Original Article

Molecular Detection of Agglutinin-like Sequence 1 Gene in *Candida albicans* that is Isolated from Diabetic Foot Patients

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Abstract

Objectives: *Candida albicans* is a microbe living within the natural human flora and is found in the upper respiratory tract, mouth, intestines, and vagina. *C. albicans* is able to cause infections that range from superficial infections of the skin to life-threatening systemic infections. **Aim of Study:** Detection of virulence gene agglutinin-like sequence (ALS) 1 by using molecular technology from clinical samples (*C. albicans*) that is isolated from ulcers of diabetic foot patients. **Materials and Methods:** This work was done on 235 patients who had diabetic foot patients admitted to the Specialized Center for Endocrinology and Diabetes (Baghdad Health Department/Rusafa) for the treatment of diabetic foot ulcers during November 2020 till March 2021. The collected samples of diabetic foot ulcers were cultured on different media (Sabouraud's dextrose agar with chloramphenicol for selective isolation and culturing of yeasts and HiCrome Candida Medium) for isolation of *C. albicans* fungus as well as automated biochemical test VITEK 2 system. The ALS1 virulence gene was detected by polymerase chain reaction using newly designed primers with a molecular size (419 bp). **Results:** Out of 235 Diabetic Foot Ulcer (DFU) cases, *C. albicans* were isolated in 20 (8.5%) patients (12 males and 8 females) of diabetic foot ulcers. In this study, the incidence of *C. albicans* infection at age [50–59 years] group was [40%], and increased at age group [60–69 years] to [55%], which represents the highest incidence of infection, then decreased in the age group [79–79 years] to [5%]). Seventy-five percent of the isolates were ALS1 gene positive. **Conclusions:** Diabetic people are more susceptible to infections due to their hyperglycemic environment and reduced immunity. The use of HiCrome *Candida* Identification Media with VITEK 2 system can help reduce the unnecessary steps of microorganism identification process. *C. albicans* infection is more common in males the females regarding diabetic foot ulceration. Majority of diabetic foot ulcers occur in older adults. ALS gene might be associated with diabetic foot ulceration.

Keywords: Agglutinin-like sequence 1, *Candida albicanis,* diabetic foot ulcers, virulence gene

Introduction

More than 20% of diabetic foot ulcers have fungal infections, and the most common causative agent is *Candida albicans*.[1,2] *C. albicans* has a number of abilities that help it cause disease, including the ability to switch phenotypes,[3] filamentation,[4] adherence,[5] and secreted hydrolases.[6] *C. albicans* produces a unique group of proteins (adhesins). Adhesion to other *C. albicans* cells, other microbes, abiotic surfaces, and host cells is mediated by these proteins. The eight agglutinin-like sequence (ALS) genes (ALS1 through ALS7 and ALS9) code for large cell surface glycoproteins, some of which aid in host surface attachment.[7-10]

Diabetic foot ulcers (DFUs) are one of the most serious and costly complications of diabetes mellitus, and they are one of the most prevalent reasons for diabetic patients to be admitted to the hospital. Diabetic foot ulcers are complex, chronic wounds that have a significant long-term influence on the morbidity, mortality, and quality of life of patients[11] and are a fairly common occurrence. Deep tissues are accessible to bacterial and fungal diseases that spread quickly once the skin's protective covering is damaged. Patients with DFUs typically require lower-limb amputations, and infection is the primary cause in more than half of the cases.

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According to reports, approximately 25% of diabetics will acquire a DFU during their lifetime.[12] Furthermore, it is estimated that lower limb is amputated every 20s due to diabetes-related complications.[13] In fact, 5% of diabetic individuals suffer foot ulcers each year, with 1% requiring amputation.[14]

Aims of the study

The aim of this study was to isolate and identify of *C. albicans* and to detect ALS1 gene in the isolated *C. albicans* using the molecular technique polymerase chain reaction (PCR) to study the relation between the availability of virulence genes ALS1 and occurrence of infection.

Materials and Methods

Sample collection

This study included a 20 *C. albicans* isolates taken from a total of (235) clinical sample of diabetic patients admitted to the Specialized Center for Endocrinology and Diabetes (Baghdad Health Department/Rusafa) for the treatment of diabetic foot ulcers during November 2020 till March 2021.

