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ORIGINAL ARTICLE

Fungal Diabetic Foot Infections

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ABSTRACT

Background: *Fungal infections play important role in pathogenesis of diabetic foot infections.* ***Objective:*** *to investigate the prevalence of fungi among patient with diabetic foot infections.* ***Methodology:*** *one hundred and twenty diabetic patients hospitalized due to foot infections were enlisted in this study. Deep tissue specimens from depth of the wound and nail samples were collected from the infected sites using the standard protocol. Laboratory identification of samples was done and pathogens were identified to the species level by morpho-physiological methods. Polymerase chain reaction was used to assess the presence of fungi in samples from infected sites.* ***Results:*** *Fungi were found in 51.7% of the patients. Candida albicans were the most predominant isolated organism (40.1%). Amphotericin B had 100% sensitivity against all Candida isolates. The most susceptible Candida species to fluconazole were C. dubliniensis and C. albicans. The most susceptible Candida species to voriconazole were C. dubliniensis. The most susceptible Candida species to itraconazole were C. dubliniensis. Of the study population, 70.0% had bacterial infection. The predominant isolates were Klebsiella spp. (32.7%). Mixed fungal and bacterial infections were seen in 20.3% of patients. Sensitivity of Pan fungal PCR was 97.4%, specificity was 92.4%.* ***Conclusion:*** *Fungal infections were more in patients with poor glycemic control. The role of antifungal agents in management of diabetic foot infections needs to be evaluated further.*

INTRODUCTION

Uncontrolled blood glucose level can cause peripheral neuropathy and impair blood circulation, especially in lower limb and this increase the risk of foot infections in diabetic patients [1]. At the same time infections are unobserved by the patients due to lack of ability of those patients to feel cuts and irritations in their feet [2].

Most of researches focus on role of bacterial microbiome in diabetic foot wound and mixed aerobic and anaerobic microbes were reported, but studies on the relationship between fungal bioburden and outcome of diabetic foot wound remain disappointing [3].

Presence of fungal mycobiome in diabetic foot wound are associated with delayed wound healing and poor outcome of the wound. Meanwhile they may form mixed biofilms with bacteria and increase severity of the wound [4], also clinical presentations of fungal infections are poor and confusing which lead to delayed diagnosis and make the situation more difficult [40].

Fungal infections of the diabetic foot includes superficial infection as tinea pedis and nail infections and deep infections as cellulites and diabetic foot ulcer infection[5].

The highest percentage of fungi that were isolated from diabetic foot wound were *Candida* species. This is explained by the fact that *Candida* are normal skin flora but with tissue injury due to internal or external trauma, they enter the wound where the environment is different from that of skin surface in many conditions as nutrition, temperature and pH, this can change *Candida* spp. metabolism and shift them from commensal to opportunistic and become more virulent[6].

Once colonization with Candida occurs they can form biofilm within the wound and disturb the synchronized tissue regeneration process [7].

METHODOLOGY

Study design:

This a descriptive cross sectional study conducted over a period of 18 month from July 2015 to December 2016 and included samples collected from feet of diabetic patients attending Diabetic foot clinic at Specialized medical hospital (SMH) and from feet of diabetic patients referred to Vascular surgery clinic and Isolation ward at Mansoura University Hospital (MUH) showing symptoms and/ or signs of infections.

Study population:

One hundred and twenty patients (80 were males and 40 were females), who were suffering from diabetes irrespective of age and sex and who were hospitalized for chronic diabetic foot lesion and whose wounds have

not already received any antibiotic or antifungal medications at time of study, were included in the study after obtaining their informed written consent. Patients with a history of malignancy, chemotherapy, or radiotherapy or who were on steroids or antifungal (topical or systemic) during the previous four weeks before sampling were excluded. Demographic details, duration of foot lesion, duration of diabetes, fasting and postprandial blood sugar were obtained. Vascular insufficiency and peripheral neuropathy were assessed. Diabetic foot was characterized according to the International Working Group on the Diabetic Foot Classifications of Diabetic Foot Infection [9].

