Sudan University of Science and Technology
College of Graduate studies

Frequency of Pulmonary Candidiasis Among Hospitalized Patients with Respiratory Tract Diseases Attending El-Shaab Teaching Hospital -Khartoum State.

تردد داء فطر المبيضات الرئوي بين المرضى المصابين بامراض الجهاز التنفسي في مستشفى الشعب التعليمي - ولايه الخرطوم.

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بسم الله الرحمن الرحيم

الآيـــــــــــــــة

قال تعالى :

{وَيَسْأَلُونَكَ عَنِ الرُّوحِ ۖ قُلِ الرُّوحُ مِنْ أَمْرِ رَبِّي وَمَا أُوتِيتُمْ مِنَ الْعِلْمِ إِلَّا قَلِيلًا}

صدق الله العظيم

سوره الاسراء الايه (85)

Dedication

To my dear mother, father, sister, brothers, supervisor, teachers and my friends for their forbearance, support and encouragement to make my dream come true.

To every person hoping to see me successful and ambition.

With my love and respect

Eman

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ABSTRACT

This was cross sectional study aimed to determine the frequency and etiology of Pulmonary Candidiasis, conducted during the period from February to June 2018 at Elshaab Teaching Hospital (Khartoum State).

Hundred hospitalized patients with respiratory tract disease suspected to have Pulmonary Candidiasis were enrolled in this study, both gender males and females were included in this study.

Sputum specimens were collected from all suspected patients, and subjected to Gram's stain, 10% potassium hydroxide (KOH) wet mount, culture and biochemical tests for identification of pathogens.

Among total 49/100 (49%) were males and 51/100 (51%) were females, their age varies from 20 years to 80 years with a mean of 44.1 ± 13.5 years

The frequency of Pulmonary Candidiasis was 9/100 (9%), and all isolates 9/9 (100%) were *Candida albicans*.

High frequency rate of Pulmonary Candidiasis was observed among patients with cancer 3/9 (34%).

The frequency of Pulmonary Candidiasis in patients with in ages between 60-80 years 6/9 (67%)

There was generally a higher frequency of Pulmonary Candidiasis especially in elderly paitents compared to other age group.

Statistical analysis showed that there was significant association between Pulmonary Candidiasis and both clinical history and age of patients, while there was no significant association between Pulmonary Candidiasis and gender of patients. *P value* (0.00, 0.001, 0.774) respectively.

المستخلص

هدفت هذه الدراسة المقطعية المستعرضة إلى تحديد تردد ومسببات داء فطرالمبيضات الرئوي ، والذي نادرا ما يهدد الحياه ويكون من مضاعفات داء المبيضات الجهازي . أجريت هذه الدراسة خلال الفترة من فبراير إلى يونيو 2018 في مستشفى الشعب التعليمي (ولايه الخرطوم).

شملت الدراسة مائة مريض من مرضى الجهاز التنفسي الذين يُشتبه في إصابتهم بداء فطر المبيضات الرئوي ، وشملت كلا الجنسين.

جمعت عينات البلغم من جميع مرضى الجهاز التنفسي ، واخضعت لصبغة غرام ، و 10 ٪ من مستحضر هيدروكسيد البوتاسيوم، الزرع والاختبارات الكيميائية الحيوية لتحديد مسببات المرض.

من المجموع وجد ان نسبة الرجال 49٪ ونسبه النساء 51٪ ، ويتراوح اعمارهم بين 20 عامًا و 80 عامًا بمتوسط 44.1 ± 13.5 سنه.

تردد داء فطر المبيضات الرئوي 100/9 (9 ٪) ، ومن كل العينات المزروعه تم التعرف على وجمعت ما بين 9 / 9 (100 ٪) مبيضه بيضاء .

لوحظ ارتفاع معدل تردد داء المبيضات الرئوي بين المرضى المصابين بالسرطان 9/3 (34 ٪).

كانت هناك نسبه تردد عالي لداء فطر المبيضات الرئوي خاصة في كبار السن بالمقارنة مع الفئات العمرية الأخرى.

تردد داء فطر المبيضات الرئوي في المرضى الذين تتراوح أعمارهم بين 60- 80 سنة 9/6 (67٪) .

واظهر التحليل الإحصائي وجود ارتباط كبير بين داء فطر المبيضات الرئوي، التاريخ السريري وعمر المرضى في حين لم يكن هناك ارتباط واضح بين داء فطر المبيضات الرئوي ونوع جنس المرضى.

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LIST OF ABBREVIATIONS

Abbreviations	Full name of abbreviations
BAL	Bronco alveolar lavage
CCR-6	C. Chemokine receptor-6
CD4+ cell	Cluster of differentiation4+ cell
CF	Cystic fibrosis
CFU	Colony forming unit
CMA	Corn meal agar
CMI	Cell mediated immunity
CLR	C-type lectin receptor
DNA	Deoxyribionucleic acid
ELISA	Enzyme linked immunosorbent assay
HIV	Human immunodeficiency virus
ICU	Intensive care unit
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
KD	Kilodalton
KOH	Potassium hydroxide

PAMPs	Pathogen associated molecular pattern
PC	Pulmonary Candidiasis
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
PMNL	Polymorphonuclear leucocyte
PNA	Peptide nucleic acid
PRR	Pattern recognition receptor
RAPDNA	Random amplified polymorphic DNA
REP	Repeat sequence amplification PCR
RFLPs	Restriction fragment length polymorphisms
RIA	Radio immunoassay
SDA	Sabouraud dexterose agar
SPSS	Statistical pakage of social science
SSP	Species
TB	Tuberculosis
Th-1	T- lymphocyte helper-1
Th-2	T -lymphocyte helper-2
TLR	Toll like receptor

CHAPTER ONE

INTRODUCTION

1. INTRODUCTION

1.1 Background

Candidiasis refer to the range of infections caused by species of fungal genus Candida, these infections can be acute or chronic, localized or systemic, disseminated Candidiasis is life threatening and the great majority of Candidiasis is caused by Candida albicans which is a common commensal organism in oropharyngeal cavity, gastrointestinal tract and vagina of humans but is capable of causing opportunistic infection following disruption of the normal flora, a breach of the mucocutaneous barrier or a defect in host cellular immunity (Naglik *et al*., 2003).

