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Original Research

Conventional Characterization and PCR based Diagnosis of Malassezia pachydermatis from Cerumen of Healthy Indian Cats

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Abstract

Malassezia pachydermatis, a non-obligatory lipophilic yeast has been standing in the focus of interest because of its highly zoonotic nature. Systemic infections in immunocompromised patients, biofilm formation and causing relapses are common and recently they have been classified as an emergent pathogen. A total of 236 cerumen samples were collected from both the ear canal of 118 adult cats without otitis externa and subjected simultaneously to both conventional and molecular tests. Samples were inoculated on Sabouraud's Dextrose Agar with 1% sterile olive oil and incubated at 37 ℃ for 2-4 weeks. Nested Polymerase Chain Reaction was performed after extracting the DNA directly from cerumen samples. The present study established nested PCR as a sensitive and rapid technique for detection of the M. pachydermatis. The study also revealed the risk of the companion animal owners because a moderately high percentage of healthy cats acted as a carrier of M. pachydermatis.

Keywords: Cats, Malassezia, Nested PCR

Introduction

Recently on basis of the molecular data, genus *Malassezia* has been revised and now it consists of 18 species: *M. pachydermatis, M. furfur, M. yamatoensis, M. globosa, M. obtusa, M. restricta, M. nana, M. sympodialis, M. slooffiae, M. caprae, M. equine, M. cuniculi, M. psittaci, M. brasiliensis, M. dermatis, M. japonica, M. vespertilionis* and *M. arunalokei* (Dawson, 2019). *M. pachydermatis* does not exhibit an absolute requirement for lipid but its growth can be enhanced by the addition of lipid substrates to SDA media (Morris *et al*., 2005).

M. pachydermatis colonizes the skin and mucosal areas of dogs, its natural host in a very low number and they have been isolated from many warm-blooded animals like cats, horses, etc (Marrero *et al*., 2017; Dawson, 2019). Infections in animals occur due to exposure to favorable environment and substrate which ultimately trigger an increase in the number of these yeasts leading to a parasitic form (Marrero *et al*., 2017). Zoonotic infections due to this yeast in immunocompromised humans have caused life-threatening systemic and invasive infections including fungemia, endocarditis, peritonitis and interstitial pneumonia (Gaitanis *et al*., 2012; Ilahi *et al*., 2017). Dog and cat owners are at high risk due to the mechanical transfer of the yeast from their pets. Few reports have traced back the human infection to their pet dogs (Fan *et al*., 2006). Thus, the potential of this organism to human exposure is quite high and poses a great risk.

Isolation and identification based on the microscopic and physiological methods (utilization of different concentration of Tween and catalase test) are used as the 'gold standard' diagnostic techniques for *Malassezia* spp (Shokohi *et al*., 2009). But it is difficult, time-consuming and often requires skilled personnel to identify yeast characteristics. Molecular tools have always been more sensitive and specific than confirming through phenotypic characters (Sugita *et al*., 2001; Affes *et al*., 2009). But the isolation of the organism in culture media and then their molecular characterization is still problematic and laborious. Therefore, the present study was aimed at isolation, conventional cultural characterization and PCR based confirmation of *M. pachydermatis* from cerumen of healthy Indian Cats.

Materials and Methods

From December 2018 to September 2019, a total of 236 cerumen samples were obtained from both the ear canals of adult domestic cats without otitis externa from Darjeeling and Kolkata, West Bengal, India (Figure 1). Of 118 cats, 67 (56.78%) were females and 51 (43.22%) were males. Only native Indian breed of cats were targeted for collection of samples for this study. The present study was carried out in the Department of Veterinary Public Health (VPH), Faculty of Veterinary and Animal Sciences, West Bengal University of Animal and Fishery Sciences (WBUAFS), Kolkata, West Bengal, India.