Specimen collection:

Clinical examinations of patients' toe nails or other infected sites in the foot were performed. The slough and necrotic tissue over the wound were surgically debrided. After a thorough wash of the wound with normal saline, a deep tissue specimen was taken from the wound bed. The specimen was collected in a sterile container, and the tissue was soaked with phosphate buffered saline[10]. The affected toenails were cleaned with 70% alcohol and nail scraping was taken with a sterile scalpel blade and collected in a clean dry paper envelope[11]. Tissue specimens were collected from the infected toe-webs[12]. This was transported to our mycology lab and Medical Microbiology and Infection Control Unit (MMICU) in Medical Microbiology and Immunology department, Faculty of Medicine, Mansoura University within 10 to 15 min for fungal and bacterial cultures.

Fungal culture and sensitivity:

The tissue specimens were sliced into tiny fragments with a sterile scalpel blade. These fragments were placed directly into two Sabourand's Dextrose Agar plates with chloramphenicol (SDA) (Oxoid, UK) plates directly and two Dermatophyte test media plates (DTM) (Liofilchem-Diagnostic, Italy). These were incubated aerobically at 25°C and 35°C and observed for 4 weeks[13]. KOH (10%) was performed, and the results were documented. Fungal species were identified morphologically [8, 14] Colonies suspected to be *Candida* were identified morphologically by Gram stain, LPCB stain, germ tube test, subculture on corn meal agar with Tween 80 (Oxoid, UK) and CHROMagar Candida plates (BD, Hungary, Germany) and biochemically using API 20 C-AUX (Bio-Merieux, France). *Aspergillus* species and other filamentous fungi were identified by slide culture on Sabourand's dextrose agar with lactophenol cotton blue staining.

Antifungal susceptibility testing for Candida and filamentous fungi were done according to three methods for antifungal susceptibility testing; M27A3 broth microdilution, M38 A2 broth microdilution [15] and M44-A[16] disk diffusion methods were used to test susceptibility of the isolated fungal species to antifungal agents; Amphotericin B (AMB), fluconazole (FLC), Itraconazole (ITC) and voriconazole (VRC).

Bacterial culture:

Part of the sterile deep tissue specimen was grinded into tiny parts, and then nutrient broth was added and incubated for 24 hours at 37˚C. Samples were vortexed well then were subcultured on nutrient agar, blood agar and MacConkey agar plates and were incubated aerobically at 37˚C for 24-48 hours[17]. Bacterial isolates were identified by standard biochemical tests, and susceptibility testing was performed according to CLSI guidelines [19].

Detection of fungal DNA in samples by Pan fungal PCR[18]:

Fungal DNA extraction from tissue was performed by Zymo DNA mini kit for DNA extraction from tissues (Zymo, USA).

The following primers (LGC Biosearch technologies, USA) were used nucleic acid amplification according to[18]. The sequences of the ITS1 primer were 5` (TCC GTA GGT GAA CCT GCG G) 3` and the sequences of the ITS2 primer 5` (GCT GCG TTC TTC ATC GAT GC) 3`.

PCRs were performed in 25-µl volumes and contained 5 µl of template DNA, 12.5 µl of Taq PCR Master Mix (TIANGEN Biotech Ltd, China), 1 µl of each primer and . The following PCR conditions were used: Initial denaturation at 96 º C for 5 min.40 cycles of: Denaturation at 94º C for 30 seconds. Annealing at 58ºC for 30 seconds. Extension at 72 C º for 30 seconds. Followed by a final extension at 72 º C for 15 min. After amplification, samples were stored at -20˚C for longer storage.[18]

Statistical analysis:

All data were collected, tabulated and statistically analyzed using SPSS version 21 for windows (SPSS Inc., Chicago, IL, USA) and Microsoft Office Excel 2010 for windows (Microsoft Cor., Redmond, WA, USA). Qualitative data were represented as proportional while quantative data were done by Kolmogorov-Smirnov test. Parametric data were expressed in mean ± standard deviation. Non parametric data were expressed in median, minimum and maximum. Normality of data was first tested by one sample K-S test. Pearson Chi-square (X^2) was used to test the association between qualitative variables, as appropriate. Mann-Whitney test was used to compare median for non- parametric variables. In addition, Cohen's Kappa (k) test was used to measure the degree of agreement between two qualitative variables. P value < 0.05 was considered as statistically significant and P value <0.001 was considered as statistically highly significant.

