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Spectrum and Prevalence of Fungi Infecting Deep Tissues of Lower-Limb Wounds in Patients with Type 2 Diabetes▽

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The prevalence rate and spectrum of fungi infecting deep tissues of diabetic lower-limb wounds (DLWs) have not been previously studied. Five hundred eighteen (382 male and 136 female) consecutive patients with type 2 diabetes hospitalized due to infected lower-limb wounds were enlisted in this study. Deep tissue (approximately 0.5- × 0.5-cm size) taken perioperatively from the wound bed was cultured for fungi. Fungi was found in 27.2% (141/518) of the study population. *Candida parapsilosis* (25.5%), *Candida tropicalis* (22.7%), *Trichosporon asahii* (12.8%), *Candida albicans* (10.6%), and *Aspergillus* species (5.0%) were the most predominant fungal isolates. Of the fungal isolates, 17.7% were resistant to itraconazole, 6.9% were resistant to amphotericin B, 6.9% were resistant to voriconazole, 3.9% were resistant to fluconazole, and 1.5% were resistant to flucytosine. Of the population, 79.7% (413/518) had bacterial infection in deep tissue. The predominant isolates were *Enterococcus faecalis* (14.1%), *Staphylococcus aureus* (12.2%), and *Pseudomonas aeruginosa* (10.8%). Mixed fungal and bacterial infections were seen in 21.4% of patients, while 5.8% had only fungal infection and 58.3% had only bacterial infections. Another 14.5% had neither bacteria nor fungi in the deep tissue. Patients with higher glycosylated hemoglobin levels had significantly more fungal infections. Our study reveals that deep-seated fungal infections are high in DLWs. In the context of delayed wound healing and amputation rates due to DLWs, it is important to study the pathogenicity of fungi in deep tissues of DLWs and their possible contribution to delayed wound healing. The role of antifungal agents in wound management needs to be evaluated further.

Diabetes is now a worldwide epidemic. Among the 191 WHO member states, India has the highest number of people with diabetes (37). Fifteen percent of patients with diabetes develop lower-extremity ulcers during their lifetimes. Diabetes is the most common cause for nontraumatic amputation of lower extremities (1, 39). Eighty-five percent of these lower-limb amputations are preceded by polymicrobial infections of the wound (23, 26, 36). Despite proper surgical and antibacterial therapy for infected diabetic lower-limb wounds (DLWs), the global long-term outcome of patients was found to be poor; only <50% of these patients had global therapeutic success (16, 22).

Fungal infections among immunocompromised patients are one of the major health concerns worldwide (5, 13, 19), but the spectrum of fungi infecting DLWs and their pathogenicity have not yet been studied thoroughly. Therefore, clinicians and surgeons treating diabetic foot wounds suspect only bacterial infections and treat them with antibacterial agents. They do not routinely send deep tissue from the wound bed for fungal culture and sensitivity, either due to lack of literature support or due to the assumption that there would not be any fungal infections in the DLWs. Surprisingly, our retrospective pilot study showed 27.9% positive fungal cultures in 318 diabetic patients with DLWs. We speculate that opportunistic fungi may invade deep into the wounds and contribute to delayed wound healing in some of the diabetic patients who are otherwise immunocompromised compared to nondiabetics.

The magnitude of fungal infections in diabetic lower extremities in India has been previously studied in a limited number of patients. Hence, we undertook this study to estimate the prevalence of fungi in DLWs and also to describe the spectrum of these fungal infections.

MATERIALS AND METHODS

Sample size estimation. As there were no available studies on prevalence of fungal infection in deep tissue of DLWs, we used our pilot study results for estimating sample size for the fungal prevalence study. Considering 95% confidence interval and 15% allowable error, the sample size (n) was calculated as 500.

Study population. All patients with type 2 diabetes (irrespective of age and sex) who were hospitalized for surgical management of lower-extremity wounds from January 2008 onward were considered for the study. Their informed consent was obtained, and demographic details, duration of lower-limb lesion, duration of diabetes, and wound assessment based on the University of Texas System of Wound Classification were documented. Glycosylated hemoglobin level (HbA1c) was measured by the high-pressure liquid chromatography (HPLC) method, and ankle brachial index (ABI), vibration perception threshold (VPT), and transcutaneous oxygen tension ($TcPO_2$) were also measured (40).

