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Research Article

Phenotypic and Genotypic Characterization of *Candida auris*, an Emerging Pathogen Isolated from Blood

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A B S T R A C T

Introduction: Candida auris has been reported from various health care settings and has recently gained importance because of its intrinsic resistance to many classes of antifungal agents and to disinfection. The outbreak potential and high mortality associated with *Candida auris* infection reinforces the need for speciation. Routine conventional methods are cumbersome and automated systems are unable to confirm up to species level.

Materials and Methods: Candida auris isolates from consecutive non-repetitive blood cultures over a 1-year period were speciated based on phenotypic, physiological and biochemical tests and VITEK. Molecular confirmation was done by **PCR-RFLP** and **MALDI-TOF**. Anti-fungal susceptibility test was performed according to CLSI guidelines (2021), using suitable controls. Virulence factors such as production of Hemolysin, Phospholipase, Esterase and Bio-film production were demonstrated. RT-PCR was used to screen the COVID-19 status using SD-Biosensor kit. Baseline data and clinical history were collected and analysed.

Results: Of 3632 blood cultures (0.77%), 28 *Candida sp*. were isolated including 9 *Candida auris,* (9/28, 32.14%). Of these 8 were from COVID-19 positive patients (88.89%), while 1 was from COVID-19 negative patient (11.11%). Two patients survived, while the remaining 7 patients succumbed to the disease.

Conclusion: The increasing incidence of Candidiasis especially during the COVID-19 pandemic has raised the concern for early speciation. Through multi-modal strategies such as quick and correct identification, active surveillance, guided reporting, stringent infection control measures and correct use of anti-fungals through proper susceptibility testing, we can prevent the occurrence and spread of new *Candida auris* cases in the future.

Keywords: *Candida auris*, Candidemia, **PCR-RFLP**, AFST, Virulence

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Introduction

Genus *Candida*, are yeast like fungi, that are present as normal human microbiota in various parts of the human body such as the mouth, gastrointestinal tract, genito urinary tract, respiratory tract and skin. They also have the potential to cause invasive mycosis. They are bio-film producers and colonize IV lines and cannulas causing Catheter Related Blood Stream Infections (CRBSI) that impede treatment.[1,2]

Candida auris, first isolated from a Japanese patient with ear infection, has now become an emerging nosocomial pathogen, with many strains being multi-drug resistant.[3] Many health-care centres have reported outbreaks, thereby being a great challenge for the infection-control practices. It causes invasive infections, mainly bloodstream, especially in immunocompromised individuals. Recently, it is also one of the leading causes of Candidemia in nosocomial settings, especially in the intensive care units.[4,6,7,8]

It is often misidentified by conventional methods and automated systems like VITEK. **MALDI-TOF**, **PCR-RFLP** and sequencing are some of the available methods to identify this yeast accurately.[3] Four major phylogenic clades have been identified in *Candida auris*. I - South Asian, II - East Asian, III - South African, IV - South American and recently, a V clade in Iran.[5]

The present study aimed to isolate *Candida* from blood cultures using both conventional and automated methods (BACTEC) over a 1-year period, (July 2020-June 2021) and to speciate the isolates based on phenotypic, physiological, biochemical tests and VITEK and to confirm them using **PCR-RFLP** and **MALDI-TOF mass spectrometry**. The study also aimed to demonstrate the anti-fungal susceptibility patterns of the confirmed isolates, demonstrate bio-film formation and other virulence factors of the isolates and to identify the potential risk factors and appropriate containment strategies to prevent the occurrence of such infections in the future.

Materials and Methods

This observational study was conducted over a 1-year period in the Mycology section, Department of Microbiology, Chettinad Hospital and Research Institute, after obtaining due Institutional Human Ethics Committee clearance (Proposal No.044/IHEC). *Candida* isolates from consecutive non-repetitive blood cultures over a 1-year period were included in our study. Table 1.

