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Evaluation of Polyclonal Antiserum Against Secretory Aspartyl Proteinase of *Candida albicans* as a Potential Serodiagnostic Tool for Invasive Candidiasis

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Abstract

Objective: *Candida albicans* (*C. albicans*) is an important agent of human Candidiasis. It expresses different virulence factors to evade host immunity and facilitate tissue invasion, including secretory aspartyl proteinases (Saps) secretion. For early and quick detection of systemic candidiasis, serological tests can be used instead of traditional blood cultures. This study aims to develop a polyvalent antiserum against (Sap) secreted by *C. albicans* and test its ability to be used as a diagnostic method in systemic *Candida* infections.

Materials and Methods: *Candida* was obtained from clinical samples, and its different species were specifically characterized, and followed by *C. albicans* extracellular protease purification. Antiserum against purified (Sap1) was prepared by immunizing two rabbits with 10 µg of purified (Sap1) protein followed by three booster doses (once/week). Prepared anti (Sap1) antibodies were tested for the detection of (Sap1) in *Candida* species extracts by western blotting technique in addition to constructing indirect ELISA using prepared anti (Sap1) antiserum.

Results: Among the tested species, *C. albicans* showed the highest extracellular proteases activity (18.6-fold with 2142 U/mg specific activity and 39% recovery). Polyclonal anti (Sap1) antiserum showed maximum ELISA titer and strong reactivity with many pathogenic *Candida* strains protein bands. Prepared antiserum had a greater binding capacity to pathogenic than non-pathogenic *Candida* strains and reacted strongly with pathogenic *Candida* strains even at low dilution.

Conclusion: Our findings suggested that the prepared anti (Sap1) antiserum showed high productivity in detecting pathogenic *Candida* and could be used in serodiagnosis of invasive candidiasis.

Keywords: *Candida albicans*, western blotting, anti (Sap1) antiserum, serodiagnosis

Introduction

Candida albicans (*C. albicans)* is a significant contributor to human candidiasis, leading to various invasive illnesses that extend from simple mucocutaneous lesions to potentially fatal bloodstream candidiasis (1).

Candida's virulence factors include adhesion surface molecules, phenotypic switching, morphological dimorphism, as well as the release of hydrolytic enzymes like proteases and phospholipases, which can help to clarify the host-pathogen interaction and provide better options for therapeutic and diagnostic interventions (2).

Secretory aspartyl proteinases (Saps) refer to a group of enzymes capable of breaking down various vital substances in the body, including albumin, immunoglobulin, and skin proteins (3).

The enzymes encoded by ten different (Saps) genes share similar functions and features, yet they exhibit differences in molecular properties, including molecular mass, isoelectric point, and the pH at which they function optimally (4).

The invasive hyphal growth of *C. albicans* relies heavily on the presence of (Saps). During the early stages

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of invasion, the production of these enzymes is elevated, potentially assisting in the breakdown of the surface barrier facilitating the formation of hyphae and subsequent penetration into the human tissues (5-7).

The presence of high antibody titers against the enzyme was identified in the sera of candidiasis patients, as it is produced by most *C. albicans* strains and other non-albicans pathogenic species, including *C. tropicalis* and *C. parapsilosis* (6).

The diagnosis of disseminated candidiasis is challenging and frequently delayed because the manifestations are non-specific. Additionally, up to half of the patients with invasive *Candida* infections show negative results in blood cultures, which may only become positive later in the infection (8,9). This renders blood culture testing unreliable and time-consuming (9). Moreover, obtaining invasive tissue samples can be difficult, particularly in severely ill patients in intensive care units. Due to the lack of early and precise diagnostic methods, treatment is often initiated late, leading to potential morbidity and fatalities (8-10). Consequently, there is an urgent medical necessity for reliable techniques that can provide accurate diagnoses (9).

