British Microbiology Research Journal
3(3): 440-447, 2013

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Evaluation of Phospholipase Activity in Biofilm Forming *Candida* Species Isolated from Intensive Care Unit Patients

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Authors' contributions

This work was carried out in collaboration between all authors. Author SD designed the study, managed the literature searchers, wrote the protocol, and wrote the first draft of the manuscript. Author SS managed the analyses of the study. All authors read and approved the final manuscript.

Research Article

Received 3rd April 2013
Accepted 12th June 2013
Published 4th July 2013

ABSTRACT

Aims: To evaluate phospholipase activity in biofilm forming *Candida* spp. isolated from patients admitted in intensive care unit of rural tertiary care hospital.
Study Design: A total of 135 biofilm forming *Candida* spp. isolated from various clinical specimens of patients admitted in ICU were included in the study.
Place and Duration of Study: Department of Microbiology, Pravara Institute of Medical Science's Rural Medical College India, between January 2010 and December 2012.
Methodology: The *Candida* isolates were identified upto species level by conventional standard mycological techniques. The biofilm formation was assessed by inoculating the isolates in conical polystyrene test tube containing Sabouraud's dextrose broth supplemented with glucose. Phospholipase activity of biofilm forming *Candida* isolates was detected by using egg yolk agar.
Results: Out of 135 biofilm forming *Candida* spp. included in the study, 60 (44.4%) isolates were *C. albicans*. Among non-*albicans Candida* (NAC) spp. *C. tropicalis* was the major isolate followed by *C. glabrata* and *C. parapsilosis*. Phospholipase production was seen in 85 (62.9%) isolates. A total 49 (81.6%) isolates of *C. albicans* showed phospholipase activity. Among NAC spp. maximum phospholipase activity was seen in *C. tropicalis* and *C. glabrata*.
Conclusion: Biofilm formed by the *Candida* spp. tend to be more resistant to antifungal

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drugs. Though *C. albicans* the most common species associated with *Candida* biofilms, the emergence of NAC spp. is of concern. NAC spp. shows varying degree of resistance either intrinsic or acquired or both to commonly used antifungal drugs. The isolation of NAC spp. from clinical specimens is no longer overlooked as these organisms are emerging pathogens. The virulence factors like biofilm formation and phospholipase activity is also noted in NAC spp. The study of these virulence factors would help in understanding the pathogenic role of NAC spp.

Keywords: Biofilm; Candida spp.; phospholipase activity; non-albicans Candida species.

1. INTRODUCTION

Healthcare–associated infection (HAI) is an ever escalating threat which needs to be expeditiously managed. Its control is an emerging concern to anyone who deals with patients i.e. from the treating physician who has to judiciously advocate antimicrobial therapy, to the clinical microbiologists who have to isolate and characterize microbes and the administrators who have to maintain cost effectiveness.

Approximately half of all cases of HAIs are device-related. Device related infections may not only require removal of device but at most of the time may be potentially fatal [1]. Among fungal etiology of HAI, *Candida* spp. predominates. *Candida* spp. is the 3rd or 4th leading cause of HAI in the United States [2].

Candida is a part of normal flora of the human body colonizing various anatomical sites like oral cavity, digestive tract, vagina and skin [3]. The transition of *Candida* from a harmless commensal to disease causing pathogen depends on the immune system of the host and virulence factors of *Candida*. Biofilm formation, hyphal switching, surface recognition and production of extracellular hydrolytic enzymes are important factors attributing to virulence of *Candida* [4,5].

