JOURNAL OF CLINICAL IMMUNOLOGY AND MICROBIOLOGY

E-ISSN: 3068-5168
Journal of Clinical Immunology & Microbiology

ATHENAEUM SCIENTIFIC PUBLISHERS

Research Article

Proteinase, Phospholipase, Hemolytic and Esterase Activities of *Candida spp.* Isolated from Pulmonary Tuberculosis Patients from Madhya Pradesh, Central India

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Citation: Yadu R, et al. Proteinase, Phospholipase, Hemolytic and Esterase Activities of *Candida Spp.* Isolated from Pulmonary Tuberculosis Patients from Madhya Pradesh, Central India. J Clin Immunol Microbiol. 2026;7(2):1-8.

https://doi.org/10.46889/JCIM.2026.7207

Received Date: 23-07-2026

Accepted Date: 15-08-2026

Published Date: 23-08-2026

Abstract

Over the past few years there has been a rapid rise in *Candida* infections accompanied with significant rise in morbidity and mortality. This study aimed to investigate the *in-vitro* production of virulence factors, such as proteinase, phospholipase, hemolytic and esterase activities in *Candida spp.* isolated from Pulmonary Tuberculosis patients from Madhya Pradesh, India. Thirty *Candida* isolates from 100 pulmonary tuberculosis patients were screened for enzymatic and hemolytic activies: *C. albicans* (17) and *C. parapsilosis* (13). Screening for enzymatic and hemolytic activities was done by YCB-BSA, Egg Yolk Plate, Tween 80 opacity test and glucose enriched blood agar medium for proteinase, phospholipase, esterase and hemolytic activity respectively. Amongst *C. parapsilosis* (n=13) 76.9% (n=10) showed proteinase activity while phospholipase, hemolytic and esterase activity was exhibited by 69.2% (n=9), 100% (n=13) and 69.2% (n=9) isolates respectively. Similarly, amongst *C. albicans* (n=17), proteinase, phospholipase, hemolytic and esterase activity was exhibited by 88.2% (n=15), 76.5% (n=13), 100% (n=17) and 47.1% (n= 8) respectively. A two tailed t test and X2 test was performed to statistically test the results A non-significant difference was observed between *C. albicans* and *C. parapsilosis* for various virulence traits studied. The X2 values obtained for proteinase, phospholipase, hemolytic and esterase activities were 0.679, 1.663,1.639 and 1.475 respectively at P>0.05.

Keywords: *Candida*; Pulmonary Tuberculosis; Proteinase; Phospholipase; Hemolytic; Esterase; Central India

Introduction

Pulmonary Tuberculosis (PTB) remains a major global health concern, particularly in developing countries such as India, where high disease burden and compromised host immunity predispose patients to secondary infections. Among these, opportunistic fungal pathogens especially species of *Candida* have emerged as significant contributors to morbidity. The prolonged use of broad-spectrum antibiotics, immunosuppression and structural lung damage in tuberculosis patients create a favorable niche for fungal colonization and subsequent infection [1-3].

Traditionally, *Candida albicans* has been regarded as the most predominant and virulent species responsible for candidiasis. However, in recent years, there has been a notable epidemiological shift toward non-albicans *Candida* non albicans species, including *Candida* parapsilosis, which are increasingly implicated in clinical infections. These species are no longer considered mere commensals but are recognized for their ability to cause invasive disease, particularly in immunocompromised individuals [4,5]. The growing prevalence of non albicans species, species underscores the need to better understand their pathogenic potential in comparison with *C. albicans*.

The pathogenicity of *Candida* species is multifactorial and largely attributed to the expression of various virulence factors that facilitate host colonization, tissue invasion and immune evasion. Among these, extracellular hydrolytic enzymes such as proteinases and phospholipases play a crucial role in degrading host cell membranes and proteins, thereby enhancing fungal penetration into host tissues [6,7]. Hemolytic activity enables the organism to acquire iron from host erythrocytes, an essential factor for survival and proliferation in iron-limited environments. Additionally, esterase activity contributes to lipid degradation and is associated with colonization and biofilm formation on host tissues and medical devices [8,9].

