

# Characterization of *Candida* Species Isolated from Cases of Lower Respiratory Tract Infection among HIV/AIDS Patients in Calabar, Nigeria\*

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## ABSTRACT

This study was designed to identify and characterize the *Candida* species isolated from lower respiratory tract infections among HIV positive patients and to determine the prevalence rates of *Candida* infections among these subjects. Two early morning expectorate sputum samples were collected from 272 HIV positive subjects visiting the ART clinics and DOTS centre with cases of lower respiratory tract infection, over a period of 14 months from May 2009 to July 2010 in Calabar. Subjects were recruited for this study upon approval by the Ethical Research Committee of the University of Calabar Teaching Hospital and obtaining written informed consent from the patients. Samples were processed by standard methods for isolation of *Candida*. Speciation was done by a germ tube test, chlamyospore production on corn meal agar and sugar fermentation and assimilation tests using the Microexpress *Candida* identification kit (Tulip, India). Out of the 544 sputum samples collected from 272 subjects, *Candida* species were isolated from 40 (14.7%) and identified after confirming the growth in the second sample. The majority of *Candida* species among the *Candida* isolates were *Candida albicans* (80%) followed by *Candida tropicalis* 5 (12.5%), *Candida dubliniensis* 2 (5.0%) and *Candida guilliermondii* 1 (2.5%). The isolation rate of *Candida* species from sputum samples was found to be highest among subjects aged 25 - 34 years, followed by those aged 15 - 24 years. Twenty (7.3%) HIV seropositive subjects had bacterial infections, while 4 (1.5%) subjects had mixed fungal and bacterial infections. This study is the first of its kind to be carried out in Calabar and the South-South geopolitical region of Nigeria, and has shown that pulmonary candidiasis is a health problem among HIV positive patients in Calabar.

**Keywords:** *Candida* Infections; Pulmonary; HIV/AIDS; Characterization

## 1. Introduction

*Candida* is a polymorphic fungus. It is a Gram positive, oval, budding yeast cell that produces pseudohyphae both in culture and in tissues and exudates [1]. They exhibit filamentous mycellial morphology in the saprophytic phase, but have typical yeast morphology in the parasitic phase, in tissue and when grown at 37°C in the laboratory [1]. They form pseudohyphae when the buds continue to grow but fail to detach, producing chains of elongated cells that are pinched or constricted at the septations between cells [2]. At temperatures below 26°C in nutritionally poor media such as cornmeal agar, it produces thick-

walled resting cells 7 to 17 mm in diameter, called chlamyospores [3]. It is a member of the normal flora of the mucous membranes in the gastrointestinal, upper respiratory and female genital tracts [4].

There are about 68 species of *Candida*, the most pathogenic is *C. albicans* [5]. The other non-*albicans* *Candida* (NAC) species include *C. dubliniensis*, *C. glabrata*, *C. guilliermondii*, *C. tropicalis*, *C. parapsilosis*, *C. krusei* and *C. lusitanae*, *C. kefyr*, *C. lipolytic* and *C. pelliculos* [6,7].

*Candida* pneumonia is one of the most challenging of all the *Candida* infections. Primary *Candida* pneumonia refers to infections limited to the lungs, while secondary *Candida* pneumonia indicates lung involvement due to

\*Conflicts of Interests: None declared.

the movement of invasive *Candida* from other areas of the body [5]. The criteria for the diagnosis of pulmonary candidiasis are still controversial. The isolation of *Candida* from culture of sputum, endotracheal aspirates, bronchoscopic samples, percutaneous lung needle aspirates and even from lung tissue may only represent colonization of the trachea-bronchial tree [4]. Despite the debate about the diagnosis of pulmonary candidiasis, the definitive diagnosis is still resting on histological demonstration of the yeast in lung tissue with associated inflammatory changes at autopsy [7,8].

