The Pharma Innovation Journal 2023; SP-12(11): 2142-2144

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; SP-12(11): 2142-2144

www.thepharmajournal.com
Received: 25-09-2023
Accepted: 30-10-2023

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Molecular identification of *Malassezia pachydermatis* isolated from dermatitis cases in dogs

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Abstract
Malassezia spp. are commensals of the normal cutaneous microbiota of humans and animals. These yeasts may become opportunistic pathogens under certain conditions and cause dermatitis and otitis externa in dogs. *Malassezia pachydermatis* is the most common cause of Malassezia in dogs. In this study skin and ear swabs from suspected cases were cultured on Modified Dixon's Agar (MDA) and the subsequent isolates obtained were initially characterized on the basis of colony characteristics, gram staining, and microscopic morphology followed by confirmation by polymerase chain reaction (PCR) targeting large subunit ribosomal RNA gene. Positive cases were treated with oral itraconazole at 5 mg/kg bodyweight, orally once daily for 28 days giving excellent clinical response and quick recovery.

Keywords: *Malassezia pachydermatis*, MDA, PCR, itraconazole

Introduction
Malassezia pachydermatis is a commensal yeast residing naturally on the skin of mammals and birds especially in sebaceous gland-rich areas. These are aerobic, non-fermentative, urease-positive organism and grows well at 35 to 37 °C. *M. pachydermatis* is a round to oval or classical peanut shape, thick-walled and up to 5 micrometres in length. These are lipophilic, non-lipid dependent, non- mycelial saprophytic yeasts. It is mostly isolated from areas of skin and ear with high pH levels [1, 2]. There are several predisposing factors responsible for malasseziosis in dogs, these are allergic dermatitis and canine atopic dermatitis, secondary to infection to flea bite, high humidity, presence of skin folds, damage to skin, altered skin pH, excessive sebum production and moisture accumulation [3, 4]. They are primarily responsible for causing otitis externa in dogs with a prevalence rate amounting to 70% [5, 6]. Clinical examination reveals affected areas having typical clinical signs on abdomen, under arm, neck, groin, between toes and on lips. Other clinical signs observed constitute skin irritation, hair loss, greasy skin and hair, bad odour, gray or white scales, redness and inflammation [7]. Malasseziosis is a prevalent disease in dogs and is complex in nature due to its ability to proliferate due to compromise of host immune system as well as yeast virulence [8]. This disease often leads to secondary complications if left untreated, hence the development of rapid, sensitive, specific diagnostic assays is of prime importance in order to formulate effective treatment and control strategies in future. Concerning this problem, the present study aimed to develop a PCR-based assay to detect *M. pachydermatis* during the early phase of infection.

Materials and Methods
Twelve dogs were presented to the Veterinary Clinical Complex, Nagpur Veterinary College, Nagpur. Which having the clinical signs of alopecia, pruritus, erythema, hyperpigmentation, foul odour and otitis externa suggestive of malasseziosis were subjected to detailed clinical examination. The samples were collected with help of sterile swabs dipped in normal saline. Skin and ear swabs were collected by rubbing the sterile swabs on the affected areas to procure loose skin scrapings and discharge from ear lesions of suspected cases of malasseziosis in dogs.
The skin and ear swabs were inoculated on Modified Dixon's Agar for the cultural examination and incubated at 37 °C for up to seven days [9]. After 7days colonies were appeared on MDA (Modified Dixon Agar), then the smear was prepared and stained with methylene blue and seen under microscope at 40x magnification.

Total DNA was extracted from the pure cultures of the isolates using conventional phenol – chloroform method [10] and Extracted DNA samples were eluted in nuclease-free water and stored at−20 °C until use. As per the prescribed protocol, the quality (absorbance ratio 260/280) was measured spectroscopically (Nanodrop, Thermo Scientific, Fisher, USA) for each extracted sample. The DNA was subjected to Polymerase chain reaction targeting subunit of ribosomal RNA gene for the confirmation of the Malassezia organism. The primers and protocol used for PCR are as follows [11].

Table 1: Primers for detection of *Malassezia* spp.

Mal 1- Forward	5'AGCGGAAAAGAAACT 3'
Mal 2 - Reverse	5'GCGCGAAGGTGTCCGAAG 3'

The sequences of LSU of the rRNA gene of *Malassezia* species which is highly conserved in all Malassezia yeast were considered to be a genus-specific PCR. The conventional PCR was performed in a 50 µl reaction mix. Amplification of the rRNA gene of Malassezia was done by using primers (Table 1) and the following thermocycling conditions; the initial denaturation was done at temperature 94 °C for 2 min, then the denaturation at the same temperature condition for 30 sec. Annealing at 55 °C for 1 min, Elongation at 72 °C for 1 min, and Final extension at 72 °C for 10 min. The electrophoresis was carried out in 1.2% agarose gel and documented [11].

