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Original Research Article

Detection of Secreted Aspartic Proteases (SAP) enzyme in the clinical isolates of *Candida* by Modified Stab Method

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ABSTRACT

Background: Mycoses has tremendously raised in the recent years, causing a wide range of infections in patients. Mycoses has occupied an important place during the COVID-19 pandemic. *Candida* species is one of the major pathogens known to mankind which is quite usually not reported. One of the most important hydrolytic enzymes which is responsible for its pathogenicity is Secreted Aspartic Protease enzyme which degrades many human proteins such as albumin, hemoglobin, keratin and secretory Immunoglobin A.
Aim: The aim of the study is to detect the presence of Secreted Aspartic Proteases (SAP) enzyme in the clinical isolates of *Candida* species.
Objectives: To isolate *Candida* species from clinical specimens. Phenotypic identification of *Candida* species. Detection of SAP enzyme by Modified Staib's Method. Clinical characterization based on the SAP enzyme production
Materials and Methods: Detecting the intensity of enzyme production by the organism helps in finding out the level of virulence exhibited which helps in clinically treating the patients with appropriate anti-fungal drugs designed to inhibit the enzyme. The detection of the Secreted Aspartic Protease enzyme was done by Modified Staib method using bovine serum albumin agar.
Result: Current study observed that 72 isolates of *Candida* species were cultured from various samples collected from the hospital setting and further testing was done to differentiate the species and to estimate their level of SAP enzyme production, out of which, 53 isolates (74%) were found to produce the enzyme in varied intensity levels,
Conclusion: The data of findings evolved from this study helps us to conclude that Secreted Aspartic Proteinase is one of the major virulence attributes of *Candida* species.

1. Introduction

Candida species are opportunistic pathogen and the most frequently isolated fungal pathogen known to cause approximately 80% of fungal infections such as oral

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thrush, cutaneous candidiasis, *candidal* vulvovaginitis etc.[1,2] *Candida* infection can vary from a minor infection such as oral thrush to fatal sepsis.[3,4] The pathogenesis of *Candida* species are multifactorial and the main virulence attributes of *Candida* include the ability to colonize and invade host mucosal layer, and secretion of hydrolytic enzymes. Even though the ability to cause disease is a complex process that involves numerous interactions between *Candida* and the host system, most research have reported and focused on the role of the Secreted Aspartyl Protease (SAP) has the virulence of the *Candida* species causing fatal infections have been reported to be associated with it.[5] Around 10 different SAP enzymes (SAP 1 – 10) were reported to be produced by *Candidaisolates*. Studies have concluded that proteinase enzymes has some functions such as nutrient acquistion, killing of cells of immune system to favour the resistance of antimicrobials, adhesion and invasion of tissue.[6,7] Extracellular proteolytic activity, first described by Staib enables *Candida* to use exogenous protein as the sole nitrogen source which helps in the presumptive identification of the enzyme production.[8,9] Secreted Aspartic Proteases (SAP), plays a major role in several infection stages and degrade many human proteins. Blocking of the stages helps in combatting the infection and to treat the infections efficiently.[10–12] The data of findings emerging out of this study helps us to determine proteinase secretion as a major virulence attribute which helps in curbing of the increasing incidence of infections caused by *Candida* species.[13–15] This study can lead to the development of novel drug therapies to neutralize the SAP enzyme effect, which can become a massive turnover in treating patients with various forms of infections caused by *Candida* species.[15,16]

2. Material and Methods

2.1. Research design

A cross sectional study was done for a period of six months. Continuous sampling method was used and 72 samples were collected during the period of March 2020 - August 2020 in the Clinical Microbiology Laboratory in Saveetha Medical College and Hospital, Chennai. The study was approved and ethically cleared by the Institutional Review Board (IRB) of SIMATS.