The increase in the number of immunocompromised patients during the last few decades, as a result of chemotherapy or HIV/AIDS has resulted in a parallel increase in the number of opportunistic infections, especially those due to *Candida* species [9,10].

Rapid identification and speciation of *Candida* species are essential in clinical laboratories. However, no single phenotypic test is highly effective in identifying *Candida* species and the combination of tests is sometimes necessary for identification. Molecular techniques have been employed to characterize *Candida* spp. Although sensitive and specific it is not cost effective for routine clinical mycology laboratories in a resource constrained setup like ours [11].

## 2. Aims and Objectives

The aim of the study was to determine the rate of isolation of *Candida* species from sputum samples of HIV positive patients with respiratory symptoms, to identify and characterize the *Candida* species using various phenotypic methods such as Germ tube test, Chlamyospore production and identification to specie level with Microexpress *Candida* identification kit (Tulip diagnostic LTD, India), according to the manufacturer's instructions.

## 3. Materials and Methods

### 3.1. Study Sites and Subjects Selection

The present study was carried out in the department of Microbiology/Parasitology, University of Calabar Teaching Hospital, Calabar. Subjects were recruited from two tertiary hospitals; the University of Calabar Teaching Hospital and Lawrence Henshaw Hospital located in Calabar municipality and Calabar south local Government areas respectively. The cases selected for the study included HIV positive subjects with respiratory symptoms who were able to produce sputum. Sputum samples showing less than 10 squamous epithelial cells and 25 or more polymorphonuclear leukocytes per low power field (100×) were included in this study.

### 3.2. Collection and Examination of Samples

A total of 272 sputum samples in duplicates were collected from May 2009 to July 2010 from the subjects. All sputum specimens were processed by Gram staining, 10% KOH mount, culture, germ tube test, detection of chlamyospore and sugars fermentation and assimilation tests. A 10% KOH mount of sputum sample showing plenty of *Candida* pseudohyphae were considered as potential pathogens and not as colonizers. The isolates were considered significant by correlation with microscopy and growth of two consecutive cultures. The *Candida* species were isolated repeatedly in pure culture from two consecutive early mornings expectorate sputum samples [4]. Speciation of *Candida* was by germ tube test, morphology on corn meal agar, sugar fermentation and assimilation tests using Microexpress *Candida* identification kits (Tulip, India) according to the manufacturer's instructions [4,12].

### 3.3. Direct Examination of the Specimens

#### 3.3.1. Gram Stain

Smears made from the most purulent: or mucopurulent part of the sputum. Smears were examined for the presence of gram positive budding yeast cells with pseudohyphae. Specimen was considered as acceptable when 25 or more polymorphonuclear leukocytes were seen per low power field (100×) with few (less than 10) squamous epithelial cells.

#### 3.3.2. KOH Mount

Smears were prepared for each sample by adding a loop full of sputum to a drop of 10% KOH on a clean, grease-free slide and placing a cover slip over it. The preparations were slightly warmed to digest the materials and examined under the microscope with X10 and X40 objective for yeast cells, pseudohyphae [12].

#### 3.3.3. Culture

The most bloody, purulent, or mucus laden portions of sputum samples were inoculated on Sabouraud Dextrose Agar (SDA) with and without chloramphenicol ( $16 \mu\text{g}\cdot\text{ml}^{-1}$ ) in duplicates incubated at  $37^\circ\text{C}$  and  $25^\circ\text{C}$ . Cultures were examined every other day for growth up to 2 weeks before discarding as negative [12,13]. The macroscopic features of the growth were opaque pasty glabrous or membranous texture often with the sweet smell, reminiscent of ripe apples. The growth was confirmed by observing the characteristic budding with pseudohyphae in the Gram stain [10]. Pure cultures of every isolate were prepared before performing any physiological test. This was done by sub-culturing individual isolates onto fresh SDA plates and incubating at  $37^\circ\text{C}$  and at room tempe-

perature (25°C - 28°C) for 24 - 48 hours [12]. Any significant bacterial growth from SDA without chloramphenicol was purified on Cystein Lactose Electrolyte Deficient (CLED) agar, for further biochemical analysis and identification [13]. Identification of isolates was based on gross morphological characteristics, detailed study of stained samples and biochemical tests [14].

