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Biofilm detection among clinical isolates of *Candida auris*

Festus Joseph.M[1], Abitha Evangelin[2], Dr. Priyadarshini Shanmugam[3], Dr. Ambujavalli Balakrishnan Thayikkannu[4*]

[1]BSc. MLT (Intern), Department of Microbiology, Chettinad Academy of Research and Education, Kelambakkam, Tamil Nadu, India
[2]Research Scholar, Department of Microbiology, Chettinad Academy of Research and Education, Kelambakkam, Tamil Nadu, India
[3]Professor & HOD, Department of Microbiology, CHRI and CARE, Kelambakkam, Tamil Nadu, India
[4]Assistant Professor, Department of Microbiology, Chettinad Academy of Research and Education, Kelambakkam, Tamil Nadu, India

***Corresponding author:** Dr. Ambujavalli Balakrishnan Thayikkannu, Assistant Professor, Department of Microbiology, Chettinad Academy of Research and Education, Kelambakkam, Tamil Nadu, India

ABSTRACT

Introduction: *Candida auris* is a yeast of concern in health care settings, due to its multi-drug resistant nature and outbreak potential. It survives on the patient's skin as commensal and in the immediate surroundings for long durations, and its existence as biofilms alarms physicians and infection control experts in treating and containing this infection.

Aim: To perform phenotypic tests to observe biofilm formation among clinical isolates of *Candida auris*.

Methodology: *Candida* species were detected and isolated from various clinical samples by conventional, automated culture methods. Isolated *Candida* species were inoculated on Selective Auris Medium to prove as *Candida auris* and they were confirmed by MALDITOF MS and were assessed for biofilm formation using Congo red agar, tube method and microtiter plate method. OD values were calculated and mean values were taken.

Results: Out of 459 isolates 20 were identified as *Candida auris* and included. 20 isolates confirmed by PCR RFLP from archives were also included along with ATCC *C.albicans* and *C.parapsilosis* as controls. 10 were biofilm producers by all three methods.

Conclusion: This study shows that biofilms can be detected easily and detection can be done concurrently to plan effective treatment, isolation and prevention strategies and improve infection control practices.

Keyword: super fungus, *Candida auris*, Selective Auris Medium, Congo red, Tube method, Microtiter plate, PCR RFLP.

INTRODUCTION

A biofilm is a microbial community of cell aggregates on a surface which, under desirable conditions forms a thick layer of colonies that can produce an extracellular matrix. These microcolonies are primarily composed of an extra cellular matrix comprising of collagens, elastin, proteoglycans, polysaccharides, and cell-binding glycoproteins.

A biofilm is produced when a pathogen tends to attach itself to moist surfaces by secreting a slimy glue-like sticky substance. This primary step is called as adhesion. A biofilm forming site can be any place with solid liquid interface, in the presence of essential nutrients. It can be also formed on natural materials, metals, plastic, also in the body and plant tissue [(1)]. The formation of biofilm occurs in five stages, starting with attachment, followed by colonization, proliferation, maturation and finally dispersion. The dispersed biofilm gets seeded to produce additional biofilms [(2)].
The first explanation for this biofilm formation dates to 17th century, when Anton Von Leeuwenhoek, the inventor of the microscope, was the first to observe an aggregation of microbes (now known as Biofilms) from samples collected from his own tooth scrapings ([3)].

"Biofilm" was termed by Bill Costerton in the year 1978.The biofilm producing pathogenic microorganisms cause chronic infection in humans and can withstand the standard antimicrobial therapy. This is due to the presence of an extra polymeric matrix which also helps in surviving adverse environmental conditions. The increased antibiotic resistance is due the expression of many genes which encode for virulence factors and a set of proteins that confer the microbial community with its character. The polymeric matrix which is produced by the biofilm producing organism prevents the entry of antimicrobial agents into the biofilm, which in turn results in treatment failure [(4)].
There are many simple methods for the identification and detection of biofilm, including microtiter plate method, tissue culture method, tube method, , Congo-red, fluorescent microscopic examination, flowcytometry, electron microscopy, genotypic methods to detect genes coding for biofilm formation etc [(5)]. It is very important to detect and treat these biofilm forming agents. Studies confirms that around 80% of recurring and chronic microbial infections are caused due to biofilm formation. The microbial cells that are capable of producing these biofilms are 10-1000 times more resistant to antibiotics than other non-biofilm producers [(6)].
Candida auris is an emerging "super fungus" which causes nosocomial infection and blood stream infections. It has many virulence qualities and is multi-drug resistant against the regularly used antifungals against other *Candida species* causing invasive infection [(7)]. In this article we document three phenotypic methods to detect biofilm production in *Candida auris.*

