

Research Article

Candidemia: A Hospital Based Study in Haematological Patients in Adult Population of Northern India

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Abstract

Background: Candidemia has become a common cause of fungal infection in bloodstream infection throughout the world. In hematological malignancies, patients have high rate of morbidity and mortality due to Candidemia. **Objective:** Early diagnosis and specific identification of Candida species in hematological patients is main purpose of the proposed study.

Methods: In present study we enrolled patients with hematological malignancies (duration November 2018- April 2022). For early diagnosis of Candidemia we did conventional method, antigen detection and PCR. We compared the conventional, automated, antigen detection test and PCR method for diagnosis of Candidemia and then we performed antifungal susceptibility testing for treatment in hematological patients.

Results: Total 229 patients were enrolled on the basis of febrile neutropenia. Total 152/229 male (66.37%) and 77/229 (33.62%) were participated in present study followed by age range from 10- 77 years (Mean=33.65, Standard deviation=16.40). Prevalence of the Candidemia was 2.1 in the present study. Five patients (2.6%) were positive from blood culture and nine patients (4.8%) were positive by PCR. Among 4 patients of AML, there were 3 Candida troicalis (3; 1.60%) and 1 Candida auris (1; 0.5%) were present followed by 1 patient of pancytopenia Candida tropicalis (1; 0.5%) present.

Conclusion: This present observational study recognizes main association of Candidemia in hematological malignancies. Automated methods are more sensitive and specific for species identification. We try to apply Non culture method like PCR and mannan antigen in routine laboratories to diagnose early stage Candidemia infection for better treatment to cure the disease.

Keywords: Candidemia; Diagnosis; Hematological Patients; Polymerase Chain Reaction; Treatment

Abbreviations

PCR: Polymerase Chain Reaction; Mn: Mannan antigen; A-Mn: Anti-Mannan antibodies; ECIL: European Conference on Infections in Leukemia; DMSO: Dimethyl Sulfoxide; MALDI-TOF: Matrix-Assisted Laser Desorption/Ionization; CLSI: Clinical Laboratory Standards Institute

Introduction

Candida is well-known, potentially pathogenic yeast and it is a frequent cause of nosocomial bloodstream infection in humans. It may lead to fungal infection, that have been associated with the prevalent cause of hospital infection [1-2]. In several nations, Candidemia (or bloodstream infection with Candida spp.) is among the top five illnesses acquired from the hospitals [3-7]. Due

to their relationship with high mortality rate and enormous medical expenses for governments and patients with fungus infection are gaining attention in the medical field. Increasing incidences of invasive systemic infections and septicemia, especially in immunocompromised patients, are accountable for the rise in fatality rates [8-10]. The geographical and temporal diversity of India influences the prevalence of different *Candida* species [11]. Among many *Candida* species, *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis* and *C. krusei* are all capable of infecting healthy hosts. When a person's immune system is compromised, these microorganisms can cause severe systemic diseases. Due to the increasing number of immunocompromised patients, the quick development of new illness medicines has heightened the risk of spreading Candidiasis. *C. albicans* is the most common cause of Candidemia, despite the fact that other *Candida* species are responsible for over 50% of bloodstream infection in certain region of the world [12]. This is because these people are more likely to have undergone surgery, spent time in critical care units, or been treated with broad-spectrum antibiotics [8,11,13,14]. In Japan (2009) and India (2013), multidrug-resistant *C. auris* was first discovered [15]. In clinical research laboratory to get an accurate diagnosis and effective treatment for *Non-Albican Candida* (NAC) species using standard method is a significant barrier [16]. There has been evidence of resistance to a number of drugs, including azoles, echinocandins, and amphotericin B. The Indian Council of Medical Research (ICMR) in 2017 emphasized the necessity of active surveillance of *C. auris* infection in Indian institutions [17]. *C. tropicalis* has emerged as a prominent agent of nosocomial infection. *C. auris* causes candidemia 40% more often than *C. albican* [18]. With *C. tropicalis* dominant, a diverse array of pathogenic agents was discovered, showing the growing pathogenic potential of NAC species. The majority of cases in India are attributable to *C. tropicalis*, but outbreaks caused by previously unidentified pathogens such as *Kodamaea ohmeri* and *Pichia anomala* have also been documented [19,20]. Echinocandins are now strongly advised for use as first-line drugs for the treatment of Candidemia regardless of primary predisposing factors [21].