Candida species are the most common fungal pathogens in humans. 8%–10% of all nosocomial infections caused by fungal pathogens, 80% are attributable to Candida species (Pendleton *et al*., 2017).

Fungal infections account for nearly 8% of all nosocomial infections, Candida is the responsible agent in 80% of the cases due to the prolonged antibiotic therapy, the more frequent use of surgery, instrumentation, and the greater number of immunocompromised patients being hospitalized, as well as the extensive use of intensive care facilities (El-Ebiary *et al*.,1997).

Candida albicans is commonly inhabits in oral and vaginal mucosa and gastrointestinal tract of human beings as one of the commensal organisms. It causes opportunistic infections in immunocompromised patients, produces allergic reactions and rarely causes morbidity and mortality (Douglas, 1988). It also causes a variety of infections that range from mucosal candidiasis to life threatening disseminated candidiasis (Dixon *et al*., 1996; O`Dwyer *et al*., 2007; Terrier *et al*., 2007).

Candida pneumonia is a rare lung infection, with the majority of cases occurring secondary to hematological dissemination of Candida organisms from a distant site, usually the gastrointestinal tract or skin (Shweihat *et al*., 2015).

Aspiration Candida pneumonia is rare, pulmonary candidiasis usually arises from a focus of infection implanted during hematogenous dissemination (Rose *et al*., 1978).

Candidiasis is an increasingly important nosocomial infection in both adults and children, especially those who are cared in intensive care units (Wisplinghoff *et al.*, 2004).

Although the infecting agent is most often part of the host's endogenous flora, nosocomial acquisition of Candida species has been described (Pfaller and Diekema, 2007).

The yeast Candida albicans is the most common human pathogenic Candida species and can cause a broad spectrum of diseases including skin, mucosal and systemic infections Candidiasis. Candida species can cause life threatening infections in immunocompromised individuals or when natural barriers are damaged, during these infections the fungus is able to colonize and multiply within almost all organs of the human body, under normal circumstances, C.albicans is a mucosal commensal, predominantly in the gastrointestinal tract of the majority of the human population and controlled by the normal microbial flora, epithelial barriers and the innate immune system while reflected by the fact that antibiotic treatments, or surgery, polytrauma, catheters and neutropenia are all considered as major risk factors for invasive Candidiasis in addition to an imbalance of the normal microbial flora, breakage of epithelial barriers or dysfunction of the immune system favour the transition of the human pathogenic yeast Candida albicans from a commensal to a pathogen (Gow and Hube, 2012).

Clinical and radiological features of fungal respiratory infections are nonspecific and have overlap with other respiratory diseases. A definitive diagnosis requires laboratory identification of the causative agent of which the most frequent one is Candida species (Badiee *et al*., 2018).

Difficulties in diagnosis of pulmonary involvement of Candida species so understanding of the frequency and pathogenesis of Candida pneumonia remains limited to indirect data collected from a relatively small number of reports. Pulmonary Candidiasis in patients with cancer occurs in tow types in cases of aspiration pneumonia in patients who are heavily colonized by Candida species in the oral cavity (primary Candida pneumonia), or as part of disseminated Candidiasis ,on the other hand Candida colonization in the upper respiratory tract is quite common in patients with cancer, therefore cultures of samples obtained from the respitratory tract including sputum and even bronchoalveolar lavage (BAL) that are positive for Candida species cannot be the sole basis for a diagnosis of invasive pulmonary infection with Candida species (Kontoyiannis *et al*., 2002).

1.2 Rationale

Candida species are the most common fungal pathogens in humans. 8%–10% of all nosocomial infections caused by fungal pathogens, 80% are attributable to Candida species (Pendleton *et al*., 2017).

Invasive C. albicans infection has emerged as a life-threatening disease in recent years and the mortality rate of invasive Candidiasis is high in critically ill host (Yan *et al*., 2013).

The increase in incidence and severity of opportunistic fungal infections such as invasive Candidiasis due to more aggressive immunosuppressive therapy and the spread of acquired immunodeficiency syndrome. Candida infections are usually due to impaired epithelial barrier functions and occur in all age groups, but are most common in the newborn and the elderly, systemic candidiasis is usually seen in patients with cell mediated immune deficiency, and those receiving aggressive cancer treatment, immunosuppression, or transplantation therapy (Gow and Hube, 2012).

Published data studied of Pulmonary Candidiasis in Sudan were scantly, also the increase in incidence and severity of opportunistic fungal infections such as Invasive Candidiasis due to more aggressive immunosuppressive therapy, the spread of acquired immunodeficiency syndrome, inappropriate use of antibiotic treatments, surgery, polytrauma, catheters and neutropenia are all considered as major risk factors for invasive candidiasis (Gow and Hube, 2012). So applying of this study will reduce complications of Pulmonary Candidiasis because early discovery of disease will easy treatment and reduce complications and bad prognosis of disease.