Speciation was done based on Gram's stain, Germ tube test, colony morphology on Saboraud's Dextrose Agar (SDA), HI Chrome agar and Dalmau's technique Figure 1,2, and 3. Biochemical tests (sugar fermentation and assimilation) and Physiological tests (temperature differentiation, growth in presence of 6.5% NaCl and 0.1% actidione, urease production using Christensen's urea agar with 2% glucose). VITEK-2 system was employed for speciation and anti-fungal susceptibility testing was done. Molecular confirmation was done by **PCR-RFLP** and **MALDI-TOF MS**.

Growth in 6.5% and 12.5% NaCl along with growth at 40ºC and 42oC were checked to get to a working identification of *C. auris,* when pseudo hyphae were not seen in Dalmau technique. This helped in the phenotypic confirmation of the yeast. Sugar assimilation and fermentation using 10% glucose, maltose, lactose, sucrose, galactose and trehalose sugars was done sequentially and their patterns were compared to aid in speciation.

Haemolysin production was detected by observing hemolysis around the colonies in 7% sheep blood agar after 48 hours of incubation Figure 2. Esterase production was demonstrated by looking for the presence of a halo around the colonies in 0.1% tween-80 agar Figure 2. Phospholipase was detected using 10% egg yolk agar by looking for the presence of precipitation zone around the colonies Figure 2.

Bio-film formation was demonstrated by both test tube method and micro-titre plate method using Sabouraud's dextrose broth with gentamicin (0.5ml/100ml) in triplicates Figure 3. The optical density in the micro-titre plate method was measured using 520 nm and 680 nm reader. The mean OD value was calculated and documented. An OD value of >=2 was taken to be positive.

Antifungal-susceptibility testing was performed conventionally in Muller Hinton Agar with 2% glucose and 0.1% Bromothymol blue, according to CLSI guidelines using suitable controls and VITEK. The anti-fungal agents tested were Amphotericin-B, Clotrimazole, Fluconazole, Itraconazole, Ketoconazole, Nystatin and Caspofungin.

PCR-RFLP and **MALDI-TOF Mass Spectrometry** were performed for confirmation of the isolates.

PCR-RFLP was performed with fresh overnight cultures on SDA Table 2, Figure 4.15 Phenol-Chloroform method was used for DNA extraction where 400 µL of lysis buffer (containing 10 mM TRIS buffer, 3% SDS (Sodium dodecyl sulphate), 1mM EDTA and 100 mM Sodium chloride) was taken in a centrifuge tube. A loopful of the isolate was mixed in the lysis buffer and heated in a water bath at 100°C for 1 minute. Phenol and Chloroform was taken in equal volumes and added to the above mixture, after which it was centrifuged at 10,000 rpm for 10 minutes. After centrifugation, the aqueous layer was transferred to another centrifuge tube and the above step was repeated by adding chloroform alone. In the final step, DNA was precipitated by adding cold isopropyl alcohol, which was then centrifuged and washed with 70% ethanol. The final pellet obtained was re suspended in 30µL TE buffer and was stored at -20°C.

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After DNA extraction, PCR was performed with the master mix (25µL PCR mix, 1µL forward primer (ITS-1), 1µL reverse primer (ITS-4) and 1µL template DNA). The volume was made up to 50µL with sterile nuclease-free water. The PCR assay was then performed (initial denaturation for 5 minutes at 95°C, denaturation for 30 seconds at 95°C, annealing for 30 seconds at 56 °C, extension for 30 seconds at 72 °C and final extension for 5 minutes at 72 °C).

RFLP (Restriction Fragment Length Polymorphism) was performed using 2µL enzyme buffer, 5 units MSP-1 (Moraxella species-1) enzyme and 10µL PCR product. All these were taken in a 200µL PCR tube and the volume was made upto 20µL with nuclease free water. This reaction mixture was incubated at 37 °C for 1 hour.

Finally, Agarose gel electrophoresis was performed with the PCR and RFLP products using 1.5% and 2.0% agarose gel respectively. Ethidium bromide stain (0.5 µg/ml) was used. It was visualized under UV light and documented.