METHODOLOGY

This prospective observational period study was conducted in the Mycology section, Department of Microbiology over a period of 6-month. IHEC approval was obtained. (IHEC -1/1554/22)

Isolation of *C. auris*

A total of 459 consecutive and non-repetitive *Candida* species which were isolated from clinical samples like blood, urine, tissue bits, HVS, pus, sputum, stool, and skin were included in this study. Out of 459, 20 isolates, were phenotypically identified as *C. auris,* and were confirmed by MALDITOF MS at JIPMER PUDUCHERRY. 20 isolates confirmed by PCR RFLP at SRIHER from archives, that were isolated from blood were also included.

Confirmation of *C. auris*

Colonies that were white to pale pink on Hi Chrome Agar were subjected to Gram stain and germ tube (GT) test. The GT negative isolates were then subjected to saline and temperature tolerance test. On Dalmau's Technique (performed using cornmeal agar with tween 80) *C.*

auris are observed as budding yeast cells they do not produce pseudo hyphae or true hyphae. Growth on Selective Auris Medium (SAM) was observed and documented.

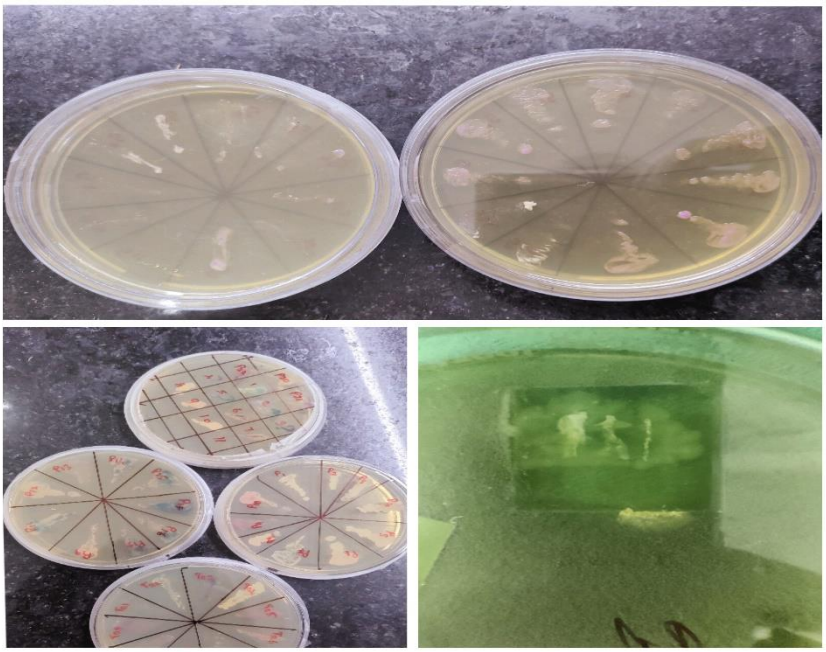

Figure 1: Growth on HI-CHROM agar, SAM (Selective Auris medium), Dalmau technique

BIOFILM DETECTION METHODS

Congo-red agar (CRA)

The 40 confirmed isolates of *C. auris* were inoculated on CRA for detection of biofilm production. CRA was prepared by combining Brain Heart Infusion (BHI) broth (37grms), sucrose (50 grams), agar (20 grams), in 900ml of distilled water. Anhydrous Congo red (0.8grms) was dissolved in 100ml distilled water. Both were separately autoclaved and then mixed, after the media cooled down and poured in Petri dishes. The inoculated isolates were incubated for 24hrs at 42°C and observed for production of black colonies that indicated biofilm production. CRA is one of the qualitative methods of biofilm detection.

ATCC *C.albicans* 14053, ATCC *C.parapsilosis* 2201 were used as control in this method.

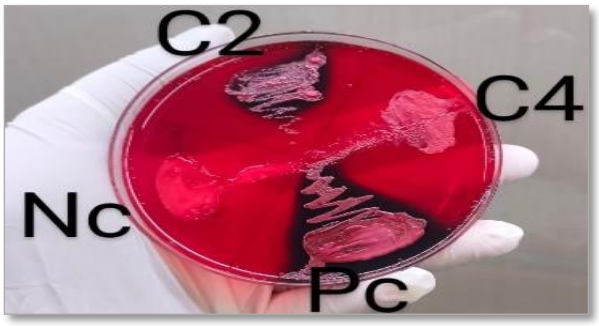

Figure 2: Growth on CRA showing black pigmented colonies with crystallized appearance were taken as positive as it represented the production of bio film. Red pigmented colonies represent non biofilm producers.