RESULTS

This study was conducted on 120 diabetic patients with foot infections, 80 (66.7%) were males and 40

(33.3%) were females. The median age of the study population was 57 years for males (range 42-66) and 59 years for females (range 38-66). Eleven (9.2%) patients were diagnosed with type 1 D.M., and 109 (90.8%) patients were diagnosed with type 2 D.M. The median duration of diabetes was 11years (range 3-32), median duration of foot lesion was 4 weeks (range 1-48), median fasting blood sugar was 270 mg/dl (range 155-400) and post prandial blood sugar was 310 mg/dl (range 200-510).

Seventy seven (64.2%) patients were on insulin therapy and 43(35.8%) patients were on oral hypoglycemic drugs, 101 patients (84.2%) had neuropathy, 16(13.3%) patients had PVD, 30(25%) patients had foot deformity, 18(15%) had done previous amputation before, 80 patients (66.7%) had history of trauma. Twenty eight patients (23.3%) had osteomyelities.

Of 120 patients, 79(65.8%) patients had diabetic foot ulcer (DFU) only, 8(6.7%) patients had interdigital toe web lesion only, 9(7.5%) patients had toe nail lesion only and 24(20.0%) had mixed foot lesion, 62 (51.7%) had FFIs; 30(25.0%) had fungal infection of DFU, 18(15.0%) had toe nail onychomycosis, 14(11.7%) had toe web fungal infection.

It was found that of 103 patients with DFU 5 (4.9%) had fungal infection only, 58(56.3%) had bacterial infection only, 25 (24.3%) had both fungal and bacterial infection and 15 (14.6%) had neither fungal nor bacterial infections in their deep tissue (Fig. 1).

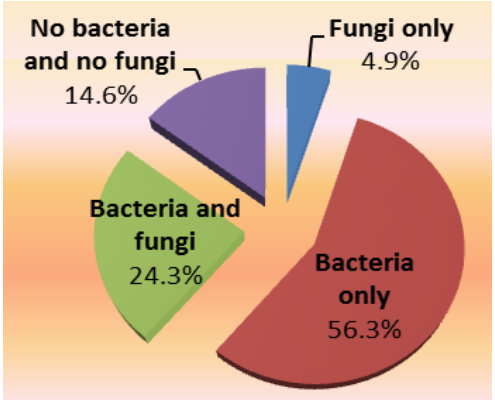

Fig. 1: Microbiological spectrum in deep tissue from DFU.

Of 30 patients with fungal infection of DFU, 18 (60.0%) were males and 12 (40.0%) were females; however this difference was not statistically significant (P value=0.160, $\chi2$=0.160). The median of age for DFU cases with positive fungal infection was 57 years (range 42-65); however fungal infection was not significantly associated with age of the patients (P=0.754, Z=0.313). Fungal infection of DFU was significantly correlated with un-controlled fasting and post prandial blood sugar and presence of osteomyelities (P <0.001) (Table 1).

Table 1: Risk factors for fungal infection in patients DFU.

Risk factors		Fungal infection				Significance	
		Positive		Negative			
		NO	%	NO	%	$\chi2$	P value
Neuropathy (n=98)		30	30.6	68	69.4	2.16	0.142
PVD (n=16)		7	43.8	9	56.3	1.96	0.161
Retinopathy (n=10)		0	0.0	10	100.0	0.42	0.519
Nephropathy (n=8)		1	12.5	7	87.5	0.03	0.871
Hypertension (n=30)		8	26.7	22	73.3	0.71	0.399
Obesity (n=28)		4	14.3	24	85.7	0.67	0.410
Smoking (n=36)		6	16.7	30	83.3	0.05	0.829
Osteomyelities (n=28)		16	57.1	12	42.9	18.59	<0.001
D.M type	**I (n=11)**	2	18.1	9	81.8	0.88	0.349
	II (n=92)	20	21.7	72	78.3	0.44	0.569
D.M duration(YS) Median(IQR)		11 (4-31)		11 (3-32)		***Z* value** 0.462	***P* value** 0.644
FBS (mg/dl) Median(IQR)		310 (300-400)		260 (155-330)		6.602	< 0.001
PPBS (mg/dl) Median(IQR)		410 (350-510)		300 (200-400)		7.347	< 0.001

Fungal infection were more common in grade 3 wounds (57.1%) with statistically significance (P <0.001, $\chi2$=15.30); however it was higher in neuroischemic ulcer (43.8%) than neuropathic ulcer (26.4%) but with statistically unsignificance (P =0.161, $\chi2$=1.962) (Table 2).

