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Original Article

Microbiology Section

Standardisation of a Semi-nested PCR for Early Identification of *Candida auris*: A Cross-sectional Study from Southern India

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ABSTRACT

Introduction: *Candida auris* has drawn more clinical attention due to its multidrug resistance. More than 90% of *C. auris* isolates are known to be resistant to all the antifungal drugs. Conventionally, the identification of *C. auris* has not been described precisely yet but one such ability of this yeast is that they can grow at an elevated temperature and fail to grow in the presence of cycloheximide and in automated approaches like VITEK 2, Matrix-assisted Laser Desorption/ionisation Time-of-flight (MALDI-TOF), Analytical Profile Index 20C AUX yeast (APIC20C-AUX) they can be misidentified as other species, which can lead to errors in the choice of antifungals.

Aim: To standardise a simple in-house semi-nested colony Polymerase Chain Reaction (PCR) for accurate identification of *C. auris*.

Materials and Methods: A cross-sectional study was conducted at the Department of Microbiology at Sri Ramachandra Institute of Higher Education and Research (SRIHER), Chennai, India, from March 2017 to February 2018. All the *Candida* isolates obtained from blood samples over a period of one year were considered for this study. All the isolates underwent conventional phenotypic identification, such as microscopy, sub-culturing on Sabouraud Dextrose Agar (SDA) with antibiotics and chromogenic medium and carbohydrate fermentation. Further genotypic identification was performed for all the isolates. Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR-RFLP) was initially performed, and the isolates that were unidentified by this method were sent for gene sequencing. *C. auris*-specific primers were designed manually. A semi-nested colony PCR was performed using the self-designed primers for all the isolates.

Results: A total of 87 *Candida* sp. were isolated and considered for this study. Phenotypic and genotypic identification was performed for the isolates. In the study, it was observed that *C. auris* was misidentified by commonly used phenotypic methods as *Candida haemulonii* and *Candida parapsilosis* and hence, further PCR-RFLP was performed to confirm the species. The isolates that still couldn't be identified by PCR-RFLP were sent for gene sequencing. All the isolates were subjected to *C. auris*-specific semi-nested PCR for identification. A 22 (25.3%) out of the 87 isolates produced amplicons of size 400 bp. The rest 65 (74.7%) isolates did not produce bands. All 22 isolates that produced bands were confirmed as *C. auris* by gene sequencing.

Conclusion: To conclude, an in-house, semi-nested colony PCR (2-hour) method for differentiating *C. auris* from *Candida haemulonii* was standardised. This method not only differentiates *C. auris* from *Candida haemulonii* but also other *Candida* species. This test will be useful in the early identification of *C. auris* in any microbiological laboratory set-up.

Keywords: *Candida haemulonii, Candida* sp. Polymerase chain reaction, Sequencing

INTRODUCTION

C. auris has emerged over the past decade as one of the most challenging Multidrug Resistant (MDR) organisms causing invasive infection in both paediatric and adult populations, leading to significant mortality and morbidity. It was first described in the year 2009 from the ear canal of a patient in Japan, but retrospective studies reveal that *C.auris* had been isolated as early as 1996 in South Korea and in 2008 from Pakistan. Subsequently, *C. auris* has been reported in more than 30 countries across six continents and is classified into five distinct clades with varying geographic distribution [1,2]. Traditionally, *Candida albicans* accounted for the majority of bloodstream and deep-seated infections leading to mortality. However, in current years, infection by Non Albicans *Candida* (NAC) such as *Candida tropicalis*, *Candida krusei*, *Candida dubliniensis*, *Candida parapsilosis* and *Candida rugosa* has increased significantly. Among these, *C. auris* has become an epidemiological threat causing nosocomial Bloodstream Infections (BSI) requiring intensive care [3]. Approximately, 50% of *C. auris-associated* candidemia cases have been reported from India, especially in Intensive Care Units (ICUs) [4]. In the year 2016, the Centres for Disease Control and Prevention (CDC) categorised *C. auris* as one of the emerging global health threats causing infection.

C. auris enters the bloodstream, disseminating to multiple organ systems, including kidneys, liver, spleen, muscles and joints causing severe infections especially in immunocompromised or critically ill patients thus posing a serious global threat. Unlike other *Candida* sp. *C. auris* has the capacity to form resilient 'dry biofilm' by adhering to the polymeric surfaces thus, making it immune to most of the disinfectants. As a consequence, this pathogen can persist for prolonged periods on inanimate objects such as door handles, bed rails, medical equipment, and patient care items leading to rapid and widespread transmission within health care facilities. Transmission typically occurs through direct contact with colonised/infected individuals or indirectly with the hands of contaminated health care workers and surfaces to patients in a hospital setting [5]. As *C. auris* has the ability to form biofilm, this pathogen has the ability to resist antifungal agents posing a clinical and public health challenge.