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The patients with a history of malignancy, chemotherapy, or radiotherapy or who were on steroids or antifungal drugs (local or systemic) were excluded from the study. A deep tissue specimen was obtained from the wounds during surgery and sent for fungal and bacterial cultures.

Specimen collection. The slough and necrotic tissue over the wound were surgically debrided in the operating theater. After a thorough wash of the wound with normal saline, a deep tissue specimen of approximately 0.5- × 0.5-cm size was taken from the wound bed. The specimen was collected in a sterile container, and the tissue was soaked with normal saline. This was transported to our microbiology lab within 10 to 15 min for further processing.

Fungal culture and sensitivity. The specimen was processed in a type IIB biological safety cabinet. The tissue was sliced into tiny fragments (about 1-mm cubes) with a sterile scalpel blade. These fragments were placed directly into two slants of Sabouraud dextrose agar with chloramphenicol and submerged slightly beneath the surface by using an inoculating needle. These slants were incubated at 30°C and 35°C and observed for 4 weeks. KOH (10%) and Gram stain examinations were performed, and the results were documented. Fungal species were identified morphologically (11, 32) and using ID32C strips (miniAPI; bioMerieux) (4, 9, 25, 30). In addition, cornmeal agar morphological study and germ tube and urease tests were also performed for identification of the yeast species. *Aspergillus* species and other filamentous fungi were identified by slide culture on potato dextrose agar (PDA) with lactophenol cotton blue staining.

Antifungal susceptibility testing for yeast was done with ATB Fungus-3 strips (miniAPI; bioMerieux) (35). MICs of <0.125 μg/ml for itraconazole (ITR), <1.0 μg/ml for voriconazole (VOR), <8.0 μg/ml for fluconazole (FLZ), and <4.0 μg/ml for flucytosine (FLCYT) were considered sensitive. For amphotericin B (AMPB), MIC values were not detected by the machine. The standard strains *Candida albicans* ATCC 90028, *Candida tropicalis* ATCC 750, *Candida parapsilosis* ATCC 22019, and *Candida krusei* ATCC 6258 were used as controls in the study. The susceptibility of filamentous fungi was not determined.

Bacterial culture. Part of the sterile deep tissue specimen was crushed or ground with a sterile mortar and pestle in the biosafety cabinet. Gram staining was done, and the crushed specimen was inoculated in thioglycolate medium. The sample was streaked on sheep blood agar (SBA) and MacConkey agar (MA). The SBA was kept in a 5% CO_2 incubator, and MA and thioglycolate were kept in an O_2 atmosphere incubator at 37°C. Bacterial isolates were identified by standard biochemical tests, and susceptibility testing was performed per CLSI guidelines.

Analysis. Data were recorded in SPSS software (version 11). The percentages of fungal and bacterial isolates were computed by applying descriptive statistics. To test the coexistence of fungi and bacteria and the statistical association of fungal infection with wound depth, the chi-square test was applied. Student's *t* test was applied to test the statistical significance of the difference in mean values of parameters such as age, duration of foot lesion, duration of diabetes, HbA1c, ABI, VPT, and $TcPO_2$ between the two groups (presence and absence of fungi).

RESULTS

Of 518 patients, 382 (73.7%) were males and 136 (26.3%) were females. The mean age of the study population was 60.8 ± 10.2 years, duration of diabetes was 193.4 ± 97.3 months, duration of lower-limb lesion was 43.7 ± 72.8 days, HbA1c was 9.8% ± 2.4%, ABI was 1.02 ± 0.51, VPT was 43.7 ± 9.6 V, and $TcPO_2$ was 33.1 ± 16.4 mm Hg.

The prevalence of fungi in deep tissue of diabetic lower-limb wounds was 27.2% (141/518 patients). Among the isolates, 76.6% (108/141) were *Candida* species, 12.8% (18/141) were *Trichosporon* species, 8.5% (12/141) were filamentous fungi, and 2.1% (3/141) were other yeasts (Table 1). The predominant species were *Candida parapsilosis* (25.5%), *Candida tropicalis* (22.7%), *Trichosporon asahii* (12.8%), *Candida albicans* (10.6%), and *Aspergillus* species (5.0%).