The samples were collected by using sterile swab from the depth of the ulcer and the surrounding area and skin scraping of the lesion. Multiple swabs and scrapings may be taken depending on the condition and size of the ulcer as shown in Figures 1-3, then the samples are transported to the laboratory for culturing. Swabs were inoculated on Sabouraud's dextrose agar with chloramphenicol for selective isolation and culturing of yeasts by excluding Gram-positive and Gram-negative bacteria. The media is made depending on the provided instructions by the manufacturing company. Swabs were rolled on a glass slide, heated to fix them, dyed using Gram's stain, and inspected for pseudohyphae budding yeasts.

Isolation of *Candida albicans*

C. albicans produces pseudohyphae, that are elongated budding structures that can be seen in clinical samples with true hyphae, blastoconidia, and yeast cells.[15] *C. albicans* may be identified quickly through germ tube development. A germ tube is the first step of hyphal development (cylindrical outgrowth) from a blastospore. Germ tube method was applied according to the procedure described by Larone.[16] HiCrome Candida Medium is a differential culture media which was used in the present study for the identification of *C. albicans* on the basis of pigmentation and color production. Different species of *Candida* produce completely different pigmentations and are differentiated easily by naked eyes.

Detection of *Candida albicans* by VITEK 2 system

VITEK 2 system and ID-YST card were used in this study to confirm the detection of *C. albicans* fungi. The VITEK ID-YST card has 64 wells that include 47 fluorescence biochemical assays.

DNA extraction from *Candida albicans* isolates

DNA extraction steps were applied using iGenomic BYF DNA Isolation Kit. Single colony was taken from the agar

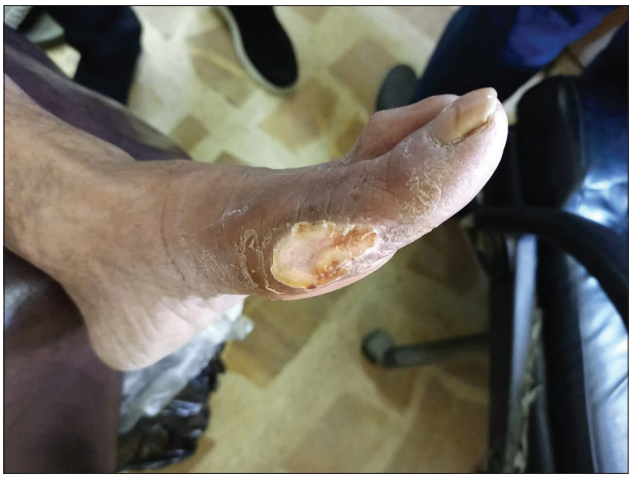

Figure 1: Superficial diabetic foot ulcer on the left foot–Wagner Grade 1

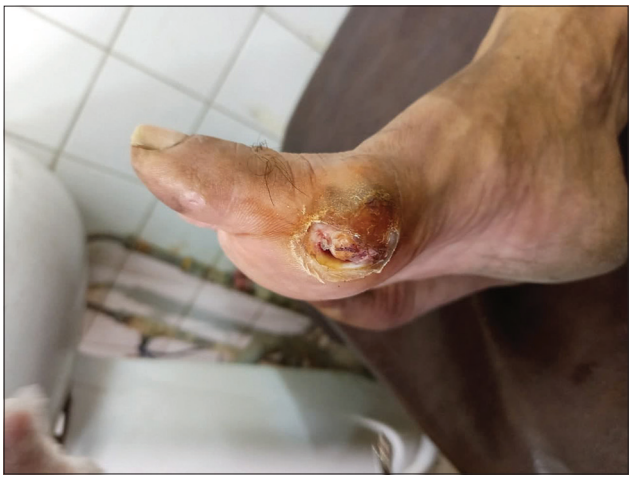

Figure 2: Deep diabetic foot ulcer on the planter region of the right foot, with infection reaching the articular capsule of metatarsophalangeal joint – Wagner Grade 3

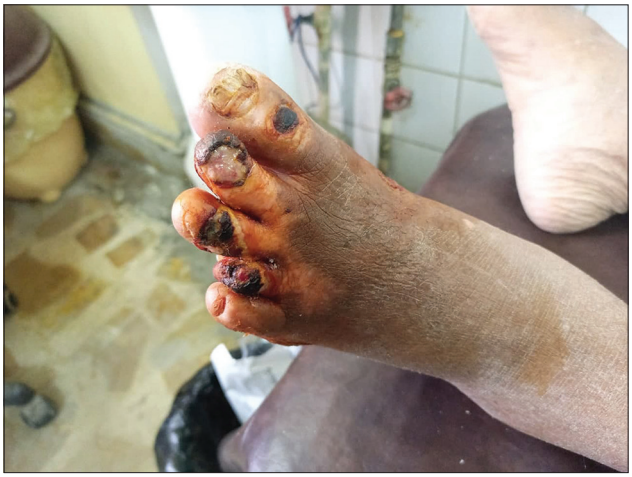

Figure 3: Multiple ischemic diabetic foot ulcers on all toes of the left foot, with red discoloration of the skin surrounding the ulcers because of using povidine iodine as a disinfectant causing scaling of the skin – Wagner Grade 4

