CORRELATE BETWEEN BIOFILM FORMATION AND AZOLE ANTIFUNGAL SUSCEPTIBILITY AGAINST PLANKTONIC AND SESSILE CANDIDA ALBICANS CLINICAL ISOLATES

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ABSTRACT

Objective: To assess the correlation between biofilm formation and azole antifungal susceptibility against plank tonic and sessile clinical isolates of *C.albicans*.

Study Design: Prospective observational study.

Place and Duration of Study: Combined Military Hospital Peshawar, from Jun 2016 to Sep 2017.

Methodology: All standard microbiological procedures were carried out according to latest Clinical & laboratory standard institute (CLSI) guidelines. After gram staining and presumptive identification on CHRO Magar Candida, the isolates were biochemically identified by API AUX Candida as *C.albicans*. Planktonic antifungal susceptibility was carried out by Kirby Bauer disk diffusion method on 300 *C.albicans* isolates. Broth microdilution method was used to determine Minimum inhibitory concentration (MICs) of plank tonic cells and micro titer assay was used for assessment of biofilm formation by *C.albicans*.

Results: In planktonic antifungal susceptibility, fluconazole was susceptible against 195 (65%) and voriconazole against 241 (80%) *C. albicans* isolates. *C. albicans* was found susceptible dose dependent (SDD) to fluconazole in 28 (9%) and to voriconazole in 21 (7%) isolates. Seventy-seven (26%) and 38 (13%) *C.albicans* isolates were found fluconazole and voriconazole resistant, respectively. Sessile antifungal susceptibility was carried out through broth micro dilution method in which 160 (53%) were susceptible, 42 (14%) were susceptible dose dependent SDD and 98 (33%) were resistance to voriconazole, and 161 (54%) were susceptible, 36 (12%) were SDD and 103 (34%) were found resistant to fluconazole. Biofilm forming isolates of *C.albicans* were observed to be 285 (95%).The *p*-value is highly significance i.e. <0.01 between the biofilm formation and azole antifungal susceptibility against plank tonic and sessile clinical isolates of *C.albicans*.

Conclusion: Plank tonic *C.albicans* clinical isolates appeared more susceptible to voriconazole than fluconazole and sessile isolates. Biofilm formation was very high among all the isolates of *C.albicans*.

Keywords: Azole antifungals, Biofilm formation, Broth microdilution, Candida albicans, Disk diffusion.

INTRODUCTION

The genus Candida is a dimorphic fungus, means that develops as bothfilamentous and yeast. It has >150 species but only fewpathogenic to human, and it is responsible for about 50-90% all candidiasis in human[1]. Candida albicans is the commonest among these. It is a part of human intestinal flora and is foundin, gastrointestinal tract in 40% of healthy adults. Candidais the leading cause of opportunistic fungal infections in human[2].

Systemic fungal infection causedby C.albicans has developed[3], an vital cause of, morbidity and mortality in immocompromised patients of AIDS, organ or bone marrow transplantation and cancer chemotherapy[4], C. albicans biofilms may establish on the surface of implanted medical devices, more so in immunocompromised hosts[5]. These include commonly the fungal infections on any type of implanted catheter, removable devices like dentures, voice prosthesis and contact lensrelatedfungal keratitis[6].

Candidiasis is of three main types,oropharyngeal or an invasive candidiasis like esophageal and genital or vulvo vaginal. Oropharyngeal candidiasis is also called thrush, candidiasis in the vagina is called yeast infection and invasive candidiasis appear when it goes to the bloodstream and tends to spread systemically[7].

Biofilms are groups of microbes found on surfaces, which can be spotted in medical, natural and industrial settings[8]. Biofilm production by *C.albicans* is a complicated process and comprises of three important steps in the process of development. Biofilm is characterized by, attachment of the yeast blastopores colonization on the substrate expansion of the yeast cells, followed by the formation of the biofilm colonization on the substrate expansion of the yeast cells, followed bytheformation of the biofilm[9,10].

Novel technological approaches have been devised to study the formation of biofilms with identification of their markers. Biofilms show reduced susceptibility to most of antimicrobial agents leading to the persistence of infection within it. Due to formation of an extra cellular framework the cells are saved from

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the environment and it becomes more difficult for antifungal to approach the yeast cells[11].

Against most antifungal agents except the lipid formulations of amphotericin B and echinocandins, Candida biofilms possess a multi-factorial broad-spectrum defense mechanisms[12]. During the early phase of formation of biofilm, the sterol composition of membrane is altered and the efflux pumps of drugs are expressed, thus contributing to the antifungal resistance against azoles[13].