Materials and Method

Sample: The present study included 30 *Candida spp.* (*C. albicans* =17) and (*C. parapsilosis* = 13) isolated from Pulmonary tuberculosis patients (Yadu et. al. 2015). The *Candida spp.* Were subjected to various assays to analyze the hydrolytic enzymes secreted by them namely proteinase, phospholipase, hemolytic activity and esterase enzyme.

Determination of Proteinase Activity

Proteinase screening was performed according to Aoki, et al. [10]. Yeast Carbon Base (YCB) agar medium supplemented with 0.1% BSA was used to examine proteinase enzyme profile. Solutions of 11.7% YCB, 0.1% BSA was sterilized by filtration through a membrane filter of 0.2 μm pore size. To prepare 200 ml of medium 20 ml of the YCB solution was added to 160 ml of 1.88% melted agar at 56°C and then 20 ml of the BSA solutions were added to the mixture. About 20 ml portions of the media thus prepared were poured into Petri dishes; these media were referred to as YCB-BSA. A very small number of cells of each strain, grown on Sabouraud Dextrose Agar at 30°C for 3 days, were inoculated on the YCB-BSA agar plates with needles. After incubation at 30°C for 14 days, the plates were fixed with 10% Trichloroacetic Acid (TCA) for 2 hours and then stained with Coomassie brilliant blue G-250. Proteinase production was detected by the appearance of proteolytic zones around the colonies (Fig. 1). Proteinase activity was measured and calculated according to the method described by Price, et al., in terms of the ratio of the diameter of the colony and that of the colony plus the precipitation zone.

$$Pz = \frac{\text{Diameter of the colony (a)}}{\text{Diameter of colony (a) + precipitation zone (b)}}$$

The *Candida* isolates were grouped in 5 classes according to the value of their Pz coefficient: Pz ≥1 (-) negative, Pz between 0.9 and 1 (+), very high Pz group; 0.89-0.80 (++) high Pz groups; 0.79-0.70 (+++) low Pz group; and Pz minor ≤0.69 (++++). According to this system a lower Pz ratio responds to a higher enzyme activity (higher virulence). The strains exhibiting (-) and (+) Pz groups were considered negative in the study.

Determination of Phospholipase Activity

The phospholipase production by the *Candida* isolates were assayed according to the egg yolk agar plate method of Price, et al., Saboraud Dextrose Agar plates containing 1M Sodium Chloride, 0.005 M Calcium Chloride and 8% sterile egg yolk emulsion were used. Each strain was spot inoculated (approx 6 mm) in triplicates [11]. The Petri dishes were incubated at 37ºC and the diameters of the colonies and the colony plus precipitation zones (Fig. 2) were measured on 3-8 days after inoculation. Three separate samples of each strain were measured to obtain the average Pz reported.

Determination of Hemolysin Activity

Hemolysin activity was evaluated with a blood assay [12,13]. Media was prepared by adding 7 ml fresh sheep blood to 100 ml SDA (Sabouraud's Dextrose Agar) supplemented with glucose at a final concentration of 3% (w/v). The final pH of medium was 5.6 + 0.2. Standard inoculums of both the test and the control *Candida* isolates {10μl, with 108 yeast cells ml saline-1} was deposited

onto the medium. A further 10µl of saline but not the yeast cells were overlaid onto the same plate. The plates were then incubated at 37°C in 5% CO_2 for 48 hrs.

After incubation, plates were examined. The ratio of the diameter of the colony to that of the translucent zone of hemolysis (in mm) (Fig. 3) was used as the extent of Hemolysin activity (Hz) by different *Candida* isolates. The assay was conducted in duplicate on three separate occasions for each yeast isolate tested. A reference strain of *Candida albicans* served as positive control. In addition, one strain each of *Streptococcus pyogenes* and *Streptococcus sanguis*, which induce beta and alpha hemolysis, were used as positive control.

Determination of Esterase Activity

The Esterase activity was evaluated with a Tween 80 opacity medium [14]. The medium was prepared by adding 5 ml of pre-autoclaved and cooled Tween 80 (hi media). The final pH was adjusted up to 6.8. A loopful of overnight culture of each *Candida* isolate grown on SDA was transferred to the Tween 80 opacity medium and spread over a circular inoculation site of approximately 10mm diameter. The inoculated agar plates were incubated aerobically at 35°C and examined daily for up to 10 days. All strains were assayed in duplicates. Detection of esterase activity on the test plates was performed by observing halos of inoculums under transmitted light (Fig. 4).