Another major concern for this pathogen is that they exhibit high rate of antifungal resistance. Most of the isolates are resistant to at least one or all the major class of antifungal drugs including echinocandins, azoles, and polyenes, thus leaving clinicians with limited treatment options. However, they are difficult to identify by conventional methods and are often misidentified as *Candida haemulonii* (most commonly), or as other *Candida* spp. sometimes even as *Cryptococcus* or *Saccharomyces cerevisiae* by common automated culture identification systems. The prevalence of infection caused by *C.auris* has not been estimated till now as the inability to identify *C.auris* using phenotypic methods [6].

Identification of *C.auris* in many health care settings are dependent on automation or Matrix-Assisted Laser Desorption Ionisation-Time of Flight Mass Spectrometry (MALDI-TOF) but, they rely on an updated database of different clades every time as it might lead to misidentification of *C. auris*, thus making it unreliable and expensive [7]. In many developing countries, laboratories still rely on conventional and biochemical methods for identification of this pathogen, increasing the risk of misdiagnosis thus leading to incorrect therapy and the subsequent development of multidrug resistance.

The present study, which will help in the early identification of this pathogen, will provide valuable insights into the prevalence and might also contribute to the global surveillance of *C. auris*. Hence, the accurate speciation of *C. auris* is important not only for choosing the appropriate antifungal agent for the treatment of patients under life-threatening circumstances caused by this pathogen, but also from the epidemiological point of view. This study aimed to design primers and standardise a semi-nested PCR for accurate identification *C. auris* by differentiating from *Candida haemulonii* which is closely related, and from other *Candida* species.

MATERIALS AND METHODS

This cross-sectional study was conducted over a period of one year. This study commenced in March 2017 and concluded in February 2018 at Department of Microbiology, Sri Ramachandra Institute of Higher Education and Research (SRIHER), Chennai, Tamil nadu, India. This study was approved by the Institutional Ethics Committee (IEC No: CSP/17/MAR/55/66).

Sample size: This study was a time bound study. 87 *Candida* isolates received in Microbiology laboratory over a period of one year from March 2017-February 2018 meeting the inclusion criteria were considered for the study.

Inclusion criteria: All the *Candida* isolates isolated from blood were considered in this study.

Exclusion criteria: The *Candida* isolated from sources other than blood was not included for this study.

Study Procedure

Phenotypic identification: Phenotypic identification was done for all the isolates. Microscopy was performed using Gram staining. Macroscopically, all the isolates were inoculated on Sabouraud's Dextrose Agar (SDA) and Malt Extract Agar (MEA) and were incubated them at varying temperatures like at 37°C, 40°C, and 42°C for 24-48 hours to observe thermotolerance. All the isolates were inoculated on chromogenic agar like Chromogenic (CHROM) agar and Triphenyl Tetrazolium Chloride (TTZ) to observe the colour variations. Sugar fermentation (2% Glucose, Galactose, Lactose, Maltose, Raffinose, Trehalose, Sucrose) were put up for all the isolates for species identification.

Genotypic identification: All the isolates were subjected to confirmation. Initially Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP) was performed for all the *Candida* isolates but it limits its identification for only a few *Candida* sp. The isolates which could not be identified were sent for gene sequencing.

PCR-RFLP: PCR was performed by using the pan-fungal primers Inter Transcribed Spacer (ITS) 1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-CCGCTTATTGATATGC-3′). RFLP was performed for further speciation using *Msp* 1, as restriction enzyme. Isolates which were not identified by PCR-RFLP were sent for gene sequencing using ITS1 primers. The sequences of these isolates were subjected to nucleotide BLAST (Basic Local Alignment Search Tool) analysis using more than two databases, compared and confirmed as accurate, based on the highest percentage of similarity [8-10].The sequences which showed 99% similarity were considered.

Standardisation of *C. auris* PCR: An in-house semi-nested colony PCR was designed and applied for all the isolates using in-house designed *C. auris* specific primers. *C. auris* - GenBank ID: MK064197 (confirmed by gene sequencing) was used as positive control and American Type Cell Culture (ATCC) *Candida albicans* 90028 as a negative control for the study.

Primer designing: The sequences of *Candida haemulonii*, *C. auris* and other *Candida* species were obtained from National Center for Biotechnology Information (NCBI) database (GenBank Accession no: LT629272.1). Multiple Sequence Alignment (MSA) was performed using MEGA X software. Based on the variation in nucleotide sequences among *C. auris* and *Candida haemulonii*, primers were designed manually.

