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Distribution of mycotic and bacterial infections among the patients with diabetic foot ulcers referred to diabetes and metabolic disorder clinic, Tehran, Iran 2011-2012

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ABSTRACT

Patients with diabetes demonstrated to be more prone to developing infections than others. Diabetic foot ulcer is a major health problem in these patients. The purpose of the study was to investigate the incidence of fungal and bacterial pathogens in diabetic foot infections. A total of 57 diabetic patients with non-healing diabetic foot infections who were referred to diabetic clinic during 2011 to 2012 were included for the study. Samples were obtained from the depth of the wound by the use of a sterile swab. Direct fresh smear, fungal and bacterial culture were performed for each patient. Fungal contaminations were confirmed by direct microscopy and/or culture and molecular method. In this study, 9 patients (15.7%) did not show any bacterial and fungal infections, 35.4 % (17/48) had yeast and 80.7% had bacterial infections whereas 31.25% showed mix infection. Candida species were the most predominantly isolated fungi (17patients) including C. albicans (30 %), C. parapsilosis (58 %) and C. glabrata (12 %). E. coli and Pseudomonas aeruginosa were the predominantly gram negative bacilli causing infections in diabetic foot ulcer. Staphylococci were the most frequent organisms encountered and S.aureous was isolated from 10 (20.8%) specimens. Other detected gram-positive bacteria were: Streptococci (4%) and Enterococcus faecalis (2%). The results of the study indicate that Candida Sp is the main yeast infection in patients with diabetic foot ulcer. As a result, mycological evaluation of the non-healing diabetic foot ulcers and suitable antifungal therapy due to biofilm formation and drug resistance should always is necessary.

INTRODUCTION

Diabetes mellitus (DM) is characterized by relative or complete insulin deficiency, a defect in insulin action or both, leading to gross defects in glucose, fat and protein metabolism.1 It is a chronic disease which affects a large part of the human population and also the main endocrine cause of morbidity and mortality all over the world. The incidence is increasing globally and the estimated number of patients with diabetes mellitus in 2030 is 366 million [1].

One of the most important complications of diabetes is diabetic ulcer which frequently results from neuropathy in conjunction with deformity and minor trauma (for example acute injury, ill-fitting shoes, walking barefoot) [2,3].

Diabetic patients are at greater risk for bacterial and fungal infections and also have an increased susceptibility to developing skin and soft tissue infections. In these patients, soft tissue and bone infection of the lower limbs is the most common cause for hospital admission and sometimes precede lower-extremity amputation. [3among dia] The rate of lower extremity amputation among diabetics is

more than 40 times that of non-diabetics [4,5,6,]. Bacterial infection of diabetic foot ulcers are polymicrobial and have aerobic and anaerobic sources that have been investigated in many other researches. [7,8,9,].But there is a little and heterogenous data about the role of mycotic infections and their prevalence in diabetic foot syndrome. The fungi involved in diabetic foot ulcers are mainly Candida spp. Filamentous fungi and yeasts are referred to as etiological agents of diabetic foot infection by some authors [10,11]. The purpose of the present study was to identify frequency of fungal and bacterial infections in ulcerated diabetic foot tissue in diabetic patient's by conventional technique and Polymerase chain reaction (PCR).

MATERIALS AND METHODS

Sample collection: The study was done over a period of 12 months, from December 2011to October 2012. Fifty seven patients with diabetes and a foot ulcer of grade 2 – 4, (based on Wagner wound classification system) who were referred to diabetes clinic (Tehran, Iran) were enrolled in the study. 42 male and 15 female with chronic diabetic foot ulcers whose wounds have not already received any antiseptic, antibiotic or surgical treatment this time were examined for this study.

The patients who had received antifungal agents in the last four weeks were excluded. They were initially presenting with diabetic foot ulcers to the Diabetes and Metabolic disorder clinic, Tehran University of Medical Sciences, Tehran, Iran.

The patient's medical history including age, sex, duration of diabetes, FBS and HbA1c level, currently therapy, presence of vascular insufficiency and/or neuropathy, nephropathy and etc. which were obtained using a questionnaire with their own consent.

Samples were obtained from the depth of the wound (taking aseptic precautions) after debridement .Samples were transported to the microbiology laboratory within an hour in sterile containers.