Serological tests that rely on detecting specific *Candida* antigens and antibodies offer a more sensitive alternative approach compared to traditional blood culture methods for the early and efficient diagnosis of systemic candidiasis (11).

Extensive application of biochemical and immunological strategies has been carried out to identify fungal antigens present in circulation. One such approach involved the creation of a more precise and sensitive diagnostic method that employed antibodies against *Candida* antigens, with a primary focus on fungal cell wall constituents (12-15). However, the low specificities and sensitivities of these antibodies rendered them ineffective for the diagnosis of *Candida* infections (16).

The use of a pathogenic factor like (Sap) as a direct serodiagnostic marker for candidiasis offers the advantage of differentiating invasive disease from mere colonization. Thus, the presence of Sap in the bloodstream could serve as an indication of active *Candida* infection (17).

An antibody-based ELISA inhibition approach has been developed to identify *C. albicans* (Saps) in serum samples, exhibiting a diagnostic sensitivity of 94% and specificity of 96% in diagnosing invasive Candidiasis (18). Consequently, the objective of this study was to produce polyclonal antiserum against (Sap) enzymes secreted by *C. albicans* and evaluate its efficacy as a diagnostic tool for invasive *Candida* infections.

Materials and Methods

Animal

Two fully mature white rabbits, aged over 12 weeks, were utilized for our study. All procedures involving the rabbits adhered to animal welfare regulations outlined in the Guide for the Care and Use of Laboratory Animals (http://oacu.od.nih.gov/regs/guide/guide.pdf) and received approval from the Ethical Committee of Tanta University, Faculty of Medicine, Egypt (approval no: 36264PR200/5/23, date: 13.05.2023).

Microorganisms (isolation of *Candida*)

Candida strains were obtained from various clinical specimens of immunocompromised patients who were hospitalized at Tanta University Hospitals. The collected samples were cultured on Sabouraud's dextrose agar (SDA) (Oxoid, England) provided with chloramphenicol (50 mg/L) and incubated under aerobic conditions at 37°C for 24-72 hours.

Identification of Different *Candida* Species

Colonies suspected to be *Candida* were further identified to species level by germ tube test, subculture on morphologic media (corn meal agar-Tween 80) (Belami Fine Chemicals, Mumbai, India), chromogenic medium (CHROMagar™ *Candida*, Paris, France) and biochemically using API 20C AUX (BioMerieux, France). To ensure accuracy, the *C. albicans* ATCC 32354 strain was used as a positive control.

The extracellular proteases and phospholipases activities were evaluated to distinguish pathogenic from non-pathogenic *Candida* strains, and the results were previously published (19). Purified *C. albicans* showed maximum protease activities. The current study was performed in parallel with the previous one, focused on purification of *C. albicans* extracellular protease with the production of polyclonal antiserum against these proteases.

Protease (Saps) Purification

C. albicans was subcultured on Sabouraud's dextrose broth. The crude extracellular supernatant was collected through centrifugation and precipitated with 80% saturated ammonium sulphate. The resulting precipitate was then resuspended in a 50 mM phosphate buffer at pH 7 and subjected to dialysis. Purification of the dialysate was carried out using formerly equilibrated Sephacryl S-200 HR, by 0.05 M acetate buffer at pH 5.6. Elution of the sample was achieved using the same buffer at a flow rate of 3 mL/min, and the A_{280} was monitored to assess total protein. To verify the presence of Saps, the collected fractions underwent further analysis using Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) (20).

Polyclonal (Sap1) Antiserum Preparation

Two rabbits weighing 1.5 ± 0.2 kg each were used for the immunization process to generate antibodies against the purified (Sap1). The immunization protocol began with a subcutaneous injection of a mixture containing 10 µg purified (Sap1) protein and complete Freund›s adjuvant in a 1:1 ratio. After one week, three additional booster doses were administered weekly, utilizing the protein emulsified with incomplete Freund's adjuvant and the collected sera were pooled and subsequently stored at -20°C (21).