MALDI-TOF Mass Spectrometry

Ethanol and formic acid extraction method was followed as per the kit protocol for the identifying the isolated strains. The obtained spectra was then analyzed using the Flex Control 3.1 software (Bruker Daltonics Inc., Billerica, MA, USA) and MALDI Biotyper OC version 3.1 (Bruker Daltonics, Bremen, Germany), which already had pre-programmed spectral software for both bacteria and yeasts. Score values obtained were analyzed. A score of 2 meant confidence up to to the species level, score of 1.7 to 1.99 meant confidence upto to the genus level and a score of <1.7 meant no probable genus identification. The final confidence value was expressed in percentage, and confidence values more than or equal to 99% indicated accurate confirmation of the strain, up to the species level.[18]

COVID-19 positivity was confirmed by subjecting the nasopharyngeal and oropharyngeal swabs to RNA extraction and then by RT-PCR using SD-biosensor kit. Baseline data such as demographics, previous medical history, laboratory parameters, microbiological findings, previous and current drug use and treatment history were collected and analyzed. The possible risk factors associated with *Candida auris* sepsis was evaluated.

Results

3632 blood samples were processed for culture and sensitivity during the one-year period. 28 *Candida sp.* (0.77%) were isolated. Out of this, 9 isolates were *Candida auris* (9/28, 32.14%) Table 1.

1727 blood samples were processed during the 1st wave from July 2020 to December 2020 of which 14 *Candida sp.* (0.81%) were isolated. Of this, only 1 isolate was *Candida auris* (1/14, 7.14 %). 1905 blood samples were processed during the 2nd wave from January to June 2021 of which 14 isolates were *Candida sp.* (0.73%). Of this, 8 isolates were *Candida auris* (8/14, 57.14 %).

C. auris showed oval to elongated budding yeast cells that did not show pseudo-hyphae on Gram's staining. Both *Candida glabrata* and *Candida auris* do not produce pseudo-hyphae. These two yeasts were differentiated by their growth on Hi Chrome agar and property of thermotolerance. Sabouraud's Dextrose Agar (SDA) showed creamy white colonies on overnight incubation at 37°C while in Hi Chrome agar it showed cream-colored smooth colonies with purple tinge, at 48 hours of incubation. This changed into purplish pink colonies upon prolonged incubation.[1]

All strains isolated, formed budding yeast cells or blastoconidia and no chlamydospores/ pseudo-hyphae. This was confirmed by Dalmau's technique, using Corn meal agar with 1% tween-80. All 9 isolates grew in hypersaline SDA (6.5% and 12.5% NaCl) and at high temperature (40°C and 42°C) conditions. They also grew in the presence of 0.1% Actidione and produced urease. Carbohydrate fermentation and assimilation tests were carried out with 2% glucose, maltose, lactose, sucrose, galactose and trehalose, where only glucose was fermented. Others showed neither fermentation nor assimilation.[1]

Anti-fungal susceptibility testing performed on Muller-Hinton agar with 2% glucose showed varying levels of resistance to Fluconazole and some strains showed resistance to all classes of antifungals, including echinocandins Table 3.[11] The highly thermo-resistant organism, can grow at temperatures between 30^0C-40^0C, sometimes even up to 42^0C. It does not grow on cycloheximide containing medium. These points can be kept in mind for easy identification of the organism.[12] Virulence testing of all the *Candida* isolates demonstrated bio-film production in 55.56% isolates, haemolysin production in 100%, esterase production in 100% and phospholipase production in 33.33% isolates. Virulence testing of the 9 *C. auris* strains showed bio-film production in 77.78% isolates, haemolysin production in 100%, esterase production in 100% and phospholipase production in 55.56% isolates.

Demographics

Among the 9 patients with *C. auris* 7 were Male and 2 were Female. Of the 7 male patients, 6 patients were COVID positive by RT PCR and 1 was negative, and both female patients were COVID positive. The mean age group was 61.78 years Figure 6.

The risk factor statistics of the isolated Candidemia strains along with *Candida auris* strains are depicted pictorially Figure 5.

The overall number and statistics of total 28 *Candida isolates* obtained during the one-year period of study is given below:

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During the period from July to December 2020, covering the 1st wave of COVID-19 pandemic in our country, of the 14 *Candida isolates*, the species identification was as follows: *C. glabrata* (5) 35.71%, *C. albicans* (4) 28.57%, *C. tropicalis* (3) 21.43%, *C. parapsilosis* (1) 7.14%, *C. auris* (1) 7.14%. Only 1 isolate of *C. auris* was obtained from blood. From January to June 2021 covering the 2nd wave of COVID-19 Pandemic 14 more isolates of *Candida* were obtained of which, 8 were *C. auris* (57.14%), 4 were *C. parapsilosis* (28.57%), 2 were *C. tropicalis* (14.29%). 8 strains of *C. auris* were obtained from blood.