Non-cultured diagnostic methods are considerably used for the identification of invasive Candidiasis which is used for routine laboratories. In previous studies, serological test, β -D glucan, Mannan antigen (Mn), Anti-Mannan antibodies (A-Mn), enolase, and arabinitol were used for the diagnosis of Invasive Candidiasis. Cell walls of *C. albican* have Mn consisting of 7% of the cell dry weight and circulating *Candida* antigen during infection [22]. The 28S rDNA region of the large ribosomal subunit (LSUD1/D2) was the target of the oligonucleotide primers NL-1 and NL-4 [24]. In this region, fungal species are exceptionally well protected. Primers P4501 and P4502 recognize the target *Candida* species' P-450 lanosterol 14-demethylase gene, according to previous research [25]. Healthcare facilities are rampant with the fungus *C. albicans*, which regularly colonizes human skin, the oral cavity, and the vagina. A patient's immune system is weakened and made more vulnerable to infection by factors like prolonged hospital stays excessive use of broad-spectrum antibiotics and chemotherapy for cancer patients, diabetes, and surgery. Patients who have central venous catheters and neutropenia are more likely to develop blood fungus infection [26]. The European Conference on Infections in Leukemia (ECIL) gives guidelines regarding diagnostic procedures and treatment therapy strategies in hematological patients [21]. The prevalence of Candidemia has increased along with the development of immunosuppressive medications and surgical techniques. Due to the absence of standardization of molecular techniques in routine laboratories, Candidemia still has a significant mortality rate (35-60%) despite medical breakthroughs. We are seeking to address the issue of diagnosing Candidemia from blood in cancer patients by contrasting the conventional method and the molecular way. The PCR approach of directly identifying *Candida* species from blood which take less time to diagnose can help to reach this goal. The mortality rate of patients with Candidemia can be reduced with early detection and treatment with a specific antifungal medicine. Finding out how prevalent Candidemia is among patients who have been admitted to the Clinical Hematology department is the aim of this study.

Methods

This prospective observational study was carried out in the department of Microbiology, King George's Medical University, Lucknow (UP), India. A total of 187 hematological malignancy patients were recruited and the blood samples were collected. This study was carried out from November 2018 to October 2021. This study was approved in 2018 by the Institutional Ethics Committee of King George's Medical University, Lucknow, Uttar Pradesh, INDIA (letter no. 223/Ethics/R. Cell-18).

a. Inclusion criteria

1. ≥ 15 years
2. Suffering from high-grade fever or with febrile neutropenia (<500 neutrophils/mm²) (3) not responding to broad-spectrum antibiotics for 5 days

b. Exclusion criteria

1. Patients received chemotherapy
2. Already used an antifungal prophylaxis course

Blood Sample Collection

Whole blood sample were withdrawn from patients with their informed consent and collected 2 ml in EDTA vial form DNA extraction and 8-10 ml of blood sample BACTECTM Mycosis IC/F blood culture bottles for convention method (Becton, Dickinson, and Company Sparks, MD 21152 USA and Benex Limited Dun Laoghaire, Ireland).

Culture bottles were incubated in BD BACTEC 9120 machine (Becton Dickinson) for 15 days unless the machine shows a positive or negative signal. Positive blood Samples were cultured on Mackonky (Himedia) and coulambia agar (5% sheep blood agar) [BIOMERIUX] plates. Plates were incubated overnight at 37°C. All isolates were identified by using microscopy, Germ Tube Test CHROMagar (Himedia, Mumbai, India), Corn meal agar (Hyphae formation) and Sugar assimilation. Blood culture was considered as negative for *Candida* after 15 days of incubation. Species Identification of *Candida* was also confirmed by Matrix-Assisted Laser Desorption/Ionization (MALDI-TOF) Mass Spectrometry (MS) Biotype CA System (Bruker DaltonicK GmbH, Germany). All *Candida* isolates were stored at -80°C in 50% glycerol till further use.