Qualitative Cross-reactivity by Western Blotting

In order to detect the presence of the (Sap1) antigen in the extracellular extract of different *Candida* species, the membrane was incubated overnight with the primary antibody, anti (Sap1) (1:400 in 5% milk/TBS-T), which was obtained from rabbits. Subsequently, the membrane underwent three rounds of washing with TBS-T, each lasting 10 minutes (22). After that, the membrane was exposed to anti-rabbit secondary antibody-HRP linked (1:500 in 5% milk/TBS-T), for an hour at room temperature. This was followed by another three rounds of washing with TBS-T, each lasting 10 minutes. To visualize the results, the blot was treated with ECLTM western blotting detection chemiluminescent substrate provided by PerkinElmer, USA, following the manufacturer's recommendations. Finally, a CCD camera-based imager known as the Chemi Doc imager from Biorad, USA, was used to capture the chemiluminescent signals.

Quantitative Cross Reactivity by Constructing Indirect ELISA Using the Prepared Antiserum

Initially, the ELISA plate wells were coated with *Candida* protease (crude sample) as the antigen, using a concentration of 1-4 µg per well, in 50 mM sodium carbonate buffer at pH 9.6. The plate was left overnight at 4°C. Subsequently, the plate washed three times with 10 mM phosphate buffer saline Tween 20 (PBST) at pH 7.4. The plate was then blocked using a blocking buffer consisting of 10 mM phosphate buffer at pH 7.4, containing 2% gelatin, for a duration of 1 hour at 37°C. After washing the plate with PBST for three times, serial dilutions of the prepared anti (Sap1) antiserum, at a volume of 100 µL per well, were added and incubated at 37°C for 1 hour. The plate was then repeatedly washed with PBST. Next, 100 µL of anti-rabbit peroxidase was added to each well and incubated at 37°C for 1 hour. The plate was washed three times with PBST before introducing 100 µL of substrate per well, composed of 0.3 mM o-phenylenediamine (OPD) in 0.1M citrate buffer at pH 5.2 containing 0.04% H_2O_2. Following a 20-minute incubation, the reaction was terminated by adding 20 µL of 2 N H_2SO_4 and the absorbance was measured at 490 nm (23).

Statistical Analysis

All tests were performed in triplicate, and the data were presented as means ± standard deviation. Statistical analysis was conducted using MS-Excel 2010. Significance was determined at a p-value of <0.05.

Results

C. albicans proteases were precipitated, dialyzed, and purified by gel filtration Sephacryl S-200 column (gel chromatography). The protease was separated in one peak as total protein, which contained three *C. albicans* proteases peaks (Figure 1). Table 1 illustrated that Sap1

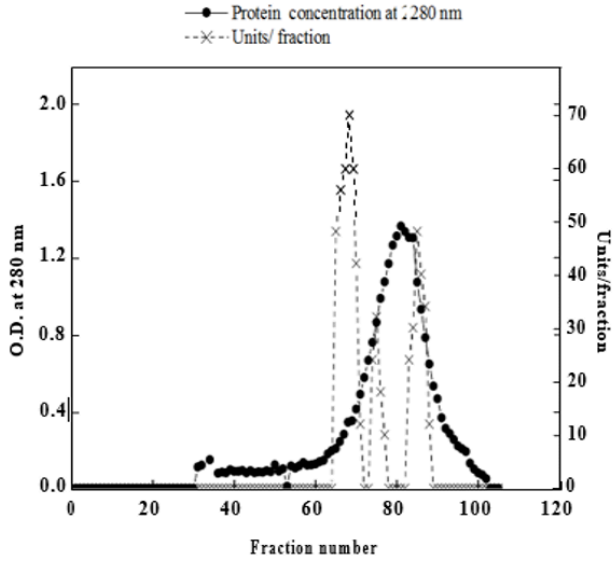

Figure 1. Elution profile of protease from *Candida albicans* on Sephacryl S-200 HR gel filtration chromatography.