DNA extraction and PCR: Deoxyribose Nucleic Acid (DNA) extraction was performed for all the isolates using the phenol-chloroform method with few modifications [11]. PCR was carried out in two stages. First, ITS region was amplified using ITS1 and ITS4 primers followed by, semi-nested PCR using ITS1 as forward primer and in-house-designed AURP (5′-AATGCAACGCCACCGCGAAG-3′) as reverse primer. Amplification conditions are as follows initial denaturation at 95°C for 10 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds, extension at 72°C for 30 seconds and final extension at 72°C for seven minutes. Amplified products were analysed by 1.5% agarose gel electrophoresis, viewed under UV transilluminator and recorded.

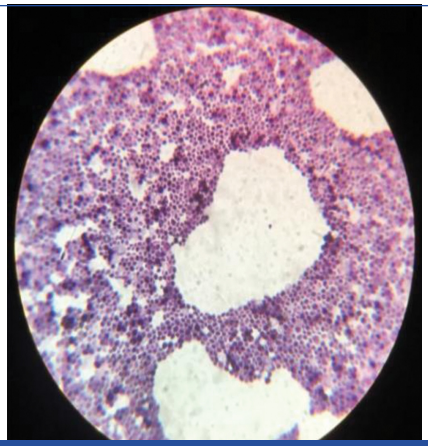
STATISTICAL ANALYSIS

Statistical analysis was done using Statistical Package for Social Sciences (SPSS) software, version 28.0 for windows. Diagnostic sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) and overall accuracy were calculated.

RESULTS

In the present study, a total of 87 *Candida* sp. were isolated. All the isolates underwent phenotypic and genotypic identification and were validated by gene sequencing.

On Gram stain, *C. auris* showed budding yeast cells with no pseudohyphae [Table/Fig-1].



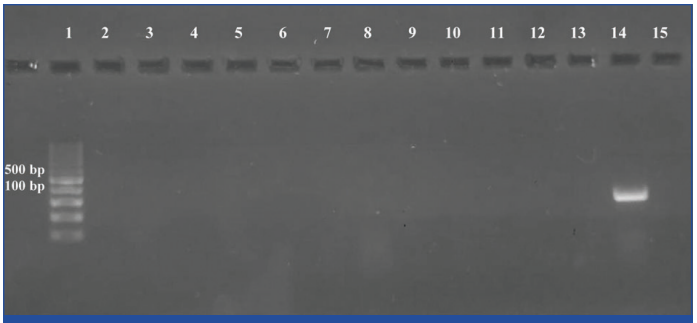
[Table/Fig-1]: Gram staining of *C. auris* under 100x magnification.

There was no significant difference observed in colony morphology compared to other *Candida* isolates. On CHROM agar, colour variation such as white, pink and purple colonies were seen [Table/Fig-2].

[Table/Fig-2]: Colour variation of *C. auris* on CHROM agar.

Temperature variation studies differentiated *C. auris* from *C. haemulonii* and other *Candida* species as they grew at 25ºC, 37ºC, 40ºC and 42ºC and were found to be thermotolerant. In sugar fermentation, all the isolates of *C. auris* fermented 2% Maltose.

All the isolates were subjected for genotypic identification. PCR-RFLP was performed to confirm the species. The isolates which couldn't be identified by PCR-RFLP were sent for gene sequencing as mentioned in [Table/Fig-3].



[Table/Fig-4]: PCR subjected to various *Candida* species (Lane 1: DNA ladder, Lane 2: *Candida albicans* ATCC 90028, Lane 3: *C.tropicalis*, Lane 4: *C.parapsilosis*, Lane 5: *C.guilliermondii*, Lane 6: *C.glabrata*, Lane 7: *C.duobushaemulonii*, Lane 8: *C.orthopsilosis*, Lane 9: *C.metapsilosis*, Lane 10: *C.famata*, Lane 11: *C.lusitaniae*, Lane 12: *C.kefyr*, Lane 13: *C.catenulata*, Lane 14: *C.auris*, Lane 15: *C.haemulonii*).

high mortality [12,13]. The epidemiological spread of *Candida auris* remains uncertain still, one of the main causes of this spread may be due to its resilient nature [14].

Studies have shown that it is difficult to distinguish *C. auris* not only from *Candida glabrata* but also from other species such as *Candida haemulonii*, *Candida guilliermondii*, *Candida famata*, *Candida parapsilosis* since all these species do not produce pseudo hyphae and no specific phenotypic characteristics are produced to differentiate them, thus posing a challenge [3,15]. Another problem with *C. auris* is that they go through morphological switching when

Phenotypic identification		Genotypic identification										
	Total (n=87)	*Candida albicans*	*Candida tropicalis*	*Candida guillier-mondii*	*Candida glabrata*	*Candida parapsi-losis*	*Candida famata*	*Candida orthop-silosis*	*Candida metapsi-losis*	*C. auris*	*Candida haemu-lonii*	*Candida pseudo-haemulonii*
Candida albicans	14	14	-	-	-	-	-	-	-	-	-	-
Candida tropicalis	22	-	22	-	-	-	-	-	-	-	-	-
Candida guilliermondii	7	-	-	7	-	-	-	-	-	-	-	-
Candida glabrata	5	-	-	-	5	-	-	-	-	-	-	-
Candida parapsilosis	16	-	-	-	-	5	3	3	2	3		
Candida sp.	23	-	-	-	1	-	-	-	-	19	2	1