1.3 Objectives

1.3.1General objective

To determine the frequency of pulmonary Candidiasis in hospitalized patients with respiratory tract disease attending El-Shaab Teaching Hospital.

1.3.2 Specific objectives

- To detect Candida species directly from sputum specimens using direct microscopical examination technique.
- To isolate Candida species from sputum specimens in Sabouraud Dexterose Agar (SDA) media and to identify Candida species from clinical isolates using conventional Candida identification tests.
- To determine the frequency and etiology of Pulmonary Candidiasis in hospitalized patients with respiratory tract disease.
- To assess possible association of clinical history, age and gender of patients with Pulmonary Candidiasis.

CHAPTER TWO

LITERAURE REVIEW

2. LITERATURE REVIEW

2.1 Pulmonary Candidiasis

2.1.1 Definition of Candidiasis

Candidiasis refer to the range of infections caused by species of fungal genus Candida, these infections can be acute or chronic, localized or systemic, disseminated Candidiasis is life threatening and the great majority of Candidiasis is caused by Candida albicans which is a common commensal organism in oropharyngeal cavity, gastrointestinal tract and vagina of humans but is capable of causing opportunistic infection following disruption of the normal flora, a breach of the mucocutaneous barrier or a defect in host cellular immunity (Naglik *et al*., 2003).

2.1.2 Definition of Pulmonary Candidiasis

Pulmonary Candidiasis can be acquired by either hematogenous dissemination causing a diffuse pneumonia or by bronchial extension in patients with oropharyngeal candidiasis, aspiration of yeasts from the oral cavity has also been reported in infants, pulmonary Candidiasis is difficult to diagnose due to non specific radiological and culture findings and most patients, especially those with granulocytopenia, present at autopsy, the presence of yeasts in alveolar lavage or sputum specimens is not specific and blood cultures may also be negative, unfortunately only histopathology can provide a definitive diagnosis and this is not always possible in patients with coagulation problems (Donghue *et al*.,2009).

2.2 Candida

2.2.1 Classification

Candidiasis is an infection caused by a yeast species of the genus *Candida*, which belongs to the Kingdom: Fungi, phylum: Ascomycota, subphylum: Saccharomycotina, class: Saccharomycetes, order: Saccharomycetales, family:

Saccharomycetacae, genus: *Candida* (Hajjeh *et al*., 2004). About 20 species are known to cause infections in humans (Moris *et al*., 2008). Includes the species *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. guilliermondii*, *C. lusitaniae*, and *C. krusei*, *C. dubliniensis*, *C. pelliculosa*, *C. kefyr*, *C. norvegensis*, *C. haemulonii*, and *Saccharomyces cervisiae* (Aittakorpi *et al*., 2012).

2.22. General characteristics

Candida yeasts have globose, ellipsoidal, cylindroidal, or elongate, occasionally ogival, triangular or lunate cells (Moris *et al*., 2008). Measuring 3-30 µm in diameter (Castellote and Soriano, 2013).

Candida albicans exists naturally as diploid yeast and until recently was thought to be asexual, as no direct observations of mating or meiosis had been reported (Carlisle and Kadosh, 2013). *C. albicans* exhibits the ability to grow as either a yeast or a mycelial form in response to different environmental factors (Kim *et al*., 2002).

Candida species reproduce asexually through a budding process in which protoplasmic protrusions or buds (blastoconidia) emerge from the mother cell and grow until they finally detach to form a new cell, the daughter cells occasionally do not detach and form chains of cells called pseudohyphae, which can be mistaken for hyphae (Castellote and Soriano, 2013).

The three primary morphological forms, blastospores, pseudohyphae and hyphae,

are all found in infected tissues, and work to date indicates that the transition between these forms is critical for pathogenesis (Andrew *et al*., 2003).

2.2.3 Habitat

Candida albicans is a natural component of the human microbiome, this fungus asymptomatically colonizes many areas of the human body, especially the skin, gastrointestinal (oral cavity or mouth of 25–50% healthy individuals, intestines) and genitourinary tracts (mainly vagina) of healthy individuals (Fox and Nobile,

2012) (Imran and Al-Shukry, 2014). *Candida* species are confined to human and warm blooded animal reservoirs; however, they can also be recovered from soil, food, water and, sometimes, air (Moris *et al*., 2008).

2.2.4 Morphology and Identification

In culture or tissue, *Candida* species grow as oval, budding yeast cells (3–6 µm in size), they also 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, unlike other species of Candida, *C.albicans* is dimorphic; in addition to yeasts and pseudohyphae, it can also produce true hyphae, On agar media or within 24 hours at 37°C or room temperature, Candida species produce soft, cream-colored colonies with a yeasty odor, Pseudohyphae are apparent as submerged growth below the agar surface, two simple morphologic tests distinguish *C.albicans*, the most common pathogen from other species of *Candida*, after incubation in serum for about 90 minutes at 37°C yeast cells of *C.albicans* will begin to form true hyphae or germ tubes and on nutritionally deficient media *C.albicans* produces large, spherical chlamydospores, Sugar fermentation and assimilation tests can be used to confirm the identification and speciate the more common Candida isolates, such as *C.tropicalis*, *C.parapsilosis*, *C.guilliermondii*, *C.kefyr*, *C.krusei*, and *C.lusitaniae*; *C.glabrata* is unique among these pathogens because it produces only yeast cells and no pseudohyphal forms (Geo *et al*., 2013).

