Scientific Foundation SPIROSKI, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2023 Jan 01; 11(A):36-40.
https://doi.org/10.3889/oamjms.2023.9602
eISSN: 1857-9655
Category: *A - Basic Sciences*
Section: *Microbiology*

Clinical Study of Coexistence of Fungal Infections in Diabetic Foot Ulcers by 18s rRNA Gene Polymerase Chain Reaction

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Edited by: Slavica Hristomanova-Mitkovska
Citation: Shalaby RS, Gaafar MM, El Basshar MA, Soliman MS, Yousry MM, Elmahdy YA. Clinical Study of Coexistence of Fungal Infections in Diabetic Foot Ulcers by 18s rRNA Gene Polymerase Chain Reaction. Open Access Maced J Med Sci. 2023 Jan 01; 11(A):36-40. https://doi.org/10.3889/oamjms.2023.9602
Keywords: Invasive fungal infection; Foot infection; Amputation; Diabetic foot ulcer
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Received: 09-Apr-2022
Revised: 25-Apr-2022
Accepted: 09-May-2022

Funding: This research did not receive any financial support
Competing Interests: The authors have declared that no competing interests exist

Abstract

BACKGROUND: Clinicians frequently ignore fungal infections in diabetic foot ulcers and do not regularly explore profound tissue from the injury for fungal culture and sympathy.

AIM: The present study aimed to detect the etiologic substances and the regularity of fungal corruptions in ulcerated diabetic foot tissue samples utilizing two important diagnostic methods, namely, conventional microbiological methods and conventional 18s rRNA gene polymerase chain reaction (PCR) for detection of fungal foot infection.

MATERIALS AND METHODS: One-hundred diabetic patients suffering from diabetic foot infections were enrolled in the present study. Deep tissue specimens from the depth of the wound were collected from the infected sites using aseptic techniques. Laboratory samples were examined, and morphophysiological methods identified pathogens to the species level. Fungi were detected in samples from infected sites using the PCR.

RESULTS: The presence of fungal infection was detected in 17 (17%) of the 100 patients recruited in our study using conventional PCR. Conventional microbiological methods, on the contrary, revealed the presence of fungal infection in 14 patients (14%). *Candida albicans* was the most isolated pathogen (71%).

CONCLUSION: In patients with chronic diabetic foot ulcers that are not responding to long-term antibiotic treatment, fungal pathogens should be considered. Early recognition of fungal corruptions in high-risk persons is serious for avoiding severe outcomes, that is, as foot amputation.

Introduction

Diabetes mellitus is the key communal endocrine illness and has reached pandemic levels. Worldwide, approximately 246 M people suffered from the illness in 2007, with projections for 2025 showing a total of 380 M patients [1]. DM patients are more inclined to skin and soft-tissue corruptions. These infections may appear throughout the progression of the disorder or as the first sign of DM demonstration [2].

Diabetic foot syndrome is a group of symbols and indications, in which neuropathy, ischemia, and infectivity are the primary pathological mechanisms. It is always related with minor trauma, foot irregularity, and incidental vascular disorder [3].

When treating diabetic foot infections, the primary concern has always been bacterial infection of diabetic foot ulcers. Nevertheless, information on the occurrence of fungal separation from diabetic patients is scarce and diverse. However, the presence of fungal infections may rise the possibility of increasing diabetic foot pattern. Furthermore, the early dealing of fungal infections in diabetic foot ulcers may decline the incapacity, morbidity, and death in diabetic patients [4]. This report targeted to measure the etiologic substances and the occurrence of fungal infections in ulcerated diabetic foot tissue samples by two important diagnostic methods; Conventional microbiological methods and conventional 18s rRNA gene polymerase chain reaction (PCR) to detect fungal foot infection.

Materials and Methods

Study population

The present study is a cross-sectional study performed in Cairo University Hospitals (Kasr El-Ainy Medicine School) and the Egyptian National Institute of Diabetes Mellitus from January 2020 to August 2021. A whole of 100 Egyptian patients with diabetic foot ulcers were enrolled in this report. All subjects were exposed to detailed history conquering concerning the duration of diabetes mellitus, previous history of diabetic foot ulcers, other chronic diseases, and prior antifungal therapy. Verbal permission was acquired from the patients for all the processes done.