The objective of this study is that the correlation between the biofilm formations by *C.albicans* isolated from clinical specimens and azole antifungal susceptibility against its planktonic and sessile forms.

METHODOLOGY

It was prospective analytical study, all clinical specimens from indoor and outdoor patients submitted to Combined Military Hospital (CMH) Peshawar-KPK for fungal culture and susceptibility testing, from September 2016 to May 2017 were included. Non-probability consecutive sampling technique was used for specimen collection. Sample size was 300 according to WHO calculator[14]. Repeat and inadequate specimens and Candida non-albicans isolates were excluded.

Fungal Culture

For obtaining a fungal growth, whole specimens were cultured aerobically on SaboraudDextrose agar (SDA) (Oxoid™) for 24-48 hours at 37°C. After a growth was available, Gram stain was done for preliminary identification of the yeast. For presumptive identification, the isolates were inoculated on CHRO Magar TM Candida (Oxoid™), which is a selective and a differential medium having chromogenic substrate, and where Candida appeared as green-colored colonies. API 20 C AUX (bio Mérieux, France) was used to differentiate biochemically *C.albicans* from other Candida species.

Antifungal Susceptibility Testing

Antifungal susceptibility testing against planktonic *C. albicans* was undertaken by Kirby Bauer disc diffusion procedure. A 0.5 McFarland standard suspension was prepared from a 24-48 hours old culture of *C. albicans*. The isolate was streaked on the plate of Mueller Hinton agar (MHA) (Oxoid™). Using Disc dispenser (Oxoid™), fluconazole (25µg) andvoriconazole (5µg) antifungal discs (Oxoid™) were applied on the surface of MHA according to CLSI M44A[8]. These plates were kept at 35°C and observed for growth after 24 to 48 hours, followed by measurement of zones of inhibition in millimeters for the antifungal discs applied. The antifungal susceptibility (susceptible S, susceptible dose-dependent SDD, and resistant R) was interpreted as per CLSI standards[9]. *C. albicans* ATCC 64548 strain was used to ensure quality control. Minimum inhibitory concentration (MIC) of both the azoles against *C.albicans* isolates was carried out in a 96-well round-bottom microtiter plate in RPMI 1640 -L-glutamines (Thermo Scientific TM) according to CLSI M27 Yeast[10]. The microdilution plates hadcoved at 35°C and were seen for visibility of any growth (fig-1). The microdilution wells were scored with, the aid of a reading mirror, the growth in every well was matched with that of the growth control (drug-free) well. After 24-48 hours clearing in turbidity was observed through the lens.

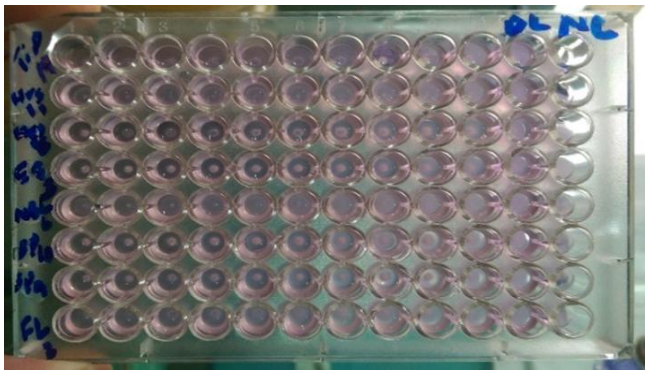

Figure-1: Optical clearness of turbidity in different wells of the microtiter plate.

Biofilm Formation

For biofilm formation, unmixed culture of *C. albicans* was developed on sabourd agar at 37°C for 24 hours. Sterile (96) well flat bottom microtiter plate was consumed. Four wells were used for every sample. The left well was used for negative control andtheright one was used as positive control. RPMI 1640-L-glutamines (Thermo Scientific TM) was included to every selected wells in the form of series. A 50µl inoculum of every sample was included in columns and 50µl of human serumwas included in the selected well. This microtiter plate was set in a vibrating incubator for 24 hours at 37°C and biofilm production was observed (fig-2).

