogs with clinical signs of pruritus, alopecia, scaly skin, erythema, foul odour, ear affections, hyperpigmentation and lichenification were found to be positive for budding yeast cells on cytological examination of clinical samples. These samples were subjected to culture on Modified Dixon agar (MDA), for the isolation of Malassezia* spp. *and the organism was isolated from eight cases. The identity of the isolates was confirmed as M. pachydermatis by polymerase chain reaction (PCR) targeting the LSU rRNA of Malassezia* spp. *followed by sequencing. Molecular characterisation of the isolates based on LSU rRNA region revealed that two types of M. pachydermatis isolates are prevalent among dogs in Kerala.*

Keywords: *Malassezia pachydermatis, Modified Dixon Agar, polymerase chain reaction*

Malassezia pachydermatis is part of the normal cutaneous microbiota of wild and domestic carnivores. Though the microorganism typically exists as a commensal, disruptions in the skin's microenvironment or host immune defences can lead to its pathogenic transformation. Over the past two decades, the opportunistic pathogenic potential of *M. pachydermatis* has become well-established. Conditions such as otitis externa and seborrheic dermatitis in dogs and cats are frequently linked to elevated numbers of *M. pachydermatis*. Though there are reports of skin infection caused by *M. pachydermatis* in dogs in Kerala (Daniel *et al*., 2021; Gagana *et al*., 2022), only a few studies have been carried out on the molecular characterisation of the pathogen. This paper reports the results of a study carried out to isolate and characterise *M. pachydermatis* from dogs with dermatitis and otitis in Kerala.

Materials and methods

Skin scrapings, impression smears and swab smears from skin and ear were collected for the study from dogs presented to the Teaching Veterinary Clinical Complex (TVCC), Pookode (n = 22), with clinical signs such as

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**Corresponding author: abhijith147@gmail.com, Ph. 9446485376*

pruritus, alopecia, scaly skin, erythema, foul odour, ear affections, hyperpigmentation and lichenification, during the period from October 2023 to August 2024. Samples were also collected from similar cases brought to the University Veterinary Hospital, Kokkalai, Thrissur (n = 9). Further, samples received from the Veterinary Hospitals in Wayanad (n = 4), Kozhikode (n = 3), Ernakulam (n = 4) and Thiruvananthapuram (n = 5) were also utilised for the study. The animals were subjected to detailed clinical examination with special emphasis on the integumentary system. Hair coat and skin of the clinical cases were thoroughly examined for the presence of primary lesions such as bulla, papule, macule, nodule, patch, pustule, vesicle, wheal and plaque and secondary lesions such as callus, lichenification, epidermal collarette, hyperkeratosis, hyperpigmentation, erosion, scar, crust, ulcer, alopecia, fissure, scale, comedone, erythema and excoriation. Dogs were examined for the distribution of lesions mainly on ventral neck, axilla, perineum, ears, ventral body, feet, lips and muzzle. Samples were collected from ear and skin and were subjected to methylene blue staining. Samples are considered as positive, if more than five cells and more than 10 cells for skin sites and the ear canal respectively, were observed in five random fields at 40X magnification (Eidi *et al*., 2011). Further, samples from suspected cases of malasseziosis received from other veterinary institutions were also utilised for the study. BiomLife® transport media (Ruhvenile Biomedical OPC Private Limited, New Delhi) was utilised for the storage and transportation of swabs when immediate processing was not possible.