Antifungal Drugs and Stock Solution

The testing of antifungal susceptibility was performed for voriconazole (Sigma Aldrich, St. Louis), Amphoterecin B (Sigma Aldrich), Fluconazole (Sigma Aldrich), Caspofungin (Sigma Aldrich) powder. A stock solution of 1600 µg/ml was prepared in Dimethyl Sulfoxide (DMSO) for all drugs and stored at -80°C till further use. The sensitivity was done according to the Clinical Laboratory Standards Institute (CLSI) guideline M27-A3 reference document for broth microdilution [23]. Antifungal susceptibility was also done with Vitek®MS (bioMerieux Inc.).

Serological Test: Sample was collected in is a sterile plain vial. Mn Ag was measured for each sample using Platelia™ *Candida* Ag Plus immunoenzymatic assay kit (Bio Rad, Platelia, Marnes, La Coquette, France). Experiment was performed according to the manufacturer's instructions. Results were calculated as sample with concentrations that are equal or greater than 125 pg/ml considered as positive, less than 62.5 pg/ml considered as Negative and Sample with concentrations between 62.5 and 125 pg/ml Intermediate.

Molecular Methods for Diagnosis of Candidemia

DNA extraction: 2 ml blood sample were withdrawn from patient immediately prior to the antifungal administration. DNA extraction was performed from Whole blood collected in EDTA vials (2 ml). DNA extraction was done according to manufacturer guidelines of the Qiagen DNA extraction kit. The above steps were performed in Laminar Air Flow (Science TECH, INDIA) Extracted DNA was stored at -20°C (VESTFROST SOLUTIONS) for amplification. DNA concentration was checked at 260-280 nm in Bio Spectrometer (Eppendorf, Germany).

Polymerase Chain Reaction (PCR)

1st Reaction-The primer forward-NL-1(5' GCATATCAATAAGCGGAGGAAAAG 3' and reverse - NL-4 (5' GGTCCGTGTTTCAAGACGG 3' (Eurofins Genomics India Pvt. Ltd.) were used, included D1/D2 region of 28S rDNA of large ribosomal subunit [21]. PCR was performed on Bio Rad C1000 touch thermal cycler in a total volume of 25µl by using a 2 x PCR master mix (Thermo Scientific, Wilmington, DE). PCR conditions with an initial denaturation at 95°C for 5 min, denaturation at 94°C for 1 min; annealing at 55°C for 30 sec and extension at 72°C for 2 min followed by 34 cycles and final extension 72°C for 5 min. PCR product was electrophoreses in an agarose gel (1.5%) for 1 h at 70 V at room temperature in TBE (Tris Borate EDTA) buffer, the gel was stained with Ethidium bromide and the bands were visualized in the gel documentation system (Bio-Rad). PCR product size was compared directly with a 100-basepairs molecular size marker (Next Gen, Genetix, India).

2nd Reaction- Positive samples from reaction 1st were further processed further. The primer forward-P45015'ATGACTGATCAAGAAATYGCTAA3' and reverse-P4502 5'TAACCTGGAGAAACYAAAAC 3' (Eurofins Genomics

India Pvt. Ltd.) were used in the current study including genus *Candida* specific for P-450 lanosterol 14 α -demethylase gene [25]. PCR was performed in a total 25 μ l volume of reaction by 2 X Master Mix (Thermo Scientific, Wilmington, DE) and PCR conditions were performed on Bio-Rad C1000 touch thermal cycler with an initial denaturation at 94°C for 3 min, denaturation at 94°C for 40 sec; annealing at 59°C for 1 min, extension at 72°C for 1 min for 34 cycles final extension at 72°C for 1 min 30 sec. Both reactions were performed ESCO PCR Cabinet (ISOCIDETM, Singapore). PCR product was electrophoreses in an agarose gel (1.5%) for 1 h at 70 V at room temperature in TBE (Tris Borate EDTA) buffer, the gel was stained with Ethidium bromide and the bands were visualized in the gel documentation system (Bio-Rad). PCR product size was compared directly with a 100-basepair molecular size marker (Next Gen, Genetix, India).

Statistical Analysis

The Chi-square test of independence was used for data analysis to compare and test the association between data. All the data were analyzed at a significant value of less than 0.05 (two-tailed). The IBM SPSS Statistics software version 21 was used for all of the analyses (IBM Corp. Armonk, N.Y., USA).