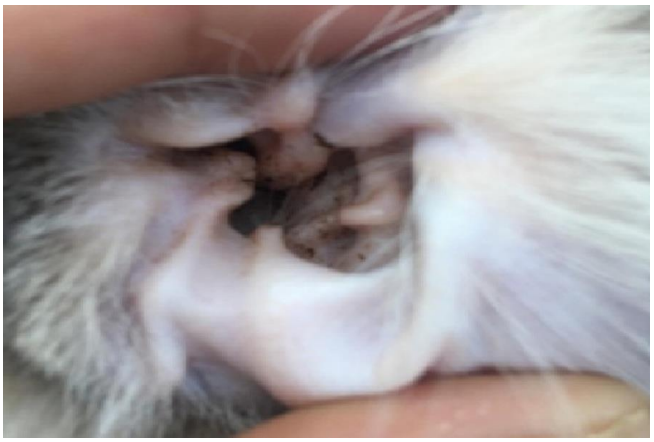

Figure 1: Showing cerumen in the external ear canal in a healthy Indian cat

Identification by Routine Methods

Samples were collected aseptically from the external ear canals using sterile cotton swabs. Samples were inoculated on SDA media (Difco™) containing cc supplement (0.05% chloramphenicol and 0.05% cycloheximide with 1%

sterile olive oil. Plates were incubated at 37°C for up to 4 weeks and observed periodically for any kind of yeast growth.

Cultures were evaluated both macro and microscopically. Subsequent transfers on SDA plates without 1% sterile olive oil were done to check the non-lipid dependent strains of *M. pachydermatis*. The growth of the isolates was also tested on SDA at 41°C (Petrov *et al*., 2013). Catalase test, urease activity and utilization of non-ionic detergents Tween 20, 40, 60 and 80 were carried out following the identification scheme described by Guillot (1996).

Identification by Molecular Method (Nested PCR)

The Nested PCR was carried out using two sets of primer which targeted internal transcribed spacer (*ITS*) of the ribosomal RNA (*rRNA*) gene. 1st set of primers were ITSIF-N (GGATCATTAGTGATTGCCTTTATA) and ITS4-R (TCCTCCGCTTATTGATATG). 2nd set of species-specific primers for *M. pachydermatis* were M.pa-F (CTGCCATACGGATGCGCAAG) and 5.8S-R (TTCGCTGCGTTCTTCATCGA) (Sugita *et al*., 2001). The gene sequence of the isolate has been deposited to GenBank database having accession number MT256206.

QIAamp DNA Mini Kit (Qiagen) for tissue was used for the isolation of DNA directly from cerumen samples with a spin-column procedure according to the protocol given by the manufacturer. PCR was carried out with 50 µl reaction buffer which consisted of 20µl of genomic DNA, 500 mM KCl, 100 mM Tris-HCL (pH-8.3), 15 mM $MgCl_2$, Triton X-100 (0.1%), 200 µM of each dNTP (dATP, dCTP, dGTP and dTTP), 2.5 U of *Taq* DNA polymerase and 30 pmol of each primer (Sigma-Aldrich) made up to a volume with pure sterile nuclease-free water (Molecular grade). The PCR was performed by using T100™ Thermal Cycler (Bio-Rad®**)** with the following protocol: initial denaturation at 94°C for 3 min, followed by 30 cycles at 94°C for 30 s, 57°C for 1 min, 72°C for 50 sec and a final extension for 72° C for 10 min. 1µl of the amplified product was added to the new reaction mixture having the same components described above. The PCR protocol consisted of initial denaturation at 94°C for 3 min followed by 30 cycles at 94°C for 30 sec, 62°C for 1 min, 72°C for 40 sec and a final extension at 72°C for 10 min (Sugita *et al*., 2001). The resulting PCR products were separated in 1% agarose gels in 0.5X Tris-borate-EDTA buffer and stained with Ethidium bromide, and then images were obtained by using a Gel Doc imaging system by Bio-Rad®.