### 3.5. Identification of *Candida* Isolates

#### 3.5.1. Germ Tube Test

All presumptive *Candida* species were subjected to germ tube test. Colonies from the purity plate were picked to perform the germ tube test. Small test tubes containing about 0.5 ml human serum were inoculated with colonies of test organisms in batches of three replicates per test. Each batch included a known positive and negative control. Inoculated tubes were incubated at 37°C for 2 - 3 hours. At the end of incubation, a drop of each serum was transferred to a clean slide, and a cover slip placed over it. These slides were examined microscopically under high power (×40) objective to detect the presence of germ tubes, which are short hyphal initials [4,9].

#### 3.5.2. Chlamydospore Formation

All *Candida* isolates were tested for the production of chlamydospores in corn meal agar (CMA) with 1% Tween 80. Subcultures on corn meal agar plates, was made from SDA purity plate by furrowing the CMA plates (cut streak method), and incubating at room temperature (25°C - 28°C) for 2 - 5 days after which they were examined for the production of thick-walled chlamydospores in a lactophenol cotton blue mount of samples taken from the furrows of the corn meal agar plates [4,15].

#### 3.5.3. Sugar Fermentation Test

All *Candida* isolates were subjected to carbohydrate fermentation and utilization test using *Candida* identification kit, Tulip Diagnostics Limited, India. Each kit contained sterile media for colorimetric identification using biochemical test and carbohydrate utilization tests based on the principle of pH change and substrate utilization designed to identify various metabolic properties of different *Candida* species, that can be used to differentiate even closely related *Candida* species. The media were inoculated by adding 100 µl of the *Candida* suspension, incubated at 20°C - 25°C and read after 24 - 48 hours incubation. The *Candidal* suspension was prepared by purifying on a Brain Heart Infusion Agar (BHIA) medium, after which a single discrete colony was picked up and streaked on to BHI agar slant for enrichment and incubated at 20°C - 25°C (room temperature) for 24 - 48 hours. The growth on the slant was washed with 2 - 3 ml

of sterile saline and the turbidity of the suspension compared with Mcfarland standard number 5. The results were interpreted as per the standards given in the result interpretation chart.

## 4. Results

A total of 272 sputum samples were collected from all the HIV positive subjects enrolled during the study. Out of which 40 (14.7%) *Candida* species were isolated and identified after confirming the growth in the second sample. The majority of *Candida* species among the *Candida* isolates were *Candida albicans* (80%) followed by *Candida tropicalis* 5 (12.5%), *Candida dubliniensis* 2 (5.0%) and *Candida guilliermondii* 1 (2.5%) (**Table 1**).

The isolation rate of *Candida* species from sputum samples was found to be highest among subjects aged 25 - 34 years, followed by those aged 15 - 24 years. No *Candida* species were isolated from subjects aged 65 - 74 years (**Table 2**). **Table 3** shows the prevalence of bacterial pathogens amongst the subjects. Twenty (7.3%) HIV seropositive subjects had bacterial infections. Out of which 11 (4.0%) were infected with *Klebsiella pneumoniae*, 6 (2.2%) with *Pseudomonas aeruginosa* and 3 (1.1%) with *Proteus specie*.

**Table 4** shows the distribution of polymicrobial infections among the HIV-positive subjects. Out of the 4 (1.5%) subjects with mixed infections, 3 (75.0%) had *Candida albicans* and *Klebsiella pneumoniae* infections, while one subject (25.0%) had *Klebsiella pneumoniae* & *Candida tropicalis* infection.