plate, then cultured on 5 ml liquid culture medium, then it was incubated at 37°C for 2–3 days. Until OD_{600} value of 0.8–1.0 on spectrophotometer. 1.5 ml of cultured yeast was transferred into 2 ml tube, then centrifuged at 13,000 rpm for 1 min, and then supernatant is discarded. The yeast pellet was resuspended in the remaining supernatant by tapping or vortexing vigorously. 200 µl Buffer MYP and 2 µl β-mercaptoethanol were added into sample tube and mixed well by vortex for 30 s or pipetting vigorously. The lysate was incubated for 15 min at 37°C. The prelysate was centrifuged at 13,000 rpm for 1 min at room temperature. Supernatant was discarded and then resuspended by vortexing or tapping of cell pellet to lysis cell perfectly. 100 µl Buffer MP and 3 µl lyticase or zymolase solution were added into spheroblast sample tube and mixed well by vortex for 30 s or pipetting vigorously. The lysate was incubated for 15 min at 37°C. The prelysate was centrifuged at 13,000 rpm for 1 min. Supernatant was discarded and then resuspended by vortexing or tapping of cell pellet to lysis cell perfectly. 200 µl Buffer MG, 10 µl Proteinase K, and 5 µl RNase A solution were added into a sample tube and aggressively vortexed. The lysate was incubated for 30 min at 65°C. 250 µl Buffer MB was added to the lysate upon complete lysis and mixed by pipetting or carefully inverting 5–6 times (not vortex). Two hundred fifty µl 80% ethanol was added to the lysate and mixed 5–6 times with pipetting or gently inverting. Seven hundred fifty µl of the mixture was pipetted from step 10 into a 2.0 ml collecting tube putted in the centrifuge tube. Centrifuged for 1 min at 13,000 rpm (RT), then the flow-through was removed. Spin column was placed into a new 2.0 ml collection tube, 700 µl Buffer MW was added to the spin column, and centrifuged at 13,000 rpm for 1 min. Flow-through was removed and centrifuged of the membrane for another minute to dry it. Flow-through was removed and collecting tubes. Spin column transferred into a new 1.5 ml tube, and 50 µl Buffer ME added directly onto the membrane. Incubated for 1 min at room temperature and then centrifuged at 13,000 rpm for 1 min to elute.

Double-stranded DNA (dsDNA) quantitation by qubit, this test is extremely selective for dsDNA over RNA, with accuracy ranging from 10 pg/µl to 100 ng/µl for initial sample concentrations. The test is carried out at room temperature, and the signal lasts for three hours. In the test, common contaminants including salts, free nucleotides, solvents, detergents, and protein are well tolerated.

Molecular Amplification of ALS gene was carried out by adding 12.5 µl from OneTaq (NEB®) mastermix, 3 µl of DNA sample, 1 µl 10 pmol/µl from primer [Table 1] and 7.5 µl of free-nuclease water. The reaction done under the optimal PCR conditions for gene as indicated in Table 2.

Table 1: The sequence forward and reverse primers of *ALS1* gene

Primer	Sequence	Product size (bp)	Reference
ALS1	F: GGATACCCAAC TTGGAATGCT R: TATGCACTTGG ATCAACGGTT	419	Newly designed

Table 2: The polymer chain reaction conditions for amplifying *ALS1* gene

Cycle number	Stage	Temperature (°C)	Time
1	Initial denaturation	94	5 min
35x	Denaturation	94	30 s
	Annealing	53	45 s
	Extension	72	45 s
1	Final extension	72	7 min

Results

The mean age range of male diabetic patients was 63 years (range 53–78 years) while the female diabetic patients' mean age was 60 years (range 54–68 years). Different age groups were subjected to this study ranging from 54 to 78 years and divided into three age groups. Table 3 shows the distribution of age groups in patients with *C. albicans* infection in respect to foot ulcers.

In the age group (50–59 years), the incidence was 40%, which increases at age group (60–69) to (55%), which represents that the highest incidence of infection there was a significant increase in the susceptibility to *C. albicans* foot ulcers in the age group (60–69). This result was statistically significant $P = 0.019$.

