Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 2, 2021, Pages. 3490 - 3500
Received 20 January 2021; Accepted 08 February 2021.

Prevalence and Characterization of Opportunistic Candidal Infection among Diabetic Foot Ulcer Patients, Puducherry

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ABSTRACT

Theaim ofour presentstudywastoestimatetheprevalence and characterization of*Candidal*infection in footulcer patientsand its virulence factors and their drug resistant pattern.Diabetic patients are more susceptible to candidiasis infection due to poor glycemic control and therapeutic dentures.A total of 100Swabs was taken from diabetic foot ulcer patients from January 2017 to June 2017.Samples were cultured onSabouraud dextrose agar (SDA) medium. *Candida* spp. were differentiated by culture on Hi CHROM agar, Sugar assimilation test, fermentation test, virulence factors and antifungal sensitivitytest.Out of 100 samplesobtainedfromdiabeticfootulcerpatients ,36(36%)werepositivefor*Candidasp*byculture.*Candida albicans*wasfoundtobethe predominantisolate followed byothers.*Candida albicans* shows more virulence activity than *non albicans*. Resistance to fluconazole was observed in our study .C.albicans was more resistant to azoles than non albicans. Our results will gives physicians the knowledge on virulence factors ,antifungal susceptibility testing and the development of newer antifungal drugs are mandatory to achieve a decrease in *Candida* infections among diabetic foot ulcer patients. Fluconazole resistance is a public health concern and the rational use of this drug is important in community.

Keywords: Diabetic Foot Ulcer, Germ tube test, CHROM agar, Drug Resistant, Antifungal Agents

INTRODUCTION

Diabetic foot ulcers (DFUs) are widely in the communitywith prevalence rates ranging from 5% to 10%. Twenty five percent of peoples with diabetes developfootulcer intheir

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lifetime.Microorganism causing infections in DFU are mostly bacteria, few studies reported the fungus and low pathogenic yeast [1,2,3]

Candida spp. is the common yeast isolated from diabetic foot ulcers with a prevalence of 5%–21% [4,5].Fluconazole used as the main option for the treatment of *Candida albicans.* [6]. Resistance to fluconazole are increasingly reported and it is apublic health issue [7,8]

Diabeticfootsyndromes are one of the maincauses of morbidity [9].Opportunistic fungal Infections was not given importance in the present scenario, compared to its bacterial portionbecause of lack of researcher.

Though, topical studies show the broad range of fungal strains in an diabetic infected foot ulcer patient, with Candida species is the commonly isolated strain. Treatment of an infected diabetes foot ulcer should encompass all the possible microbiologicalcauses, to provide efficient and specific treatment to thepatients [10].Therefore the main objective of our present study ,find out the prevalence and characterization of candida infectionsin diabetic foot ulcer patients.

MATERIALS AND METHODS:

This was a prospective study done on diabetic patients with DFU who visited the Out Patientsurgical department at tertiary care hospital Puducherry from January 2017 to June 2017. The study was carried out after institutional human ethical committee clearance. Patients with diabetic foot ulcers visiting our OPD were included in this study. Patients treated with antifungal therapy, chemotherapyand corticosteroids were excluded.

A total of 100 samples were collected from diabetic foot ulcer patients were studied. Two tissue samples were collected from deep ulcer,place the tissue in normal saline and sent to laboratory for further processing.

Microscopic examination of tissues was done.First tissue was placedin 10% KOH, second tissue used for fungal culture with Sabouraud’s dextrose agar(SDA) supplemented with Chloramphenicol and cycloheximide, incubated at 30°Cfor 4 weeks.

Based on Colony Morphology, Gram stain was performed to rule out the bacterial isolates.

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IDENTIFICATION OF CANDIDA SPECIES:

HiCHROM Agar plates were incubated at 25°C -30°C for 24-48 hours. Species were identified based on the colour of the colony.

Candida albicans- Light green

Candida glabrata- cream to white

Candida krusei- purple fuzzy - and blue to purple

Candida tropicalis - Candida tropicalis.

GERM TUBE TEST

Take 0.5 ml of human serumin a test tube and inoculate 2-3 isolated colony, incubate at 37ºC for 2 hours. Observe the germ tube formation under the microscope after 2 hours.

CORN MEAL AGAR FOR CHLAMYDOSPORE FORMATION (DALMAU PLATE)

Divide the Corn meal Agar plate into 4 parts. Using a needle, touch the isolated colony and then make 2-3 streaks. Place a cover glass to the control part. This will provide anaerobic environment. Plates are incubated at 25ºC for 2-5 days. Place the plate in microscope and focus the edge of the cover glass under the 40X objective. Observe morphological features of candida species.

SUGAR FERMENTATION: Prepare sugar fermentation medium.Add 2% of sugar to the mediumand place sterile Durham's tube for gas production.

Inoculate each sugar fermentation broth with 0.1 ml of inoculum. Incubate the tubes at 25ºC up to 1 week. Examine the tubes every 48-72hrs period for theacid and gas production in Durham's tube.Production of gas in the tube is taken as fermentation positive, acid production indicate that carbohydrate is assimilated.