**Table 5** shows the specific characteristics of isolates of *Candida* species established with Microxpress *Candida* identification kit (Tulip diagnostics LTD, India).

## 5. Discussion

The diagnosis of pulmonary candidiasis is difficult not because it is difficult to demonstrate the organism in clinical samples but because isolation of *Candida* species in sputum does not necessarily mean that a person is suffering from pulmonary candidiasis. A good clinical cor-

**Table 1. Distribution of *Candida* isolates among subjects.**

<i>Candida</i> species	No of isolates (n = 272)	Percentage of isolates (%)
<i>Candida albicans</i>	32	80
<i>Candida tropicalis</i>	5	12.5
<i>Candida dubliniensis</i>	2	5.0
<i>Candida guilliermondii</i>	1	2.5
Total	40	14.7

**Table 2. Age and gender distribution of *Candida* species among subjects.**

Age (years)	Gender	<i>Candida</i> species	Total (%)
15 - 24	Female (F)	5 <i>Candida albicans</i> 2 <i>Candida tropicalis</i>	9 (22.5)
	Male (M)	2 <i>Candida albicans</i>	
25 - 34	Female (F)	12 <i>Candida albicans</i> 3 <i>Candida tropicalis</i>	19 (47.5)
	Male (M)	3 <i>Candida albicans</i> 1 <i>Candida guilliermondii</i>	
35 - 44	Female (F)	2 <i>Candida albicans</i>	4 (10.0)
	Male (M)	1 <i>Candida albicans</i> 1 <i>Candida dubliniensis</i>	
45 - 54	Female (F)	4 <i>Candida albicans</i>	7 (17.5)
	Male (M)	2 <i>Candida albicans</i> 1 <i>Candida dubliniensis</i>	
55 - 64	Female (F)	-	1 (2.5)
	Male (M)	1 <i>Candida albicans</i>	
65 - 74	Female (F)	-	0 (0.0)
	Male (M)	-	
Total			40 (100)

Denotes no *Candida* species was isolated.

**Table 3. Distribution of bacterial pathogens from sputum samples of subjects.**

Bacteria pathogens	No of isolates (n = 272)
<i>K. pneumonia</i>	11 (4.0)
<i>P. aeruginosa</i>	6 (2.2)
<i>Proteus</i> specie	3 (1.1)
Total	20 (7.3)

**Table 4. Polymicrobial isolation from HIV positive subjects.**

Types of isolates	No of isolates (%) (n = 272)
<i>Candida albicans</i> & <i>Klebsiella pneumonia</i>	3 (75.0)
<i>Candida tropicalis</i> & <i>klebsiella pneumonia</i>	1 (25.0)
Total	4 (1.5%)

relation is therefore necessary to ascertain the clinical significance of the isolate [4].

The total number of *Candida* species isolated during the study was 40 (14.7%). These is higher than the 12.1% prevalence reported in India by Jha *et al.* [3], but considerably lower than 48% prevalence reported in New York,

USA by Diaz-Fuentes *et al.* [7]. The low prevalence rate of candidiasis recorded in this study as compared to the 48% reported in America could have been due to the fact that Diaz-Fuentes *et al.* [7] recovered the *Candida* isolates at autopsy from lung tissues which is a definitive diagnostic technique for pulmonary candidiasis. It is likely that none of their isolates were lost. *Candida* species generally remains the most implicated opportunistic fungal pathogen in HIV/AIDS infection probably because it is an endogenous opportunist.

*Candida albicans* were found to be the most common species with 32 (80%) isolates. This was followed in descending order of prevalence by *C. tropicalis* 5 (12.5%), *C. dubliniensis* 2 (5.0%) and *C. guilliermondii* 1 (2.5%) (**Table 1**). Aluyi *et al.* [16] in Edo state, Nigeria found the distribution of *Candida* species among patients to be as follows: *C. albicans* (49.3%), *C. stellatoidea* (25.4%) and *C. parapsilosis* (25.3%). A multicenter surveillance study conducted in Quebec, Canada in 2001 reported a distribution pattern for *Candida* as follows: *C. albicans* (64%), *C. tropicalis* (17%), *C. parapsilosis* (8%), *C. glabrata* (6%) and *C. krusei* (2%) [17]. The findings of the present study are different from the above studies. This could be due to variation in geographical distribution of various *Candida* species.