Results

A total of 229 patients suspected of Candidemia with different hematological malignancies were enrolled in this study. Out of 229 patients, 152 were male and 77 were female ranging from 10 years to 77 years. The mean ($\pm$ SD) age of the study group was 33.65 ($\pm$ 16.40) and the mean neutrophil count was 4.85 (4.19). All patients had received broad-range antibiotics for more than 72 hrs but did not respond to antibiotics.

We found 5 positive candida patients during this study from November 2018 to April 2022. Most common disease was AML 121 (52.8%), ALL 57 (24.8%) followed by aplastic anemia 19 (8.2%), Non-Hodgkin's Lymphoma 10 (4.3%), Multiple Myeloma 6 (2.6%) and pancytopenia 7 (3.0%) and 7 (3.0%) other hematological malignancies. The patient's shows symptoms like cough, chest pain, Pleural rub, weakness, Body ache, breathlessness, dyspnea, vomiting, and hemoptysis with hematological malignancies [Fig. 1-3].

5 (2.1%) patients' sample was positive for blood culture (Table 1). *Candida* was positive in 11 (4.8%) patients detected by polymerase chain reaction including 5 patients with positive blood culture. 13 (5.6%) serum samples were showing positive results for Mn Ag value. In those 5 patients, Candidemia was confirmed by blood culture and non-culture method. The Demographical data, clinical assessment and outcome interpreted of total 13 patients are shown in Table 2 and 3.

Budding yeast was seen under microscopy. For species identification dalamau technique, CCA, and sugar assimilation was performed. *C. tropicalis* (4; 2.1%) was the most common species followed by *C. auris* (1; 0.5%) in the blood of Cancer patients. *C. tropicalis* was identified in 24-48 by producing color on Candida Chrome agar. Sugar assimilation and CMA was done to identify other non-albican candida species like *C. auris* were took 48-72 hrs. A conventional phenotypic method was also confirmed by the Maldi-TOF.

Antifungal susceptibility was performed through the broth microdilution method for *C. tropicalis* and *C. auris* [1,4]. Four isolates of *Candida tropicalis* were sensitive for drug VRC (80%), AMP (80%), FLU (60%), CAS (80%) and resistant pattern for FLU (20%). *Candida auris* was resistant towards AMP (20%), FLU (20%) and sensitive for CAS (20%). 4 isolates of *C. tropicalis* were sensitive to drug VRC (MIC range- 0.125 μ g/ml), AMP (MIC range- 1 μ g/ml), FLU (MIC range- 1 μ g/ml), CAS (MIC range- 0.25 μ g/ml) and 1 resistant pattern towards FLU (MIC range- 4 μ g/ml). *C. auris* was resistant towards AMP (MIC range- 2 μ g/ml), FLU (MIC range- 64 μ g/ml) and sensitive towards CAS (MIC range- 0.5 μ g/ml). MIC results were read after 24 hrs. Conventional methods give final results minimum of 5-7 days after the blood culture was positive.

Antifungal susceptibility and species identification were also confirmed by with Vitek® MS (bioMerieux Inc.). The automated method for diagnosis of Candidemia, Maldi- TOF, and Vitek® MS (24 hrs) was less time-consuming rather than conventional methods.

The mean ($\pm$ SD) age of the PCR, Mn Ag and blood culture positive for candidemia was 37.91 (16.78) years, 39.00 (17.03) years and 40.80 (18.18) years respectively. Blood culture positive variables are significantly (p -value < 0.05) associated with each other PCR-positive patients and Mn Ag. 11 Out of 229 patients' blood was positive for fungal infection, primer NL-1 and NL-4 were able to amplify the D1/D2 region of 28S r- DNA of large ribosomal subunit which target rDNA yielding Product of PCR give 600 bp related to all fungus (Fig. 1). 5 out of 11 patients' blood was positive for candida specific Primer P4501 and P4502 identify the P-450 lanosterol 14 α -demethylase gene which is target Candida species genes single band of around 350 bp [Fig. 2]. 13 patients were positive for Mannan antigen for candida. The sensitivity and specificity of PCR 100% and 97.3% and Mannan Ag was 100% and 96.4% respectively. In combination sensitivity and specificity of blood culture and PCR 55.5% (95% CI: 21.20%- 86.30%) and 100% (95% CI: 97.9%- 100 %). In current study higher sensitivity 84.6% was obtained in combination of PCR and Mn Ag.