2.2. Sample selection and speciation

Samples collected from the various In-Patient Departments of Saveetha Medical College and Hospital which were sent to the laboratory for culturing were processed under standard protocols. *Candida* species isolated from those samples were selected for the study. The isolates were identified and speciated using automated system (VITEK 2 COMPACT) and manual methods such as germ tube test, CHROM agar *Candida* (HiMedia Laboratories, Chennai, Tamil Nadu) and sugar fermentation test.[17]

2.3. Proteinase detection

The Candida proteinase detection was done by Modified Staib method using Bovine Serum Albumin (BSA) agar.[18,19] Yeast Carbon Base agar (Sigma-Aldrich Chemicals, Banglore, Karnataka) which was prepared according to the manufacture's instruction and autoclaved. After cooling down to 50^0C, 1 ml of 1% Bovine Serum Albumin (Thermo Fisher Scientific, Bengaluru, Karnataka) solution was added to 100 ml of Yeast Carbon Base and poured into sterile petri plates. 10 μl aliquots of yeast cell suspension (approximately 10^8cells/ml) was added to the wells punched onto the surface of the BSA agar prepared and incubated at 37^0C for 48 hours. 20% trichloroacetic acid (HiMedia Laboratories) was used to fix the plates for 20 minutes. Then the plates were flooded with 1.25% amidoblack (Sigma-Aldrich Chemicals, Banglore, Karnataka) stain for 15 minutes. After 15 minutes, the stain was poured off and rinsed with sterile distilled water to remove the excess stain sticking onto the agar surface. Then the plates were decolorized using 15% glacial acetic acid (HiMedia Laboratories, Chennai, Tamil Nadu) for 2 hours, by replacing the glacial acetic acid at an interval of every 30 min. The plates were examined for proteolytic zones.[20] The diameter of the zones formed were measured. Then the proteolytic activity was calculated using the formula:

$$PZ = \frac{Diameter\ of\ the\ well}{Zone\ Diameter + Well\ Diameter}$$

When the calculated proteolytic zone (PZ) value is equal to 1 (PZ=1) then the test strain has no proteolytic activity evidenced. If the proteolytic zone value is less than or equal to 0.25 (PZ≤0.25) then strong proteolytic activity was detected and a value more than 0.25 & less than or equal to 0.50 (PZ= 0.25 to 0.50) means moderate level of proteolytic activity was detected. A proteolytic value of more than 0.50 & less than or equal to 0.99 (PZ=0.50 to 0.99) interprets that the test strain exhibits a weak proteolytic activity.[21]

3. Results

3.1. Distribution of candida species

Out of 72 samples collected during this study period, *Candida glabrata* (26.4%) was isolated many times, followed by *Candidatropicalis* (23.6%) & *Candida albicans* (23.6%) on the second position and then by *Candida* parapsilosis (16.7%) and *Candida krusei* (9.7%) in order. (Table 1)

Table 1: Distribution of candida species

Candida albicans	***Candida glabrat* a**	***Candida krusei***	***Candida parasilosis***	***Candida tropicalis***
17 (23.6%)	19 (26.4%)	7 (9.7%)	12(16.7%)	17 (23.6%)

3.2. Distribution of Candida species from various clinical samples

In this study, out of 72 *Candida* isolates, most of the *Candida* species were isolated from urine (51%), followed by the blood, endotracheal aspirate, broncho alveolar lavage, high vaginal swab, sputum, stool, pus, wound swab and at last ear swab. (Figure 1)

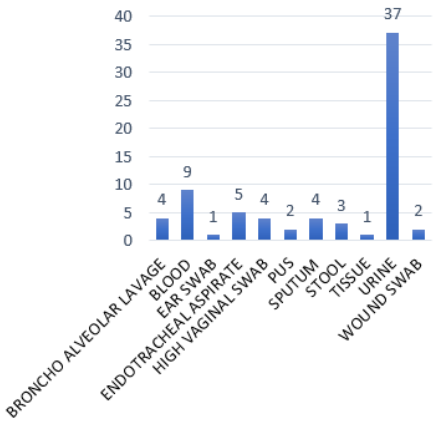

Figure 1: Sample wise distribution

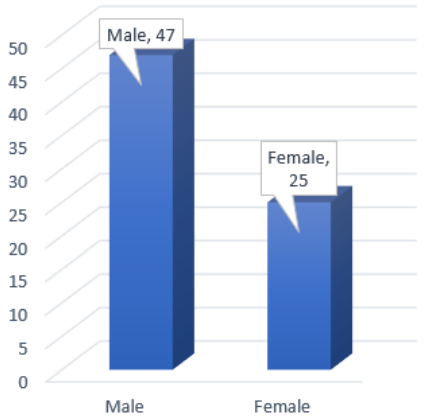

Figure 2: Gender wise distribution

3.3. Gender wise distribution

Out of 72 patients in this study, male patients (65%) were the highest to get infections caused by *Candida.* (Figure 2)

