Molecular characterization of some types of fungi isolated from wound patients in Kirkuk city hospitals.

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Abstract

Background: The current study aims to diagnose fungi isolated from wounds using traditional and molecular methods, study virulence factors, and test biofilm formation. Chronic wound infection results from several factors such as repeated infections, chronic diseases, or disorders of the immune system. **Materials and methods:** This research included 250 patients in Kirkuk City. Direct laboratory examination of the samples was performed using 10% KOH, and laboratory examination after culturing the samples on Sabouraud Dextrose Agar medium. **Results**: Three types of yeast were diagnosed *Candida albicans*, *Candida*, *tropicalis*, and *Candida krusei*. The most frequent of them was *C. albicans*, with 27 isolates (29.67%) *C. tropicalis* 22 isolates 24.18%, and *C. krusei* 7 isolates 7.69%. The diagnosis of the isolated yeasts was confirmed based on PCR technology.

Conclusions: The study showed that diabetic foot wound infection was the most common and that *Candida* spp. is the primary cause of these infections.

Keywords: Molecular diagnosis, *Candida* spp, infection, virulence factors.

Introduction

Chronic wound infection manifests as prolonged inflammation and delayed healing, persisting for over six weeks. Contributing factors include recurrent infections, chronic illnesses, and immune system disorders. Effective treatment necessitates thorough evaluation to identify underlying causes and implement a tailored treatment strategy (1). The estimated prevalence of chronic wounds ranges from 1% to 4% (2). The interaction between bacteria and fungi in chronic wounds is one of the factors that contribute to persistent inflammation and delayed healing. These multiple communities of organisms can challenge the immune system and affect tissue regeneration, making it necessary for appropriate medical intervention to promote the healing process (3-5). Although *C. albicans* is considered a more isolated pathogen, Non-albicans candida such as *C. glabrata*, *C. tropicalis*, and *C. krusei* are considered opportunistic pathogens and have demonstrated more resistance to antifungal agents (6,7). Analyzes of historical data show that the cost of treating non-healing wounds may range between $28.1 and $96.8 billion (6). So, the report indicates that the value of wound treatment and care is approximately US$ 18.22 billion and is expected to reach US$ 26.24 billion globally in 2023. This includes more than 38 million. chronic wound infections resulting from treatment failure to heal wounds, and are associated with poor prognosis (8). Studies indicate that the wound microbiome, which arises as a result of colonization by bacteria and fungi, is a factor that hinders the healing process and contributes to the development of chronic wounds via community microbiota processes (9). Fungal species were

identified in 23% of chronic wounds studied (915 cases), which included diabetic foot ulcers, non-healing surgical wounds, pressure ulcers, and venous ulcers. It is noted that *Candida* spp yeast species appear to have the highest prevalence (10,11). Data indicates that the prevalence of fungal wounds in diabetic patients ranges between 9% and 40.1%. The common fungal species in these cases are *Candida albicans*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei*, followed by *Aspergillus flavus*, *guillermondii*, , *Aspergillus niger*, and *Aspergillus fusarium* (12). Based on the analysis of fungal prevalence in 152 lower extremity ulcers and surrounding skin, 6% of ulcer samples and 27.6% of skin samples tested positive for fungi. Common fungal species include *C. albicans*, *C. parapsilosis*, and *Candida tropicalis* (13).

Materials and methods

Sample collection:

The current study was conducted in Kirkuk Governorate during the period from September to December 2023, collecting 250 samples from patients with chronic wound infections at Kirkuk General Hospital, Azadi Teaching Hospital, and the Burn Center of Azadi Teaching Hospital. Personal protective equipment was used during sample collection, and swabs containing the Ames preservative were used. Samples were taken from the festering infection areas and transferred to the Mycology Laboratory at the College of Science to isolate and diagnose fungi and some virulence factors using traditional methods and detect yeasts using molecular methods.