Identification of Candidemia by the conventional method was 100% accurate when correlate with the non-culture methods. Conventional methods are time taking and need an expert in mycology to do all tests. There was a difference in time taken by conventional, automated, and molecular methods Table 4.

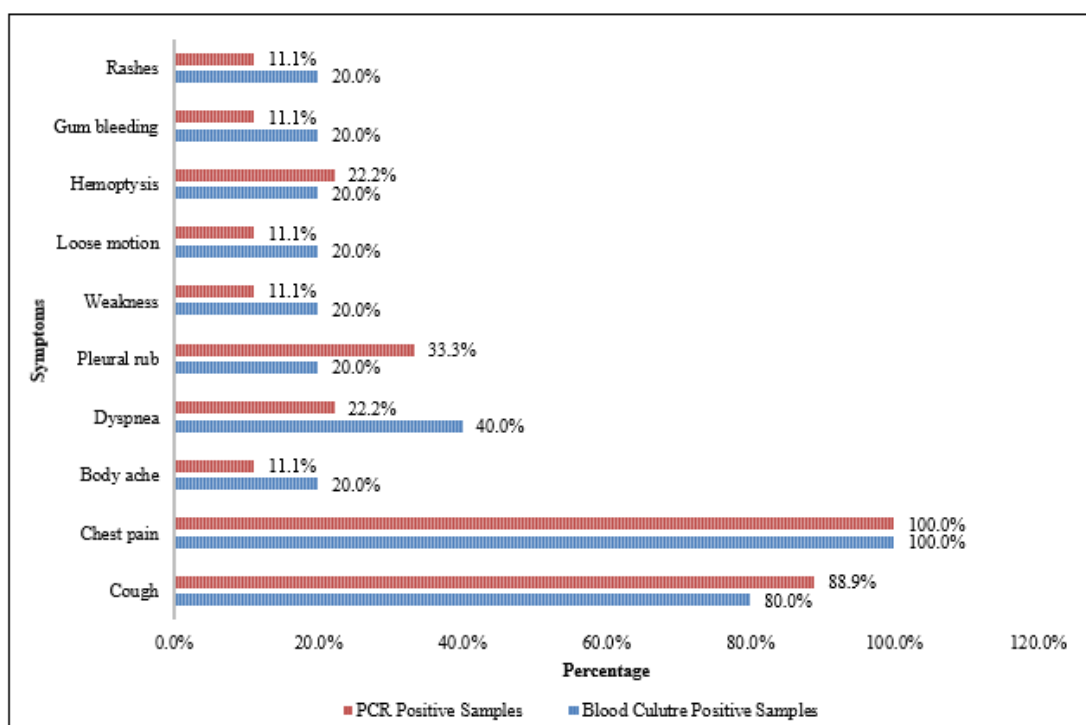


Figure 1: Clinical Features and association with Candidemia.

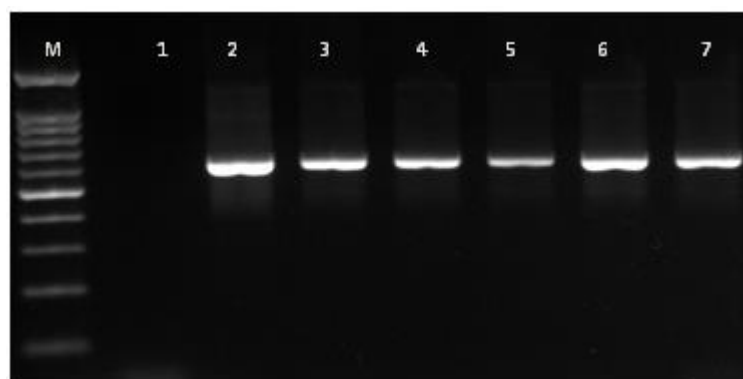


Figure 2: Lane M showing 100 bp DNA Ladder, lane 1 as a negative control, lane 2 showing 600 bp amplified PCR product of fungus DNA, Lane 3,4,5,6,7 showing 600 bp amplified PCR product of fungal DNA.