Biofilms are produced when microorganism adhere to a surface and produce extracellular polymers [1]. *Candida* can form biofilm on most of medical devices like stents, shunts, implants, endotracheal tubes, pacemakers and catheters [6]. The strong adherent character of biofilm-producing ability of *Candida* on medical devices makes them a persistent source of infection [7]. Among hydrolytic enzymes, phospholipase plays a pivotal role in pathogenicity of *Candida*. Phospholipase enzyme facilitates the adherence [8] and invasion of the host cells by *Candida* spp [9]. Therefore phospholipase production in *Candida* spp. can serve as an important parameter to distinguish invasive pathogenic strain from non-invasive colonizing strains [5]. Though there are studies available on biofilm production and phospholipase activity in *Candida* isolates, there is a paucity of studies between the co-relation of these two virulence factors.

The present study was conducted with an aim to evaluate phospholipase activity in biofilm forming *Candida* spp. isolated from patients admitted in intensive care unit of rural tertiary care hospital.

2. MATERIALS AND METHODS

The present study is part of a Ph.D thesis and was approved by the Institutional Ethics Committee (Registration No.FN.32/2010). A total of 135 biofilm forming *Candida* spp.

isolated from patients admitted in the ICU were included in study. The *Candida* isolates were identified upto species level by assessing the formation of germ tubes, sugar assimilation and fermentation and colony color on chrom agar. Hi- *Candida* identification kit (Himedia Laboratories Pvt. Ltd Mumbai, India) supplemented the speciation of the *Candida* isolates [10].

The biofilm formation was assessed by visual method described by Yigit et al. [11]. The isolate to be tested for production of biofilm was inoculated in conical polystyrene test tube containing Sabouraud's dextrose broth supplemented with glucose (final concentration 8%). The tubes were incubated at 35ºC for 48 h. After incubation the broth from the tubes were gently aspirated using Pasteur pipette. The tubes were twice washed with distilled water to remove non-adherent cells. The tubes were stained with 2% safranin for 10 min. Excess of stain was removed by rinsing with distilled water and the tubes were examined for the presence of adherent layer. The isolate was considered positive for biofilm formation when a visible film was seen on the wall and bottom of the tube. The formation of ring at the liquid interface was not considered as an indication of biofilm production. *Staphylococcus epidermidis* ATCC 35984 served as positive control.

Phospholipase activity of biofilm forming *Candida* isolates was detected by method of Samaranayake et al. [12]. Egg yolk agar was used for demonstration of phospholipase production. This medium contained Sabouraud's dextrose agar (SDA) (13.0 g), NaCl (11.7 g), $CaCl_2$ (0.11 g) and 10% sterile egg yolk and distilled water (184 ml). The components were mixed and sterilized using autoclave prior to addition of egg yolk. The egg yolk was centrifuged at 500 rpm for 10 min at room temperature and 20 ml of the supernatant was added to the sterilized medium.

Standard inoculum of the test and control *Candida* (*C. albicans* ATCC 10231) [5 µl, with 10^8 yeast cells (ml saline)$^{-1}$] were deposited onto the egg yolk agar medium and left to dry at room temperature. The culture plate was incubated at 37^0 C for 48 h.

The presence of visible precipitation zone around the colony indicated phospholipase production. The value of phospholipase activity (Pz) was measured by the ratio of the diameter of the colony to the total diameter of the colony plus the precipitation zone.

A Pz value of 1 denoted no activity, and less than one (Pz < 1) indicated the phospholipase activity. The lower the Pz value, the higher the enzymatic activity. To minimise the experimental error the assay was conducted in duplicate on three separate occasions for each *Candida* isolate.

3. RESULTS AND DISCUSSION

Table 1 shows the sample wise distribution of biofilm forming *Candida* species. A total of 135 biofilm forming *Candida* spp. were isolated from different clinical specimens. Majority of the isolates were obtained from urine samples, followed by blood cultures. *C. albicans* was isolated from 60 (44.4%) samples. Non-*albicans Candida* (NAC) spp. were isolated from 75 (55.5%) clinical specimens.Among NAC spp., *C. tropicalis*was the major isolate followed by *C. glabrata, C. parapsilosis, C. krusei* and C. kefyr.