Among the COVID positive patients, the *Candida* isolates (n=17) were as follows: *C. auris* (8) 47.06%, *C. tropicalis* (3) 17.65%, *C. parapsilosis* (3) 17.65%, *C. glabrata* (2) 11.76%, *C. albicans* (1) 5.88%. Among the Non-COVID patients, the *Candida isolates* (n=11) were: *C. glabrata* (3) 27.27%, *C. albicans* (3) 27.27%, *C. tropicalis* (2) 18.18%, *C. parapsilosis* (2) 18.18%, *C. auris* (1) 9.09%. Figure 6.

The most prevalent isolate among COVID-19 positive patients was *C. auris* (47.06%) and among COVID-19 negative patients it was both *C. glabrata* and *C. albicans* (27.27% each). Thus, out of the 9 isolates of *C. auris* obtained 8 were from COVID-19 positive patients (88.89%), while 1 strain was from a COVID-19 negative patient (11.11%).

The overall percentage of sensitivity of the *Candida isolates* to antifungal agents was Fluconazole 33.33%, Ketoconazole 48.15%, Itraconazole 33.33% and Amphotericin-B 100%. Species wise sensitivity is depicted in Table 3.

The most sensitive species was *C. tropicalis* - Fluconazole 75%, Ketoconazole 75%, Itraconazole 50% and Amphotericin-B 100%. The most resistant species was *C. auris* - Fluconazole 0%, Ketoconazole 50%, Itraconazole 50% and Amphotericin-B 100%. The 9 isolated strains of *C. auris* were tested for Echinocandin - Caspofungin sensitivity by MIC. It was seen that 6 strains (66.67%) were sensitive, while the remaining 3 strains (33.33%) showed resistance.

The therapeutic outcome of 19 patients (67.86%) was good while 9 (32.14%) succumbed to the disease. Among *C.auris* strains, only 2 patients (22.22%) survived, while the remaining 7 patients (77.78%) succumbed to the disease.

Table 1.Table Showing the Demographics of the Total Blood Samples Processed and *Candida sp.* Isolated among 1st Wave, 2nd Wave and Overall, 1 Year Period

	1st Wave (July to Dec 2020)	2nd Wave (Jan to June 2021)	Total
Total Blood Samples	1727	1905	3632
Candida sp.	14	14	28
Candida auris	1	8	9

Table 2.Table Showing the Size of PCR Product and RFLP Product of the different *Candida sp.* Isolated

Candida Species	Size of PCR Product (BP)	Size of RFLP Product (BP)
Candida albicans	535	297,238
Candida tropicalis	524	340,184
Candida glabrata	871	557,314
Candida parapsilosis	520	520
Candida auris	400	400

Table 3.Table Showing % Sensitivity of the different *Candida sp.* Isolated to Various Antifungals that were Tested

Percentage Sensitivity of the Isolates				
Candida species	Fluco-nazole	Ketoco-nazole	Itraco-nazole	Amphot-ericin-B
Candida albicans	75 %	75 %	50%	100%
Candida tropicalis	75 %	100%	75%	100%
Candida glabrata	75 %	75%	50%	100%
Candida parapsilosis	50 %	75%	50%	100%
Candida auris	0 %	50 %	50%	100%

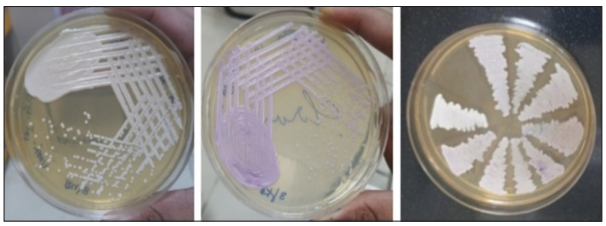

Figure 1.*C. auris* on SDA and CHROM agar Showing Creamy White Colonies and Purplish Colonies Upon Prolonged Incubation, >48 Hours, Respectively. CHROM agar Plate Showing all 9 Strains of *C. auris* with Cream Coloured, Smooth Colonies with Purple Tinge, at 48 Hurs of Incubation