3.4. Age wise distribution

Above 50 years age group (49%) have the highest frequency to get culture positivity for *Candida* followed by the 30 to 50 years age group (28 %) and 1 to 30 years age group (23%).

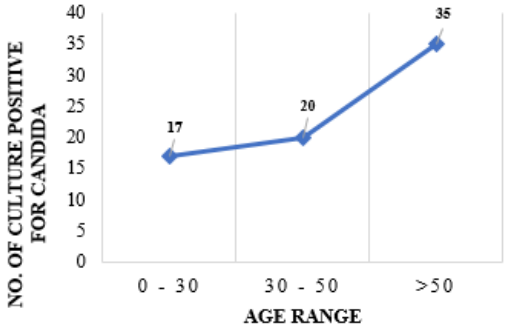

Figure 3: Age wise distribution

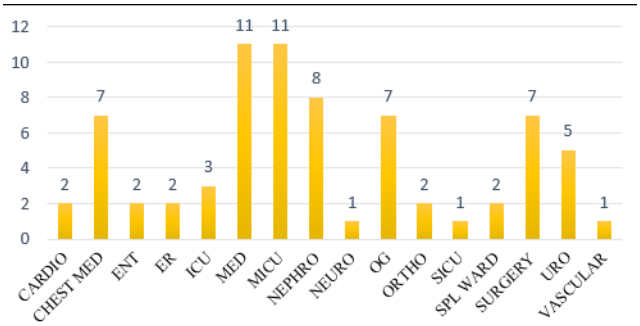

Figure 4: Ward wise distribution

3.5. Ward wise distribution

Candidal infection cases are highly seen in Medicine ICU and general ward (15%). Nephrology wards (11%) see the second highest number of cases, followed by the Surgery, Obstetrics Gynecology and the Chest Medicine (9%) in the third position. (Figure 4)

3.6. Distribution based on proteolytic activity

Out of the 72 isolates, 19 isolates (26%) were found to be negative and 53 isolates (74%) were found to be positive. In the 53 positive isolates 11 isolates (20%) were found to be strong positive, 29 isolates (55%) were found to be moderate positive and 13 isolates (25%) were found to be weak positive.

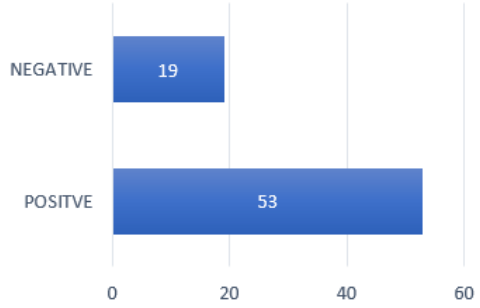

Figure 5: Proteolytic activity

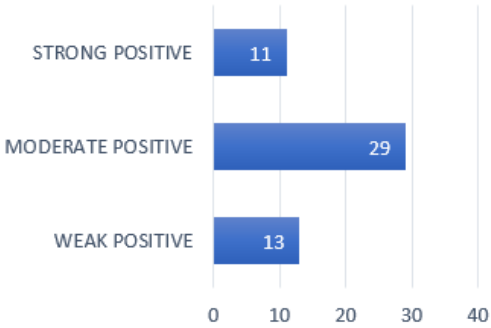

Figure 6: Proteolytic activity measure in positive samples

3.7. Distribution based on the proteolytic activity in species level

In this study, *Candida glabrata* (42%) has the highest number of negative strains followed by *Candida albicans* (26%). *Candida tropicalis* (26%) have the highest number of positive strains followed by the *Candida albicans* (23%). *Candida albicans* (46%) have the highest number of strong positive strains. *Candida tropicalis* (27%) have the highest number of moderate positive strains. *Candida glabrata* (31%) and *Candida tropicalis* (31%) have the highest number of weak positive strains.