Isolation of fungi: The phenotypic characteristics of colonies growing on SDA medium are determined by observing their color, shape, texture, odor, and height. The colonies appeared smooth and convex. Creamy white, characteristic yeast, the cells are spherical or oval and contain spores and pseudohyphae (14). This is followed by microscopic examination by preparing a glass slide for the colonies, where a portion of the colony is taken with a vector, placed on a glass slide, stained with lactophenol blue dye, and then examined with an(X40) light microscope to observe the pseudo mycelium and giant spores (15,16).

Identification

Yeast biofilm formation test

In this study, the composition of the yeast biosynthesis was detected using a microtiter plate with 96 marked holes. The yeast was introduced into a tube containing 2 ml of YPD (Yeast Peptone Dextrose) broth using a loop and then incubated at 37°C for 24 hours. Following this incubation period, all the tubes were diluted by 20%. :1 Using YPD prepared with 1% glucose, each hole of the microplate was filled with 200µl of the final prepared solution, then the microplate was covered with coverslips and incubated at 37°C for 24 hours. After incubating for 24 hours, the medium in the plate is removed and washed twice with PBS (Phosphate Puffer solution), then turned over and left to dry. The microplate is stained by adding 200 µl of Crystal Violet formula to each well and waiting for 20 minutes. The microplate is washed twice with PBS and then stirred until it dries. finally, add acetone to each hole and wait about 10 minutes, then read the result at 450 nm with a reader ELISA (17).

Germ Tube Formation Test:

A portion of the colony is placed in a test tube containing 0.5 ml of serum and then incubated at 27°C for 2-3 hours. Following incubation, a serum drop is withdrawn, put on a glass slide, covered, and examined under a microscope to observe germ tube formation (18).

Corn Meal Agar with Tween 80 (CMA):

It was prepared according to the manufacturer's instructions (Merck, Germany), and Tween 80 was added to the basal medium to stimulate the production of chlamydospore.

Chrome Candida Differential Agar (CCA):

Candida species were distinguished using agar chromogenic medium, where colors are formed through the interaction of isolates with the medium (19). From the SDA medium, a portion of the Candida colony was taken using a sterile Loop carrier at 24 hours of growth on the SDA medium and streaked on the CCA medium. The plates were incubated for 24-48 hours at a temperature of 37, which led to the production of colonies of different colors.

Molecular diagnosis of yeasts

A polymerase chain reaction was conducted to investigate the yeast *Candida* spp. Fungal DNA was extracted and the ITS target region was amplified using the primers (ITS1) and (ITS4). Accordance with (20).

Results and discussion

Laboratory culture results showed that the rate of infection of chronic wounds with molds and yeasts was 36.4%, with a rate of (91) positive samples out of a total of (250) samples. (56) were infected with yeasts (61.54%), (35) were infected with Aspergillus (38.46%). Three species of yeast have been identified: *Candida albicans*, *C. tropicalis*, and *C. krusei*, the most common of which is *C. albicans*, with 27 isolates, or 29.67%, and *C. tropicalis*, with 22 isolates, or 24.18%. And *C. krusei*, with 7 isolates, representing a rate of 7.69%. As for *Aspergillus* spp, the most common is *A. fumigatus*. There were 18 isolates, or 19.78%, *A. niger*, 13 isolates, or 14.29%, and *A. flavus*, 4 isolates, or 4.39%. The study showed that diabetic foot wounds are the most common infection, and that the most common isolate was *Candida* spp, which causes wound infections. The study also indicated the spread of *Candida albicans* as the most common isolate in wounds. This is what researcher Doud and his group concluded (10). Followed by *Aspergillus* species, and this result was consistent with the result reached by researcher Helal and her group (21).

In this study, three different types of *Candida* spp and some types of *Aspergillus* were isolated from patients, as shown in Table (1). These species were diagnosed using morphological, microscopic, and biochemical tests and diagnosis. Several team methods were used. Between yeast and mold. CCA, a differential method, was used to differentiate *Candida* spp by color. The study indicated that (29.27%) were bright sky colonies of the Candida fungus on Chrome Agar, where all types of *C. albicans* came to form germ tubes and chlamydia spores and were able to grow at a temperature of 45 °C. The urease test result was negative, followed by (24.18%) *C. tropicalis* with a metallic blue appearance. The urease test was negative, and growth did not begin at 45 °C. As for *C. krusei* (7.69%), its colonies showed a pink color, and all non-albicans *Candida* spp did not form the germ tube and did not form chlamydospores. These results are consistent with those of (10,22) Although they did not agree with (23,24). who suggested that *C. tropicalis* is the second or third most identifiable fungal species as shown in Table (2) and Figures (1,2,3,4)