Different duration of diagnosis was subjected to this study ranging from 1 week to 6 years. There were divided into three groups. Table 4 shows the duration of diabetic foot ulcers in patients with Candida albicans infection. In the group (<1 month), the incidence was 10%, which increases at group (1 month to 1 year) to (20%), the incidence in the infection highly increases at group (more than 1 year) reaching to (70%), which represents the highest incidence. This result was statistically significant, $P = 0.02$.

Sabouraud's dextrose agar culture: all samples were cultivated on Sabouraud's Dextrose agar with chloramphenicol, as shown in Figure 4a. After cultivation the white creamy colonies that were cultured on Sabouraud's Dextrose Agar were subculture on HiCrome Candida differential agar to identify of *C. albicans*. As shown in Figure 4b.

As shown in Table 5 (75%) of the isolates were ALS1 gene positive, this result was statistically significant ($P = 0.025$) as shown in Figure 5.

Fifteen out of twenty isolates of *C. albicans* were positive for ALS1 gene. PCR product of this gene was 419 bp Figure 6.

Discussion

This study included 20 (8.5%) pure *C. albicans* isolates (twelve patients were males and eight patients were females) out of 235

Table 3: Age distribution with *Candida albicans* infection

50-59 years		60-69 years		70-79 years		Total	
Count	Percentage	Count	Percentage	Count	Percentage	Count	Percentage
8	40.0	11	55.0	1	5.0	20	100.0

P: 0.019 (significant)

Table 4: Distribution of the duration of diagnosis with diabetic foot ulcer

<1 month		1 month-1 year		More than 1 year		Total	
Count	Percentage	Count	Percentage	Count	Percentage	Count	Percentage
2	10.0	4	20.0	14	70.0	20	100.0

P: 0.02 (significant)

Table 5: Virulence genes detected

	Positive		Negative		Total		*P*
	Count	Percentage	Count	Percentage	Count	Percentage	
ALS1	15	75.0	5	25.0	20	100.0	0.025 Significance

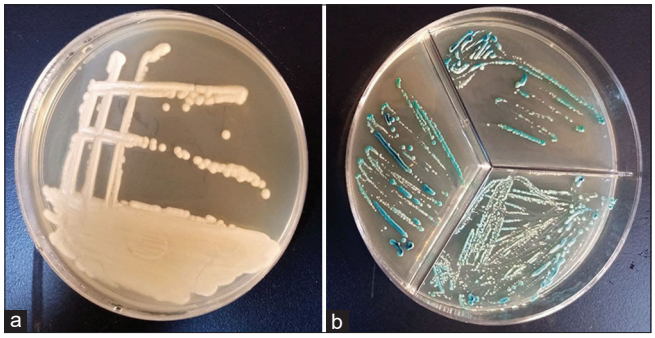

Figure 4: (a) Represents creamy white *Candida albicans* colonies cultured on Sabouraud's Dextrose agar containing Chloramphenicol. (b) Represents green colonies of *Candida albicans* subcultured on HiCrome agar

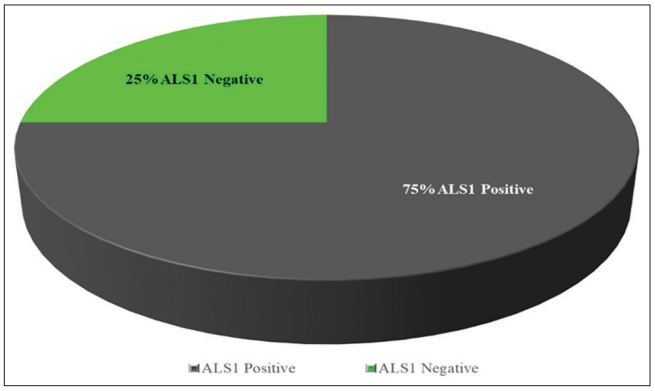

Figure 5: Distribution of agglutinin-like sequence 1 gene

diabetic foot ulcer patients. Similar findings were found by Fata *et al*., who found that 11 (9%) *C. albicans* cultures of infected diabetic foot ulcers of 120 patients[1] and (7.5%) similarly by Kalshetti *et al*.,[17] while others disagreed with these results who found that the incidence was as low as 2.9% of the total infected deep tissues of the wounds of lower limb in patients diagnosed with Type 2 Diabetes and with *C. albicans* positive isolates.[18] Also another study disagreed but with increased incidence as high as 30.7% of isolates of *C. albicans* from diabetic foot infections.[2]