The isolation rate of *Candida* species from sputum samples was found to be highest among subjects aged 25 - 34 years, followed by those aged 15 - 24 years. No *Candida* species were isolated from subjects aged 65 - 74 years (**Table 2**). Jha *et al.* [3], found the isolation rates of *Candida* species highest among subjects in the seventh decade of life followed by the fourth and fifth decade. In this study, the high prevalence of *Candida* species among subjects in the second and third decade of life could be attributed to the high sexual activity of subjects in this age group which leads to high HIV/AIDS transmission with resultant immunosuppression and opportunistic infections which include *Candida* infections.

Bhalla [18] noted that pneumonia of bacterial origin occur at a rate many times higher in the HIV infected patients than in the general population. In this study, the 20 (7.3%) prevalence of bacterial isolates is lower than the 44.28% reported in Hyderabad by Shailaja *et al.* [19], with *K. pneumoniae* as the most prevalent etiologic agent. This study also encountered *K. pneumoniae* as the most prevalent bacterial agent. Tchamran [20] in his study on lung diseases of bacterial origin in HIV infected individuals in African adults, noted that 81% of infections were due to *S. pneumoniae* and reported it to be the most offending pathogen in HIV reactive patients. The difference in prevalence rates may be ascribed to the types of culture media used during the present study. Sabouraud Dextrose Agar was the primary isolation medium. *Streptococcus pneumoniae* and other fastidious organisms do

**Table 5. Characterization of *Candida* species with Microexpress *Candida* identification kit.**

SPECIES	Urease utilisation	Melibiose utilisation	Lactose utilization	Maltose utilization	Sucrose utilization	Galactose utilization	Cellobiose utilisation	Inositol utilization	Xylose utilization	Dulcitol utilization	Raffinose utilization
<i>C. albicans</i>	-	-	-	+	-	+	-	-	+	-	-
<i>C. dubliniensis</i>	-	-	-	+	-	+	-	-	V	-	-
<i>C. tropicalis</i>	-	-	-	+	+	V	+	-	+	-	-
<i>C. guilliemondii</i>	-	+	-	-	+	V	+	-	+	+	+

Key: + = positive; - = negative; v = variable.

not grow on this media, thus such organisms were not isolated.

Also the polymicrobial etiology (mixed *Candida* and bacterial infections) in 4 (1.5%) of the HIV reactive subjects indicates the severity and the effect of immunosuppression in this group.

## 6. Conclusion

Pulmonary candidiasis is a health problem among HIV positive patients in Calabar, Nigeria. Microexpress candida identification kit has been found to be useful in the identification of *Candida* to species level, as PCR is expensive and not easily assessable. Periodic study of pulmonary *Candida* infections among HIV infected patients is the present day's need, to know the changing pattern in incidence of *Candida* species.

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## List of Abbreviations

### Abbreviation/Symbol Meaning

HIV: Human immunodeficiency syndrome  
 AIDS: Acquired immune deficiency syndrome  
 ART: Antiretroviral therapy  
 DOTS: Directly Observed Treatment Short Course  
 KOH: Potassium hydroxide  
 SDA: Sabouraud Dextrose Agar  
 CMA: Corn meal agar  
 BHIA: Brain Heart Infusion Agar  
 °C: Degree Celsius  
 $\chi^2$ : Chi Square  
 mg: Milligramme  
 ml: Milliliter  
 L: Litre  
 $\mu$ l: Microliter  
 g: Gram  
 %: Percent  
 No: Number  
 TB: Tuberculosis