Table (1) shows the distribution of fungal infections according to the types of wounds

Factors causing injury	Diabetic foot ulcers	Bedsore	Surgical wounds	Burn wounds	Total samples Positive	percentage
Candida albicans	11	7	6	3	27	29.67
Candida tropicalis	8	7	4	3	22	24.18
Candida krusei	4	2	0	1	7	7.69
Aspergillus fumegatus	7	5	3	3	18	19.78
Aspergillus niger	4	4	3	2	13	14.29
Aspergillus flavus	1	2	1	0	4	4.39
the total	35	27	17	12	91	%100

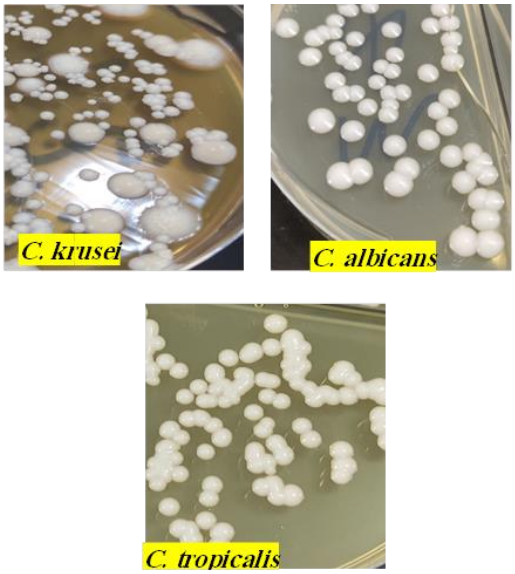

Figure (1): *Candida spp.* colonies on SDA medium at 37°C for a period of (24-48)

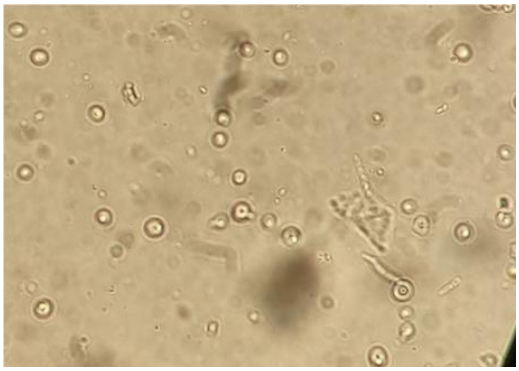

Figure (2): Germ tube production under force (40X Power)

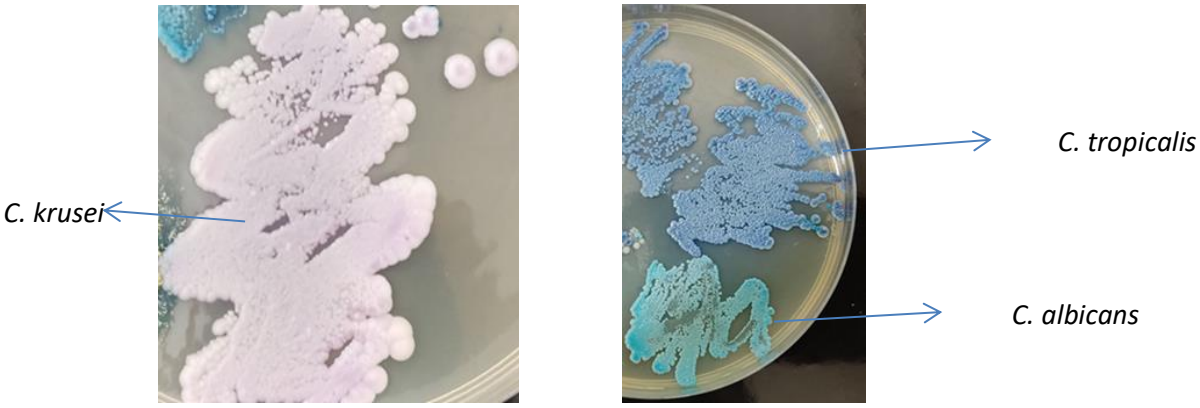

Figure (3): Growth of *Candida* species on (CCA)