Table 2: Relation between fungal infections and grade and type of DFU.

Ulcer grade		Presence of fungi	
		No.	**%**
Grade II (n=69)		**12**	**17.4**
Grade III (n=28)		**16**	**57.1**
Grade IV (n=6)		**2**	**33.3**
Total (n= 103)		**30**	**29.1**
Significance	***P* value**	**< 0.001**	
	χ2	**15.30**	
Type of ulcer		**Presence of fungi**	
		No.	**%**
Neuropathic (n=87)		**23**	**26.4**
Neuroischemic (n=16)		**7**	**43.8**
Total (n= 103)		**30**	**29.1**
Significance	***P* value**	0.161	
	χ2	1.962	

The prevalence of fungi in deep tissues of DFU was 29.1% (30/103). About 36 fungal species were isolated from 88 patients with infected DFU and the isolation rate was 0.4(36/88). The predominant species were *C. albicans* (47.2%) followed by *C. krusei* (13.9%). (Table 3).

Table 3: Number and percentage of fungi isolated from deep tissue of DFU. (Total No=103).

Fungal Species	Frequency (No. of isolates)	Percentage (%)
C. albicans	17	47.2
C.krusei	5	13.9
C.dubliniensis	3	8.3
C.galabrata	1	2.8
C. tropicalis	1	2.8
A. flavus	3	8.3
A. fumigatus	1	2.8
Mucor	2	5.6
Penicillium spp.	1	2.8
Basidiobolus ranarum	1	2.8
Cladosporium spp.	1	2.8
Total No. of fungal isolates	36	100

This study found that the prevalence of fungal toeweb infection was 63.6% (14/22). About 18 fungal species were isolated from 14 patient and the isolation rate was 1.3 (18/14). The predominant species were *C. albicans* (55.6%) followed by *C. krusei* (16.7%). Fungal infection in toe web had 1.4 times more increase in fungal infection of DFU (Odds ratio=1.411).

The prevalence of fungal toenail infection among patients with toenail lesion was 85.7% (18/21). About 23 fungal species were isolated from 18 patients with infected toe nail and the isolation rate was 1.3 (23/18). The predominant species were *A. flavus* (44.4%) followed by *A. niger* (21.7%) and *C. albicans* (17.4%) (Table 4). It was found that patients with fungal infection in toe nails had 2.6 times increase in fungal infection of DFU more than patients without fungal infection in toe nails (Odds ratio=2.592).

Table 4: Number and percentage of fungal pathogens isolated from toe nail lesion:

Fungal Species	Frequency (No. of isolates)	Percentage (%)
A. flavus	8	44.4
A. fumigatus	3	13.0
A.niger	5	21.7
C. albicans	4	17.4
C.galabrata	1	4.3
Penicillium spp.	1	4.3
Fusarium spp.	1	4.3
Total No. of fungal isolates	23	100

The prevalence of bacteria in deep tissues of DFU was 80.6% (83/103). About 142 bacterial isolates were isolated from 83 patients with infected DFU and the isolation rate was 1.7 (142/83). The most commonly isolated bacteria were *Klebsiella* spp. (33.1%) followed by *Proteus* spp. (17.6%) (Table 5). Among 83 patients who had bacterial infections, 59 (71.1%) patient had only single bacterial infection in the deep tissue, while 24 (28.9%) patient had two different types of bacteria coexisting in the wound.

Table 5: Prevalence of bacteria isolated from patient with DFU. (Total No=103).

	Bacterial isolates	Frequency (No. of isolates)	Percentage (%)
Gram-negative bacteria N= 115 (81.0%)	*Klebsiella spp.*	47	33.1
	Proteus spp.	25	17.6
	Escherichia coli	24	16.9
	Pseudomonas spp.	14	9.8
	Enterobacter spp.	3	2.1
	Citrobacter spp.	2	1.4
Gram-positive bacteria N=27 (23.5%)	*Staphylococcus aureus*	18	12.7
	MRSA	8	5.6
	Group D streptococci	1	0.7
Total No. of bacterial isolates	142		100 %

The prevalence of bacterial infection of toeweb space was (27.3%). About 8 bacterial species were isolated from 6 patient and the isolation rate was 1.3 (8/6). The predominant species were *Pseudomonas* spp. (37.5%).