The necrotic areas of the tissues were mounted on KOH and also inoculated into Sabouraud's Dextrose Agar (SDA) (HiMedia Ltd, Mumbai). Specimens for bacteriological study were cultured in the following agar media: sheep blood, chocolate, and MacConkey agar. The fungal samples were incubated at 37 °C for 1 weak and evaluated daily for growth of fungal microorganism. The bacterial culture plates were incubated aerobically at 37°C (under 5% CO2, chocolate blood agar) and examined at 24 and 48h. For anaerobic cultures, the specimens were inoculated into blood agar .This media was incubated in Gas Pak (BBL) jars at 37°C and examined after 48 and 96 h of incubation. Aerobic bacteria were identified according to standard methods [12] Anaerobic bacteria were identified by techniques described previously [13].

The colonies were identified on the basis of their macroscopic and microscopic (slide culture) features. Yeast samples were cultured in Chrom agar (HiMedia, India) for isolation and identification of *Candida* spp.

Molecular identification of yeast

All isolates of *Candida* species were examined by molecular method of PCR-RFLP, as following steps.

DNA extraction : Genomic DNA from clinical isolates and standard species of *candida* were extracted by glass bead and lysis solution according to previous described method [14]. briefly about 10 mm3 of a fresh colony was transferred to a 1.5 ml eppendorf tube and then 300 µl of lysis buffer containing(100 mM Tris pH 8, 10 mM pH 8, 100 mM NaCl, 1% SDS, Triton 2% X-100), 300 µl of phenol: chloroform (1:1) and 200 µl of glass beads, with a diameter of 1 mm, were added and the tube was vigorously shaken for 60 minutes, the sample was centrifuged for 5 minutes at 5000 rpm. The supernatant was transferred to a clean tube and 400 µl of chloroform was added. After centrifuging as the previous conditions, the aqueous phase was transferred to a clean tube and then 1 volume of cold isopropanol and 5 of 3M sodium acetate (pH: 5.2) were added and was kept at -20 °C for 10 minutes. After that, the sample was washed by 70% ethanol. Then 30 µl distilled water was added and the sample was kept at -20°C.

PCR amplification: PCR was performed to amplify ITS1-5.8SITS2 segment in ribosomal DNA. For this, ITS1-5.8S-ITS2 universal primers were used. The sequences of ITS1 and ITS2 were 5'-TCCGTAGGTGAACCTGCGG-3' and 5'-TCCTCCGCTTATTGATATGC-3' respectively. PCR reaction was performed with the following components: 2.5 µl of 10x PCR buffer, 1.5mM MgCl2, 0.5 µl of 10 mM dNTPs, 0.4µM Primers, 1.25 units of Taq polymerase (Sinagene, Iran), 1 µl of template DNA and molecular grade dH2o up to 25 µl. The reactions were performed in a Thermo cycler (Bio Rad). Thirty amplification cycles were performed in the thermo cycler (Bio Rad) after initial DNA denaturation at 95 °C for 5 min. Each cycle consisted of a denaturation step at 95°C for 30 s, an

annealing step at 55°C for 30 s, and an extension step at 72°C for 1 min, with a final extension at 72°C for 5 min following the last cycle.

Identification of *Candida* species using Restriction Fragment lengths Polymerase Assay (RFLP):
RFLP differential pattern were used in order to distinguish the *Candida* isolates. Restriction enzyme *Msp*I (Fermentas) were used for cutting the amplified DNAs of *Candida* spp .Digestion of amplified ITS fragments produced different size fragments for *Candida* species. For digestion, 10 µl of each PCR product was directly digested with by 5 U (1 µl) of the restriction enzyme *Msp*I, 1.5 µl of the digestion buffer, and dH2o up to 13 µl incubated at 37°C for 180 min. The digested fragments electrophoresed through 1.8% agarose gel and then visualized by ethidium bromide staining [15].

RESULTS

Among the 57 patients with diabetic foot infections from wound units, 42(73.7%) were male and 15(26.31%) female patients, aged between 38 to 74 years. Duration of diabetes was >10 years in 82.4%, 5-10 years in 12.2% and <5 years in 5.3% population.

Fungal and bacterial growth:
Fungal Infections: The most fungal species isolated from diabetic foot ulcers were *Candida* spp. 9 patients (15.7%) did not have any bacterial and fungal infections in their samples. The frequency of yeast infections was 35.4% out of the 48 patients considered which pure fungal infection were found in 2 (4.16%) of patients whereas in 15 (31.25%) patients mix infection (bacterial and fungal infections) were occurred .

Table 1 shows the molecular identification scheme for yeast cultures adopted in this study and indicates the size of PCR product with ITS1-ITS2 primers for different standard species of *Candida* before and after digestion with *MspI*. The most fungal species isolated from diabetic foot ulcers were *Candida* spp (Table 2). Molecular evaluation confirmed *Candida* spp infection in 35.4% of the patients. *C. albicans* (30%), *C. parapsilosis* (58%), *C. glabrata* (12%) were obtained. PCR products were given 500 to 600 bp fragments by using ITS1 and ITS2 primers and revealed in a 1.5% agarose gel electrophoresis after staining in 0.50 mg/ml of ethidium bromide.