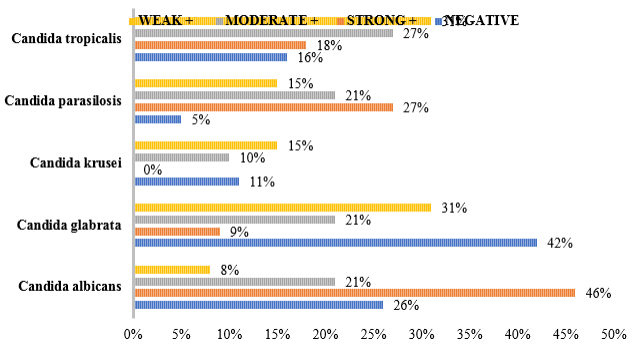

Figure 7: Proteolytic activity in species level

4. Discussion

A total of 72 *Candida* isolates were isolated from various sample over a period of three months, in the Clinical Microbiology Laboratory of Saveetha Medical College and Hospital, Chennai. In our study *Candida* species were isolated more from male patients 47 (65%) than in female patients 25 (35%). Among the 72 isolates, 23% of isolates were from the age group of 1 to 30 years, 28% of isolates were from the age group 30 to 50 years and 49% of the isolates were from above 50 years age group. Hence infection caused by *Candida* species were predominant in the older age group. Out of 72 isolates, 17 isolates were *Candida tropicalis* (24%), 17 isolates were *Candida albicans* (24%), 19 isolates were *Candida glabrata* (26%), 12 isolates were *Candida parapsilosis* (17%) and 7 isolates were *Candida krusei* (10%). *Candida glabrata* was isolated in a higher frequency than other species during the period of study.

A similar study was conducted by Vinitha Mohan Das et al. which reported that *Candida krusei* (39%) was highly isolated than other species from blood samples.[22] This is discordant with the present study. This discrepancy in highly isolated species may be considered as being relevant as the isolates were isolated only from blood samples in their study, whereas in our study the isolates were isolated from various clinical samples. *Candida* species were isolated from various clinical samples such as broncho alveolar lavage (5%), blood (13%), ear swab (1%), endotracheal aspirate (7%), high vaginal swab (5%), pus (3%), sputum (5%), stool (4%), tissue (1%), urine (51%) and wound swab (3%). This signifies that candiduric is more prevalent followed by candidemia. *Candida* infection cases were seen in higher amount in Medicine ward and ICU (15%) followed by the Surgery, Chest Medicine and Obstetrics & Gynecology departments (9%).Out of 72 isolates, 53 isolates were found to produce SAP enzyme (74%) and 19 isolates were non-SAP enzyme producers (26%). Among the 53 positive isolates, 11 isolates were strong positive (20%), 29 isolates were moderate positive (55%) and 13 isolates were weak positive (25%). *Candida tropicalis* isolates shows higher rate of positivity for SAP enzyme in the present study.

The results of positivity for SAP enzyme production were found to be concordant with the findings of Mohan das V et al, (74.56%) for proteinase production.[23]

Santosh Patil et al. study demonstrates that *Candida albicans* shows a higher rate of positivity (88.23%) which is discordant to the present study due to the difference in the number of the species tested.[24]

5. Conclusion

The data of findings evolved from this study helps us to conclude that Secreted Aspartic Proteinase is one of the

major virulence attributes of *Candida* species. Out of the 72 clinical isolates obtained about 74% of the isolates were tested positive for SAP production which indicates its pathogenicity nature and it is known to cause a greater number of urinary tract infections (51%) followed by blood stream infections. Further research on SAP enzyme, helps us to develop novel drugs which can be used as a potent inhibitor of SAP thus curbing majority of *Candida* related mycoses.[25]

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The authors declare that no funds or grants or external support were received during the study.

7. Conflict of Interest

The authors confirms that there is no relevant financial or non-financial information to disclose.

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