



International Journal Of
**Recent Scientific
Research**

ISSN: 0976-3031
Volume: 7(3) March -2016

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THE OFFICIAL PUBLICATION OF
INTERNATIONAL JOURNAL OF RECENT SCIENTIFIC RESEARCH (IJRSR)
<http://www.recentscientific.com/> recentscientific@gmail.com



ISSN: 0976-3031

Available Online at <http://www.recentscientific.com>

International Journal of Recent Scientific Research
Vol. 7, Issue, 3, pp. 9383-9386, March, 2016

International Journal
of Recent Scientific
Research

RESEARCH ARTICLE

INCIDENCE OF *M.JAPONICAIN* MODERATE DANDRUFF CONDITION IN SOUTH INDIA AND ITS SENSITIVITY AGAINST TEA TREE OIL, GERANIOL, CITRONELLOL AND KETOCONAZOLE

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ARTICLE INFO

Article History:

Received 06th December, 2015

Received in revised form 14th January, 2016

Accepted 23rd February, 2016

Published online 28th March, 2016

Keywords:

Malassezia, PCR-RFLP, DNA sequencing, Tea tree oil, Geraniol, Citronellol

ABSTRACT

Background: Many previous study reports over the years recorded the prevalence of different *Malassezia* species among various geographical conditions.

Objective: To report the incidence of rarely isolated *Malassezia japonica* and also to identify its susceptibility towards essential oils and Ketoconazole.

Methods: Flakes were collected by rubbing the swabs over the dandruff scalp. *Malassezia* spp were isolated by plating on Modified Leeming-Notman agar medium and Modified Dixon agar. Isolated *Malassezia* yeasts were further identified by PCR-RFLP and confirmed by DNA sequencing. Susceptibility testing was done by micro tube dilution method.

Results: 60 isolates were taken from 45 subjects and among that one isolate is identified as *Malassezia japonica* and rest 50 other *Malassezia* isolates were reported separately. Susceptibility of the *M.japonica* ranges from 396.9 mcg/ml to 500 mcg/ml for the tested tea tree oil, geraniol, citronellol and for ketoconazole it is 3.12 mcg/ml. These tested essential oils can be considered as natural actives to manage dandruff.

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INTRODUCTION

Malassezia species are the commensal yeasts of the normal human skin. They are known to produce clinical disease under conditions that permit massive growth of the fungus. *Malassezia* is associated with clinical infections such as pityriasis versicolor (PV), folliculitis, seborrheic dermatitis, some forms of atopic dermatitis, Confluent and reticulate papillomatosis and even systemic infections. (Cabanes, 2014).

Currently, *Malassezia* genus has been expanded to include 14 species comprising *M.furfur*, *M.pachydermatis*, *M.symptodialis*, *M.obtusa*, *M.restricta*, *M.slooffiae*, *M.globosa*, *M. dermatis*, *M.equina*, *M.japonica*, *M.yamatoensis*, *M.nana*, *M.caprae* and *M.cuniculi*. The *Malassezia* species can be identified using various physical, chemical and metabolic characteristics. (Gucho *et al.*, 1996). Molecular biology toolkits using rRNA sequence analysis and DNA comparisons have been of great use in identification and classification of the *Malassezia* spp.

M.japonica was first reported in Japan from atopic dermatitis patients as well as healthy subjects (Sugita *et al.*, 2003) but reported rarely among rest of the world. In India *M.japonica* has been isolated from psoriasis vulgaris patients (Rudramurthy *et al.*, 2014) and reported the phenotypic and molecular characterization (Honnar *et al.*, 2015)

The major problem faced during the management of infectious microorganisms is the paucity of new drugs (Finch and Hunter, 2006). Prior art indicates that the drug resistance has been a major threat across the globe and have presented a scientific challenge and an economic opportunity for the researchers and the pharmaceuticals to bring out new drugs to which the microbes can be susceptible (Liss and Batchelor, 1987). In this regard, we have checked the presence of a relatively newer species, *M.japonica* isolated from mild to moderate dandruff conditioned subjects living in Bengaluru region. The isolated strains were confirmed with molecular biology toolkits and DNA gene sequencing.