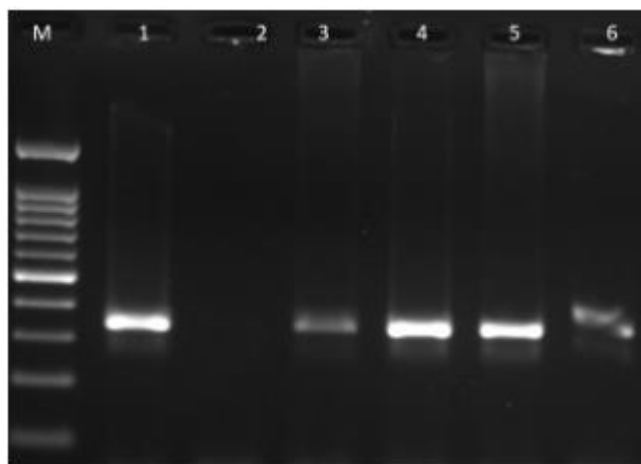


Figure 3: Lane M showing 100 bp DNA Ladder, lane 1 showing 350 bp amplified PCR product of Candida, Lane 2 showing as a negative control. Lane 3,4,5,6 showing 350 bp amplified PCR product of Candida.

Characteristic	Total (n=229)	Possible IFD (n=13)	%	Probable IFD (n=220)	%	Proven IFD (n=5)	%
Age, median (year)							
Malignancy							
Multiple myeloma	06	01	7.69	6	2.72	01	20
Pancytopenia	07	01	7.69	7	3.18	00	00
Aplastic Anemia	19	00	00	19	8.63	00	00
Immune Thrombocytopenia purprra	01	00	00	1	0.45	00	00
CML with Blast	01	00	00	1	0.45	00	00
Philadelphia acute leukemia	01	00	00	1	0.45	00	00
Hematologic Malignancy							
ALL	57	03	23.07	54	24.54	00	00
AML	121	6	46.15	115	52.27	04	80
Non Hodgkins Lymphoma	10	00	00	10	4.45	00	00
Myelodysplastic Syndrome	02	00	00	2	0.90	00	00
Auto immune hemolytic anemia	01	00	00	1	0.45	00	00
Mantle Cell lymphoma	01	00	00	1	0.45	00	00
Hemophilia A	01	00	00	1	0.45	00	00
Hodgkins Lymphoma	01	00	00	1	0.45	00	00

Table 1: Demographic profile of underlying disease of hematological patients enrolled in the study.

S. No.	Age/Sex	Type of Cancer	Clinical Presentation	Culture	PCR Results	Mn Ag Results	Specimen	Antifungal Treatment	Outcome
1.	65Y/M	Multiple myeloma	Pleural rub, lesion in spleen	<i>Candida tropicalis</i>	Positive	1.2	Blood	AmphoterecinB	Death
2.	45Y/M	AML	Chest pain	<i>Candida tropicalis</i>	Positive	0.38	Blood	Posaconazoles	Discharge
3.	40Y/M	AML	Fever, Chest Pain, Cough,	<i>Candida tropicalis</i>	Positive	1.2	Blood	Posaconazole	Death
4.	40Y/M	AML	Pleural rub, Chest Pain, Cough	<i>Candida tropicalis</i>	Positive	0.4	Blood	AmphoterecinB	Death

5.	14Y/F	AML	Cough, fever, chest pain	<i>Candida auris</i>	Positive	1.3	Blood	Caspofungin	Death
PCR are polymerase chain reaction. MnAg values are in pg/ml.									

Table 2: Characteristic of hematological patients with blood culture positive for *Candida* spp.

S. No.	Age/Sex	Type of Cancer	Clinical Presentation	Blood Culture	PCR Results	Mn Ag Results	Specimen	Antifungal Treatment	Outcome
1.	17Y/M	AML	Chest pain, Cough	Negative	Positive	0.321	Blood	Caspofungin	Death
2.	26Y/M	T- ALL	Pleural rub	Negative	Positive	0.901	Blood	Lipo-AmphoB	Death
3.	27Y/F	AML	Cough	Negative	Positive	0.289	Blood	Lipo-Ampho	Discharge
4.	65Y/M	B-ALL	Fever	Negative	Positive	1.246	Blood	Lipo- Amhoterecin	Death
5.	36Y/F	ALL	Fever	Negative	Positive	0.299	Blood	Posaconazole	Discharge
6.	42Y/F	Pancytopenia	Febrile Neutropenia	Negative	Positive	2.416	Blood	AmphoB	Death
7.	62 Y/M	AML	Fever	Negative	Negative	0.288	Blood	Posaconazole	Death
8.	28Y/F	AML	Fever, cough	Negative	Negative	0.298	Blood	Voriconazole	Discharge
MnAg values are in pg/ml. AML: Acute Myeloid leukemia									