Results

A total of 30 *Candida* isolates recovered from pulmonary tuberculosis patients were analyzed for the production of key virulence factors, including proteinase, phospholipase, hemolytic and esterase activities. The isolates comprised *Candida albicans* (n = 17) and *Candida* parapsilosis (n = 13). Among *C. albicans* (Table 1), proteinase activity was observed in 88.2% (15/17) of isolates, while phospholipase activity was detected in 76.5% (13/17). All isolates (100%) exhibited hemolytic activity, whereas esterase activity was comparatively lower, detected in 47.1% (8/17) of isolates. In contrast, *C. parapsilosis* (Table 2) demonstrated proteinase activity in 76.9% (10/13) of isolates and phospholipase activity in 69.2% (9/13). Similar to *C. albicans*, all isolates of *C. parapsilosis* 69.2% (9/13) showed hemolytic activity. Notably, esterase activity was higher in *C. parapsilosis*, observed in 69.2% (9/13) of isolates. Comparative analysis between the two species revealed that *C. albicans* exhibited relatively higher proteinase and phospholipase activities, whereas *C. parapsilosis* showed increased esterase activity. Hemolytic activity was uniformly present across both species, indicating a conserved virulence trait. Statistical evaluation using a two-tailed t-test and Chi-square (χ^2) test demonstrated no significant difference between *C. albicans* and *C. parapsilosis* with respect to any of the virulence factors studied ($P > 0.05$). The χ^2 values obtained for proteinase, phospholipase, hemolytic and esterase activities were 0.679, 1.663, 1.639 and 1.475, respectively, further confirming that the observed variations between species were not statistically significant.

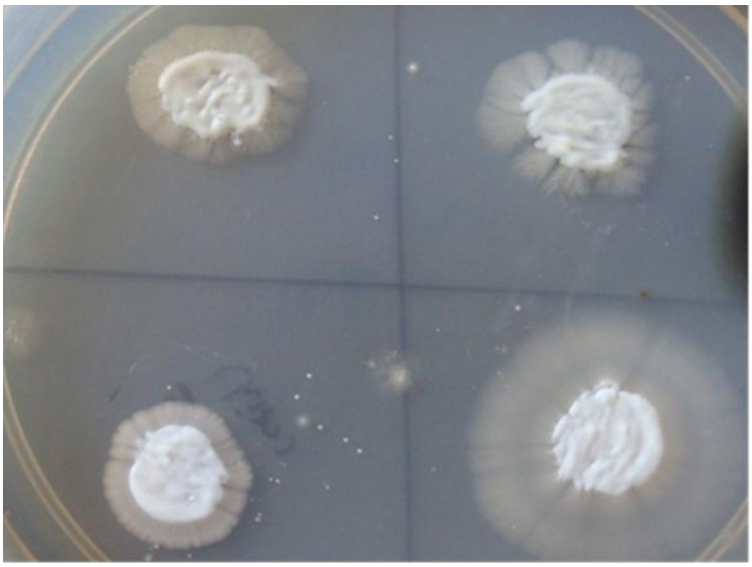

Figure 1: Showing Proteinase enzyme activity secreted by *C. albicans* strains isolated from Pulmonary Tuberculosis patients on YCB-BSA Agar plates.

Figure 2: Showing Phospholipase enzyme activity secreted by *C. albicans* strains isolated from Pulmonary Tuberculosis patients on Egg yolk agar medium.