2.2.5 Antigenic Structure

The use of adsorbed antisera have defined two serotypes of *C.albicans*: A (which includes *C.tropicalis*) and B. During infection, cell wall components, such as mannans, glucans, other polysaccharides and glycoproteins, as well as enzymes are released, these macromolecules typically elicit innate host defenses and Th1 and Th2 immune responses, for example sera from patients with systemic *candidiasis* often contain detectable antibodies to candidal enolase, secretory proteases and heat shock proteins (Geo *et al*., 2013).

2.2.6 Cell wall organization of *Candida* species

Electron microscopy of thin sections of the *C.albicans* cell wall shows layers which appear to be derived from differential abundances of cell wall constituents, the number of layers observed is variable and seems to be related to both strains and methodology, the inner layer enriched for chitin and polysaccharide matrix, is more electron translucent than outer layers, which are enriched for manno protein, the translucent layer was of a similar size, with a thin electron dense layer adjacent to the cell membrane (Chaffin, 2008). Structurally, the outer portion of the cell wall appears to have perpendicularly aligned fibrils that differ in length with surface hydrophobicity, the integrity of the cell wall is clearly of paramount importance to the survival of the organism, yeasts have developed mechanisms as cell wall integrity pathways to respond to stress that threatens the cell wall, except for noting some individual genes that respond to certain stresses as cell wall loss (Chaffin, 2008).

2.3 Pathogenicity

The ability of *C. albicans* to infect such diverse host niches is supported by a wide range of virulence factors and fitness attributes. A number of attributes, including the morphological transition between yeast and hyphal forms, the expression of adhesions and invasins on the cell surface, thigmotropism, the formation of biofilms, phenotypic switching and the secretion of hydrolytic enzymes are considered virulence factors. Additionally, fitness attributes include rapid adaptation to fluctuations in environmental pH, metabolic flexibility, powerful nutrient acquisition systems and robust stress response machineries (Nicholls *et al*., 2011).

2.4 Immunity

Host immune recognition of *Candida* occurs via several mechanisms comprising innate and adaptive immunity**,** both innate resistance and acquired immunity play some role in maintaining *C. albicans* in the commensal state and

protecting the systemic circulation, polymorphonuclear leukocytes (PMNL) are critical for protection against systemic infections, whereas cell-mediated immunity (CMI) by Th1-type $CD4^+$ T-cells is important for protection against mucosal infections (Fidel, 2002).

Candida specific antibodies although present are controversial relative to a role in protection or eradication of infection (Fidel, 2002).

2.4.1 Innate Immune Recognition of *Candida*

Innate immune recognition of *Candida* occurs via the recognition of pathogen associated molecular patterns (PAMPs). PAMPs are motifs or molecules that are common between different types of fungi. Unlike antigens, individual PAMPs are not specific to a single *Candida* species but rather are shared between many different species and fungal genera. These microbial PAMPs are recognized by host germ line encoded pattern recognition receptors (PRRs) and provide a preprogrammed mode of fungal recognition, allowing for instant recognition of common fungal components (Janeway and Medzhitov, 2002).

C.albicans activates the innate immune system specifically *C.albicans* induces proinflammatory cytokine production in various cell types *via* many receptors, such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), also promotes phagocytosis via CLRs on macrophages (Kiyoura and Tamai, 2015).

2.4.2 Antimicrobial peptides against *C. albicans*

I. LL-37

Human LL-37 is an antimicrobial peptide that is chemotactic for neutrophils and monocytes, also disrupts the cell membrane of *C. albicans* and causes an efflux of nucleotides and proteins with molecular masses of up to 40 kDa (Nicholls *et al*., 2011).

II. Defensin

The defensin family, produced by epithelial cells, acts against gram-positive and gram-negative bacteria, fungi, and enveloped viruses in vitro, six human

defensins have been identified and are present in gingival epithelia, saliva, and gingival crevicular fluid .In particular, human defensins 2 and 3 demonstrate antifungal activity against *C. albicans* (Kiyoura and Tamai,2015).

III. Histatin

Histatins are basic histidine rich proteins secreted in human parotid and submandibular sublingual saliva in humans, histatin 5 is secreted by human salivary glands and has the most potent fungicidal activity against *C.albicans* (Nicholls *et al*., 2011)

2.4.3 daptive Immune Responses

The adaptive arm of the anti *Candida* response is initiated through the recruitment of dendritic cells or Langerhans cells and macrophages during innate immunity. During infection, epithelial cells initiate host defence via the production of multiple proinflammatory molecules, including CCL20 and β-defensin 2 which act as chemoattractants to recruit mucosal homing CCR6 expressing dendritic cells (Bonifazi *et al*., 2009).

Dendritic cells recognise *Candida* through PRRs (pattern recognition receptors) including TLRs (toll like receptors), dectin-1, dectin-2, which results in fungal ingestion, dendritic cell activation and trafficking to the local lymph nodes. In the lymph node, dendritic cells will present processed fungal antigens to naive and memory T cells, initiating adaptive immunity. However, following PRR recognition, different dendritic cell subsets can be activated via distinct signalling pathways to shape T cell responses against *Candida* infections (Bonifazi *et al*., 2009).

2.5 Diagnosis

2.5.1 Microscopical examination

Direct microscopic examination of clinical specimens containing Candida reveals budding yeast cells (blastoconidia) 2 to 4 μM in diameter and /or pseudohyphae, true septate hyphae may also be produced. The blastoconidia , septate hyphae and pseudohyphae are strongly gram’s positive. It is advisable

to report the approximate number of such forms, in that the presence of large numbers in a fresh clinical specimen may be of diagnostic significance (Forbes, 2007).