Table 1. Purification of protease from *C. albicans*

Steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Crude extract	2300	20.0	115	1.00	100.0
Ammonium sulphateprecipitation	1840	12.0	153	1.33	80.0
Dialysis	1820	11.6	156	1.36	79.0
Sephacryl S-200	900	0.4	2142	18.6	39.0
Protease I Protease II	220	2.0	110	0.96	9.5
Protease II	520	1.4	371	3.20	22.6

was purified to 18.6-fold with specific activity of 2142 U/mg and recovery of 39%.

The Analysis of Proteins Using SDS-PAGE

SDS-PAGE examination of the obtained proteins after each purification step exhibited a prominent protein band with a molecular weight ranging from 50 to 55 kDa. This band was observed in the fractions pooled after Sephacryl S-200 HR gel filtration chromatography, indicating the presence of a partially purified form I protease, as shown in Figure 2.

Qualitative Cross-Reactivity by Western Blotting

The prepared antiserum showed maximum ELISA titer (1:5000) at the 7th week after injection. In Western blotting technique, the isolated (Sap) enzyme showed a single precipitin band, which confirmed its purity (Figure 3). The prepared antiserum showed strong reactivity with many protein bands ranging from ~55 to 50 kDa of some pathogenic *Candida* species, including *C. albicans, C. glabrata*, *C. tropicalis*, C. *dubliniesis* and *C. parapsilosis* but not to other proteases of non-pathogenic strains and commercial pepsin enzyme (Figure 3). This observation demonstrates that the proteases of tested pathogenic *Candida* strains are immunologically similar to the purified (Sap) enzyme.

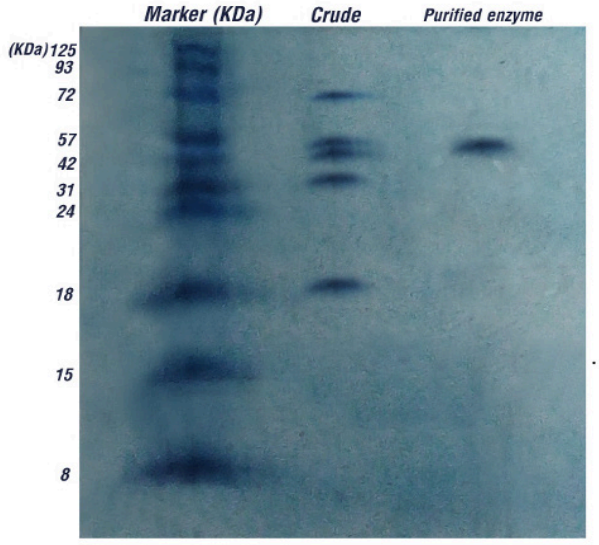

Figure 2. SDS-PAGE gel electrophoresis of partially purified protease from *C. albicans*. The separated bands were visualized after Coomassi brilliant blue R- 250 staining.

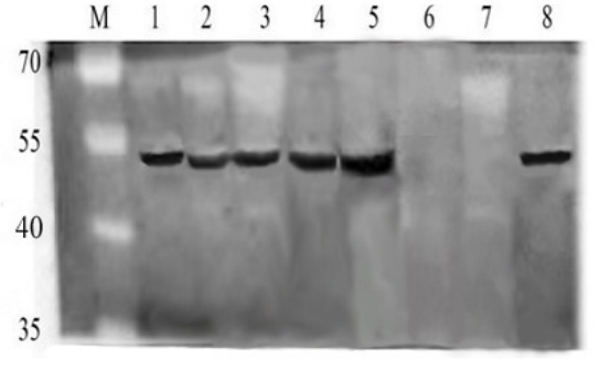

Figure 3. The immunoblot patterns of the purified (Sap) enzyme, pathogenic and non-pathogenic *Candida* species and other proteases as developed using the prepared rabbit anti-SAP at a dilution of 1: 400. The samples: (M) Molecular mass marker, (1) *C. albicans*, (2) *C. glabrata*, (3) *C. tropicalis*, (4) *C. dubliniesis*, (5) *C. parapsilosis*, (6) Non-pathogenic *Candida* species, (7) Commercial pepsin enzyme, (8) isolated (Sap) enzyme.