Results and Discussion

Following isolation of *Malassezia* on MDA, the colonies were identified. The colonies appeared cream to buff coloured with a smooth convex surface (Fig. 1). Similar to our study, [12] successfully isolated *Malassezia* on MDA at 37 °C for five days with a higher isolation rate compared to Sabouraud Dextrose Agar. Isolated colonies were stained with gram staining purple coloured oval round bodies were found under 40x magnification. The immersion smear of exudate from skin lesions stained with methylene blue stain revealed "Foot Print "shaped cells morphology (Fig. 2). Similar to our findings, microscopic examination of colonies in a previous study revealed dark blue colored footprint shaped organism on Gram's staining [9]. Further, the extracted DNA from the isolates was amplified by the conventional PCR method and the amplicon size obtained was between 541 bp to 579 bp, and the samples were confirmed positive for the malasseziosis (Fig. 3). A study [13] revealed that all the twelve isolates were positive for the PCR targeting LSU region of *Malassezia* spp. In our study, the positive cases of dogs suffering from malasseziosis were treated with oral itraconazole at dose rate of 5 mg/kg orally OID for 28 days. This designed treatment gave excellent results and good clinical response. In a similar study, it was reported that itraconazole given at 5 mg/ kg bodyweight daily for three weeks had good efficacy against Malassezia infection in dogs [14].

Fig 1: Colonies of *M. pachydermatis* seen on MDA agar

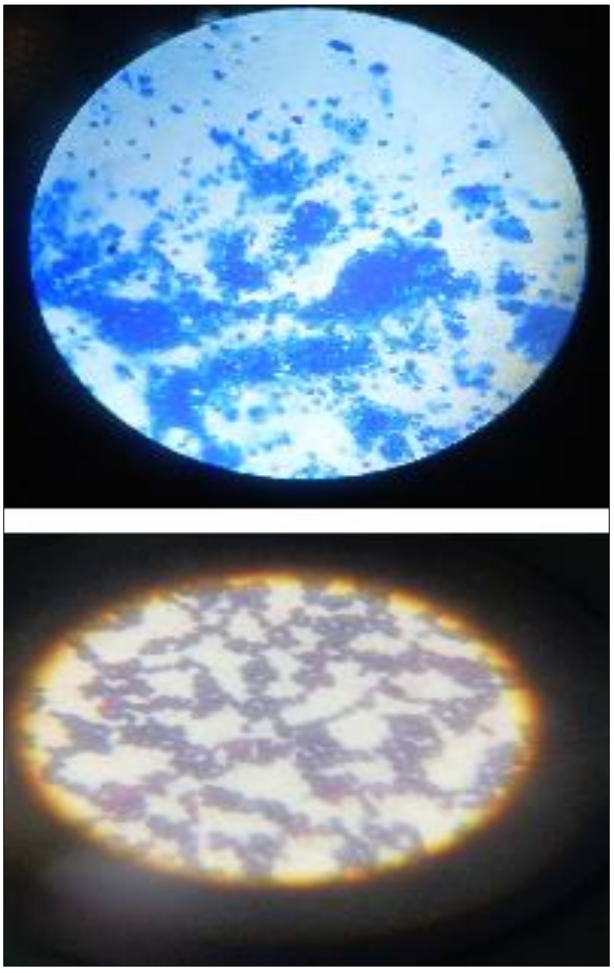

Fig 2: Staining Characteristics of *M.pachydermatis* seen by (A) Methylene Blue dye and (B) Gram Staining

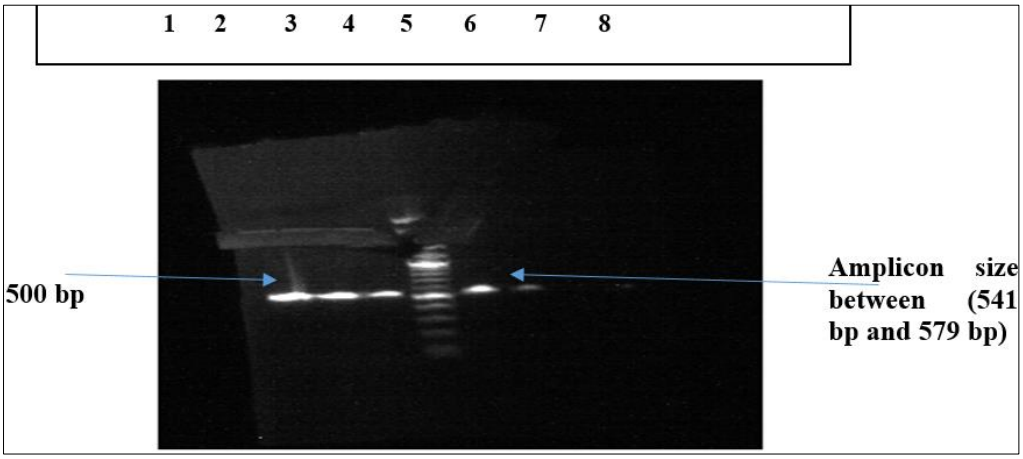

Fig 3: Molecular detection of *Malassezia* spp. (Lane 1 –Negative control; Lane 2, 3, 4, 6 & 7– Isolates of *Malassezia* spp.; Lane 8 – negative sample, Lane 5 –100 bp ladder

Conclusion

The conclusions derived from this study suggested that early detection of *Malassezia pachydermatis* in suspected dogs is significant for devising a treatment plan to cure the disease. The Cultural and molecular characterization of *M. Pachydermatis* using effective diagnostic assays can prove to be beneficial to clinicians in curbing this disease. The confirmed cases of malasezziosis-infected dogs when treated with oral itraconazole at a dose rate of 5mg/kg orally OID for 28 days gave excellent results and incited good clinical response.

Acknowledgment

All Authors are thankful to the Department of Veterinary Microbiology and Animal Biotechnology T & R cell, Department of Maharashtra Animal & Fishery Sciences University, Seminary Hills, Nagpur-440006, India for the provision of financial support and research facility required to conduct this research.

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