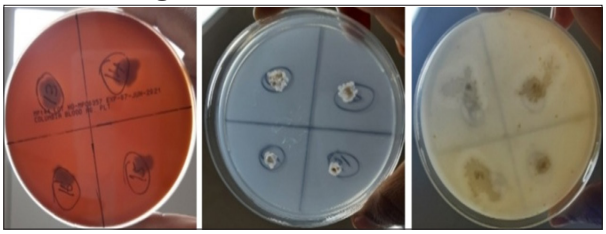

Figure 2.Virulence Assessment of the *C. auris* Strains-Hemolysin, Esterase and Phospholipase Respectively

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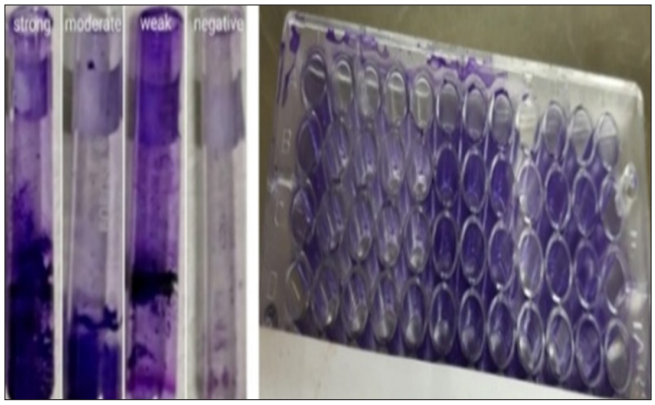

Figure 3.Biofilm Production of the *C. auris* Isolates Demonstrated using Test-tube Method and Micro-Titre Plate Method Respectively

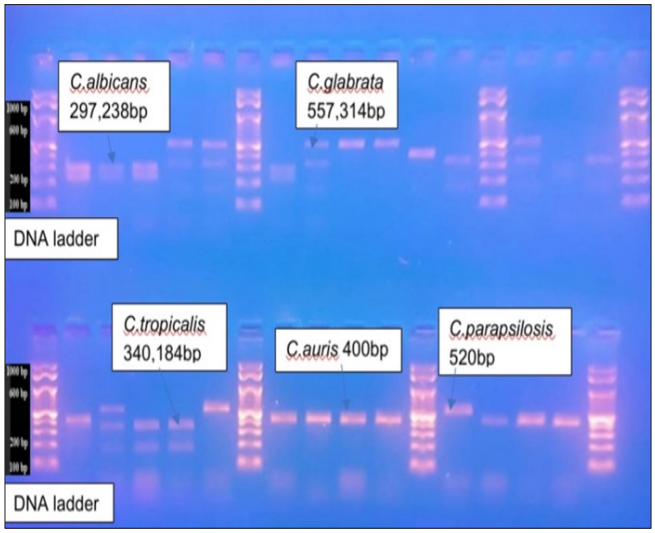

Figure 4.Molecular Confirmation of all the Isolated Strains of *Candida* Including the 9 *C. auris* Strains using PCR-RFLP Image shows PCR-RFLP Product Showing Representation on 1% Agarose gel Electrophoresis, eg: RFLP Product Isolated at 400 base Pairs is Suggestive of *Candida auris*

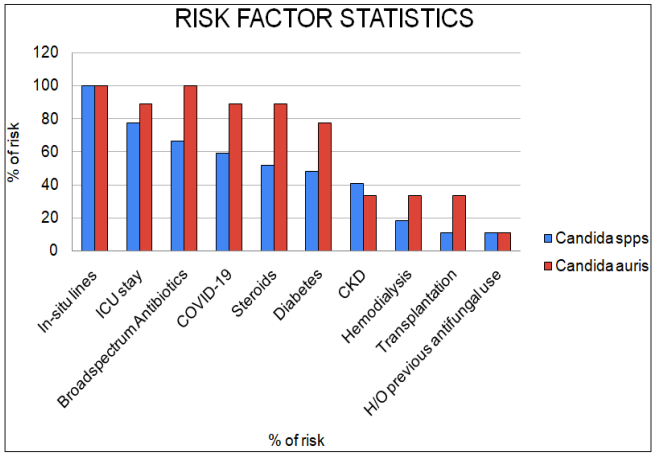

Figure 5.Risk Factor Statistics of *C. auris* and other *Candida sp.* Strains Isolated during the 1 Year Period