Sample collection

Sterile normal saline was used to clean the diabetic foot ulcer, and a sample was taken from the depth of the ulcer through a sterile scalpel. Tissue samples were gathered in sterile plastic cups comprising approximately 4–5 mL of normal saline. Plastic cups were sealed, labeled, and taken to microbiology laboratory once the samples were collected. Under sterile conditions, tissue blocks received at the microbiology laboratory were taken out of the sample collection cups. Part of the tissue was investigated using conventional microbiological laboratory methods. The remaining portion was stored in a sterile container at −80°C for use in conventional PCR.

Conventional microbiological laboratory techniques

A portion of the tissue specimen was examined with 10% KOH. The tissue specimen was cultured on Sabouraud Chloramphenicol Agar (*HiMedia*), incubated at 37°C, and investigated twice a week, up to 4 weeks, before being declared negative.

The colonies' direct film was used to identify the isolated pathogen after growth was observed on culture media. Any growth of *Candida* species was cultivated on Chrom agar *(Hi media)* to diagnose subspecies [5]. Concomitant with fungal pathogens separation, any bacterial growth was investigated by culturing tissue specimens on blood and McConkey agar (*Oxoid*). They were investigated every day for 48 h. Any positive growth is investigated using appropriate selective cultures and conventional biochemical reactions to identify species of isolated pathogen.

Measuring of fungal DNA in samples by Pan fungal PCR18

DNA extraction was performed by QIAamp DNA Mini and QIAamp DNA Blood Mini Kit extraction kit and Lysozyme mix solution (Bio Basic). Conventional PCR was performed on the extracted DNA from all tissue samples. For amplification, two oligonucleotide Pan-fungal primers were used [6]. The ITS region primers use conserved regions of the 18S (ITS 1) with the following sequence (5' TCCGTAGGTGAACCTGCGG) and the 28S (ITS 4) with the sequence (5'GCTGCGTTCTTCATCGATGC) rRNA genes to amplify the intervening 5.8S gene. The computerized thermocycler was programmed for the following conditions: Thermal cycling was performed at 95°C for 5 min denaturation, monitored by 50°C for 30-s Annealing, and then 72°C for 4 min Elongation. *Positive control* of *Candida* spp. (confirmed by *VITEK 2 compact system*) was used to detect reagent efficiency with each run – detection of PCR Amplification Products Using Gel Electrophoresis and Ultra-Violet Light Trans-illumination.

Statistical methods

Data were coded and inserted by the Statistical Package for the Social Sciences version 26 (IBM Corp., Armonk, NY, USA). Data were shortened by mean and standard deviation for quantitative variables and frequencies (number of cases) and relative frequencies (percentages) for categorical variables. Comparisons among groups were done by the unpaired t-test [7]. For associating categorical data, Chi-square test was done. The exact test was utilized instead when the predictable frequency was <5 [7]. $p < 0.05$ were measured statistically significant. Kappa measure of agreement was utilized to analysis the conformity among the categorical variables.

Results

A whole of 100 patients were enrolled: 61 males (61%) and 39 females (39%). The incidence of fungal infection in diabetic foot ulcers using both conventional microbiological methods and conventional PCR was higher in males than females, yet, it was not statistically significant. The age of the patients fluctuated from 40 to 70 years, with a mean of 56.8. The duration of diabetes fluctuated from 5 to 20 years, with a mean of 13.7. Diabetes mellitus was present in 62% of our study population for more than 10 years. A significant correlation was detected between the occurrence of fungal poison in diabetic foot ulcers in relation to age and the duration of diabetes in the patients, with older age and long duration, specifically >10 years, being more prone to develop fungal infectivity in diabetic foot ulcers. This correlation was statistically significant with $p < 0.001$. Our findings assessed the occurrence of fungal infectivity in diabetic foot ulcers. Using conventional PCR, we found that 17 of the 100 patients (17%) had a fungal infection, whereas using conventional microbiological methods, 14 patients (14%) were found to have a fungal infection. A significant agreement was found between conventional microbiological methods and conventional PCR. Kappa measure of agreement was utilized to examine agreement among the categorical variables. Both diagnostic methods showed consistent results with a Kappa value of 0.886, which was statistically significant with $p < 0.001$.

The conventional method revealed 14 cases of fungal infection; they were all PCR positive as well. From the 86 cases that were negative by conventional methods, 83 cases (96.5%) were negative by PCR, and three cases (3.5%) were positive by PCR, as depicted in Table 1.