This plate was washed with approximately 100µl PBS (phosphate buffer saline) after 24 hours of incubation at 37C and 100µl of a 0.1% solution of crystal violet (CV) in water were included in every well. This plate included at room temperature for 10-15 min and washed twice. It was turned upside down and left to dry for a few hours. About 100µl of 30% acetic acid in water was included inevery well of the microtiter plate to solubilize CV and it was incubated at room temperature for 10-15 min. Solubilized CV 100µl was put to a

new flat bottom microtiter dish. Absorbance was quantified in a plate reader (Bio Tek–Elx800) at 620 nm.

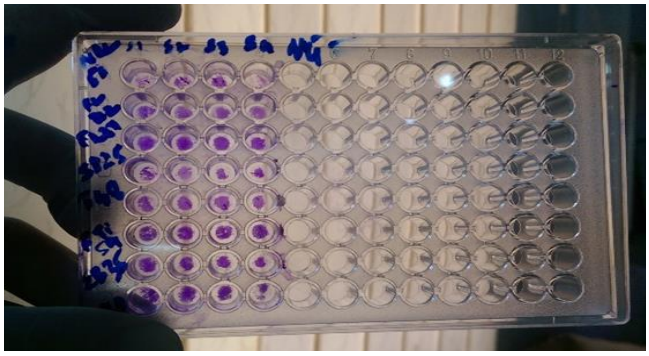

Figure-2: Biofilm production by C.albicans in a microtiter plate after CV staining.

RESULTS

CHRO Magar Candida accurately distinguished >92% of our Candida strains. After identifying biochemically by API AUX Candida, a total of 300 isolates of *C.albicans* were used to test forin vitro antifungal susceptibility against planktonic *C.albicans* and then its biofilm production. Planktonic antifungal susceptibility was proceeded disk diffusion procedure which appeared that *C.albicans* was susceptible voriconazole in 240 (80%) and fluconazole in 195 (65%) isolates. *C. albicans* was found susceptible dose dependent (SDD) voriconazole in 21 (7%) and fluconazole in 28 (9%) isolates respectively. Seventy-seven (26%) and 38 (13%) isolates of *C.albicans* were resistant voriconazole and fluconazole, respectively (fig-3).

Sessile antifungal susceptibility of *C.albicans* isolates was performed through broth microdilution procedure, in which 161 (54%) isolates had susceptible, 36 (12%) were SDD and 148 (49%) haveresistant to fluconazole, and 160 (53%) isolates were susceptible, 42 (14%) were SDD and 98 (33%) were resistant to voriconazole (fig-4). Among 300 *C.albicans*, 285 (95%) were biofilm producer and 15 (5%) were not biofilm producer

Level of significance was applied between the two variables i.e. among fluconazole and voriconazole for plankton and sessile *C.albicans* as shown in table.

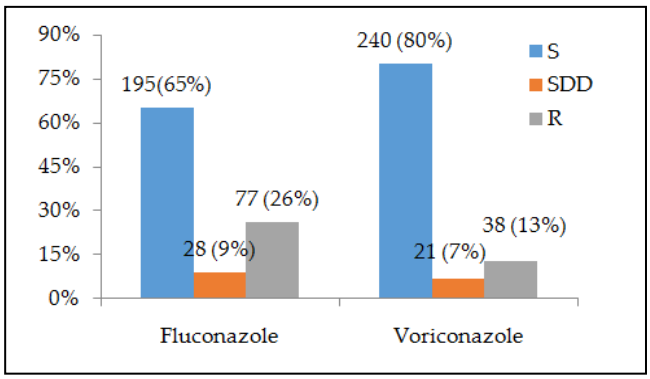

Figure-3: Planktonic antifungal susceptibility of fluconazole and voriconazole by disk diffusion procedure.

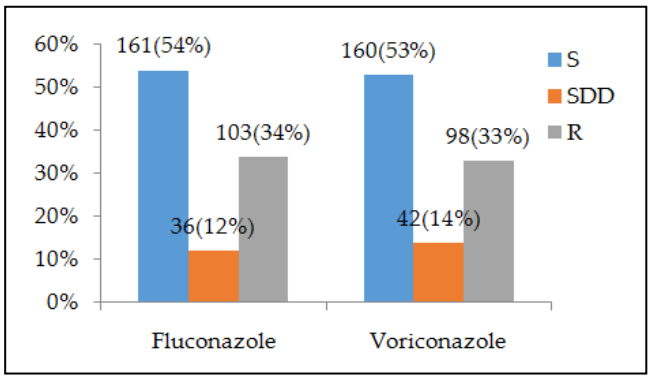

Figure-4: Sessile antifungal susceptibility against fluconazole and voriconazoleby microdilution method.