Quantitative Cross-Reactivity by ELISA

Low concentrations of the pathogenic and non-pathogenic *Candida* protease from 1 to 4 µg/100 µL were analyzed against the prepared antiserum at a dilution of 1:500, as presented in Figure 4 and Table 2. The prepared antiserum had a greater binding capacity to the pathogenic than the non-pathogenic *Candida* strains. Furthermore,

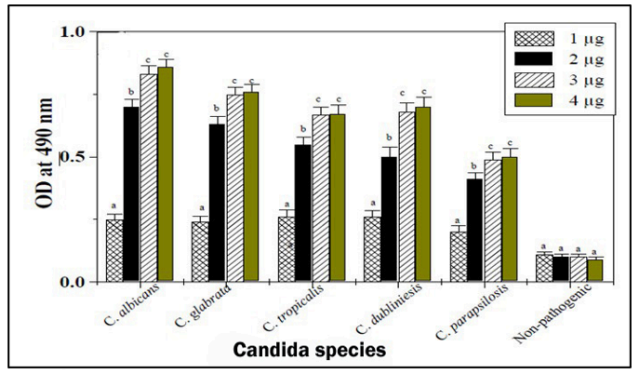

Figure 4. Effects of different concentrations of the pathogenic and non-pathogenic *Candida* strains (1-4 µg/mL) on the binding of the prepared anti (Sap1) antibodies at a dilution of 1: 500, as assayed by ELISA. Values with different superscript letters within the same species were significantly different at (p=0.017).

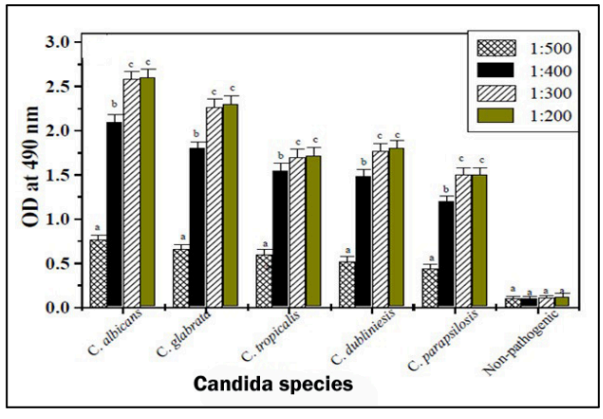

Figure 5. Effects of different dilutions of the prepared anti-(Sap) (1:200-1:500) on the pathogenic and non-pathogenic *Candida* strains at concentration of 2 µg, as assayed by ELISA. Values are presented as means ± SD (n=3). Values with different superscript letters within the same strain were significantly different at (p=0.009).

Table 2. ELISA results using 2 µg protein (prepared antibody) against different *Candida* protease enzyme protein concentration

Species	Different *Candida* protease enzyme protein concentration			
	1 µg/100 µL	2 µg/100 µL	3 µg/100 µL	4 µg/100 µL
C. albicans	0.250 ± 0.016	0.700 ± 0.011	0.860 ± 0.022	0.820 ± 0.027
C. glabrata	0.240 ± 0.014	0.630 ± 0.018	0.750 ± 0.016	0.760 ± 0.012
C. tropicalis	0.258 ± 0.009	0.518 ± 0.011	0.570 ± 0.019	0.570 ± 0.010
C. dubliniesis	0.260 ± 0.019	0.497 ± 0.032	0.580 ± 0.019	0.600 ± 0.011
C. parapcilosis	0.187 ± 0.002	0.412 ± 0.006	0.490 ± 0.011	0.480 ± 0.009
Non- pathogenic	0.120 ± 0.015	0.100 ± 0.012	0.100 ± 0.005	0.110 ± 0.006