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A common strategy to forestall antibiotic resistance is to use new compounds with chemical structures that are not based on existing synthetic antimicrobial agents (Shah, 2005). In this regard, the susceptibility of the isolated species *M.japonicato* tea tree oil, geraniol and citronellol these essential oils basically contains mixture of various mono and sesquiterpenes, minimum inhibitory concentration were checked and discussed with sigma grade ketoconazole, as standard control.

MATERIALS AND METHODS

Study Subject and Study site

Forty five subjects with mild to moderate dandruff conditions were selected during 2014, winter season in Bengaluru region. Based on the visible flakes found in the scalp, the volunteers were categorized as mild to moderate dandruff subjects by the dermatologist. Formal consent was taken from all volunteers before initiation of the study, and a detailed study protocol approval was taken from an Independent Ethics Committee.

Volunteers were advised not to wash their scalp, nor apply any antidandruff oil for three days prior to the day of sampling. Scalp samples were collected with a sterile swab dipped in 0.01% Tween 80 rubbed over one-inch area.

Scalp samples were enriched for a period of four weeks at 30°C in Dixon broth containing chloramphenicol and cycloheximide. From the enriched Dixon broth, cultures were grown in Leeming and Notman agar (LMNA) (Leeming et al., 1987) and Dixon agar (Midgley et al., 1989). For identification, pure cultures in Dixon agar were transferred and maintained at Department of Medical Microbiology and Dermatology, Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh.

Microscopic and Physiological Characteristics

Colonies were dull, wrinkled over entire edge on Leeming and Notman agar and by methylene blue staining, the cell shape looks ellipsoidal and bud pattern as sympodial when observed under light microscope. For catalase test, few drops of hydrogen peroxide was added on isolated colony and instant production of gas bubbles was observed.

Molecular characteristics

Confirmation of *Malassezia japonica* species' level molecular characterization was carried out by sequencing of the 26S rDNA by amplifying this region with universal primers NL-1 (59-GCATATCAATAAGCGGAGGAAAAG-39) and NL-4 (59 GGTCCGTGTTTCAAGACGG-39) (Kurtzman & Robnett, 1997). Sequencing of the ITS2 region of rDNA gene was carried out with the same primer pairs, ITS3 and ITS4, as used for PCR-RFLP (Gaitanis et al., 2006; Rudramurthy et al., 2014). Additionally, amplification of the intergenic spacer 1 (IGS1) region of rDNA was performed with the primer pairs 26SF (59-ATCCTTTGCAGACGACTTGA- 39) and 5SR (59-AGCTTGACTTCGACGATCGG-39) (Sugita et al., 2002). Purification of amplified gene products (26S, ITS2 and IGS1

regions of rRNA gene) was performed using gel extraction kit (QIAquick; Qiagen). Sequencing PCR of both the strands of the DNA was performed using the above-mentioned primers and the BigDye Terminator Cycle Sequencing kit, version 3.1 (Applied Biosystems). The PCR products were purified and analysed on an ABI 3130 Genetic Analyzer (Applied Biosystems). The sequences were analysed using Bionumerics software (version 7.1; Applied Maths), and compared with sequences in the GenBank DNA database (<http://www.ncbi.nlm.nih.gov/Genbank/index.html>) and the CBS-KNAW Fungal Biodiversity Centre database (<http://www.cbs.knaw.nl/>).

Microdilution method

Stock solutions were prepared by dissolving 10mg of sample in 2 ml DMSO and 3ml dixon broth. 100µL of sample was serially diluted in the microtiter plate to obtain the concentration ranges from 1000 mcg/ml to 0.976 mcg/ml and then dosed with 100µL of the inoculum (10^4 to 10^5 cell per ml) in each well of the micro - titre plates except the test control. The micro- titre plates were incubated at 32 °C for 3 days in BOD incubator at controlled humidity to prevent the medium evaporation and better fungal growth (Nascente et al., Margarita et al.,).

Owing to the opacity of the modified Leeming-Notman medium, the susceptibility or the resistances of the organisms were determined by the non-turbid metric method by adding 50 microliters of 0.1% Alamar blue. After 24- hour incubation at 32 °C, the plates were visually read. Due to the metabolic activity of *Malassezia* spp, the color changes from blue to pink was observed. The lowest concentration of the drug preventing the color changes from blue to pink was considered as the MIC (Floressia et al., 2014).