PCR-RFLP patterns of various *Candida* spp are shown in Fig.2 and 3**.**

Table 1: Sizes of ITS1-ITS2 PCR product for *Candida* species, before and after digestion by the restriction enzyme *Msp*I.

Candida species	***size of ITS1-ITS2(bp)***	***size of MspI digestion(bp)***
C. albicans	535	297, 338
C. glabrata	871	557, 314
C. tropicalis	524	340, 184
C. krusei	510	261, 249
C. parapsilosis	520	520

Table2. *Candida* species isolated from diabetic foot ulcer samples

Candida species	Frequency %
C.Parapsiolsis	58
C.Albicans	30
C.glabrata	12

Table3: Bacterial *species* isolated from diabetic foot ulcer samples

Bacteria species	Number (Percentage)
E.coli	16 (33.3)
Pseudomonas aeruginosa	5 (10.4)
Proteus	4 (8.3)
Klebsiella Spp	3 (6.25)
S.aureos	10 (20.8)
Acinetobacter	2 (4.1)
Entrobacter	2 (4.1)
Serattia	3 (6.25)
Other gram positive cocci	6 (12.5)
No growth	9 (15.7)
Total	57

Figure1. Bacterial isolates from wound samples of diabetic patients

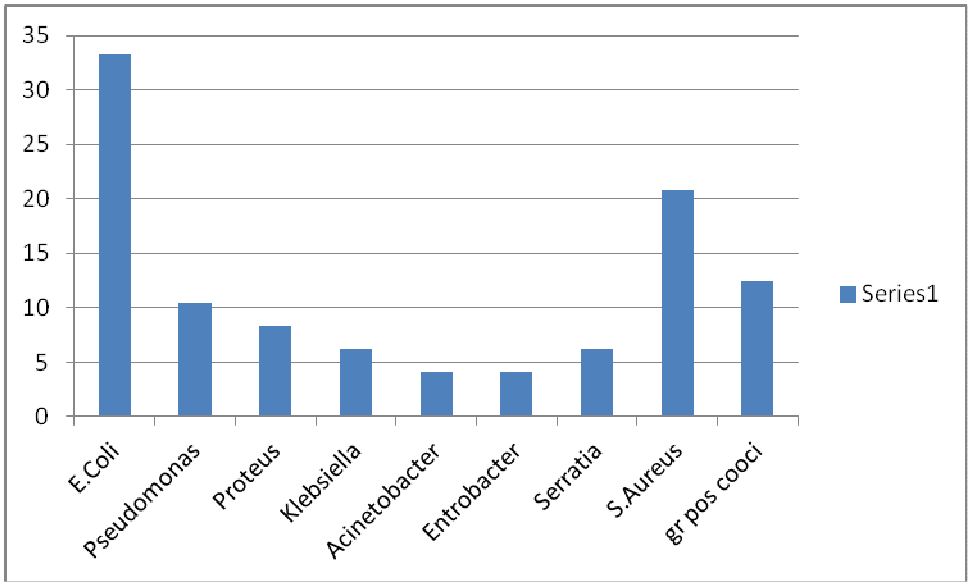


Figure 2.PCR-RFLP patterns of *Candida albicans* after digest with *MSP1*
Line 1-7:*C.albicans* isolated from patient,M:Marker 100bp

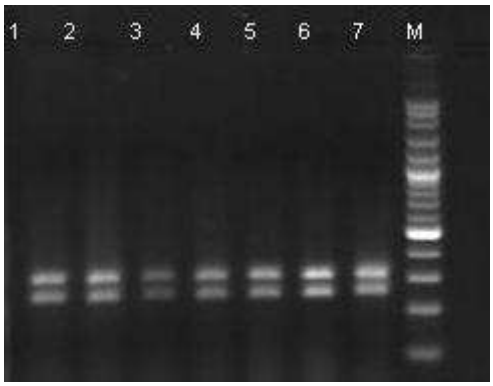
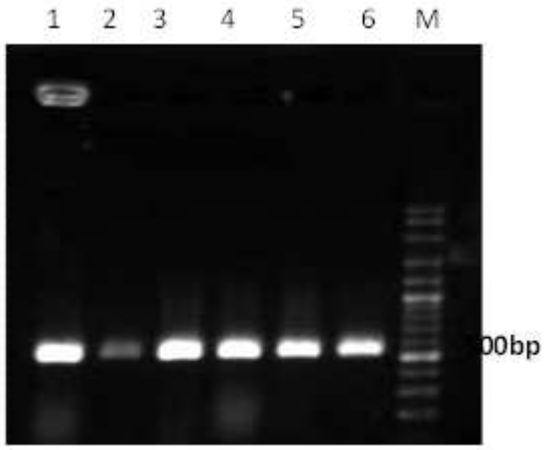