Sensitivity to fluconazole (FLZ), itraconazole (ITR), voriconazole (VOR), flucytosine (FLCYT), and amphotericin B (AMPB) was tested for 130 yeast isolates. The resistance rate of the fungal isolates was 1.5% (2/130) for FLCYT, 3.9% (5/130) for FLZ, 6.9% (9/130) for AMPB, 6.9% (9/130) for VOR, and 17.7% (23/130) for ITR. Of the 32 *Candida tropicalis* and 15 *Candida albicans* isolates, 3 of the former and 2 of the latter were resistant to FLZ. One each of the 32 *Candida tropicalis* and 18 *Trichosporon asahii* isolates were resistant to FLCYT. Similarly, resistance to AMPB was seen in 3 isolates of *Candida parapsilosis*, 2 isolates of *Zygosaccharomyces* species, and one each of *Candida tropicalis*, *Trichosporon asahii*, *Candida guilliermondii*, and *Candida lusitaniae*. VOR resistance was seen in *Candida tropicalis* (3/32), *Trichosporon asahii* (1/18), *Candida albicans* (2/15), *Candida glabrata* (2/4), and *Zygosaccharomyces* species (1/3). *Candida tropicalis* showed a high incidence of resistance to ITR (9/32).

TABLE 1. Spectrum of fungi isolated from deep tissue of diabetic foot wounds

Sample no.	Species	Frequency (no. of isolates)	Percentage
1	*Candida parapsilosis*	36	25.5
2	*C. tropicalis*	32	22.7
3	*T. asahii*	18	12.8
4	*C. albicans*	15	10.6
5	*Aspergillus* sp.	7	5.0
6	*C. guilliermondii*	4	2.8
7	Non-*albicans Candida* sp.	4	2.8
8	*C. glabrata*	4	2.8
9	*Fusarium* sp.	4	2.8
10	*Candida sake*	4	2.8
11	*Zygosaccharomyces* sp.	3	2.1
12	*Kodamaea ohmeri*	3	2.1
13	*Candida globosa*	2	1.4
14	*C. krusei*	1	0.7
15	*Penicillium* sp.	1	0.7
16	*C. lusitaniae*	1	0.7
17	*Candida famata*	1	0.7
18	*Candida melibiosica*	1	0.7
Total		141	100.0

An analysis was performed to look for patients who had purely bacterial or fungal infections, mixed bacterial and fungal infections, or neither bacteria nor fungi in DLWs. It was found that 5.8% (30/518) of these patients had only fungal infections, while 58.3% (302/518) of patients had only bacterial infections, whereas 14.5% (75/518) of patients had neither fungal nor bacterial infections and 21.4% (111/518) had both bacteria and fungi in their deep tissue (Fig. 1).

The prevalence of bacteria in deep tissues of DLWs was 79.7% (413/518). About 607 bacterial isolates were cultured from 413 patients; hence, the isolation rate was 1.5 (607/413). Among these, 55.3% were Gram negative and 44.7% were Gram positive. The predominant bacteria cultured were *Enterococcus faecalis* (14.1%), *Staphylococcus aureus* (12.2%), *Pseudomonas aeruginosa* (10.8%), *Klebsiella pneumoniae* (7.9%), *Escherichia coli* (7.7%), and coagulase-negative staphylococci (5.8%) (Table 2).

Among patients who had bacterial infections, 47.5% (246/518) had only single bacterial colonization in the deep tissue, while 27.0% (140/518) patient had two different types of bacteria coexisting in the wound and 5.2% (27/518) had three types of bacteria in the deep tissue (Table 3). About 20.3% (105/518) of the total patients had no bacterial growth in the deep tissue. The presence of coexistent fungal infection was the same in patients who had one, two, or three bacterial

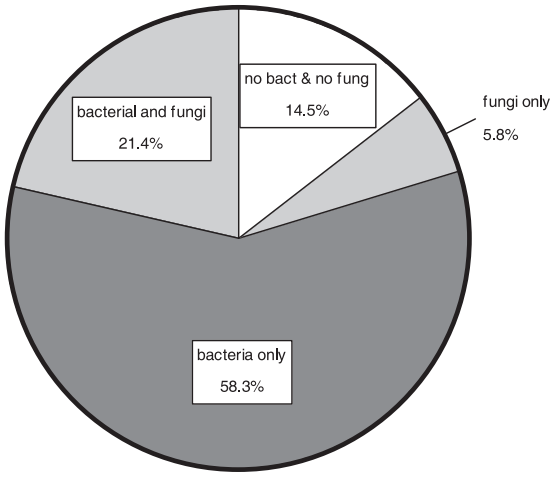

FIG. 1. Microbial flora in deep tissue of diabetic lower-limb wounds.

species isolated ($P = 0.12$). Among the bacterial isolates, the predominant isolates were Gram negative (70.46%). On analysis, we found no significant correlation between fungal infection and Gram-positive or Gram-negative bacteria ($P = 0.81$) isolated from the deep tissue.