RESULTS

Salient features and the molecular characteristics of *Malassezia japonica* isolate is shown in Table 1. Figure 1 depicts the micrograph of *M.japonica* isolate. Results of species identification by PCR-RFLP were in similar with the results of ITS2, 26S and IGS1 regions of rDNA sequences (Fig.2). NCBI BLAST analysis of both the sequences of the 26S rDNA and the ITS2 region of rDNA had more than 99% identity with the sequences of the *M.japonica* type strain, CBS9348, whereas the IGS1 region had 95% identity with the type strain of *M.japonica* (M 9966, AB105063).

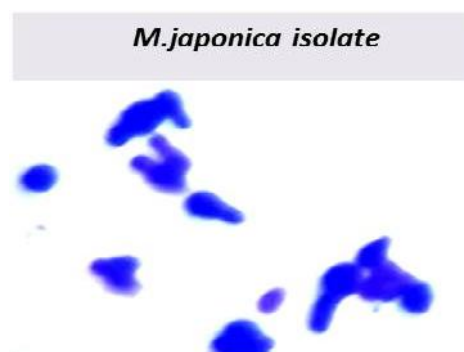


Figure 1 Micrograph of isolated *Malassezia japonica*

Table 1 *M.japonica* isolated volunteer details

Volunteer code	Age	Gender	Colony morphology	Dandruff severity
K36	21	Male	Dull and wrinkled	Moderate

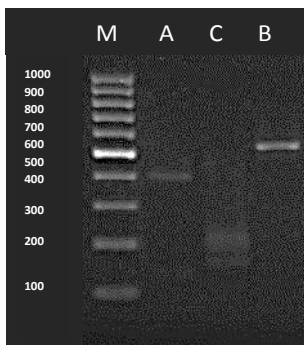


Figure-2 Agarose gel picture of PCR-RFLP profile of *M. japonica* species after digestion with restriction enzymes- AluI, BanI, MspAI.

M= Molecular marker, A=AluI, B=BanI, C=MspAI

Antimicrobial susceptibility of the *M.japonica* isolate is given in Table 2. *M.japonica* isolates showed susceptibility towards essential oils which ranges from 396.9 mcg/ml to 500 mcg/ml and 3.12 mcg/ml for Ketoconazole.

Table 2 Minimum Inhibitory Concentration (mcg/ml) of essential oils against *M. japonica*

Strain	MIC (mcg/ml) #			
	Tea tree oil	Geraniol	Citronellol	Ketoconazole
<i>M.japonica</i> isolate	500.0	396.9	500.0	3.12
<i>M.japonica</i> -CBS-9348	500.0	500.0	500.0	0.78

Average of 3 replicate determinations

DISCUSSION

M. globosa, *M. sympodialis*, *M.furfur* were reported to be mostly isolated *Malassezia* spp among dermatoses conditions (Erchiga *et al.*, 2000; Gupta *et al.*, 2001; Kim *et al.*, 2000). Several studies in the past have observed differences with respect incidences of *Malassezia* species from both healthy and diseased individuals, but *M. japonica* is hardly reported across the globe. A very few studies have reported the occurrence of *M. japonica* in Psoriasis vulgaris patients (Rudramurthy *et al.*, 2014, Sugita *et al.*, 2003).

Lack of standard culture medium for primary isolation, variations in the technique of isolation and recovery, and finally, incomplete description of phenotypic tests for identification of *M. japonica* have limited the description of this species. However, these strains and the data generated from such studies might serve as an important source to further assess the pathogenicity, resistance and susceptibility of the strains against various antibiotics/drugs/compounds, etc. Although several studies have been reported on the antifungal activity of essential oil, very limited information is available on use of essential oils on fungal species on human host such as *Malassezia* species (Naeini *et al* 2011).