Tube method

A standard *C. auris* inoculum was added to 10ml of Sabouraud's Dextrose Broth (SDB) and incubated for 48 hrs. After incubation, the broth was decanted carefully and the tubes were washed using PBS (Phosphate Buffered Saline), and then stained with 0.1% of crystal violet for 10 mins. The tubes were left for dry and observed for biofilm formation that can be identified by the stain deposits on the walls and in the bottom of the test tubes. Tube method is also a qualitative method for the detection of bio film. ATCC *C. albicans* (14053) and *C. parapsilosis* (22019) served as controls

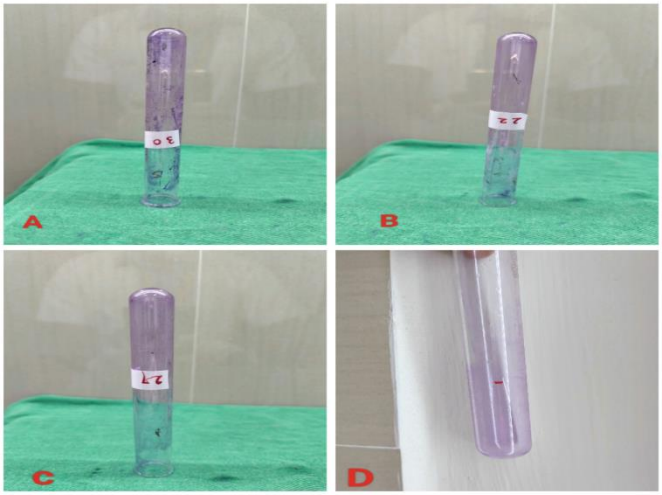

Figure 3: The above image represent **(A):** strong production of biofilm, **(B)** moderate production of biofilm**(C)** weak production of biofilm, **(D)** no production of biofilm.

Microtiter plate method

1 (one) McFarland suspension of the 40 isolates were inoculated in SD Broth, (1ml per Eppendorf vial) and kept for 24 hours incubation. The Microtiter Plate was filled with 180µl of SDB and 20µl of incubated suspension in each well. The wells were covered and incubated for 24 hours at 37□C. The contents were decanted and rinsed carefully thrice with PBS. The washed wells were stained with 0.1% of Crystal violet, dried and read in ELISA reader at 450nm and 650nm respectively. The mean values were calculated. ATCC *C. albicans* (14053) and *C. parapsilosis* (22019) were included as controls

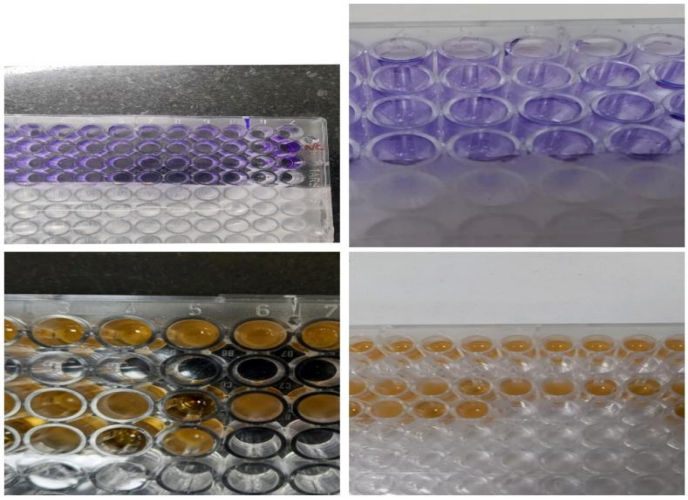

Figure 4: Microtiter plate method

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RESULTS

In total 459 isolates was included for this study out of 459 isolates 20 was identified as *C. auris* 20 isolates from archives were also included. C.*auris* grows well on SAM, in 12.5% salinity and upto 42□C. The confirmed isolates were tested for biofilm production.