Figure 3.PCR-RFLP patterns of *Candida Parapsiolsia* after digest with *MSP1*
Line 1-6:*C.Parapsiolsis* isolated from patient,M:Marker 100bp



Bacterial Infections: The distribution of bacterial species causing infections in diabetic patients is shown in Table 3 and Fig1.

A wide variety of gram-negative bacteria including 8 different species were predictable as the etiologic agents of infections.

Clearly, *E. coli* was the predominant pathogen and 16(33.3%) samples were identified as *E. coli*. The second one was *Pseudomonas aeruginosa* which was isolated from 10.4% followed by *Klebsiella pneumoniae* (6.25%), and *Proteus mirabilis* (8.3%). *Enterobacter, Acinetobacter, and Serattia* were found in 4.1%, 4.1% and 6.25% of the cases respectively. S*taphylococci Spp.* were the most frequent gram-positive bacteria and *S.aureous* were isolated from 10 (20.8%) of the specimens. Other detected gram-positive bacteria were: *Streptococci* (4%), *Enterococcus faecalis* (2%).

DISCUSSION

Diabetic foot problems is one of most serious complication of diabetes that results in high economic burden so more effort is required to find better way to manage it. Numerous investigations have been approved the bacteriology of diabetic foot ulcers. Most diabetic foot lesions are known to have a polymicrobial etiology . But there are a few reports on the incidence of fungal pathogens in diabetic foot infections which progress following systemic antifungal therapy [16-20].

Mucocutaneous *Candida* infections occur more frequently among patients with diabetes mellitus, especially in those with poorly controlled disease and sometimes Candida infection is the early sign of undiagnosed diabetes [21].

It had been suggested that fungal infections are involved in pathogenesis of diabetic foot ulcers [22]. In consequence, the aims of this study were to describe the incidence of fungal and mixed fungal - bacterial infections in diabetic foot ulcer detected by micobiological and molecular methods and to determine the most criteria for diagnosing fungal ulcer infection such as *candida* spp
Our study is comparable to the other studies. Our result reveal that *Candida* spp are the most frequent isolated fungi, including C. *albicans* (30%) and *C. parapsilosis* (58%) and *C.glabrata*(12%). Similarly, Nair et al [19] reported high prevalence of *Candida spp*. [C albicans (46%) and C. tropicalis (27%)].DF mashad . The presence of other species of Candida (C. guilliermondii, C, krusei, C, tropicalis, C, fornata, C, kefyr and C, glabrata) has been reported in diabetic patients by other investigators [23,24].

According to our finding in the present study in agreement with the other reports, *Candida parapsilosis* was the most frequently isolated fungal species from diabetic foot ulcers

The importance of *Candida Parapsilosis* is obvious due to its different potent virulence factor. The capacities of different *C. parapsilosis* isolates to develop infection in various tissues may be influenced by their ability to form biofilm.

That it confers significant resistance to antifungal therapy by limiting the penetration of substances through the matrix and protecting cells from host immune responses. Biofilm-forming *C. albicans*, *C. parapsilosis* and *C. glabrata* isolates have been associated with significantly higher mortality rates in patients at an Italian university hospital compared to patient isolates incapable of forming biofilm (70.0% versus 45.7%, respectively). Specifically for *C. parapsilosis*, the mortality rate for isolates forming biofilm in vitro was 71.4%, as opposed to 28% for biofilm-deficient isolates[25].

Studies showed that in patients with *Diabetes mellitus*, soft tissue and bone infection of the lower limbs is the most common indication for hospital admission [3]. Among the 150 microbes causing infections in diabetic patients, a total of 40 (26.7%) were identified as *E. coli*. *Pseudomonas aeruginosa* were the second bacterial species causing infections[26]. Diaz-Colodrero *et al*[27] evaluated foot infection in diabetic patients and recognized that gram-positive cocci (predominantly *Staphylococcus aureus* and *Enterococcus*) were detected in 56% patients and 43% of the cases were infected by gram-negative *Bacilli*. Similarly in this study, our result indicated that *Staphylococci* and *Streptococci* account for 33.3% of the foot infections but gram-negative bacteria, in particular *Pseudomonas aeruginosa* and *E.Coli* occur in 43.7% of the cases.