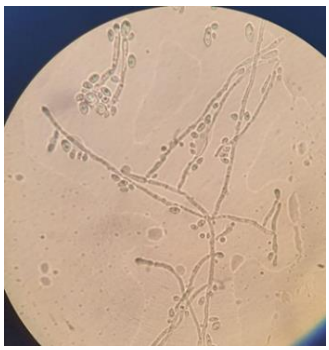

Figure (4) chlamydiosporo formation under force (40X Power)

Table 2: Diagnostic and biochemical tests for Candida yeasts isolated during the study

Candida spp	Coloring of yeasts on CHROM agar medium	Formation of germination tubes	Formation of chlamydial spores	Growth at a temperature of 45°C	Urease test
C.albicans	light green	+	+	+	-
C. tropicalis	metallic blue	-	-	-	-
C.krusei	pink	-	-	-	-

Biofilm formation test

The results showed the ability of *C. albicans* yeast to produce biofilm at a rate of 85%, and this percentage agreed with what researcher Al-Obaidi reached in 2022, (18). where she recorded a rate of 90%, it did not agree with the result that Shin and his group came up with in his study, where the production rate was 8% biofilm for *C. albicans* (25). While the percentage of biofilm production of *C. tropicalis* yeast was 68%, it agreed with the findings of researchers Marak & Dhanashree 2018, where the biofilm production percentage was 58% (23). The *C. krusei* yeast biofilm production rate was 57%, and the result was consistent with what the researchers reported (23). the rate of biofilm production by this yeast reached 61%, as shown in Table (3) and Figure (5).

Molecular diagnosis of yeasts

We relied on molecular methods using PCR technology, which was called the golden method due to its high accuracy and specificity in diagnosis (26). The study in which *Candida* spp was diagnosed showed that the concentrations of the extracted DNA were measured using a NanoDrop spectrometer at two wavelengths (260) and (280) nm and the purity of the extracted DNA reached (1.82-1.98). Using the universal primer pair specific to fungi (ITS and ITS4), which amplifies a region, which is the inner transcribed space (ITS) region, which contains (ITS2-SrDNA-5.8-ITS1), which is a special region for testing all types of fungi, we note that it produced different sizes ranging from (510 - 535) years ago, based on the length difference between the ITS1 and ITS4 regions in the DNA of *Candida* species. The

results of the PCR reactions showed that the size of *C. albicans* was about 535 bp, while the size of *C. tropicalis* was 524 bp, and the size of *C. krusie* was 510 bp. The species size was as shown in the PCR reaction products in Figure (6) and Table (4). These results are consistent with the findings of researcher Habib and his group (27) who mentioned that these species were the size of a piece of DNA itself. The fungi differ from each other in the sizes of the ITS region of the DNA, and the PCR reaction parts with primers can target this region, which includes S (5.8) and S (28) of the rDNA, as well as S (18).