Results and Discussion

A total of 97 *M. pachydermatis* isolates were found positive culturally from a pool of 236 samples giving an overall prevalence of 41.1% in the study population. Many researchers have reported this kind of prevalence in dogs with dermatitis (Rasamalla and Kumar 2017) and healthy dogs (Girao *et al*., 2006; Ravikant *et al*., 2015), but this type of research is not well studied in healthy pet cats and only a few reports are available which show a low prevalence (Cafarchia *et al*., 2005). The high prevalence of this yeast might be due to hot and humid weather and geographical variation in the study region which is supported by findings of Reddy and Kumari 2015. Of 97 isolates, 54 isolates and 43 isolates were obtained from female and male cats respectively. No sex predisposition was seen which was similar to work carried out by other authors (Crespo *et al*., 2002; Cafarchia *et al*., 2005). *M. pachydermatis* isolates had pale yellowish, cream-colored colonies which were typically smooth to slightly wrinkled (Figure 2). Microscopic examination with Gram's staining revealed small, ellipsoidal and sometimes spherical budding yeast cells (Figure 3). 16 isolates and 7 isolates from female and male cats respectively also grew on SDA without 1% sterile olive oil which reveals the non-lipid dependent nature of these isolates. All the isolates were urease positive on Christensen's media after 48-hour incubation at 37°C and produced effervescence on glass slide due to the liberation of free oxygen when a drop of hydrogen peroxide was placed on it which makes the organism catalase positive. All the isolates utilized tween 20, 40, 60 and 80 and grew on SDA containing 1% sterile olive oil at 41°C. Molecular identification by Nested PCR assay confirmed 117 (49.58%) isolates as *M. pachydermatis* indicating a sensitivity of 8.48% more than conventional methods. The isolates generated a band at 220 bp which was specific for *M. pachydermatis*. In Figure 4, Lane 1 and 2 contains a 100bp DNA ladder (Promega™) and positive control departmental isolate respectively. Lane 3-8 consisted of test samples.

Figure 2: Cream colored colonies of *M. pachydermatis* on SDA plate

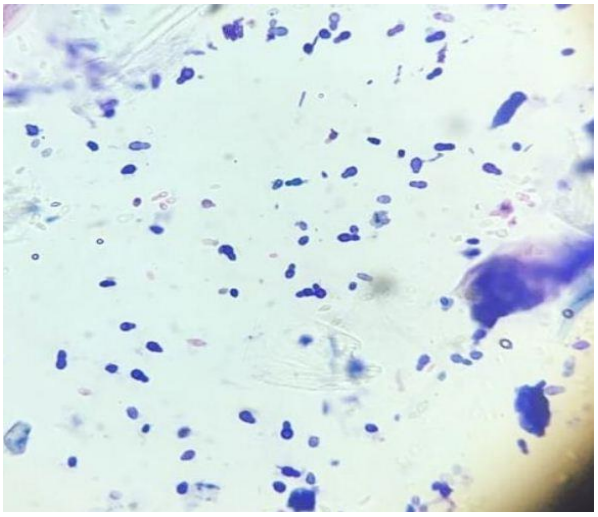

Figure 3: Gram's staining of the isolates revealing small, ellipsoidal and spherical budding yeast cells (100X)

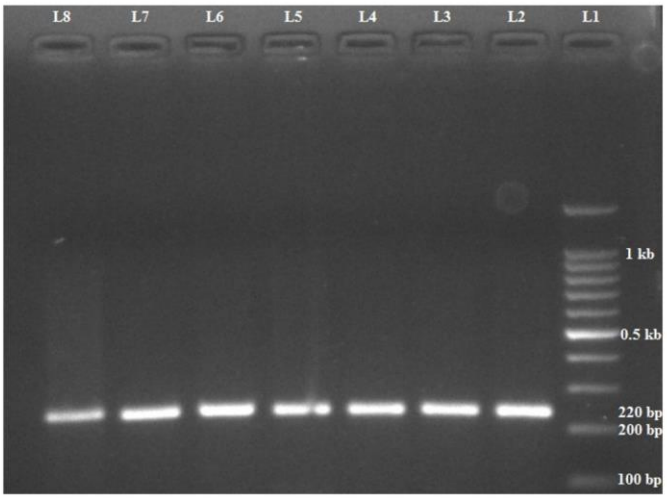

Figure 4: Gel documentation showing nested PCR products (220 bp). Lane 1, 100 bp ladder; Lane 2, Positive control; Lane 3-8, Test samples

Conclusion

Nested PCR assay for identification of *M. pachydermatis* is highly sensitive and specific and the results can be obtained within a day which serves great importance in a hospital or laboratory setting where time and treatment are of importance. The high prevalence of this yeast from pet cats which was seen in the present study can pose a significant public health risk to humans especially immunocompromised owners and neonates as outbreaks are not uncommon due to *M. pachydermatis*.

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Conflict of Interests

There is no conflict of interest.

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