Although some authors have explained the benefit of systemic antifungal therapy, without revascularization producers during the period of antifungal therapy the influence of antifungal therapy on protracted diabetic foot ulcers is still unclear. *Candida* infection should be always tested and aggressively treated, since in our experience it is related to a severe prognosis in with polymicrobial infected diabetic foot ulcers. Because of multidrug-resistance of Candida Spp, susceptibility tests for common used antifungal agents should be performed, whenever available.

It is recommended that our result will make easier for clinician to treat fungal and mixed fungal-bacterial infections in diabetic foot ulcers and fungal infection should always be considered in appropriate treatment of diabetic foot ulcers.

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REFERENCES

[1] Wild S, Roglie G, Green A , *Diabetes Care* ,**2004**, 27, 1047-53.
[2] Mayser P, Hensel J, Thoma W,*Exp Clin Endocrinol Diabetes*, **2004**, 112, 264-8.
[3] Apelqvist J, Bakker K, van Houtum WH, Schaper NC, *Diabetes Metab Res Rev*, **2008**, 24,1-7.
[4] Heald AH, O'Halloran DJ, Richards K, *Diabet Med*, **2001**, 18(7), 567-72.
[5] Bansal E, Garg A, Bhatia S, Attri AK, Chander J, *Indian J Pathol Microbiol*, **2008**, 5 1,204-8.
[6] Nair S, Peter S, Sasidharan A, Sistla S, Unni AK, *J Culture Coll*, **2006**, 5, 85-9.
[7] Hohaus K, Vennewald I, Wollina U , *Mycoses*, **2003**, 46, 337-9.
[8] Gupta AK, Konnikov N, MacDonald P, *Br J Derm*, **1998**, 139, 665-71.
[9] Mlinaric-Missoni E, Kalenic S, VazicBabic V, *Acta Dermatovenerol Croat*, **2005**, 13, 85-92.
[10] Tekeli A, Dolapci I, Emral R, Cesur S, *Mycoses*,**2004**, 47, 315-8.
[11] Eckhard M, Lengler A, Liersch J, Bretzel RG, Mayser P, *Mycoses*, **2007**, 50 , 14-9.
[12] Hawkey PM, Lewis DA , Medical bacteriology, a practical approach. Oxford: IRL Press, **1989**.
[13] Holdeman LU, Cato EP, Moore WEC , *Anaerobe Laboratory Manual*, 4th edition. Virginia Polytechnic Institute and State University, Blacksburg, **1977**.
[14] Yamada Y , Makimura K, Merhendi H, Ueda K, Nishiyama Y , *J Infec Dis*,**2002**, 55,122-5.
[15] Mirhendi H, Makimura K, Khoramizadeh M, Yamaguchi H, *J Med Microbiol*,**2006**,47,225-9.
[16] Bader, M., A. K. Jafri, T. Krueger, V. Kumar, *Scand. J Infect Dis*,**2003,** 35, 895-896.
[17] Bauters TG, Dhont MA, Temmerman MI , *Am J Obstet Gynecol*,**2002**,187,569-74.
[18] Missoni E M, RadeNaderal S J, Chromatogr, *Analyt. Technol. Biomed. Life Sci.*, **2005,**822 , 118-123.
[19] Lai C S, Lin C K,Chou H J, *Plast. Reconstr. Surg*,**1993**, **92** (3), 532-536.
[20] Peter S, Sasidharan A, Sistla S, *J culture collection* , **2006**, *85-89.*
[21] Eke N, gangrene F, *Br J Surg* ,**2000**, 87, 718.
[22] Joshi N, Caputo GM, Weitekamp MR, Karchmer AW,*N Engl J Med*, **1999**, 341, 1906-99.
[23] Heald A H, Ohalloran, K, Richards F, Webb S , Jenkis S, *Diab Med*., **2001**, 18 (7), 567-572.
[24] Fata S, Modaghegh MH, Faizi R, Najafzadeh MJ, Afzalaghaee M, Ghasemi M, Mohammadian M, Naseri A, Meshkat M, Fata A , *Jundishapur J Microbiol*. **2011**, 4(1), 11-6.
[25] Tumbarello M B,Posteraro E M,Trecarichi B, Fiori M, Rossi R, Porta K, *J Clin. Microbiol*,**2007**, 45,1843–1850.
[26] S0liman M, Sheikh EL, Ayman K, *Med. Sci*, **2000**,8 , 41-48.
[27] Diaz Colodrero G, Altclas J, Jasovich A, Mikaelian G, Fiks G, Caro E**,***Enterm Infect Microbiol Clin* **1992**, 10(8), 451-455.