Phospholipase production was seen in 85 (62.9%) isolates. A total of 49 (81.6%) isolates of *C. albicans* showed phospholipase activity. Among NAC spp. maximum phospholipase activity was seen in *C. tropicalis* and *C. glabrata* (Fig. 1).

Candida spp. is the most important cause of opportunistic mycotic infection worldwide [13]. The expanding population of HIV infected patients, increase in the use of broad spectrum antibiotics, cytotoxic chemotherapies and transplantation have increased the incidence of candidiasis [14]. *Candida* is one of the major cause of morbitidy and mortality in HIV/AIDS patients and recipients of solid organ transplants [3]. Therefore it can be rightly said that *Candida* detect immunocompromised status of host more faster than the physician with the help of diagnostic tests.

The versatility of *Candida* in getting adapted to a variety of habitat for the growth is an important pathogenic attribute [3]. Adherence of *Candida* to medical devices often leads to the formation of biofilms. As *Candida* biofilm demonstrate high resistance to antifungal drugs their eradication is difficult [1]. The recent research on *Candida* pathogenicity is focused on the prevention and management of biofilms [15].

In our study, biofilm formation was noted in both *C. albicans* and NAC spp. Out of 135 biofilm forming *Candida* spp. isolated from ICU patients, 75 (55.5%) belonged to NAC spp. This finding was consistent with studies conducted by Singhai et al. [7] and Mohandas et al. [8]. In contrast to our observation, Nerurkar et al. [16] in their study on *Candida* spp reported less biofilm forming ability in NAC spp. like *C. glabrata* and *C. parapsilosis*.The shift in etiology of candidiasis from *C. albicans* to NAC spp. have been documented by other researchers [17,18]. NAC spp. can no longer be considered as non-pathogenic or dismissed as contaminant. Among NAC spp. *C.tropicalis*, C. *glabrata* and *C. parapsilosis* are important cause of HAI [19].

Constitutive and inducible hydrolytic enzymes of *Candida* aid the invasion of host tissues by deranging the host cell membranes constituents [9]. *Candida* secretes hydrolytic enzymes such as lipases, phospholipases and proteinases [9]. Phospholipase hydrolyze the phospholipid of the host cell membrane leading to lysis of cell and alternation of surface characteristics. These events facilitate the establishment of infection [5]. In *Candida* phospholipase activity is concentrated at the growing tip of the pseudohyphae [9]. In *Candida* the location and extent of biofilm formation depends on various other virulence factors [7]. In the present study, we evaluated the phospholipase activity in biofilm forming *Candida* spp. Maximum phospholipase activity was seen in *C. albicans* (81.6%). High phospholipase activity in *C. albicans* was also observed by Tsang et al [20] and Thangam et al [21]. Among NAC spp. maximum phospholipase activity was noted in *C. tropicalis* (65.5%). Thangam et al. [21] also reported the increased phospholipase activity in *C. tropicalis* isolates among NAC spp. In contrast to this Samaranayake et al. [10] reported no phospholipase activity in *C. tropicalis*. *C. tropicalis* is reported as the most common emerging pathogen from the NAC spp.

Table 1. Distribution of Candida species among clinical specimens

Specimen	Total No. of isolates	*C. albicans* (%)	*C. tropicalis* (%)	*C. glabrata* (%)	*C. parapsilosis* (%)	*C. krusei* (%)	*C. kefyr* (%)
Urine	51	22 (43.1)	13 (25.4)	06 (11.7)	02 (3.9)	03 (5.8)	05 (9.8)
Blood	34	16 (47.1)	08 (23.5)	04 (11.7)	03 (8.8)	02 (5.8)	01 (2.9)
Foley's catheter tip	26	12 (46.1)	05 (19.2)	03 (11.5)	01 (3.8)	03 (11.5)	02 (7.6)
Endotracheal tube	14	05 (35.7)	02 (14.2)	02 (14.2)	03 (21.4)	02 (14.2)	-
Sputum	06	03 (50)	-	01 (16.6)	02 (33.3)	-	-
Pleural fluid	03	01 (33.3)	01 (33.3)	-	01 (33.3)	-	-
Cerebrospinal fluid	01	01 (100)	-	-	-	-	-
Total	135	60 (44.4)	29 (21.4)	16 (11.8)	12 (8.8)	10 (7.4)	08 (5.9)