DISCUSSION

A proper and prompt diagnosis of *C.albicans* infection as a pathogenic yeast playsa major role for its definitive treatment. Germ tube test and Gram staining are still the easiest and reliable methods for the recognition of Candida spp. CHRO Magar Candida is used to be a rapidand reliable procedure for recognition of Candida species as compared to API 20 C AUX[15]. CHRO Magar Candida correctly identified 92% of our *C.albicans* strains which is in consistence with the result of astudy done by a researcher in 2012[16].

Forthe treatment of superficial or systemic Candida infections, the important classes of antifungal drugs used are azoles, echinocandins and polyenes[17]. Azoles were used here because they are reliable and easilyaccessible antifungal agents. Fluconazole and

Table: Correlation between plankton and Sessile antifungal susceptibilityin clinical isolates.

	Fluconazole			Voriconazole			*p*-value
	Sensitive	SDD	Resistance	Sensitive	SDD	Resistance	
Plankton antifungal susceptibility test	195	28	77	240	22	38	<0.01
Sessile antifungal susceptibility test	161	36	103	160	42	98	<0.01

voriconazole are mostly used to treat candida or other fungal infections due to their good fungi static effect. Their action is by blocking the ergo sterol production and targeting the enzyme lanosterol 14-demethylase (related to the ERG11 gene), thereby, leads tocollection of intermediates of toxic sterol pathway[18].

Our results of antifungal susceptibility appeared significantly lower susceptibility of azole antifungal agents againstboth planktonic and sessile *C.albicans* like other studies. Results of planktonic antifungal susceptibility appeared that fluconazole was susceptible against 195 (65%) and that of voriconazole was against 241 (80%) *C.albicans* isolates[19]. The susceptibility of voriconazole against *C.albicans* is higher in most of the studies as voriconazole is a novel azole with more strict binding to the sterol 14 α-demethylase, thereby more constructive inhibiting ergo sterol synthesis[20]. Twenty-eight (9%) and 21 (7%) isolates of *C.albicans* were showed that it were susceptible dose-dependent (SDD) to fluconazole and voriconazole, respectively. Seventy-seven (26%) and 38 (13%) isolates were found resistant to fluconazole and voriconazole respectively. One of the study was carried out in the United Kingdom, on different confines of Candida species, shows comparable figures of 206 (76%) *C.albicans* isolates as susceptible, 25 as SDD and 39 (14%) as resistant to fluconazole by using CLSI-validated methods. A similar study carried out in Singapore and China found that 28.1% of Candida confines were susceptible, 8.4% were SDD, while 63.6% confines were resistant to fluconazole.

Antifungal susceptibility testing of planktonic cells against fluconazole and voriconazole was performed by NCCLS M-27A broth microdilution by doing MICs in this study. In a similar study, 74% of *C.albicans* isolated from blood stream were resistant to fluconazole and 8.5% were resistant to voriconazole[21].

Biofilms are more commonly formed adherent to solid surfaces can also be produced even in edges of liquid to air. Aquatic environments, biomaterials, artificial structures and mammalian tissuesand plant are the environments most commonly colonized by the biofilms. Almost all species of C.albicanspossess the capability of biofilm formation. Candida biofilm resistance phenomenon was for the first explained in 1995 for *C.albicans* by Hawser and Douglas (1995). Lower resistance to azole antifungals has been shown in planktonic conditions as compared to *C.albicans* biofilm experimentally, as shown in our study[2]. Biofilm formation with associated antifungal resistance is increasing by the yeardue to increase in the use of indwelling medical devices. Our data found that 95% of all the isolated Candida albicanswere biofilm producer, which was in conformance with a study in 2017 showing 90% of all Candida albicans as biofilm producer. The slight increase in biofilm production in this study is attributable out being clinical specimens.

RECOMMENDATIONS

Candida Biofilm producing infections are an emerging problem. Using the variables for biofilm production, needed an intervention strategies can be implemented to lower biofilm-based Candidiasis, as there is a high antifungal resistance watched in biofilm-producing strains in this study. There is also a need to search for newerand effective antifungal agents with potent anti-biofilm abilities due to the increasing resistance to conventional antifungal drugs like azoles, and also due to their high probability of biofilm formation on implanted medical devices and tissues.

CONCLUSION

Planktonic *C.albicans* clinical confines were more susceptible to voriconazole than fluconazole and sessile isolates. Biofilm formation was very high among all the isolates of *C.albicans.*

CONFLICT OF INTEREST

This study has no conflict of interest to be declared by any author.

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