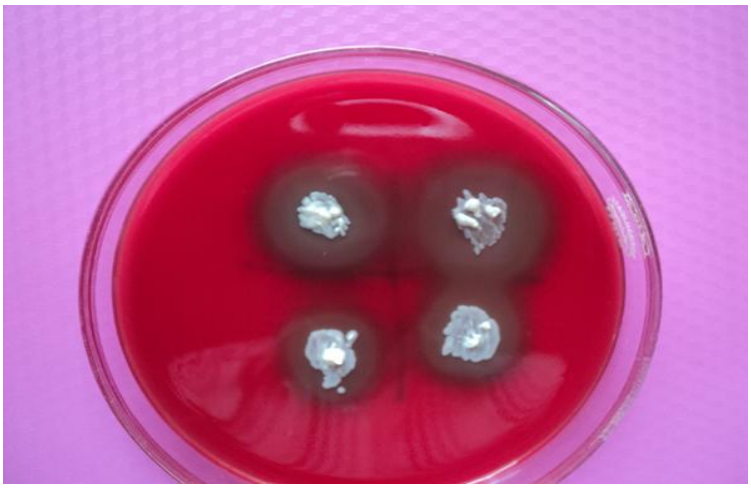

Figure 3: Showing Hemolytic activity of *C. albicans* strains isolated from Pulmonary Tuberculosis patients on Blood agar plates.

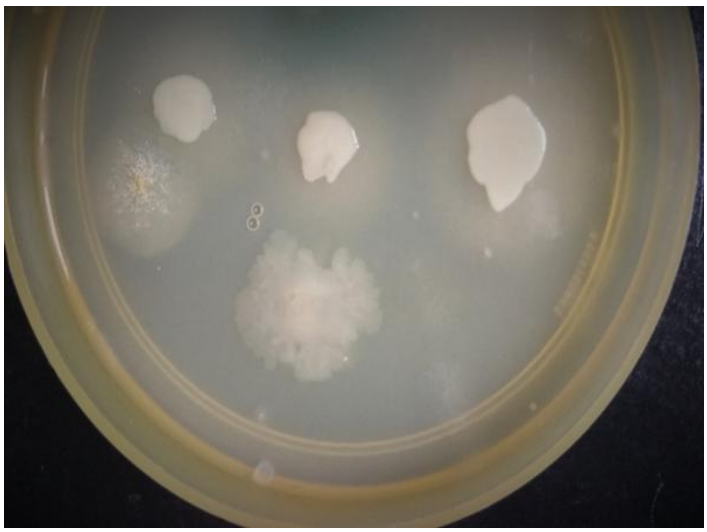

Figure 4: Showing Esterase enzyme activity of *C. albicans* strains isolated from Pulmonary Tuberculosis patients on Tween 80 opacity medium.

Enzyme	Positive	%	Pz Range	Avg Pz ± SD
Proteinase	15	88.2	0.51-1.2	0.67±0.1867

Phospholipase	15	88.2	0.49-1.2	0.70±0.2053
Hemolytic	15	88.2	0.33-0.9	0.49±0.1947
Esterase	8	47.1	0.47-1	0.84±0.1849

Table 1: Showing enzymatic profile of *C. albicans* (n=17).

Enzyme	Positive	%	Pz Range	Avg Pz ± SD
Proteinase	10	76.9	0.56-1.1	0.79±0.1793
Phospholipase	9	69.2	0.42-1.1	0.75±0.2148
Hemolytic	13	100	0.3-0.71	0.46±0.1303
Esterase	9	69.2	0.5-1	0.78-0.1754

Table 2: Showing enzymatic profile of *C. parapsilosis* (n=13).

Statistical Analysis

A two tailed t test and X^2 test was performed to statistically test the results A non-significant difference was observed between *C. albicans* and *C. parapsilosis* for various virulence traits studied. The X^2 values obtained for proteinase, phospholipase, hemolytic and esterase activities were 0.679, 1.663,1.639 and 1.475 respectively at $P>0.05$.

Discussion

The present study provides insights into the virulence attributes of *Candida* species isolated from pulmonary tuberculosis patients, emphasizing their potential role as opportunistic pathogens in immunocompromised individuals. The high prevalence of enzymatic virulence factors observed in both *Candida albicans* and *Candida* parapsilosis underscores their adaptive mechanisms for host invasion and persistence. Proteinase and phospholipase activities, which were more pronounced in *C. albicans*, are well-established determinants of pathogenicity, facilitating degradation of host proteins, disruption of epithelial barriers and evasion of immune defenses [15]. These findings are consistent with earlier and recent studies highlighting the dominant virulence potential of *C. albicans* due to its robust enzymatic arsenal and morphological plasticity [8,9,16].