Table 3: Characteristic of patients with PCR and Mannan Ag positive for IFIS.

Test	Sensitivity	Specificity	PPV	NPV
Blood culture Mn Ag assay	38.46	100	100	96.43
Blood Culture and PCR	45.45	100	100	97.32
PCR and Mannan Antigen Assay	84.62	100	100	99.08

Table 4: Sensitivity, specificity, positive predictive value and negative predictive values of blood culture, mannan Ag assay and conventional PCR in combination for the diagnosis of IFIs.

Discussion and Conclusion

Candidemia is the fourth most common cause of all hospital-based blood stream infections which mainly affects immunocompromised patients [27]. Immediate commencement of suitable antifungal treatment is required for controlling invasive *Candida* infections, consequently rapid and early diagnosis is a requirement for ameliorating (better, improve) the prediction of invasive Candidiasis. The present study focuses on the comparison of diagnostic methods for identification of Candidemia to minimize the time and accurate species-specific is needed. Non- culture methods are less time-consuming than blood culture or another automated method. The performance of Mn Ag and PCR was evaluated and compared with blood culture as a golden standard for diagnosis of Candidemia [28]. Previous studies suggested that specificity of Ag diagnosis was increased in patients during *Candida* infection [28].

In our present study, 2.1% (5/229) of blood culture samples received from suspected Candidemia cases were positive for 4°C. *tropicalis* (2.1%) and 1 isolate for *C. auris* (0.5%) in hematological patients in 3-year duration. The prevalence of Candidemia in our study is 2.6% in hematological patients. In other studies prevalence of Candidemia varies in hematological patients ranging from 1.6%,10% and 22.9% [11,13,20,30,31]. *C. tropicalis* was reported as the most frequent cause of Candidemia in the present study [16,18]. *C. tropicalis* was sensitive to drug VRC, AMP, FLU, CAS, and 1 resistant pattern towards FLU [23,32]. *C. auris* was resistant to AMP, FLU and sensitive towards CAS [15,23,34]. The prevalence rate depends on the geographical conditions in a particular area and also depends on the availability of diagnostic procedures in a particular laboratory. *C. tropicalis* and *C. auris*

were identified by Maldi-TOF MS with 100% accuracy [33,34]. The effectiveness of conventional PCR is more than the blood culture method used in mycology laboratories.

In present study, Mn Ag positive (13/229) was find low threshold values (125 pg/ml), with high threshold values (500 pg/ml). Similar findings were made by Mokaddas, et al., who discovered that when the Ag threshold was set at 500, there was no discernible rise in the diagnosis of individuals with mucosal *Candida* colonisation in their Ag levels [35]. Children in the ICU who potentially develop IC were studied in a study by Rao et al., and it was discovered that Ag was 100% sensitive [36].

In immune compromised patients, caspofungin is an ideal antifungal drug in minimum concentration for treatment of patients on chemotherapy. Prior use of antifungal drugs for a long time is also a reason for the shifting of *C. albican* species to Non albicans *Candida* and the drug resistance pattern of *Candida* is different.

Declaration of Patient Consent

All authors certify that the relevant patient information was obtained by consent forms. In the form the patient(s) have/ has given her/his/their consent for her/his/theirs. The patients understand that their identities will not be published or disclosed.

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Conflict of Interest

The author has no conflict of interest to declare.

Availability of Data

All data generated during and/or analysed during the current study are not publicly available due to maintained confidentiality of the patients, but are available from the corresponding author on reasonable request.

Ethics Approval

This study was approved in 2018 by the Institutional Ethics Committee of King George's Medical University, Lucknow, Uttar Pradesh, INDIA (letter no. 223/Ethics/R. Cell-18).

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