Candida species was the only fungal pathogen isolated using traditional microbiological methods. *Candida albicans* was the key commonly separated

Table 1: The association between using polymerase chain reaction to detect fungal infections and using conventional microbiological methods

Conventional PCR	Conventional microbiological methods		
	Positive (n = 14), n (%)	Negative (n = 86), n (%)	Kappa value
Positive	14 (100.0)	3 (3.5)	0.0886
Negative	0	83 (96.5)	

PCR: Polymerase chain reaction.

species (71.4%), tailed by *Candida tropicalis* (21.4%), and finally *Candida glabrata* (7.1%). Diabetic foot ulcer is known for its polymicrobial nature. We investigated the association between different isolated bacterial pathogens and the presence of fungal infection in the same patient. This association was to identify the most prevalent bacterial pathogen associated with fungal infectivity in diabetic foot ulcers. The prevalence of bacteria in our samples was 80% (80/100). A total of 126 bacterial isolates were isolated from 100 patients with infected DFU, yielding a 1.26 (126/100) isolation rate. The most isolated bacteria were *Proteus* species. (37.3%) followed by *Pseuodomonas* spp. (30.16%), as depicted in Table 2,

Table 2: Incidence of different bacterial pathogens isolated from diabetic foot infections

Isolated pathogen	Number of isolates, n (%)
Proteus species	47 (37.30)
Pseudomonas	38 (30.16)
Escherichia coli	15 (11.90)
Staphylococcus aureus	7 (5.56)
Citrobacter	4 (3.17)
Provedencia	1 (0.79)
Mixed Gram-negative	14 (11.11)
Total number of bacterial isolates	126 (100)

whereas the most common bacterial pathogen isolated with fungal infection was *Proteus* species (47%). There was a *statistically significant* association between the attendance of *Proteus* species and the presence of fungal infection, as shown in Table 3. A high incidence of fungal infection was also found with *Pseudomonas* spp., yet this association was not statistically significant.

Table 3: Correlation between isolated bacterial pathogens and presence of fungal infection

Isolated pathogen	Number of isolates, n (%)	*Candida* spp.
Staphylococcus aureus	7 (5.56)	3
Proteus	47 (37.30)	12
Pseudomonas	38 (30.16)	9
Escherichia coli	15 (11.90)	4
Providencia	1 (0.79)	0
Citrobacter	4 (3.17)	0
Mixed Gram-negative	14 (11.11)	1

Discussion

Diabetes mellitus is a chronic metabolic disease caused by relative or complete insulin deficiency, leading to significant glucose, fat, and protein metabolism defects. The high regularity of infections in diabetic patients is instigated by a hyperglycemic environment that causes impairment in both cell-mediated immunity and humoral immunity thus causing an increase in the risk of bacterial and fungal infection [8]. According to the findings of recent studies, fungal infectivity, mainly Candida fungus, is communal in most diabetic patients. If fungal infections in diabetic foot ulcers are not treated promptly, they can direct to fatal complications such as amputation of the foot.

In the present study, we targeted to focus on fungal infection in diabetic foot ulcers by adopting two crucial diagnostic methods, "Conventional microbiological methods and Conventional 18s rRNA gene PCR," to detect fungal foot infection.

A total of 100 patients were enrolled: 61 males (61%) and 39 females (39%). The incidence of fungal infection in diabetic foot ulcers was upper in males than females, yet, it was not statistically significant ($p > 0.05$). Most studies reported that males are at higher risk of developing diabetic foot ulcers. This may be explained, by the higher incidence of diabetes mellitus in males than females. In addition, males are at a higher risk of experiencing foot trauma than females [9].

A study by Sujatha *et al*. also indicated that fungal infection in DFU was higher among males than females; however, the association was also insignificant ($p > 0.05$) [10]. Another study by Raza and Anurshetru, 2017 and Fata *et al*., conducted on 100 and 120, repetitively, cases of diabetic foot ulcers demonstrated a high incidence of infection in males (71%) and (71.6%) than females (29%) and (28.4%) with significant $p < 0.05$ [4], [11].