The samples were cultured on Modified Dixon agar (MDA) (Himedia, India) for the isolation of *Malassezia* spp. as described by Marin *et al.* (2018). The plates were incubated at 37^{0}C and examined for the presence of growth daily. Smears prepared from colonies suggestive of *Malassezia* spp. were stained by Gram's method and observed under oil immersion objective. Colonies suggestive of *Malassezia* spp. were subcultured on Sabouraud Dextrose agar (SDA) (Himedia, India) for purification and subjected to PCR for confirmation of *Malassezia* spp. For DNA extraction, pure colonies were suspended in 500 µL of nuclease-free water (NFW) and centrifuged at 10,000 rpm for 10 min. The supernatant was discarded and the cell pellet was resuspended in 600 µL of lyticase lysis buffer containing 1.2 M of sorbitol, 10 mM of EDTA and 50 mM of tris hydrochloride with 10 mM beta-mercaptoethanol and 10 µL of lyticase (20000IU/mL) (Sigma Aldrich, USA) and incubated for one hour at room temperature to facilitate cell lysis. The DNA extraction was carried out by conventional phenol - chloroform method (Sambrook and Russell, 2001) and the extracted DNA was stored at -20 ^{0}C. The primers Malup (5′AGCGGAGGAAAAGAAACT3′) and Maldown (5′GCGCGAAGGTGTCCGAAG3′), were used to amplify a portion of the large subunit (LSU) ribosomal RNA (rRNA) of *Malassezia* spp. (Guillot *et al*., 2000). The PCR program consisted of an initial denaturation step at 95 ^{0}C for 3 min, followed by 34 cycles of denaturation at 94 ^{0}C for 30 sec, annealing at 55 ^{0}C for 1 min, and extension at 72 ^{0}C for 1 min and a final extension step of 5 min at 72 ^{0}C. The PCR products were resolved by submarine gel electrophoresis carried out at 100 V for 45 min. The amplified products were visualized in a gel documentation system (Igene, New Delhi) under UV illumination and outsourced to M/s GeneSpec Private Limited, Kochi, India, for Sanger sequencing. The identity of the sequences was confirmed using Basic Local Alignment Search Tool (BLAST) hosted by the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST). The phylogenetic analysis was carried out using MEGA11 software. The evolutionary history was inferred using Maximum Likelihood method. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the sequences analysed. The evolutionary distances used to infer the phylogenetic trees were computed using the Kimura 2 parameter model.

Results and discussion

The clinical signs of malasseziosis in dogs observed during the study included pruritus, erythema, hyperpigmentation, lichenification, traumatic alopecia, scaling, rancid fat odour and otitis externa (Figs. 1 and 2). These findings were in accordance with Mircean *et al.* (2010), Sharma *et al.* (2017) and Bond *et al.* (2020) who observed pruritus, erythema, hyperpigmentation, lichenification, malodour, traumatic alopecia and otitis externa as clinical manifestations in dogs with malasseziosis.

A total of 47 dogs with clinical signs suggestive of malasseziosis were found to be positive for budding yeast cells on cytological evaluation of clinical samples collected from the affected dogs. Impression smears from lesions stained with methylene blue revealed the presence of dark blue coloured, footprint-shaped budding yeast cells

Fig. 1. Erythema

Fig. 2. Scaling and lichenification

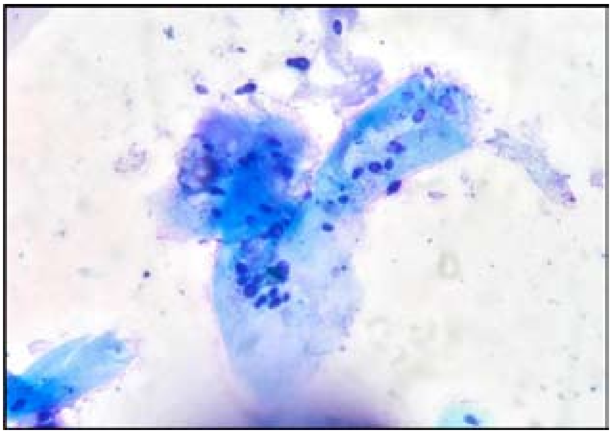

Fig. 3. Skin impression smear stained using methylene blue stain revealing foot print shaped yeast (1000X)

(Fig. 3). Mircean *et al*. (2010) and Reddy and Sivajyothi (2016) reported that impression smears from wet lesions and tape strip impression smears from dry lesions of malassezia dermatitis could be utilised for cytological diagnostic evaluation. De Abreu *et al*. (2023) and Rakesh *et al.* (2023) reported that cytological examination was the preferred diagnostic method for identifying and counting *Malassezia* spp. in clinical samples and it could be useful for monitoring therapeutic progress until the skin lesions healed and was a practical and cost-effective procedure for identification of malassezia yeasts.