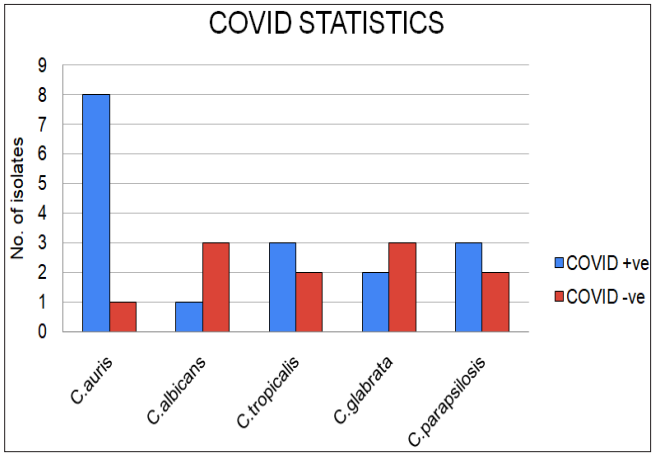

Figure 6.COVID-19 Positivity Statistics of *C. auris* and other *Candida sp.* Strains Isolated during the 1 Year Period

Discussion

Candida auris is a leading cause of *Candidemia*, in nosocomial settings particularly in the ICU's. Recent studies also show high prevalence of *C. auris* among in-patients in the wards as well.[9] Due to its high colonization rates in skin and environmental surfaces, the organism spreads through humans and fomites causing outbreaks. Other important properties such as high thermotolerance, bio-film production, increased drug resistance to multiple classes of anti-fungals and osmotic stability contribute to the enhanced virulence and persistence in the environment. This adversely affects the clinical outcomes, contributing to increased mortality rates.[10]

The increased resistance rates of the fungus have been attributed to several factors: target alteration, target over-expression, drug expulsion, bio-film mediated resistance etc. Several Azole resistance genes have been identified in the various clades of the organism.[13] Therefore starting empirical treatment with Amphotericin-B or Echinocandins is rather preferred than Fluconazole. CLSI guidelines 2020, says that Fluconazole can be used as a surrogate marker for checking susceptibility to second generation tri-azoles.[21] Isolates resistant to Fluconazole can sometimes respond to other Azoles.

All multi-drug resistant yeasts need not be *Candida auris* and all *Candida auris* strains need not be multi-drug resistant. For example, in our study, all 9 strains of *Candida auris* were sensitive to Amphotericin-B (100%) while none was sensitive to Fluconazole (0%). Among the Echinocandins, Caspofungin sensitivity was shown by 6 strains (66.67%) while the remaining 3 strains (33.33%) were resistant. Echinocandins are the main class of anti-fungal agents prescribed for treatment, though *C.auris* strains resistant to all classes of anti-fungals including Echinocandins have been isolated. This severely limits the treatment options.

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Liposomal Amphotericin-B and Voriconazole have been tried as alternatives, especially for the very severe cases. This is usually guided by in-vitro susceptibility testing.[13]

Marilyn M Ninan et al. have shown that *C. auris* strains identified as resistant to Amphotericin-B by the VITEK2 system (100%), were found to be only 77% resistant to Amphotericin-B by Micro-broth dilution method. The authors also say that automated methods can overestimate resistance especially for Amphotericin-B.[20] Therefore conventional AFST methods like Micro-broth dilution or E-strip can be used, as in our study to confirm susceptibility patterns.