Tea tree oil, Geraniol and Citronellol are commonly used as fragrance components in consumer products but they also have antimicrobial and anti-inflammatory property (de Cassia da Silveira e Sa *et al.*, 2013). Tea tree oil, essential oil of *Melaleuca alternifolia* L., is very often used in the cosmetic

application and claimed to be an effective antidandruff compound at concentration below 5% (Lee *et al.*, 2013). One of the earlier studies has shown that 5% tea tree oil to be effective in the treatment of mild to moderate dandruff (Carson *et al.*, 2006). Cutaneous application of geranium oil inhibited the cellular inflammation and it is suggested during aromatherapy massages for suppressing inflammatory symptoms (Maruyama N, *et al.*, 2005). Khosravi *et al* (2015) studied the distribution pattern and population size of *Malassezia* species in dogs with atopic dermatitis (AD) and the inhibitory efficacy of *Zataria multiflora*, *Thymus kotschyanus*, *Mentha spicata*, *Artemisia sieberi*, *Rosmarinus officinalis* and *Heracleum persicum* essential oils against pathogenic *Malassezia* isolates. Study was conducted against eight different *Malassezia* species (*Malassezia pachydermatis*, *M. globosa*, *M. restricta*, *M. sloofiae*, *M. furfur*, *M. nana*, *M. obtusa* and *M. sympodialis*). Antifungal susceptibility test revealed the inhibitory efficacy of essential oils on pathogenic *Malassezia* isolates with MIC values ranging from 30 to 850 mg/ml. Among the tested oils, *Z. multiflora* and *T. kotschyanus* exhibited the highest inhibitory effects. (Khosravi *et al*, 2015) Another study conducted by Selvakumar *et al.*, (2012) demonstrated the establishment of a natural compound from *Coleus amboinicus* and *Eucalyptus globules* as an antidandruff agent against *M.furfur* which can be explored for the production of potential antifungal drug and novel pharmaceutical and cosmeceutical leads. Published value for Ketoconazole against *M.japonica* is 0.016 mcg/ml (H. R. Ashbee 2007).

In the current study we have reported the antifungal activity of tea tree oil, geraniol and citronellol against *M. japonica* for the first time. MIC values of essential oils were found to be between 396.9 mcg/ml to 500 mcg/ml against the tested *M.japonica* strains.

Our current study corroborates with the findings reported for *M.furfur* by Nenoff *et al*, (1996). MIC value of tea tree oil was reported in range of 556.2 and 4,450.0 mcg/ml against the tested *M. furfur* strains.

Antifungal susceptibility pattern of *M.japonica* isolate and *M.japonica* CBS 9348 standard strain were more or less similar in the range of 0.78 mcg/ml to 3.18 mcg/ml (Table 2). This also correlates well with the susceptibility pattern of other *Malassezia* isolates from 4 different species (*M. furfur*, *M. sympodialis*, *M. obtusa* and *M. globosa*) against 4 different azoles (fluconazole, itraconazole, ketoconazole and voriconazole), Miranda *et al*, 2006. For the 95 *Malassezia* strains, the MIC ranges reported were in the range of <0.03–4 mcg/ml for ketoconazole.

CONCLUSION

This study describes a relatively rarely isolated species, *M. japonica*, isolated from the scalp of the individuals suffering from mild to moderate level of dandruff in south India region. We have also reported alternate approaches to conventional treatment where certain essential oils can be explored at a concentration which is safe and efficacious. Currently, not much information is available to establish role of *M. japonica* in

dandruff or other dermatological conditions. There is a merit in conducting in-depth study in this direction to unravel this issue. The antifungal therapy in *Malassezia* infections demands vigilant screening for the choice of drugs especially in cases of resistance to antifungal treatment or recurrent infections. Anti*Malassezia* activity of the tea tree oil, geraniol and citronellol appear like promising candidates in development of over the counter drug or cosmetic preparations in the management of dandruff. Therapeutic benefits of these essential oil based preparations need to be investigated through clinical studies, and potent natural actives need to be utilized effectively within the permitted concentration.

Acknowledgement

The authors would like to acknowledge Dr.ArunalokeChakrabarti, Dr.Shivaprakash M. Rudramurthy and Mr.PrasannaHonnarof Mycology Division, Department of Medical Microbiology, PGIMER, Chandigarh, for theirvaluable trainings and guidance in identification of the organisms.

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How to cite this article:

Kavitha K et al.2016, Incidence of *M.Japonicain* Moderate Dandruff Condition in South India and its Sensitivity Against Tea Tree oil, Geraniol, Citronellol And Ketoconazole. *Int J Recent Sci Res*. 7(3), pp. 9383-9386.

T.SSN 0976-3031



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