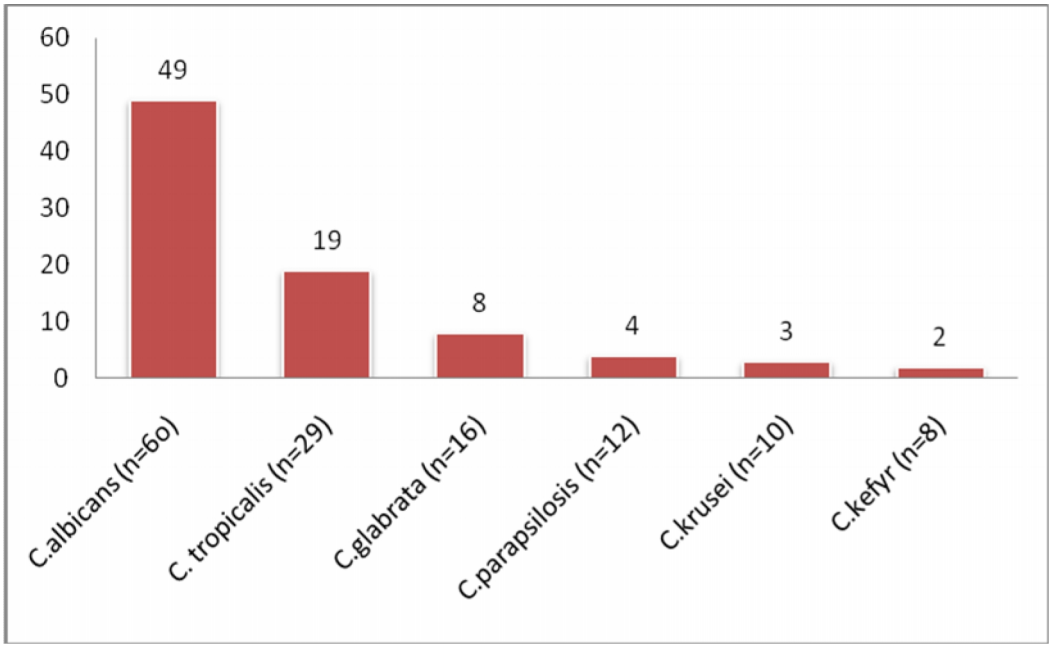

Fig. 1. Phospholipase activity in biofilm forming *Candida* isolates

4. CONCLUSION

Biofilm formed by the *Candida* spp. tend to be more resistant to antifungal drugs. Though *C. albicans* the most common species associated with *Candida* biofilms, the emergence of NAC spp. is of concern. NAC spp. shows varying degree of resistance either intrinsic or acquired or both to commonly used antifungal drugs. The isolation of NAC spp. from clinical specimens now not overlooked as these organism are considered as emerging pathogens. The virulence factors like biofilm formation and phospholipase activity is also noted in NAC spp. The study of these virulence factors would help in understanding the pathogenic role of NAC spp.

ETHICAL APPROVAL

The present was approved by the institutional Ethics Committee (Registration No. 32/2010).

ACKNOWLEDGEMENTS

This study was conducted under the aegis of Laboratory, Department of Microbiology, Rural Medical College. We are grateful to the management of Rural Medical College and Rural Hospital of Pravara Institute of Medical Sciences, Deemed University, Loni, Maharashtra, India for their encouragement and support throughout the study. We also thank the technical staff of Department of Microbiology for their assistance in the study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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