In exudates or tissues, budding yeasts and pseudohyphae appear gram-positive and can be visualized by using calcofluor white staining (Levinson, 2014).

2.5.2 Culture Media

The most frequently used primary isolation medium for *Candida* is SDA which permitting growth of *Candida* **(**Odds, 1991). Incorporation of antibiotics into SDA will further increase its selectivity (Marsh and Martin, 2009). Typically SDA is incubated aerobically at 37°C for 24–48 hrs. *Candida* develops as cream, smooth, pasty convex colonies on SDA and differentiation between species is rarely possible (Baveja, 2010).

In recent years, other differential media have been developed that allow identification of certain *Candida* species based on colony appearance and colour following primary culture. The advantage of such media is that the presence of multiple *Candida* species in a single infection can be determined which can be important in selecting subsequent treatment options (Marsh and Martin, 2009). Examples of these include Pagano-Levin agar or commercially available chromogenic agars namely CHROMagar *Candida*, Albicans ID, Fluroplate, or Candichrom albicans (Williams and Lewis, 2000).

2.5.3 Identification of *Candida* species

Identification of yeasts based on primary culture media can be confirmed through a variety of supplemental tests traditionally based on morphological and physiological characteristics of the isolates (Raju and Rajappa, 2011).

2.5.4. Morphological criteria

The germ tube test is the standard laboratory method for identifying *C. albicans*. The test involves the induction of hyphal outgrowths (germ tubes) when subcultured in horse serum at 37°C for 2–4 hours. Approximately 95% of *C.*

albicans isolates produce germ tubes, a property also shared by *C. stellatoidea* and *C. dubliniensis* (Williams and Lewis, 2000).

C. albicans and *C. dubliniensis* can also be identified from other species based on their ability to produce morphological features known as chlamydospores. Chlamydospores are refractile, spherical structures generated at the termini of hyphae following culture of isolates on a nutritionally poor medium such as cornmeal agar. Isolates are inoculated in a cross hatch pattern on the agar and overlaid with a sterile coverslip. Agars are incubated for 24–48 hours at 37°C and then examined microscopically for chlamydospore presence (Marsh and Martin, 2009).

2.5.5 Physiological criteria and biochemical identification

Biochemical identification of *Candida* species is largely based on carbohydrate utilization. Traditional testing would have involved culture of test isolates on a basal agar lacking a carbon source. Carbohydrate solutions would then be placed within wells of the seeded agar or upon filter paper discs located on the agar surface. Growth in the vicinity of the carbon source would indicate utilization. Commercial systems are based on the same principle but test carbohydrates are housed in plastic wells located on a test strip. Growth in each well is read by changes in turbidity or colour changes in certain kit systems. Numerical codes obtained from the test results are used to identify the test organism based on database comparison (Ellepola and Morrison, 2005).

2.5.6 Serology of Candidiasis

Serological tests are frequently used to ascertain the clinical significance of *Candida* species isolates. Rising titers of lgG antibodies to *C. albicans* may reflect invasive candidiasis in immunocompetent individuals, the detection of IgA and IgM antibodies is important to identify an acute infection, immunosuppressed individuals often show variability in antibody production and in such a case the use of an antigen detection test is recommended, tests like enzyme linked immunosorbent assay (ELISA) and radio immuno assay (RIA)

for detection of candidial antigen, either cell-wall mannan or cytoplasmic constituents (Raju and Rajappa, 2011).

Serological diagnosis is often delayed and the tests still lack sensitivity and specificity. Furthermore, antibody production in immunocompromised patients is variable, making diagnosis complicated (Wahyuningsih et al., 2000). This is due to the fact that fungal antigens and metabolites are often cleared rapidly from the circulation and the presence of antibodies does not always imply a *Candida* infection, especially in patients with serious underlying disease or who are taking immunosuppressive drugs (Yeo and Wong, 2002).

2.5.7 Molecular based identification methods

Identification by analysis of genetic variability is a more stable approach than using methods based on phenotypic criteria, for the identification of *Candida* based on genetic variation are analyses of electrophoretic karyotype differences and restriction fragment length polymorphisms (RFLPs) using gel electrophoresis or DNA-DNA hybridization (Williams and Lewis, 2000).

Species-specific PCR approaches have also been used for *Candida* species identification, several target genes have been reported for *Candida* species discrimination, although those most frequently amplified are the sequences of the ribosomal RNA operon. Identification can be obtained based on PCR product sizes obtained following gel electrophoresis resolution, or PCR product sequence variation determined either by direct sequencing or through the use of restriction fragment analysis following cutting of PCR sequences with restriction endonucleases (Marsh and Martin, 2009).

Fluorescence in situ hybridization with peptide nucleic acid method (PNA Fish) is a new detection technique which targets highly conserved species-specific sequences in the abundant rRNA of living *C. albicans*. Individual cells can be detected directly without the need for amplification (Shepard et al., 2008). This technique achieves a sensitivity of 98 –100%, with a specificity of 100%,

allowing for the discrimination of *C. albicans* from the phenotypically similar *C. dubliniensis* (Trnovsky et al., 2008)

Molecular-based technology can also be used to identify strains of *Candida* species although the use of techniques such as Pulsed Field Gel Electrophoresis (PFGE), Random Amplified Polymorphic DNA (RAPD) analysis, and repeat sequence amplification PCR (REP) are largely reserved for epidemiological investigations in research of oral candidiasis (Marsh and Martin, 2009).