Majority of the diabetic foot ulcers patients with *C. albicans* were men in the age range 60 years to 69 years (11 patients) similar findings have been reported by another studies.[1,19]

The present sample of DFU patients reveals that there was an increasing ratio in males to females that came with agreement in the studies[20,21] Maybe because of increased rate of outdoor activity among male patients in comparison to females, the male preponderance was higher, in which increases their chances of getting trauma to the foot by any agents not noticed.[22] Ribu found that men were lower self-care had a foot ulcer.[23] Similarly, foot self-care deficit, characterized by the following points which include not regularly drying feet and between toes, not checking feet for injuries, walking barefoot, poor hygiene behaviors, and not appropriate trimmed nails, were significantly higher among men, although men presented a lower prevalence of feet scaling and use of inappropriate shoes when compared to women. With regard to lifestyle, men presented less healthy habits, such as not adhering to a proper diet and taking laboratory exams to check for lipid profile at the frequency recommended.[24] But in a study that was conducted in Kenya disagrees,[25] as females are more than males included in their study, a possible explanation for this difference is that some patients refused to participate were not included in the study and only 61 patients included in the study.

In this study, the data show that the incidence in the infection highly increases at group (more than 1 year of diagnosis with diabetes) reaching to (70%) of the 20 patients, which is in agreement with Heald *et al*.[26] and Raiesi *et al*.[27]

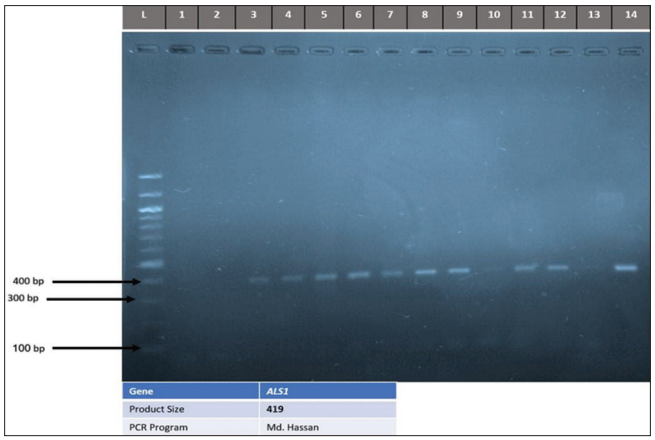

Figure 6: Gel electrophoresis of extracted DNA from *Candida albicans* isolates to detect agglutinin-like sequence 1 gene on 2% agarose gel at 7volt/cm for 70 min

This result highlights the risk factor of developing diabetic foot ulcers in older age patients.[28] The researchers discovered a link between age and the incidence of DFUs. An increased risk of angiopathy has been linked to increasing age. People with age more than 40 years appear to be at higher risk of developing angiopathy.[29,30] Wound healing in DFU patients was more challenging in older persons, according to Jeffcoate.[31] This finding might be attributed to a loss in vascular function as people age, causing infection to occur more frequently in old age than in youth.[32]

The most important *C. albicans* adhesins virulence genes are ALS proteins (ALS1-7 and ALS9).[33] ALS1 was reported as the most commonly expressed genes of the ALS gene family.[34] In this study, it has been found that 75% of *C. albicans* isolates reveals the presence of ALS1 gene, this result agrees with[34] results when they found that 69% of *C. albicans* isolates of vulvovaginal candidiasis are ALS1 positive, and Goulart *et al*.[35] were they found 73.68% of *C. albicans* isolates from vaginal infection are ALS1 positive, and Roudbary *et al*.[36] were they found 83% of *C. albicans* isolates from vaginal samples are ALS1 positive. But disagree with Ardehali *et al*.,[37] who found that 92% of *C. albicans* isolates of mostly blood and urine are ALS1 positive, and Mohammed *et al*.,[38] were they found 100% of *C. albicans* isolates from oral and vaginal infection are ALS1 positive. Those variations might be associated with the number of samples studied or with the virulence of the strains analyzed and also with the methodology employed.

Conclusions

Diabetic people are more susceptible to infections due to their hyperglycemic environment and reduced immunity. If a fungal infection in a diabetic patient is not handled appropriately, it can lead to catastrophic complications such as amputation of the foot. The use of HiCrome *Candida* Identification Media with VITEK 2 system can help reduce the unnecessary steps of microorganism identification process. *C. albicans* infection is more common in males the females regarding diabetic foot ulceration. Majority of diabetic foot ulcers occurring in older adults. ALS gene might be associated with diabetic foot ulceration.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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