The mean age for the patients in our report was 56 years old, with a least age of 40 years old and supreme age of 70 years old. In our study, there was a statistically significant relationship ($p < 0.05$) between an elevation in the incidence of fungal infection in DFU and an increase in age. Rasoulpoor *et al*. reached a similar finding in their systematic review analysis, stating that the rate of fungal infection in DFU was higher in elderly individuals [12]. Another study published in 2017 by Sugandhi and Prasanth found that patients in the 51–60 years old age group were more susceptible to fungal infection in DFU due to their physical health conditions [13]. Diabetes duration also acts a vital function in the creation of diabetic foot ulcer and the increase in the occurrence of fungal infection. In our study, we found out that the longer the duration of diabetes, the greater the probabilities of developing a fungal infection in DFU. This association was statistically significant ($p < 0.05$), 62% of our study population had diabetes mellitus for more than 10 years. Our positive cases (76%) had DM for more than 15 years. Ozturk *et al*., also illustrated that fungal infection in diabetic foot ulcers is strongly associated with the increase in duration of diabetes, as the highest incidence of fungal infection in their study was associated with mean diabetes mellitus duration of (12–15 years) [14]. According to Abilash *et al*., who studied fungal infection in DFU, the longer the duration of DM specifically >20 years, the higher the incidence of fungal infection [15].

We assessed the occurrence of fungal infection in diabetic foot ulcers; out of the 100 patients who were investigated, 17 patients (17%) were found to have fungal infections using conventional PCR. In contrast, 14 patients (14%) showed the presence of fungal infection using conventional microbiological methods.

Candida species was the only isolated fungus using conventional microbiological methods, with *C. albicans* (71.4%) as the key common separated species, tailed by *C. tropicalis* (21.4%) and *C. glabrata* (7.1%). Our results are consistent with the study of Kalshetti *et al*., 2017 on 80 patients. About 17.5% of 80 patients had a positive fungal culture. *C. albicans* (42.85%) was the most isolated yeast from positive fungal cases, followed by *C. tropicalis (*21.42%) and *C. glabrata* (14.28%) [8]. In another study by Raiesi *et al*., on 122 diabetic patients, 19.1% of patients had a fungal infection in diabetic foot ulcers. *C. albicans* was the commonest isolated species [16].

In addition, a recent systematic review and meta-analysis study by Rasoulpoor *et al.* was conducted on 13 eligible studies on the occurrence of Candida infection in diabetic patients. The study data included 1384 samples from patients ranging in age from 18 to 87 years. According to the study, the prevalence of *C. albicans* skin infection in patients with type 2 diabetes was (11.4%) [12].

Diabetic foot ulcers were poly-microbial in most of our study cases. The most frequent isolated organism was *Proteus* spp. (37%) and *Pseudomonas* spp. (30%), followed by *Escherichia coli* (11.9%) and *Staphylococcus aureus* (5.5%). Comparable results were reported in a study by Kareliya *et al*.; the most prevalent isolated pathogens were *Pseudomonas aeruginosa*, *S. aureus* (MSSA)*, Klebsiella*, and *Proteus* species [17]. Another study by El Nagar *et al*., conducted on deep tissue samples from diabetic foot ulcers showed that most of the samples showed a high prevalence of bacterial infection (83/103). The key regularly separated bacteria were *Klebsiella* spp. (33.1%) and tailed by *Proteus* spp. (17.6%) [18]. Furthermore, a report by Sanniyasi *et al*., on (105) patients with diabetic foot ulcers demonstrated that the most common isolated Gram-negative bacteria was *Pseudomonas spp.,* while the most common Gram-positive isolated bacteria were *S. aureus* [19]. In our study, we correlated the association between different isolated bacterial pathogens and the incidence of fungal infection in diabetic foot ulcers to detect the most prevalent bacterial pathogen associated with fungal infection in diabetic foot ulcers. The most common bacterial organism that grew in association with fungal infection was *Proteus* spp. The correlation among them was statistically significant ($p < 0.05$). A high incidence of fungal infection was also associated with *Pseudomonas* spp., but it was *not* statistically significant. A report by Sanniyasi *et al*., illustrated that the key communal bacterial organism isolated with fungal infection was *Pseudomonas* [19].

Conclusion

Diabetic foot ulcers infection is particularly challenging to any microbiology or molecular laboratory, as it is usually polymicrobial and requires multiple investigations and appropriate knowledge to reach the pathogenic organism. The mycological evaluation must be considered and required in patients with long-standing, resistant diabetic foot ulcer infections. We do believe that our findings have vital consequences for preventing and recognizing mycotic foot disease in diabetic feet and suggest that diabetic foot patients be observed for fungal infections.

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