Table 1: Biofilm production by phenotypic methods

Method	Positive/total	Percentage
CRA	10/40	25%
Test tube method	10/40	25%
Micro titre plate method	10/40	25%

The same biofilm producing isolates could be detected by all three methods

Table 2: Congo red agar method

Method	Positive/total	Percentage
CRA	10/40	25%

Table 2: Type of Biofilm production by Congo red agar method

Table 3: Test tube method

Type of bio film	No/total produced	Percentage
Strong	4/40	1%
Intermediate	4/40	1%
Weak	2/40	0.5%
Non- Biofilm producers	30/40	75%

Out of the 40 isolates 10 of them have stained with 0.1% methylene blue, and had detectable bio films by test tube method.

Table 4: Microtitre Plate Method

Optical density	Highest	Lowest
450nm	2.1487	0.0626
620nm	2.1634	0.0773

Table 4: Biofilm production by microtiter plate method

The optical density of the biofilm produced by the 40 isolates was noted and the highest, lowest, their mean values were calculated. The highest and the lowest values are tabulated in Table 4 25% of the isolates obtained from blood were biofilm producers.

The biofilm forming ability among candidemia isolates has been documented to range from 16% to 100% (S Tulasidas 2018). We observed 25% biofilm producers among 40, probably because the 20 confirmed isolates from archives were from patients with Candidemia.

DISCUSSION

C.auris is a yeast like fungus listed as critical in the WHO priority pathogen list. It can colonize humans and exist in the immediate patient environment and on devices and fomites for a long duration and need stronger disinfectants and longer contact periods. (8).

In clinical settings, hospitalized patients have several risk factors such as lines both central and peripheral, those on long term anti microbials, on drug that have anti-anaerobic activity, steroid therapy, immunosuppresants, uncontrolled diabetics with or without keto acidosis, transplant recipients(both solid organ or bonemarrow) patients with chronic kidney disease (CKD), patients with cancer, HIV etc are prone to fungal infections, and many of these risk factors are inevitable for clinicians caring for them.It is therefore very vital to have clinical suscpicion of biofilm forming *C. auris* persistors and easy methods to confirm the same

A porcine (pig) skin model was developed to simulate *C. auris* skin colonization. *C. auris* formed High-Burden biofilms. In addition, synthetic sweat medium was developed to simulate axillary skin environment, *C. auris* was observed to form multilayered biofilms that resisted mechanical desiccation. Saline tolerance, an important virulence mechanism of *C.auris* probably assists its establishment as skin colonizers. *C. auris* is known to produce biofilm ≈ 30-fold > than C. *albicans* which is known as good biofilm producer. It was observed that after 14 days post desiccation, Mark V. Horton et al. stated that *C. auris* grew readily from biofilms whereas *C. albicans* biofilm are not viable.
Similar phenotypes are shared by *C. auris* and other *Candida species*, including *Candida haemulonii* and *Candida duobushaemulonii*. When the traditional techniques are used, these species can be erroneously identified as *C. auris*, resulting in inappropriate treatment and insufficient infection control measures. The use of SAM (Selective Medium for Isolation and Detection of Candida auris) Sourav Das et al helps greatly in early detection.

Methods such as CRA, Test tube method, microtiter plate methods are classified as traditional methods and ultra sound, Electron microscopy, detection of genes for PCR, photobioreactor, sequencing technologies are the new methods that can be used but these new methods are not cost efficient thus it cannot be used in small labs. Therefore, we need the classic and traditional methods which are easy to perform, cost effective, and the discarding process is also simple [(13)]. The simplest method to perform is growth on Congo red agar, which can be performed along with routine phenotypic tests to look for biofilm producing isolates. This is valuable in patients with device associated hospital acquired infection. Results can be read by 24-48 hours. Interpretation is easy, no staining or handling and discarding is also easy (autoclaving) and hence suggested.

CONCLUSION

C. auris is known to produce biofilm approximately 30-fold more than C. *albicans.*Further studies on *C auris* biofilms will help understanding its unique ability to persist on skin and in health care settings that could serve as a constant source of spread and outbreaks .Policies on patient isolation and intervention strategies to eliminate *C. auris* biofilms from colonized skin will help control the spread of this pathogen.

Speciation of Candida should be adopted by labs as there is a steady shift towards non albican species, that show both higher MIC for antifungals and resistance. Under reporting of *C.auris* delays recognizing the true public heath burden and threat.
This organism has a noteworthy virulence capacity that merits further studies to assess if the biofilm forming ability is also clade related.The need for preventive methods like patient screening can be emphasized to prevent infections and in spread of this agent.

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CONFLICT OF INTEREST

None

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