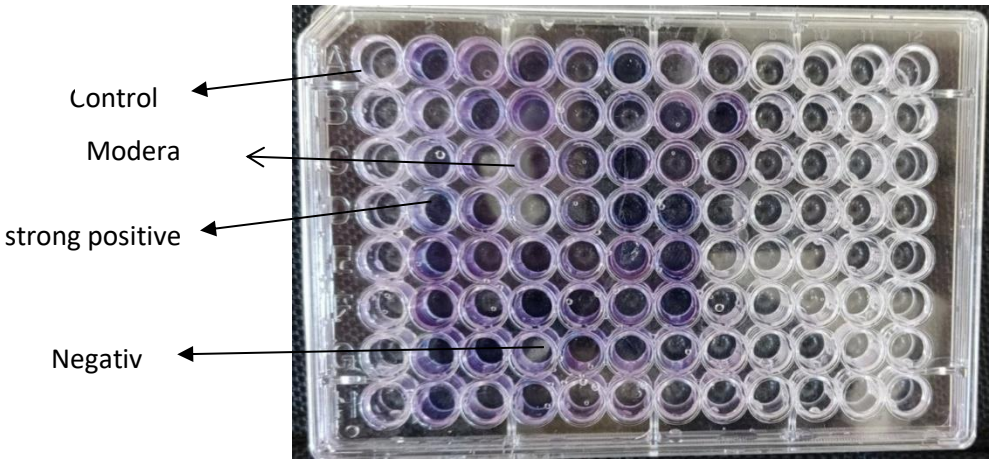

Figure (5) Biofilm activity test in standard microplates for *Candida* spp isolates

Table (3) shows the numbers of biofilm-forming *Candida* spp.

Yeasts	number of isolates	negative	Moderate positive	strong positive	percentage
Candida albicans	27	4	9	14	85%
Candida tropicalis	22	7	5	10	68%
Candida krusei	7	3	1	3	57%

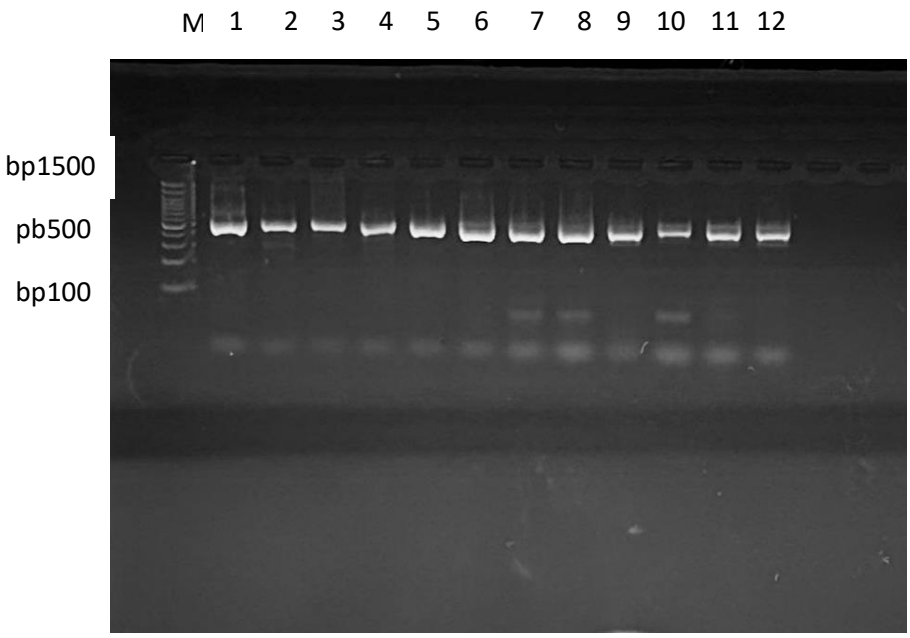

Figure (6) Electrophoresis results using an agarose gel by amplifying the ITS gene of *Candida* spp., where it represents: M Marker bp (100-1500).

Table (4): Types of yeasts resulting from the chain reaction polymerase

Candida species	Isolation number	ITS1-ITS4 size (bp)
Candida albicans	1	535
	2	
	3	
	4	
Candida tropicalis	5	524
	10	
	11	
	12	
Candida krusei	6	510
	7	
	8	
	9	

Conclusions

Yeasts and molds were successfully isolated and identified from wound infection patients. The study showed that the incidence of diabetic foot injury was the most common. The current study concluded that *Candida* species are among the most common infection causes. Results indicated that the most common Candida species isolated was *C. albicans*. In addition, this result was supported by the results of molecular diagnosis.

Conflict of interest

There are no conflicts of interest.

Ethical approval:

The ethical approval was obtained from the College of Science, the University of Kirkuk ethical committee, and the Ministry of Health.

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The authors have not obtained any source of funding.

Data Sharing Statement

All data are available upon reasonable request to the corresponding author

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