Fig. 4. Colonies of *Malassezia pachydermatis* on MDA

On MDA, growth was observed from the second day of incubation onwards. Cream to buff-coloured colonies with smooth, convex surface suggestive of *Malassezia* spp. were obtained in eight samples (Fig. 4). Koike *et al*. (2013), Manna *et al*. (2015), Rathnapriya *et al*. (2016) and Marin *et al.* (2018) had used MDA for the isolation of malassezia yeasts and had observed smooth, convex surfaced malassezia colonies with colour ranging from cream to buff. Microscopically, dark blue coloured foot print shaped organisms suggestive of *Malassezia* spp. were observed in smears stained by Gram's technique (Fig 5). Rathnapriya *et al*. (2016) and Daniel *et al.* (2022) also observed dark blue coloured foot print shaped organisms of *Malassezia* spp. on microscopical examination of smears made from colonies obtained on MDA. David *et al.* (2003) noted that *M. pachydermatis* reproduced through enteroblastic budding, where the bud emerged from a broad base and was consistently located at the same pole of the cell, resulting in monopolar budding. This gave the typical microscopical appearance for *M. pachydermatis*.

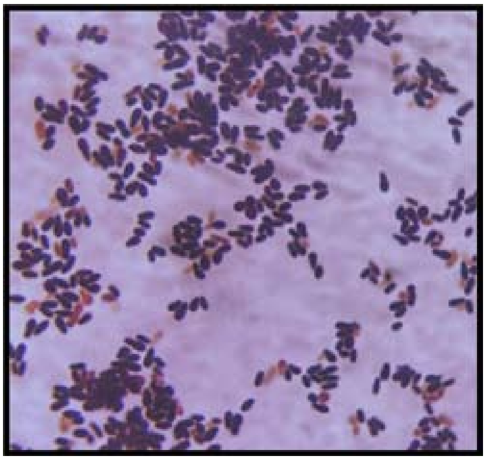

Fig. 5. Smear made from colonies of *Malassezia spp.* stained by Gram's technique (1000X)

In PCR, amplicons of approximately 550 bp were observed. No amplicons were found in the no template control (Fig. 6). Polymerase chain reaction based on LSU rRNA has been employed by many researchers for the molecular detection of *Malassezia* spp. (Gupta *et al.,* 2000; Morris *et al.*, 2005; Mirhendi *et al.*, 2009; Daniel *et al.*, 2022) as it has several highly conserved regions that facilitate the accurate sequence alignment. On BLAST

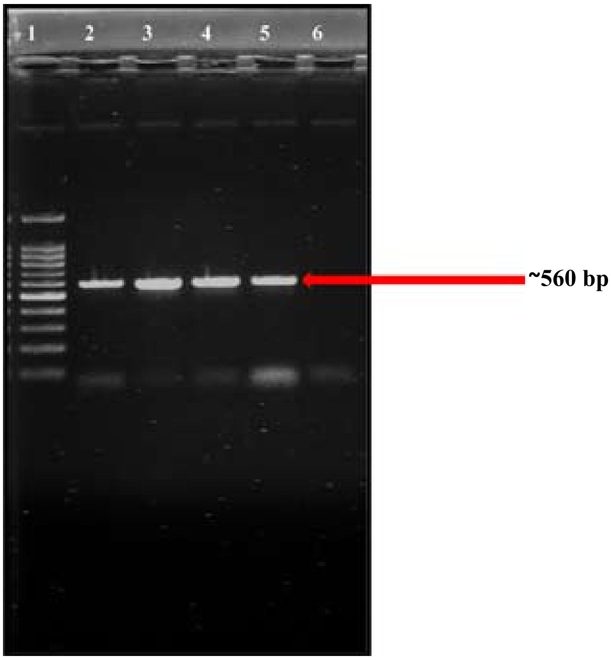

Fig. 6. Molecular detection of *Malassezia* spp. (Lane 1: 100 bp ladder, Lane 2-5: *Malassezia* spp. (approximately 550 bp), Lane 6: No template control)