On analyzing the depth of DLWs of the study population, we found that 40.3% (209/518) of patients had a wound extending up to muscles, 45.4% (235/518) of patients had wounds extending up to the tendon or capsule, and 14.3% of patients had wounds extending up to the adjacent joint or bone (Table 4). Though fungal isolates were more common in grade 2 wounds than in grade 1 or grade 3 wounds, the difference was not statistically significant ($P = 0.59$).

Fungal infection was significantly associated with the glycosylated hemoglobin level ($P = 0.04$) of the patients but not with the age, sex, duration of diabetes, duration of foot lesion, ABI, VPT, or $TcPO_2$ (Table 5).

TABLE 2. Spectrum of bacteria isolated from deep tissue of diabetic lower-extremity wounds ($n = 518$)

Sample no.	Species[a]	Frequency (no. of isolates)	Percentage
1	*Enterococcus faecalis*	73	14.1
2	*Staphylococcus aureus*	63	12.2
3	*Pseudomonas aeruginosa*	60	10.8
4	*Klebsiella pneumoniae*	41	7.9
5	*E. coli*	40	7.7
6	Coagulase-negative *Staphylococcus* sp.	30	5.8
7	Nonfermenting Gram-negative bacilli	24	4.7
8	*Enterobacter* sp.	16	3.1
9	Beta-hemolytic *Streptococcus*	15	2.9
10	*Proteus mirabilis*	10	1.9
11	*Proteus vulgaris*	10	1.9
12	*Streptococcus* sp.	8	1.5
13	*Citrobacter freundii*	7	1.4
16	MDR *Pseudomonas aeruginosa*	4	0.8
17	MRSA	4	0.8
18	Diphtheroid sp.	3	0.6
19	*Citrobacter diversus*	2	0.4
20	*Serratia* sp.	2	0.4
21	Gram-positive bacilli	2	0.4
22	MDR *E. coli*	2	0.4
23	*Morganella morganii*	1	0.2
Total		413	79.7

[a] Abbreviations: MDR, multidrug resistant; MRSA, methicillin-resistant *S. aureus*.

TABLE 3. Coexistence of fungal and bacterial infections in deep tissues of diabetic lower-limb wounds[a]

Samples with no. of bacterial strains isolated from deep tissue	Positive fungal culture	
	No.	Percentage
One isolate ($n = 246$)	57	23.2
Two isolates ($n = 140$)	46	32.9
Three isolates ($n = 27$)	8	29.6
Total ($n = 413$)	111	26.9

[a] $P = 0.12$.

DISCUSSION

The incidence of diabetes is increasing rapidly. Diabetic foot ulcers affect millions of people worldwide and impose tremendous medical, psychosocial, and financial losses or burdens. Patient care for diabetic foot ulceration is complex and necessitates multiprofessional collaboration to provide comprehensive wound care. Eighty-five percent of the lower-limb amputations in diabetics are preceded by polymicrobial infections of the wounds. Many studies have been done on the prevalence and spectrum of bacterial infections, the role of systemic/local antibiotics, and their effect on wound healing. However, the magnitude of fungal infections in diabetic foot wounds is an area which has received very little attention. Studies have shown that toe web dermatophyte infection provides a hospitable niche for subsequent colonization by bacteria. Exacerbation of a mild dermatophyte infection (dermatophytosis simplex) can arise in the occlusive environment of the toe web space. Fungal infection induces damage to the stratum corneum, which allows overgrowth of resident bacteria and maceration, itching, and often malodor at the site (15, 20).