Sensitivity to AMB, FLC and VRC was tested on 49 *Candida* isolates according to M 27A3 broth microdilution method. The resistance rate was 8.2% (4/49) for AMB, 28.6% (14/49) for FLC and 13.3% (6/49) for VRC and according to M44-A disk diffusion method; the resistance rate was 6.1% (3/49) for AMB, 38.8% (19/49) for FLC, 10.2% (5/49) for VRC and 24.5% (12/49) for ITC.

The overall agreement agreement between M 27A3 broth microdilution and M44-A Disk diffusion methods in determining the susceptibility to AMB and FLC was very good (κ=1.00 and 0.898) respectively and was moderate for VRC (κ=0.664).

Sensitivity to AMB and VRC was tested on 28 filamentous fungi isolates according to M38 A2 broth microdilution method. The resistance rate was 35.7% (10/28) for AMB and 17.9% (5/28) for VRC.

In this study the most sensitive antibiotics for Gram negative bacteria isolated from diabetic foot lesion were imipenem and levofloxacin, while the most resistant were ceftrixone, cefipime, ceftazidime, aztronam, tobramycin, and colistin. The most sensitive antibiotics for Gram positive bacteria isolated from diabetic foot lesion were vancomycin, while the most resistant were ampicillin, cefazolin, cefalexin and cefuroxime.

This study found that 75 (41.2%) samples were positive by both culture and PCR, 97 (53.3%) samples were negative by both methods, 2 (1.1%) samples were culture positive and PCR negative, meanwhile 8 (4.4) samples were PCR positive and culture negative. Sensitivity of PCR was 97.4%, specificity was 92.4% and this was statistically highly significant (P<0.001).

DISCUSSION

Patient care for DFIs is complex and needs multi-professional collaboration. In 85% of lower-limb amputations cases in diabetic patients are preceded by mixed infections of foot wounds [20].

Many studies concentrate on role of bacteria in diabetic foot wound and mixed aerobic and anaerobic microbes were reported, but studies on the association between fungal infection and outcome of diabetic foot wound remain unsatisfactory [21].

Fifteen percent of Egyptian populations from age of 10 to 79 years old have diabetes. The rate of foot problems in Egypt is very high and associated with subsequent amputation with percentage reach to 20% of cases of diabetes related hospital admission [22].

One hundred and eighty two samples were collected from 120 diabetic patients with foot infections over period of one and half year, they include 119 tissue specimen from DFU, 34 toe nail specimen and 29 tissue specimen from toe web infection.

In our study; of 103 patients who had DFU, 72 were males and 31 were females. Males were found to have greater risk of developing DFU than females; this result is supported by the results of another study conducted by [23] who found a considerable association between males and risk of DFU. This may be explained by that males are known to have higher foot pressure than females; also peripheral neuropathy that found more often in males than females could share in this difference.

The median age of the patients with DFU was 57 years for males (range 42-66) and 61 years for females (range 42-66). The percentage of diabetic foot problems increased markdely with age. This is supported by [24] who documented that elderly patients had major risk to develop foot ulcer due to peripheral neuropathy, vasculopathy and poor vision, also [25] identified DFU to be associated with patients > 50 years.

In our study there was clear and significant association between the DFIs and the degree of glycemic control, which is agreed with study done by [26] also long duration of diabetes, was significantly associated with DFIs. [27] had reported great association between development of DFU and prolonged duration of diabetes. This may be explained by long duration of diabetes and poor glycemic control increase rate of diabetic foot problems.

In our study, among the 120 diabetic patients, 57.5% were mycologically confirmed to have fungal foot infections (FFIs), 25.0% had fungal infection of DFU, 17.5% had toe web fungal infection and 15.0% had toe nail onychomycosis. Fungal infection of DFU was the commonest type of FFIs among the study population. In a study done by [28] 66% of patients had FFIs and nail infections was the commonest type of FFIs, this difference may be due to high number of patients included in their study and different geographical area.

In our study 36 fungal species were isolated from DFU. The predominant species were *C. albicans* (47.2%) followed by *C. krusei* (13.9).

Our result were near to the finding of [29] who reported *C. albicans* (63.63%) as the commonest fungal species isolated from DFU followed by *C. tropicalis* (18.18%).

In contrast to our study [30] had reported *C. parapsilosis* (45.5%) as the predominant isolate in DFU followed by *C. tropicalis* (22.7%).