ASSIMILATION TEST: Suspend a heavy inoculum of a yeast culture that has been subculture on sugar free medium in 2ml of Yeast Nitrogen Base.Place the carbohydrateimpregnated discs

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onto the agar surface.Incubate the plates at 37 ºC for 3-4 days. The presence of growth around the disc is considered as positive.

VIRULENCE FACTORS OF CANDIDA SPECIES

BIOFILM PRODUCTION TEST

Suspend a loopful of colony from the SDA was inoculated into a tube containing 10ml Sabouraud's liquid medium traces supplemented with glucose (to the final awareness of 8%). The tubes were incubated at37°C for 24 h, and then the broth is aspirated out and the walls of the tubes were stained with saffron. **Results:** Negative (0), Weak positive (1+), Moderate positive (2+) ,Strong positive (3+).

PHOSPHOLIPASE ESTIMATION TEST

Inoculating 10 µL aliquots of the yeast suspension into the wells punched onto the surface of the egg yolk medium. The diameter of the precipitation zone around the well was measured after incubation at 37°C for 48 h. **Results:** Ps = 1, negative Phospholipase activity; Pz = 0.64–0.99, positive ,Phospholipase activity; and Pz ≤ 0.63, very strong .

HEMOLYTIC ACTIVITY TEST

Add 7 ml aseptically collected fresh sheep blood to 100 ml SDA supplemented with glucose at a very last awareness of 3% (w/v). 10 µL of preferred inoculum prepared from both the take a look at and the manipulate.

Results: Diameter of the colony to the translucent zone of hemolysis.

ESTERASE ACTIVITY TEST

Ten microliters of previously prepared suspension from each isolate emerge as carefully deposited on the Tween-80 opacity test medium; this changed into then incubated at 37 °C for 10 days in aerobic. Results:

Positive - Presence of a halo surround the inoculation site

Negative – Absences of a halo around the inoculation site

ANTIFUNGAL SUSCEPTIBILITY TEST

The antifungal sensitivity testing of yeast isolates was carried out using the disk diffusion method as per CLSI guidelines. Mueller Hinton agar supplemented with 2% glucose and 0.5µg/ml methylene blue was used for sensitivity testing. Inoculums was prepared by 3-4 isolated yeast isolates.Inoculum suspension was adjusted to 0.5McFarland standard.Inoculate the

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Muller hinter agar with a suspension using a sterile cotton swab by lawn culture method.The plates were allowed to dry and antifungal discs were placed onto the surface of inoculated agar plate.

RESULTS AND DISCUSSION

Table 1:Gender distribution of Candidiasis in DFU cases

Total Sample	**Positive Candida Cases in DFU**	**Male**	**Female**
100	36(36%)	26(72.2%)	10(27.7%)

Out of 100 DFU cases, Candida species were isolated in **36(36%)** patients. It was more significant in males 26(72.2%) than females10(27.7%).Age of the patients from 40 to 69 years were more infected.

Table:2Prevalence of *Candida sp*in diabetic foot ulcer

S.No	**Total Positive Cases(n=100)**	**% prevalence of candida species**
1	36	36%

Out of 100 samples collected from patients with diabetic foot ulcer 36(36%) isolates of candida were isolated.

Table 3: Distribution of candida species in diabetic foot ulcer

Candida species	**No of culture positive cases(n=36)**	**Percentage%**
C.albicans	18	50%
C.tropicalis	10	27.7%
C. parapsilosis	5	13.8%
C.krusei	3	8.3%

Amongthe36*Candida* isolatesobtained from 100samples,*Candidaalbicans*was foundtobetheprimespecies.Outof 36*Candida* isolates, 18(50%)*C. albicans* ,10(27.7%)*C.tropicalis,5(13.8%) C. parapsilosis*and 3(8.3%) C.krusei were isolated in DFU cases.

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TABLE 4:Biofilm Production by Candida sp

Biofilm	***C.albicans(n=18)***	***C.tropicalis(n=10)***	***C. parapsilosis(n=5)***	***C.krusei(n=3)***
Negative	5	1	0	0
Weekly positive	2	3	1	1
Moderate Positive	5	3	1	1
Strong positive	6	3	3	1

Above table 4 shows, *Candida albicans* shows higher positivity rate for biofilm production than*Nonalbicans*.

Table 5 :Phospholipase production by Candida sp

Phospholipase Production	***C.albicans(n=18)***	***C.tropicalis(n=10)***	***C. parapsilosis(n=5)***	***C.krusei(n=3)***
Negative	**2**	**2**	**1**	**0**
Positive	12	6	3	3
Strong Positive	4	2	1	0

In this Study,Candida albicans is the major Phopholipase producers than Non albicans shown in Table 5.