Mlinaric Missoni et al. from Croatia had reported the fungal incidence in tissue biopsy specimens of 22 diabetic patients who had clinical evidence of fungal infections (12, 24). The predominant isolates were *C. parapsilosis* (45.5%), *C. tropicalis* (22.7%), *C. albicans* (9.1%), and *C. glabrata* (9.1%). Bansal et al. from India had reported 9% isolation of fungi from superficial swabs taken from 103 patients with diabetic foot wounds

TABLE 4. Fungal infections and depth of diabetic foot lesions[a]

Wound grade[b]	Presence of fungi	
	No.	Percentage
II (muscle only) ($n = 209$)	54	25.8
III (tendon and capsule) ($n = 235$)	69	29.4
IV (joint and bone) ($n = 74$)	18	24.3
Total ($n = 518$)	141	27.2

[a] $P = 0.59$.
[b] University of Texas wound classification.

TABLE 5. Association of deep tissue fungal infection with age, duration of diabetes, glycemic status, duration of lesion, and neurovascular status of the lower limb of the patients with diabetic lower-limb wounds

Sample type and value	Age (yr)	Wound duration (days)	HbA1c (%)	ABI (ratio)	VPT (V)	$TcPO_2$ (mm Hg)	Diabetes duration (mo)
Without fungi							
n	377	364	152	273	279	224	274
Mean	60.6	41.2	9.5	1.0	43.6	33.1	190.0
SD	10.0	73.0	2.4	0.57	9.3	16.1	95.7
With fungi							
n	141	136	58	102	107	86	101
Mean	61.2	50.2	10.3	0.9	43.9	32.8	201.9
SD	10.8	71.7	2.2	0.3	10.1	17.0	101.5
P value[a]	0.57	0.22	0.04	0.35	0.80	0.86	0.30
Total							
n	518	500	210	375	386	310	375
Mean	60.7	43.6	9.7	1.0	43.7	33.0	193.2
SD	10.2	72.7	2.3	0.5	9.5	16.3	97.3

[a] *P* values are for comparisons of samples with fungi and samples without fungi.

(2). The predominant species were *C. tropicalis* (29%), *C. albicans* (14%), and *C. guilliermondii* (7%), followed by *Aspergillus flavus* (21%), *Aspergillus niger* (14%), and *Fusarium* species (14%). The same spectrum of fungi was isolated from immunocompromised patients' blood by Pfaller et al. and Bedini et al. (3, 28). Though these studies confirm pathogenic fungal infections in DLWs, the spectrum of fungi and their prevalence in deep tissue of the wounds have not been explored properly.

Our study shows high prevalence and a wide spectrum of fungi (18 different species) in deep tissues of DLWs compared to the previous studies. Among these, 89.4% are yeasts and 10.6% are filamentous fungi. The isolates obtained by us from deep tissue of DLWs were similar to the spectrum of species isolated from bloodstream samples by Gonzalez et al. (11). *C. parapsilosis* emerged as the most common fungal isolate in our study. Studies have reported that *C. parapsilosis* has dramatically increased in significance and prevalence over the past 2 decades and is known to be one of the leading causes of invasive candidal disease (34). *Aspergillus* infections are to be considered the differential diagnosis of slowly progressive destructive wound infections (13). *Aspergillus* species have been found as the most common filamentous fungi isolated from DLWs of our patients.

Sensitivity to FLZ, ITR, VOR, FLCYT, and AMPB was tested for 130 yeast isolates. *In vitro* susceptibility was highest to FLCYT and lowest to ITR for these isolates. We observed intra- and interspecies variations in susceptibility to antifungal agents. Similar results were reported by Lass-Florl et al. (18). In their study, AMPB and posaconazole were found to be active against most of the pathogens, including species that cause rare and difficult-to-treat infections. In our study, about 6.9% of the yeasts showed resistance to AMPB. *C. parapsilosis* and *Zygosaccharomyces* species were the most common species showing resistance to AMPB. We did not ask patients about their past exposure to AMPB. This might have provided an insight into the cause of resistance. Resistance to FLZ was found to be low (3.9%) in our study, which is consistent with the results from the study by Tan et al. (33). In their study, resistance was observed in 3.2% of all *Candida* infections in the bloodstream (33). In the same study, about 37.5% of the fungal isolates were resistant to ITR, whereas the rate was only 17.7% in our study. In our study, resistance of *Candida tropicalis* to FLZ was 6.25%, whereas it was 2.7% in the study by Tan et al. (33). Though there have been reports on itraconazole-resistant and fluconazole-susceptible isolates of *Candida* species, these remain unconfirmed (38). Further studies are required in this regard. With the past series of studies, it is observed that the susceptibility of *Candida* species to antifungal agents varies over time and among countries and regions (27). Case-to-case treatment with culture-specific antifungal therapy would provide greater benefit than presumptive therapy.