Our study also reported that fungal isolates were more common in grade 3 wounds than in grade 2 or grade 4 wounds and there was high statistically significant relationship between grade of ulcer and presence of fungal infection in DFU (P value was

<0.001). Fungal infections were more common in neuroischemic ulcer than neuropathic ulcer; however there was no statistically significant relationship between type of ulcer and presence of fungal infection (P value was 0.161).

In our study among 21 patients who had toe nail lesion 85.7% had toe nail onychomycosis, this result was in line with previous study done by [31]. We isolate about 23 fungal species from 34 toe nail specimens. The predominant species were *A. flavus* (44.4%) followed by *A. niger* (21.7%).

Our result agreed with the finding of [32] who reported *A. flavus* (77.3%) as the most common fungus isolated from toe nail onycomycosis in diabetes patients followed by *A. niger* (2.6%).

In our study there was statistically significant correlation between fungal infection in toe nails and fungal infection of DFU (P value was 0.020). Patients with fungal infection in toe nails had 2.6 times increase in fungal infection of DFU more than patients without fungal infection in toe nails (Odds ratio=2.592).This was agreed with a study done by [33] who reported that fungal infection of toe nail was significantly increased in patients with DFU (P value was <0.05).

In our study the prevalence of fungal toeweb infection was (63.6%) and about 18 fungal species were isolated. The predominant species were *C. albicans* (55.6%) followed by *C. krusei* (16.7%).

In contrast to our study [34] have reported *C. parapsilosis* (11.6%) and *Trichophyton mentagrophytes* (3.1%) as the most frequently isolated fungi from toe web of diabetic patients, also [35] have reported *T. rubrum* (19.3%), *T. mentagrophytes* (14.0%) and *T. tonsurans* (3.5%) as most common fungi isolated from toe web of diabetic patients.

The present study showed that the overall agreement between M27A3 broth microdilution method and M44-A Disk diffusion methods in determining *Candida* species susceptibility to amphotericin B was very good and this was statistically significant (P value was 0.003). These results were not in agreement with a study done by [36] and reported the high false positive results of disk diffusion method in testing *Candida* susceptibility when compared to M 27A3 method.

In this study susceptibility to amphotericin B was 100% in all *A. niger* and *A. fumigatus* isolates, while resistance to amphotericin B was 100% in all *Fusarium spp.*, *Penicillium* spp., *Mucor*, *Cladosporium* spp. and *Basidiobolus ranarum* isolates. Susceptibility to voriconazole was 100% in all *A. flavus* and *A. niger*, *A. fumigates*, *Mucor* and *Basidiobolus ranarum* isolates; however it was 100% resistant in all *Cladosporium* spp. isolates. Our results agreed with the result of [37].

The incidence of bacteria in our study in deep tissues of DFU was 80.6%. About 142 bacterial isolates were isolated. The most commonly isolated bacteria were *Klebsiella* spp. accounting for (33.1%), followed by *Proteus* spp. (17.6%).

Our results were similar to those obtained by [38] they reported *Klebsiella* spp., as the most commonly isolated organisms (24.4 %). In contrast to our study [39] had reported polymicrobial nature in DFU (62.7%) of patients while (37.2%) of patients had monomicrobial infection. *S. aureus* was the most predominant (38.4%) isolate followed by *P. aeruginosa* (17.5%) and *P. mirabilis* (14%).

In our study we observe that mixed bacterial and fungal infection were found in 20.3% of patients; of 31 *C. albicans* strains that we isolated from diabetic foot lesion, 3 were found in patients who had *Pseudomonas* spp. infection in their foot, 15 *C. albicans* isolates were found in presence of *S. aureus*. [7] agree with our results. This may be explained by that *S. aureus* and *C. albicans* appear to be initially synergistic.

In this study, 4.4% of samples were positive by PCR only, positive samples by both culture and PCR were 41.2%, and negative samples by both methods were 53.3%; however 1.1% samples were culture positive and PCR negative.

In our study sensitivity of PCR and culture was 97.4% and 90.4% respectively while specificity of PCR and culture was 92.4% and 98.0% respectively. Our PCR results also were near to that reported by [40] who found sensitivity of PCR was 100% and specificity was 79%.

CONCLUSION

Fungal infections in foot of diabetic patients should not be ignored as they potentially have a role in the pathogenesis of foot ulceration.

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