Table 6:Esterase production by Candida sp

Esterase Production	***C.albicans(n=18)***	***C.tropicalis(n=10)***	***C. parapsilosis(n=5)***	***C.krusei(n=3)***
Negative	8	3	1	0
Positive	10	7	4	3

In our Study, Esterase production is higher in 14 (78%)Non albicans than 10(56%)Candida albicansdescribed in Table 6.

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Table7: Haemolyase production by *Candida sp*

Haemolyase Production	*C.albicans(n=18)*	*C.tropicalis(n=10)*	*C. parapsilosis(n=5)*	*C.krusei(n=3)*
Negative	1	1	0	1
Positive	17	9	5	2

In our current Study, haemolyase production is equal in both Non albicans and Candida albicans described in Table7 .

Table8 :Antifungal Resistant Pattern (%) of *Candida species*

Anti-Fungal Drug	*Candida albicans*(n=18)	*Candida tropicalis*(n=10)	*Candida parapsilosis*(n=5)	*Candida krusei(n=3)*
Fluconazole	12(66.6%)	4(50%)	2(40%)	1(33%)
Amphotericin B	8(44.4%)	2(25%)	2(40%)	0(0%)
Voriconazole	6(33.3%)	2(25%)	1(20%)	0(0%)
Itraconazole	4(22.2%)	0(0%)	0(0%)	0(0%)

Out of 18*C. albicans isolated*, 12(66.6%) was resistant to fluconazole and 18 Non albicans sp isolated ,7(44%) were resistant to fluconazole.

DISCUSSION

About17% of diabetic patientsdevelop footulcer in their lifetime. It is one of the major causeof hospitalizationfordiabetic patients. 85% Polymicrobialinfectionsof ulcerare responsible forlimbamputationin thediabeticpatients[11].Severalstudies havebeen conducted onthebacterial infections of footulcer.

Literature references onfungalinfections arevery limited still[12]. Thus,little dataisavailableon *Candida* Co-infection in diabetic f o o t ulcer [13].

In our presentstudy, among 100 DFU cases, 36 (36%)Candida species were isolated . It was more significant in males 26(72.2%) than females10(27.7).Diabetic foot ulcer patients age range from 40 to 69 years. It hasbeenobserved that57.5% weremalesand42.5%were females

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which is similartotheresult[1 4]. Theaccommodating resultswas shown inother similar studies by [15,16],males leadsinhaving diabeteswithfoot infections when compared to females.

Inthepresentstudy, weanalyzedthe fungalco-infectionin foot ulcer. Amongthe36*Candida* isolatesfrom 100samples,*Candidaalbicans*was foundtobethecommonspecies. Outof 36 *Candida* isolates, 18(50%)*C. albicans* ,10(27.7%)*C.tropicalis,5(13.8%) C. parapsilosis*and 3(8.3%) C.krusei were isolated in DFU cases. Correlates withreportby [17]showed theprevalenceof 19.9%.Ourresultsarehigher than those reported[18,19].Our results compared with [20] this study,49%*C.albicans*,23% *C.tropicalis* and 18% *C.parapsilosis*, reported a similar finding. Even though the connection of Candida sp with diabetic foot ulcer relates [21,22]

In our present study,*Candida albicans* shows higher positivity rate for biofilm production than *nonalbicans*. our results compared with similar finding [23].

Phospholipases are a group of enzymes produced by *Candida species* that primarily help in digesting the phospholipids of the host cells leading to cell lyses.In our study,*Candida albicans* is the major phospholipase producer,followed by correlated with similar study [24]

Esterase acts as a virulence character among clinical isolates of *Candida*.Mechanism of virulence is due to the cytotoxic effects of esterase in the host tissues.Our study find a higher production of esterase among *Non albicans* than *Candida albicans* compared with C.P.G Kumar.T et al[25]

Haemolyase is one of the important virulence factors contributing to pathogenicity of *Candida*.It activates complement and opsonize surface of RBC.In our study Haemolyase production was noted equally in both *albicans* and non *albicans***.**

Out of 18 *C. albicans isolated*, 12(66.6%) was resistant to fluconazole and 18 Non albicans sp isolated ,7(44%) were resistant to fluconazole. Antifungal drugs resistant *to Candida sp*was seen in Amphotericin B drug followed by Voriconazole. Our present study showsC.albicans more resistant to azoles than non albicans these results correlatewith [26].Resistance to antifungal agents was comparable to previous studies with amphotericin resistance 7%,

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flucytosine 7.9%, and voriconazole 4%. It is unclear at present ,due to limited use of these agents in the community compared to fluconazole [27]

CONCLUSION

Our results shows a *Candida* Sp resistant to fluconazole in DFU is a major concern due to inappropriate use of drugs in diabetes patients. Due to lack of oral antifungal agents for treating fungal infections, which makes it important to prevent spread of resistance.

Increased production of virulence factor among candida and non candidaalbians become common .Our study recommends to screen biofilm production ,and other virulence factor testing as a routine screening for Candida and non albicans isolates

Increase in resistance is a major public health concern for the use of fluconazole in the community.Our results will make physicians easier to treat fungal and mixed infections of diabetic foot ulcers, andencourage further research into these infections.

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