2.6 Treatment and Prevention

Fluconazole is the drug of choice for oropharyngeal or esophageal thrush, itraconazole and voriconazole are also effective, caspofungin or micafungin can also be used for esophageal candidiasis, treatment of skin infections consists of topical antifungal drugs (e.g., clotrimazole or nystatin), candida vaginitis is treated either with topical (intravaginal) azole drugs, such as clotrimazole or miconazole, or with oral fluconazole, mucocutaneous candidiasis can be controlled by ketoconazole, treatment of disseminated candidiasis consists of either amphotericin B or fluconazole, liposomal amphotericin B should be used in patients with preexisting kidney damage, treatment of candidal infections with antifungal drugs should be supplemented by reduction of predisposing factors, certain candidal infections (e.g., thrush) can be prevented by oral clotrimazole troches, buccal miconazole tablets, or nystatin, fluconazole is useful in preventing candidal infections in high-risk patients, such as those undergoing bone marrow transplantation and premature infants, Micafungin can also be used, there is no vaccine (Levinson,2014).

2.7 previous studies

Fungi identification was successful in 49/96 (51%) patients. The Candida species growth was observed in the culture of 28/96 (29.2%). Aspergillus species were isolated from 7 patients (7.3%). The most frequent species identified were Candida albicans, C. glabrata, C. krusei, Aspergillus flavus, and A. fumigatus. Pneumocystis jirovecii immunofluorescence staining was positive in 23.9% of the patients with more than five cysts and 42.7% of the patients with less than five cysts. By real time PCR, P. jirovecii was detected in 54.2% of the patients (Badiee et al., 2018).

Fifty-nine (49.2%) yeast colonies were identified from the total of 120 BAL specimens. Twenty-nine isolated yeasts; including 17 (58.6%) of C. albicans/dubliniensis complex and 12 (41.4%) of non albicans isolates produced pseudohypha or blastoconidia in direct smear with a mean colony count of 42000 CFU/mL. C. albicans with the frequency of 15 (42.9%) were the most common isolated yeasts, whereas C. dubliniensis was identified in two non HIV patients. Fifty-nine (49.2%) yeast colonies were identified from the total of 120 BAL specimens. Twenty-nine isolated yeasts; including 17 (58.6%) of C. albicans/dubliniensis complex and 12 (41.4%) of non albicans isolates produced pseudohypha or blastoconidia in direct smear with a mean colony count of 42000 CFU/mL. C. albicans with the frequency of 15 (42.9%) were the most common isolated yeasts, whereas C. dubliniensis was identified in two non HIV patients (Kianipour et al., 2018).

Over the 4-year study period, of the 803 patients meeting study inclusion criteria in the six study centers, 214 patients (26.6%) had respiratory tract Candida colonization. *Candida albicans* was the most common species (68.7%), followed by *Candida glabrata* (20.1%) and *Candida tropicalis* (13.1%), extra pulmonary Candida colonization was more common in exposed patients. Exposed patients had longer ICU and hospital stays but similar mortality to

unexposed patients. The matched exposed/unexposed nested cohort study identified bronchial
Candida colonization as an independent risk factor for pneumonia (Azoulay et al., 2006).
A total of 140 patients were included. Ninety four cases of pulmonary fungal infection (67%) were community acquired. The most frequently encountered fungi were Aspergillus species
(57%), followed by Cryptococcus species (21%) and Candida species (14%). There were 72
patients with acute invasive fungal infection, with a mortality rate of 67%. Multivariate logistc regression analysis showed that nosocomial infection (pvalue =0.014) and respiratory failure (p value= 0.001) were significantly and independently associated with death of acute
invasive fungal infection (Chen et al., 2001).
During the 3-year study period, 634 cases of Candida infections occurred among the 17,797 patients who entered the ICU with a cancer diagnosis. Among them, C. albicans accounted for 544 cases (85.8%) and 90 cases (14.2%) were classified as non Candida albicans (Choi et al., 2017).
A total of 422 Candida isolates were recovered from sputa or oropharyngeal swabs of 56 CF patients during the 30 month study period. Depending of the time frame of each patient, a mean of 5.3 (range 1–21) isolates were found in the respiratory samples, of which 332 (78.8%) from 53 patients were identified as C.albicans.nThe three other most frequent Candida spp. found in specimens were; C.glabrata (31 isolates from 9 patients), C.dubliniensis (22 isolates from 7 patients) and C.parapsilosis 20 isolates from 6 patients (Muthig et al., 2010).
A total of 676 patients with cancer underwent autopsy during the study period. Of those, 254 patients (38%) had histopathologic evidence of pneumonia. Histologically proven PC was diagnosed in 36 (14%) of these 254 patients. A total of 146 patients with pneumonia at autopsy had matching sputum cultures

(110 patients) and/or BAL cultures (85 patients) performed during the preceding 4 weeks and were analyzed further. For 5 patients, autopsy was limited to the chest. Of the remaining 31 patients with PC, 13 patients (42%) had evidence of disseminated candidiasis; 1 patient who did not have PC had a kidney abscesses due to Candida krusei. Ten (77%) of 13 patients with disseminated candidiasis involving the lungs had a underlying hematologic malignancy. A comparison of some clinical parameters of the patients who had and patients who did not have PC. Sputum cultures, BAL cultures, or both that were positive for Candida species were seen in 30 (83%) of the 36 cases of PC. However, they were also seen in 46% of the cases without histopathologic evidence of PC at autopsy (Kontoyiannis et al., 2002).