Values are presented as means ± SD (n=3), SD: Standard deviation

Table 3. ELISA results using 2 µg *Candida* protease enzyme protein against different dilutions of prepared antibody

Species	Different dilutions of prepared antibody			
	(1:500)	(1:400)	(1:300)	(1:200)
C. albicans	1.125 ± 0.110	2.100 ± 0.078	2.580 ± 0.156	2.870 ± 0.191
C. glabrata	0.936 ± 0.098	1.890 ± 0.124	2.250 ± 0.110	2.280 ± 0.085
C. tropicalis	0.774 ± 0.065	1.554 ± 0.075	1.710 ± 0.130	1.710 ± 0.068
C. dubliniensis	0.650 ± 0.131	1.491 ± 0.222	1.740 ± 0.133	1.800 ± 0.079
C. parapcilosis	0.561 ± 0.015	1.236 ± 0.042	1.470 ± 0.077	1.440 ± 0.062
Non-pathogenic	0.360 ± 0.105	0.300 ± 0.085	0.300 ± 0.032	0.330 ± 0.042

Values are presented as means ± SD (n=3), SD: Standard deviation

the increase of coated pathogenic strains concentrations was significantly related to the increase in the binding affinity of the prepared anti (Sap1) antibodies (p=0.017) at concentrations of 1, 2, and 3 µg/mL. However, no change in the binding capacity of the prepared anti (Sap1) antibodies was noticed with increasing the non-pathogenic *Candida* strain concentrations.

Additionally, different dilutions of the prepared antiserum from 1:200 to 1:500 were tested against the pathogenic and non-pathogenic *Candida* strains at the concentration of 2 µg/mL (Figure 5 and Table 3). The results showed that the prepared antiserum reacted strongly with the pathogenic *Candida* strains even at high dilution of 1:500 but not to the non-pathogenic strain even at low dilution of 1:200, suggesting high degree of the specificity and sensitivity of the prepared anti (Sap1) for discrimination between the pathogenic and non-pathogenic *Candida* strains.

Discussion

Candida is a type of organism that typically exists as a commensal, but under certain circumstances, it can turn pathogenic, leading to opportunistic fungal infections (24). Interestingly, it has now emerged as the fourth most common cause of bloodstream infections acquired in healthcare settings (24).

In *C. albicans*, a variety of virulence factors have been identified, including Saps and phospholipases. Among these factors, Saps are considered to play a crucial role in the pathogenicity of *Candida* (25).

The objective of this study was to use the purified Sap from the *C. albicans* strains which exhibited the highest level of the enzyme among the examined species (19), in an attempt for the production of anti (Sap1) antiserum for possible use as an immunodiagnostic tool for invasive *Candida* infections.

The purification procedure involved 80% ammonium sulphate precipitation, which helped to improve protease purification and concentration. The recovery percent of forms I, II, and III was 39%, 9.5%, and 22.6% with a specific activity of 2142, 110, and 371 U/mg, respectively. These results were in line with the evidence that were published by Morrison et al. (26), who reported that total yield of purified aspartic protease from *C. albicans* recovered was 1295 UL^{-1} of culture medium.

Generally, the molecular mass of Sap1 to Sap10 proteins secreted from *Candida* species are between 35 and 50 kDa in size (7). In our study, the molecular weight of the partially purified form I protease produced from *C. albicans* was estimated to be 50 kDa by running on SDS-PEGE. These findings are consistent with the results reported by El Moudni et al. (27), who found that the native metallopeptidase enzyme of *C. albicans* had a molecular weight of 52 kDa as demonstrated by SDS-PAGE.