[Table/Fig-3]: Phenotypic and genotypic identification of Candida isolates.

the isolates were subjected to first stage ITS and second stage *C. auris* specific semi-nested PCR for identification. A total of 22 (25.3%) out of the 87 isolates produced amplicons of size 400 bp at first stage and 300 bp in the second stage, respectively. The rest 65 (74.7%) isolates produced bands in first stage PCR (appropriate DNA extraction and confirming it as a fungi) but not in the second stage PCR. All the 22 (25.3%) isolates which produced bands in second stage PCR were confirmed as *C. auris* by gene sequencing.

The specificity of semi-nested colony PCR was tested with genomic DNA extracted using ATCC *Candida albicans* 90028 and gene sequenced archived isolates of *Candida lusitaniae*, *Candida kefyr* and *Candida catenulata.* The rest 65 (74.7%) isolates which did not produce bands in *C. auris* specific PCR were sequenced and used for analysing the specificity. No bands were observed for *Candida haemulonii* and other *Candida* isolates [Table/Fig-4]. This shows that the designed primer is specific for *C. auris*. The sequences were submitted in GenBank (Accession numbers: MK064207, MK064206, MK064198, MK064201, MK064194, MK064195, MK064196, MK064200, MK064199, MK064202, MK064203, MK064204, MK064205, MK064208). The sensitivity, specificity, PPV, NPV and overall accuracy of the assay was 100%.

DISCUSSION

C. auris has been reported as a 'superbug' resistant to most of the conventional drugs with a diverse set of risk factors causing grown on differential media making it difficult to differentiate from other *Candida* sp [16]. In the study, out of 87 isolates, 22 were identified as *C. auris*, based on the results of gene sequencing. Though, *C. auris* were misidentified by commonly used phenotypic methods, in this study all the isolates of *C. auris* fermented 2% Maltose and grew at 42ºC demonstrating it to be thermotolerant yeast, differentiating it from *Candida haemulonii* and other *Candida* species. This simple preliminary test could give a clue that the organism is *C. auris.*

Due to the limited number of biochemical and other conventional tests, a molecular identification test for *C. auris* has become important. Standard automated systems are dependent on reference databases and can misidentify *C. auris* as *C. haemulonii*, *Rhodotorula glutinis*, *Candida sake* or *Saccharomyces cerevisiae* [7,17]. DNA sequencing provides accurate identification, but it demands technicality, is time consuming, expensive and is limited to a few diagnostic labs [18]. In the current study, authors have designed primers based on ITS sequences of *C. auris* by semi-nested colony PCR. The primers designed in our study were highly specific with a corresponding band size of 300 bp. A study by Theill L et al., has designed a single tube PCR to identify *C. auris* from *Candida haemulonii* [19]. Similar studies by Kordalewska M et al., Leach L et al., developed rapid and accurate Real time as well as colony PCR for identification of *C. auris* and its closely related species. Two other studies have designed multiplex PCR for identification of *C.auris* from other species by designing two sets

of primers using unique (glycosylphosphatidylinositol) GPI protein encoding genes [20,21]. One such study by Yamamoto M et al., have designed rapid detection of *C.auris* using three sets of primers targets by Loop-Mediated Isothermal Amplification (LAMP) assay [22]. However, in current study the range of differentiation was not limited to closely related species but a wide range of unrelated *Candida* species also. The PCR standardised in our lab was done using a ready to use master mix and only one external primer compared with the above-mentioned studies Also, this single tube colony-based PCR identifies *C. auris* without the requirement of expensive instruments and a huge microbiological set-up. This test is highly specific and cost-effective and does not require technical expertise, is easy to perform and gives results within two hours after the growth of the culture. *C. auris* is known to be resistant to the commonly used azoles, hence this will help the clinician to choose one among the three echinocandins and prevent morbidity and mortality. Future prospective can focus on expanding this test in epidemiological surveys and to test the specificity in all four clades as it will be essential for improving the management of invasive candidiasis caused by this species.

Limitation(s)

The study had a small sample size and the present study was conducted using a single site (blood) and from one centre.

CONCLUSION(S)

In conclusion, an in-house semi-nested colony PCR to differentiate *C. auris* from *C. haemulonii,* and other *Candida* species will be helpful in early identification of *C. auris* in any microbiological laboratory with minimal expertise and resource limited settings. Further, this assay will also aid in the epidemiological surveillance as this primer is specifically designed for *C. auris*.

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