However, the relatively high expression of these enzymes in *C. parapsilosis* observed in this study reinforces the growing recognition of non-albicans *Candida* species as clinically significant pathogens. Over the past decade, there has been a notable epidemiological shift toward non-albicans species, particularly in immunocompromised populations, including tuberculosis patients [4,5]. The comparable levels of proteinase and phospholipase activity between the two species, although not statistically significant, suggest that *C. parapsilosis* possesses considerable invasive potential, challenging the traditional perception of its lower virulence.

One of the most striking findings of this study was the universal presence of hemolytic activity in all isolates of both species. Hemolysins play a crucial role in iron acquisition, which is essential for fungal survival and proliferation within the host. The consistent expression of this virulence factor indicates that iron scavenging is a fundamental and conserved mechanism among *Candida* species. Recent studies have emphasized the importance of hemolytic activity in enhancing fungal fitness, particularly in host environments where free iron availability is limited [13]. This may be particularly relevant in pulmonary tuberculosis patients, where tissue damage and altered immune responses create a favorable niche for opportunistic fungal colonization.

Interestingly, esterase activity was found to be higher in *C. parapsilosis* compared to *C. albicans*. Esterases are associated with lipid metabolism and play a significant role in colonization, especially on medical devices and host tissues rich in lipids. This observation aligns with reports suggesting that *C. parapsilosis* exhibits enhanced biofilm-forming ability and surface adherence, particularly in nosocomial settings [5,17]. The increased esterase activity may therefore contribute to its persistence and pathogenicity, especially in chronic or device-associated infections.

Despite observable differences in the frequency of virulence factors, statistical analysis revealed no significant differences between *C. albicans* and *C. parapsilosis* ($P > 0.05$). This finding suggests that both species possess comparable pathogenic potential in the context of pulmonary tuberculosis. The lack of statistical significance may also be influenced by the relatively small sample size; however, it highlights an important clinical implication that non-albicans *Candida* species should not be underestimated in

terms of virulence. Similar observations have been reported in recent comparative studies, where differences in virulence traits among *Candida* species were found to be subtle and often influenced by host factors rather than species alone [18].

The coexistence of *Candida* species in pulmonary tuberculosis patients raises important concerns regarding disease progression and management. Tuberculosis-induced immunosuppression, along with prolonged antibiotic therapy, creates an environment conducive to fungal colonization and infection. Studies have demonstrated that fungal co-infections can complicate clinical outcomes, delay recovery and increase morbidity [1]. Therefore, the detection and characterization of virulence factors in *Candida* isolates from such patients are crucial for understanding their pathogenic role and guiding appropriate therapeutic strategies.

The findings of the present investigation are further supported by recent studies demonstrating that extracellular hydrolytic enzymes remain central to *Candida* pathogenicity across diverse clinical settings. A study on clinical isolates reported that proteinase and phospholipase production was significantly higher in *C. albicans* compared to non-albicans species, reinforcing the classical understanding of its dominant virulence profile [19]. However, the same study also highlighted that non-albicans *Candida* species exhibit substantial enzymatic activity, suggesting that they are equally capable of establishing infection under favorable host conditions. This aligns with the present study, where *C. parapsilosis* demonstrated considerable enzymatic activity, albeit with no statistically significant difference compared to *C. albicans*.

A similar trend has been observed in comparative studies evaluating multiple virulence determinants, including proteinase, phospholipase, hydrophobicity and biofilm formation. These studies report that although *C. albicans* often shows the highest enzymatic activity, a considerable proportion of non-albicans species also produce these enzymes at clinically relevant levels, contributing to their pathogenic potential [20]. This supports the present findings and further strengthens the argument that virulence is not species-restricted but rather multifactorial and influenced by both microbial and host determinants.

The results of the current study are also in close agreement with the work of Nawange, et al., who evaluated extracellular proteinase and phospholipase activities in Indian clinical isolates of *Candida* [21]. Their study demonstrated a significant association between enzyme production, source of isolation and antifungal susceptibility patterns, suggesting that higher enzymatic activity may correlate with increased pathogenicity and drug resistance. The comparable enzymatic profiles observed between *C. albicans* and non-albicans species in their study further validate the findings reported here, emphasizing the need to consider both groups in clinical diagnosis and management.