Invasive candidiasis has nonspecific symptoms which make the diagnosis more difficult and result in failure to respond to several antimicrobial agents (28). Besides, the traditional microbiological diagnostic methods lack sensitivity and specificity (28,29). Therefore, several studies have been carried out to establish precise serological techniques that enable the rapid and accurate diagnosis of invasive candidiasis (30,31).

Failure of detecting antibodies against *Candida* antigen in the serum of patients with systemic candidiasis was due to inability to produce antibodies in immunocompromised patients, or the serum was tested early before the production of antibodies. Moreover, healthy individuals may have high titer of antibodies against their commensal *Candida* (15). Consequently, numerous antibodies targeting *Candida* cell wall components have been developed, but none of them have demonstrated effectiveness in diagnosing *Candida* infections due to their low specificity and sensitivity (15). As a result, many attempts for the detection of the candida antigens in circulation by biochemical and immunological methods were previously reported (31).

Mannan and β-D-glucan, two cell wall antigens, appeared to be encouraging targets (32). Immune assays identifying mannan and anti-mannan antibodies were actually the first used tests with sensitivity and specificity not more than 55% and 65%, respectively, when both assays were used concurrently (32,33).

In terms of β-D-glucan detection, which is another component of *Candida* cell wall, it is employed as a diagnostic method for invasive candidiasis, offering a sensitivity of 92% and a specificity of 81% (34). However, a major drawback of such approach is the presence of β-D-glucan in some fungi other than *Candida*, such as *Aspergillus* spp. and *Pneumocystis jirovecii*, which raises concerns about its reliability in diagnosing *Candida* infections (35).

The production of aspartic protease is widespread among most pathogenic *Candida* strains, particularly *C. albicans*. It is considered one of the key contributors for the pathogenicity of these strains (36). The utilization of Sap as a marker for candidiasis provides an advantage in distinguishing between invasive disease and mere colonization. Therefore, the detection of Sap in the bloodstream can be indicative of an ongoing candida infection (17).

In the present study, the prepared anti (Sap1) showed a strong cross-reactivity to the five tested pathogenic *Candida* species strains and not to other non-pathogenic strain by ELISA as well as Western blotting techniques. However, it did not react with the non-pathogenic *Candida* strain. These findings align with the observation made by Zevenhoven-Dobbe et al. (37), who stated that polyclonal antisera, composed of a variety of immunoglobulin molecules originating from different B-cell lines, had the potential to recognize multiple epitopes of the target protein, making them a preferable choice over monoclonal antibodies.

Conclusion

This study could be useful for the production of efficient antiserum against immunogenic *C. albicans* (Sap) enzyme. In addition, the prepared anti (Sap1) antiserum could be of value as a diagnostic method for active candidiasis, and to some extent may be preferred over monoclonal antibodies as it may reduce both time and labor costs. Moreover, the cross reactivity with other *Candida* species helps to diagnose Candidiasis caused by the *Candida* species other than albicans. Using the prepared antiserum, the detection of Sap in patients' samples facilitated the early diagnosis of invasive candidiasis before microbiological results were obtained. However, large scale studies including more samples with different concentrations are needed to validate this method for general use.

Ethics

Ethics Committee Approval: All procedures involving the rabbits adhered to animal welfare regulations outlined in the Guide for the Care and Use of Laboratory Animals (http://oacu.od.nih.gov/regs/guide/guide.pdf) and received approval from the Ethical Committee of Tanta University, Faculty of Medicine, Egypt (approval no: 36264PR200/5/23, date: 13.05.2023).

Informed Consent: Informed consent from all patients were obtained.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: M.A.A., M.I.G., Concept: M.M.G., T.M.M., Design: T.M.M., Data Collection or Processing: M.A.A., M.M.G., Analysis or Interpretation: M.M.G., T.M.M., M.A.A., Literature Search: M.M.G., M.A.A., Writing: M.M.G., M.A.A.

Conflict of Interest: No conflict of interest was declared by the authors.

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