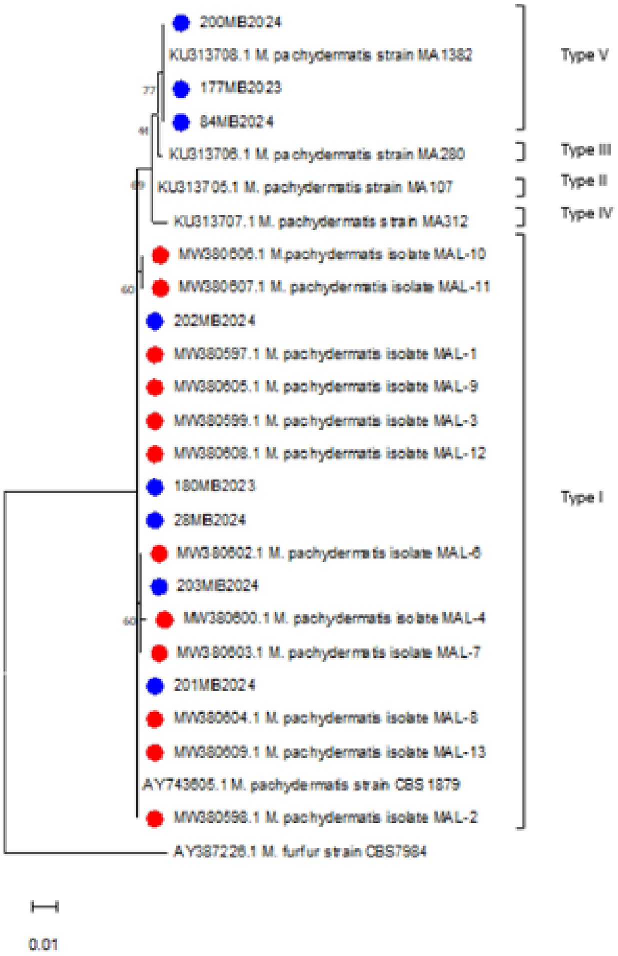

Fig. 7. Phylogenetic tree constructed using the LSU rRNA sequences of *Malassezia pachydermatis* isolates

(Legend: Blue filled circles indicate isolates obtained in the study, red filled circles indicate isolates reported previously from Kerala)

analysis, all the eight sequences showed 98.94 to 100 per cent similarity with the sequences of *M. pachydermatis*. The most common *Malassezia* spp. in dogs is reported to be *M. pachydermatis* (Meason-Smith *et al*., 2020). However, malasseziosis in dogs can also be caused by other species including *M. arunalokei, M. restricta, M. sympodialis, M. obtusa, M. nana, M. slooffiae, M. furfur* and *M. globosa* (Hobi *et al*., 2022). On the other hand, *M. pachydermatis* had been isolated from other animals such as cat, goat, pig, horse, cow, bear and humans (Hadina *et al.,* 2023).

Molecular characterisation of *M. pachydermatis* isolates were carried out by comparing the sequences of the LSU rRNA region obtained in the study with sequences of other isolates reported from Kerala and with reference sequences. The LSU rRNA region and D1D2 portion of the LSU rRNA region has been used by many researchers for the molecular characterisation of *Malassezia* spp. Puig *et al.* (2016) and Hadina *et al.* (2023) carried out the molecular characterisation of *M. pachydermatis* isolates and reported that based on sequence of LSU rRNA/D1D2 region, five sequence types (Type I to V) could be detected. From the phylogenetic tree, it was observed that the isolates obtained in the present study clustered into two groups. The isolates 28MIB2024 (Wayanad district), 180MIB2023 (Thrissur district), 201MIB2024, 202MIB2024 and 203MIB2024 (Thiruvananthapuram district) clustered with Type I sequences and isolates 84MIB2024, 177MIB2023 (Wayanad district) and 200MIB2024 (Thiruvananthapuram district) clustered with Type V sequences, revealing diversity in the *M. pachydermatis* sequence types prevalent in Kerala (Fig 7). From the results, it could be concluded that the predominant sequence type of *M. pachydermatis* in Kerala is Type I. In a previous study carried out by Daniel *et al.* (2022) in Kerala, all the isolates obtained from Wayanad district were of Type I. But in this study, in addition to Type I sequences, *M. pachydermatis* with Type V sequences were also isolated from Wayanad which indicated that the diversity of the pathogen in the district is evolving. Puig *et al.* (2016) based on analysis of sequences of D1D2 region which is a part of LSU region grouped *M. pachydermatis* isolates obtained from dogs into Types I, III and V. In another study conducted by Hadina *et al*. (2023), the LSU types of *M. pachydermatis* reported from dogs were also Types I, III and V.

Conclusion

Malasseziosis is a relatively common dermatological disorder in dogs in Kerala and is caused by *Malassezia pachydermatis*. The isolates of *M. pachydermatis* prevalent in Kerala fall into two types, Type I and V, based on the sequence of the LSU rRNA region. While microscopical examination can be used for diagnosis of the condition, molecular characterisation can throw light on the species and diversity of the isolates prevalent in a geographical area.

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Conflict of interest

The authors declare that they have no conflict of interest.

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