In addition to *Candida*, other pathogenic yeasts such as *Cryptococcus neoformans* have also been shown to produce extracellular enzymes contributing to virulence. A regional study by Gumasta, et al., reported the presence of extracellular protease and DNase activities in both clinical and environmental isolates of *Cryptococcus neoformans*, indicating that enzyme-mediated pathogenic mechanisms are conserved across different fungal genera [22]. This broader perspective highlights that enzyme production is a universal virulence strategy among pathogenic yeasts, reinforcing the biological significance of the findings in the present study.

Furthermore, studies on immunocompromised patient populations have consistently demonstrated that enzyme production is markedly higher in clinical isolates compared to commensal strains, suggesting a direct role in disease progression [23]. The elevated expression of proteinase and phospholipase in pathogenic isolates enhances tissue invasion, immune evasion and nutrient acquisition, particularly in compromised hosts such as tuberculosis patients. This observation is particularly relevant in the present study population, where underlying pulmonary pathology may facilitate fungal colonization and persistence.

The role of enzymatic virulence factors extends beyond invasion to include biofilm formation and surface adherence. It has been shown that hydrolytic enzyme production is often associated with increased biofilm-forming ability, which enhances resistance to antifungal agents and host immune responses [20]. In this context, the relatively higher esterase activity observed in *C. parapsilosis* in the present study may indicate its enhanced capacity for colonization and persistence, particularly on mucosal surfaces or medical devices.

Taken together, these findings highlight that while *C. albicans* remains a key pathogen, non-albicans *Candida* species such as *C. parapsilosis* are emerging as equally important opportunistic pathogens, possessing comparable virulence attributes [24]. The

absence of statistically significant differences in enzyme production between species in the present study further emphasizes that clinical management should not rely solely on species identification but must also consider functional virulence characteristics.

Overall, the findings of this study contribute to the growing body of evidence that both *C. albicans* and *C. parapsilosis* exhibit significant virulence capabilities, with no substantial difference in their pathogenic potential. This underscores the need for comprehensive diagnostic and surveillance approaches that consider both albicans and non-albicans *Candida* species in clinical settings. Future studies with larger sample sizes and molecular characterization of virulence genes would further enhance our understanding of the pathogenic mechanisms and epidemiology of *Candida* infections in tuberculosis patients [25,26].

Conclusion

The present study demonstrates that both *Candida albicans* and *Candida* parapsilosis isolates obtained from pulmonary tuberculosis patients possess a wide array of virulence factors, including proteinase, phospholipase, hemolytic and esterase activities, which are crucial for host invasion and survival. Although *C. albicans* exhibited relatively higher proteinase and phospholipase activity and *C. parapsilosis* showed greater esterase activity, statistical analysis revealed no significant difference between the two species ($P > 0.05$), indicating comparable pathogenic potential.

The universal expression of hemolytic activity among all isolates highlights the importance of iron acquisition mechanisms in the pathogenesis of *Candida* infections. The presence of multiple virulence traits in both species underscores their ability to act as opportunistic pathogens, particularly in immunocompromised conditions such as pulmonary tuberculosis. These findings emphasize that non-albicans *Candida* species, especially *C. parapsilosis*, should not be underestimated in clinical settings, as they exhibit virulence characteristics similar to *C. albicans*. Therefore, accurate identification and routine screening of virulence factors in *Candida* isolates are essential for effective clinical management. Further large-scale studies incorporating molecular approaches are recommended to better understand the epidemiology and pathogenic mechanisms of *Candida* infections in tuberculosis patients and to inform targeted therapeutic strategies.

Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

Funding Statement

The present research has been funded by Madhya Pradesh Council of Science and Technology as part of the Major Research Project.

Acknowledgement

The authors express their gratitude to Madhya Pradesh Council of Science and Technology for granting aid and also to management of respective colleges and Center for Medical Mycology for providing research facilities. The authors also express gratitude to Shri Arvind Kavishwar, Biostatistician (Retired), NIRTH, Jabalpur for assisting in statistical analysis of the study.

Data Availability Statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Ethical Statement

The project did not meet the definition of human subject research under the purview of the IRB according to federal regulations and therefore was exempt.

Informed Consent Statement

Informed consent was obtained from all participants included in the study.

Authors' Contributions

All authors contributed equally to this paper.

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