Both conventional and automated systems like VITEK 2 can lead to misidentification. *C. auris* is commonly misidentified as *C. haemulonii*, *C. duobushaemulonii*, *C. famata*, *C. lusitaniae*, *Rhodoturula glutinis* and *Saccharomyces cerevisiae* by VITEK.[3] In our study, 2 isolates identified as *C. famata* through VITEK, were found to be *C. auris* strains through **PCR-RFLP**. The *C. auris* VITEK ID's of the remaining 7 strains matched with the **PCR-RFLP** ID's. Hence **MALDI-TOF**, **PCR-RFLP** and Sequencing are some of the reliable methods to accurately identify this organism.[14,15]

The appearance of pale pink colonies in CHROM agar along with the presence of just cells and no pseudo-hyphae via Dalmau technique should raise the suspicion of *Candida auris* for smaller laboratories that do not have latest automated methods and sequencing techniques.[20] Also, a Selective Auris Medium (SAM) developed by Das S et al., makes it easier for the reliable and rapid identification of *C. auris*.[22] Thus it is possible to come to a preliminary conclusion based on phenotypic methods alone, although confirmation is warranted.

The common risk factors for the *C. auris* strains noted in the patients were: In-situ lines (100%), ICU stay (88.89%), Broad spectrum antibiotics (100%), COVID-19 (88.89%), Steroids (88.89%), Diabetes (77.78%), CKD (33.33%), Hemodialysis (33.33%), Transplantation (33.33%) and history of previous antifungal use (11.11%). It is seen that patients with *C.auris* candidemia had more predisposing risk factors, including COVID-19 positivity, when compared with other patients who had *non- C.auris* candidemia. Virulence testing of the 9 *C. auris* strains showed bio-film production in 77.78% isolates, haemolysin production in 100%, esterase production in 100% and phospholipase production in 55.56% isolates. This was also higher when compared with the virulence testing of other *Candida sp*. The yeast has high colonization rates in skin, plastics and other environmental surfaces, thereby remaining viable for a longer time period, when compared with other *Candida sp.* This can be attributed to the increased incidence of infections in patients with prolonged hospital stay.[20]

After identification in the laboratory, a critical alert was reported immediately to the consultant and care team, without any delay. This ensured early enforcement of isolation and infection control measures, so as to limit the transmission. The entire care giving team is educated on the infection control measures that have to be implemented periodically.[9,16]

Contact and droplet precautions, restricting the number of visitors, dedicated care team assigned for the patients, all horizontal measures such as hand hygiene, decolonization of all patients in high-risk areas using topical chlorhexidine, antibiotic stewardship, thorough environmental cleaning etc to be strengthened across the hospital, proper use of PPE (gloves, aprons, and gowns) by the health care personnel, chlorhexidine body washes, wipes and mouth washes for proper isolation with enhanced surface cleaning, terminal cleaning and disinfection of the contact areas every day, shared devices and materials to be disinfected properly before being used by another patient, environmental disinfection using sodium hypochlorite everyday and terminal cleaning of the contact areas and rooms after patient discharge.[9]

The therapeutic outcome of the *C. auris* infected patients proved to be very fatal in our study. Of the 9 patients, only 2 patients (22.22%) survived, while the remaining 7 patients (77.78%) succumbed to the disease. This can be attributed to the highly infectious and fatal nature of *C. auris*. Though some studies had mortality rates as low as 18%, some studies showed mortality rates ranging from 33-72%.[20] This can be very well reduced, by early and accurate identification, early reporting along with strict infection control practices and contact precautions.

Conclusion

Thus, during our one-year period study, we have had 9 confirmed isolates of *Candida auris* from blood. This study aimed at highlighting the importance of: increased outbreaks of *C. auris* in nosocomial settings, both in ICU's and wards, the highly transmissible nature, multi-drug resistance and thermotolerance of the yeast, difficulties and misinterpretations in identifying the yeast correctly, proper reporting of the identified yeast, appropriate speciation and virulence assessment including bio-film formation, anti-fungal susceptibility testing according to proper guidelines and controls, use of right antifungals for treatment and finally appropriate containment strategies to prevent the occurrence of such infections in the future. It is possible for the early and reliable identification of *Candida auris* based on phenotypic methods alone, which however should be confirmed by molecular/proteomic methods.[17]

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Conflict of Interest: None

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