Patients were categorized as having positive pulmonary biopsy cultures for Candida spp. or otherwise. Ten patients (40%) had at least one pulmonary biopsy sample yielding Candida spp.; 15 did not (60%). Only two patients (8%) had definite pulmonary candidiasis. Growth of Candida species in any count was seen in 10 of 25 (40%) patients in at least one pulmonary biopsy sample. A total of 470 microorganisms were isolated in any count from 280 of 375 (77%) lung biopsy samples in all 25 patients. Forty percent (190 of 470) of the isolates were gram-negative bacilli, of which 72% (137 microorganisms) were Pseudomonas spp. Thirty-eight percent of the isolates (178 microorganisms) were gram-positive cocci, and 12% (56 microorganisms) included non pathogenic microorganisms (e.g., Neisseria spp., Staphylococcus epidermidis, Streptococcus sanguis). Ten percent (46 of 470) of the isolates represented fungi and yeasts: Candida spp. (40 isolates corresponding to 10 patients; 9%) and Aspergillus fumigatus (six isolates; 1%) (El-ebiary et al., 1997).

Atotal of 385 consecutive immunocompetent patients with ICUAP were assessed, according to the presence or absence of Candida spp. in lower respiratory tract samples. Candida spp. was isolated in at least one sample in 82 (21%) patients. Patients with Candida spp. had higher severity scores and organ

dysfunction at admission and at onset of pneumonia. In multivariate analysis, previous surgery, diabetes mellitus and higher Simplified Acute Physiology Score II at ICU admission independently predicted isolation of Candida spp. There were no significant differences in the rate of specific a etiological pathogens, the systemic inflammatory response, and length of stay between patients with and without Candida species (Terraneo et al., 2016).

Candida co-infection was observed in 30 (40%) of patients with pulmonary tuberculosis. Candida albicans was the most common isolate observed in 50% of the patients with co-infection, followed by C. tropicalis (20%) and C. glabrata (20%). Candida co-infection was found in 62.5% of female patients, while it was observed in only 29.4% of the male patients (Kali et al., 2013).

CHAPTER THREE

MATERIAL AND METHODS

3. MATERIAL AND METHODS

3.1 Study design

This study was a cross sectional study and hospital based study.

3.2 Study area

This study was conducted at Elshaab Teaching Hospital and the practical part was carried out in Research Laboratory in Sudan University of Science and Technology.

3.3 Study duration

Study was carried out during 5 months in the period from February to June 2018.

3.4 Study population

Hundred hospitalized patients with respiratory tract disease, both gender and their age varies from 20 years to 80 years, were verbally consented.

3.5 Inclusion criteria

Patients with symptoms of respiratory diseases and patients with diseases predispose individual to oppurtunstic Pulmonary Candidiasis.

3.6 Exclusion criteria

Patients with symptoms of respiratory diseases and patients with diseases predispose individual to oppurtunstic Pulmonary Candidiasis treated with anti fungal agent.

3.7 Sample size

One hundred hospitalized patients with respiratory tract disease.

3.8 Data collection

Personal and clinical data was collected from patients by direct interviewing questionnaire from each subject (Appendix 1).

3.9 Ethical consideration

Permission to carry out the study was obtained from the Ethical Committee of College of Medical Laboratory Science, Sudan University of Science and Technology and verbal consent was taken from all participants in this study.

3.10 Specimens collection

Sputum specimens were collected and obtained using a non invasive method before antibiotics therapies were started.

Early morning expectorated sputum specimens were collected by deep couph in wide mouth, sterile universal container before eating and washing of mouth. Sputum specimens were transported in ice bag and processed as soon as possible (Ssengooba *et al*., 2012).

3.11 Specimens processing

3.11.1 Macroscopical examination

The color (yellow, green or red) and consistency (purulent, mucoid, bloody or mucopurulant) of sputum specimens were observed and recorded (Ssengooba *et al*., 2012).

3.11.2 Microscopical examination

Sputum specimens were examined microscopically by 10% potassium hydroxide (KOH) wet mount and Gram's staining for the presence of budding yeast and pseudo hyphae of *Candida* species.

3.11.2.1 Direct Potassium Hydroxide preparation

Two drops of 10% KOH was placed in glass slide, purulant part of sputum specimen was selected and mixed with a drop of KOH in glass slide, cover slip was placed into preparation and incubated 3-5 minute in wet petridish and examined under microscope (using X10 and X40) for budding yeast and pseudohyphae of Candida species (appendix 2) (Aslam *et al*., 2008) (Appendix 5) .

3.11.2.2 Direct Gram's stain

One drop of sterile normal saline was placed into glass slide and purulent part of sputum was selected and placed into glass slide, preparation was mixed gently to make smear then smear was left to air dry, fixed with alcohol, and covered with crystal violet stain for one minute. Then washed by tap water, and then covered with lougol's iodine one minute. Iodine was washed off, and smear was decolourized with acetone ethanol alcohol for few seconds and washed by tap water. Safranin was added for two minutes, washed off with tap water and let to air dry and microscopically examined using oil immersion objective (X100) to observed yeast cell morphology, size, gram positive reaction and presence of pus cells, epithelial cells (appendix 3) (Srinivasan *et al*., 2012).

3.11.3 Culture

3.11.3.1 Preparation of Sabouraud's dextrose agar plates

The Sabouraud's Dextrose agar consists of Sabouraud's dextrose broth (pH, 5.6) and 1.5 % agar. This was autoclaved and cooled to 45Ċ. It was mixed thoroughly and dispensed into plates without air bubbles (Bahavan et *al*., 2010).