Chronic wounds have a complex microbiological environment with a mixed flora that changes over time. Coagulase-negative staphylococci, *Streptococcus* species, *Corynebacterium* species, and *Staphylococcus aureus* populate the wound initially before facultative anaerobic Gram-negative bacilli, such as *E. coli*, *Klebsiella*, or *Proteus* species, take up residence, usually days or even weeks later. The longer that an ulcer remains unhealed, the more likely it is that it will acquire multiple aerobic organisms, as well as a significant anaerobic population. Chronic wounds have a statistically higher proportion of anaerobes than do acute wounds (17). Dowd et al. found 30% of anaerobes in diabetic wounds but 62% in pressure wounds (7). Our study points to the fact that the deep tissue of the DLWs must be cultured for fungi and bacteria, as diabetic patients may present with sterile wounds or have purely bacterial or fungal infections or mixed bacterial and fungal infections. This raises interesting questions about the contribution of these deep tissue fungal infections to the delay in the healing of DLWs, especially in those few patients who had only fungal infections. The bacteria that we isolated from DLWs were predominantly Gram negative, which was consistent with the work of Lipsky et al. (21) and Citron et al. (6).

Some bacteria work together in microbial synergy. In mixed aerobic-anaerobic infections, microbial synergy frequently exists. The effect of synergy between two bacteria can be devastating for the host, especially if the synergy fosters a rapidly destructive necrotizing fasciitis. Less invasive microorganisms

like coliforms can be synergistic with more virulent ones and play a crucial role in wound infection (17). The synergy between bacteria and fungi may have a role in wound healing. Studies have shown that when two opportunistic pathogens, *P. aeruginosa* and *C. albicans*, are found together, the former forms a dense biofilm on the filaments of the latter and kills the fungus (14). We also observed the same in our study. Of the 15 *C. albicans* strains that we isolated from DLWs, none were found in patients who had *P. aeruginosa* infection in their wounds. Two of 15 *C. albicans* isolates were found in the presence of *Staphylococcus aureus* in our study. Preliminary studies have shown that *Staphylococcus aureus* and *C. albicans* appear to be initially synergistic (31). Further work is definitely warranted to prove synergy of fungi and bacteria in chronic wounds.

Compared to PCR or bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP), culture-based methods were found insufficient for characterizing complex polymicrobial communities in chronic wounds (7, 8). By using molecular methods, a wide range of bacteria, including fastidious anaerobic bacteria in chronic wounds, were identified that were not observed using culture-based methods (29).

The limitation of our study is that we used only classical methods and not molecular methods to identify fungi and bacteria from DLWs. We also did not do susceptibility testing for filamentous fungi and culture for anaerobic bacteria.

In summary, our study revealed that there is a high prevalence of fungal infection in deep tissues of diabetic lower-extremity wounds. About one in four diabetic patients with lower-limb wounds harbored a deep tissue fungal infection. These fungi were found to infect wounds either alone or in conjunction with bacteria. Patients with poor glycemic control had significantly higher fungal infection, but no statistically significant association of fungal infections was observed with patient age, sex, ABI, VPT, $TcPO_2$, depth of the wound, and duration of diabetes and limb lesion. Similar findings were made by Gadepalli et al., wherein the multidrug-resistant bacterial organisms (MDROs) showed significant association with poor glycemic control (10). Most of the yeast isolates were susceptible to FLCYT and FLZ compared to the other antifungal agents tested.

More research on evaluating, studying, and treating chronic DLW pathogenic biofilms is required. Application of molecular biology-based diagnostic tools would provide better understanding of the wound's ecology and would allow clinicians to better manage the wounds and improve the prognosis for the patient. Understanding the mechanisms of adhesion and signaling involved in bacterial-fungal interactions may lead to development of novel therapeutic strategies for chronic DLWs. More studies are to be done to assess the role of antifungal agents in diabetic foot wound healing. Diabetic foot infections require careful attention and coordinated management by a multidisciplinary foot care team, which includes an infectious disease specialist and a medical microbiologist. Reinforcing preventive actions and educating patients about the importance of glycemic control, use of appropriate footwear at all times, avoidance of foot trauma, daily self-examination of the foot, and early reporting to a health professional of any change in the foot would minimize morbidity and mortality due to diabetic foot complications.

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