3.11.3.2 Inoculation of specimens into Sabouraud's dextrose agar plates

The specimens were inoculated into Sabouraud's dextrose agar plates. Purulent part of sputum was inoculated in two Sabouraud's dextrose agar plates using wire loop under aseptic condition incubated at 37Ċ and 25Ċ in aerobic condition for overnight incubation. The suspected isolates of *Candida* were sub cultured onto Sabouraud's agar plates. Colonial morphology was commented and indirect microscopy and special test were done for full identification (Aslam *et al*., 2008) (Appendix 5).

3.11.4 Indirect Gram's stain

One drop of sterile normal saline was placed into glass slide and a part of white creamy colonies was selected and placed into glass slide, preparation was mixed gently to make smear then smear was left to air dry, fixed with alcohol, and

covered with crystal violet stain for one minute. Then washed by tap water, and then covered with lougol's iodine one minute. Iodine was washed off, and smear was decolourized with acetone ethanol alcohol for few seconds and washed by tap water. Safranin was added for two minutes, washed off with tap water and let to air dry and microscopically examined (Srinivasan *et al*., 2012) (Appendix 5) .

3.11.5 Germ tube test

Test proves yeast germination, and its characteristic for the detection of *Candida albicans*. This is rapid test for presumptive identification of *C. albicans*. One ml of serum was added into small vitek tube by using a Pasteur pipette, colony of yeast was touched by sterile wire loop and emulsified it in the serum. The tube was mixed and incubated at 37Ċ for 2-4 hours but no longer, the a drop of the serum was transferred to a slide for examination, cover slip was added and examined microscopically using X40 objective. Germ tubes are appendages half the width and 3 to 4 times the length of the yeast cell from which they arise. There is no constriction between the yeast cell and the germination tube. Presence of short lateral filament (germ tube) for *C.albicans* indicate positive test. Presence of yeast cell only for non *C.albicans* indicte negative test (Bahavan *et al*., 2010) (Appendix 5).

3.11.6 Chlamydospore formation test

By using sterile inoculating needle or loop, appropriate yeast colony was touched and immediately scraped or cut "X" through prepared corn meal agar (CMA) in the middle on one half of the agar plate. Using sterile forceps, sterile cover slip was centered over the cross of one of the "X" patterns. Plate was inverted and incubated up to 3 days (72 hours) at 25± 2Ċ. Plates were examined daily for the development of chlamydospores with the aid of dissecting or stage microscope.The "X" without cover slip serves as a growth control. The result seen by microscopic examination of the yeast under the cover slip revealed chlamydospore that appear as terminal double walled

spheres on the pseudohyphae which indicated positive result (Bahavan *et al*., 2010) (Appendix 5).

3.12 Data analysis

The collected data was checked and analyzed using the computer software program SPSS (Statistical Package of Social Science) version 20 for windows and by using chi square test , p value, mean ± STD and frequency (sig value ≤ 0.05).

CHAPTER FOUR

RESULTS

4. RESULTS

A total number of hundred sputum specimens were collected from respiratory tract disease patients at Elshaab Teaching Hospital during the period from February to June 2018, 51% were female and 49% were male with male female ratio 1:1 and the age varies from 20 years to 80 years with mean of age 44.1 years ± 13.5 years (figure 1).

Sputum specimens were collected from 38/100 (38%) patients with Pneumonia, 23/100 (23%) patients with TB, 18/100 (18%) patients with Asthma, 12/100 (12%) patients with Cystic fibrosis, 5/100 (5%) patients with cancer, 3/100 (3%) patients with malignant effusion, and only 1/100 (1%) patient with HIV (figure 2).

Candida species was isolated from nine 9/100 (9%) sputum specimens of respiratory tract diseases patients (figure 3).

The results revealed that, 9/100 (9%) samples were positive for Pulmonary Candidiasis, while 91/100 (91%) samples were negative.

Among the total, the frequency of Pulmonary Candidiasis was 9/100 (9%), and among total isolates 9/9 (100%) were *Candida albicans*.

Three of nine infected patients 3/9 (34%) had cancer, two patients 2/9 (22%) had malignant effusion, also two patients 2/9 (22%) had TB, while one patient 1/9 (11%) had HIV and one patient 1/9 (11%) had cystic fibrosis (Figure3).

According to clinical history of patients, most of patients with Pulmonary Candidiasis were patients with malignancy. Over one half of patients 5/9 (56%) had cancer or malignant effusion as an underlying illness. The rest of patients two 2/9 (22%) had TB, one patient had 1/9 (11%) HIV and one patient 1/9 (11%) had cystic fibrosis.

Statistical analysis showed that there was significant association between *Pulmonary Candidiasis* and clinical history of patients (Table 1) *P value* (0.00).

According to age group, the frequency of in patients with in ages between 60-80 years 6/9 (67%) and in patients with in age between 40-59 years 2/9 (22%) followed by 1/9 (11%) in patient witn in age between 20-39 years.

Statistical analysis showed that there was significant association between Pulmonary Candidiasis and age (Table 2) *P value* (0.001).

According to gender, 4/9 (44%) of infected patients with Pulmonary Candidiasis were male and 5/9 (56%) of infected patients with Pulmonary Candidiasis were female.

The result of statistical analysis revealed that there was no significant association between Pulmonary Candidiasis and gender (Table 3) *P value* (0.774).

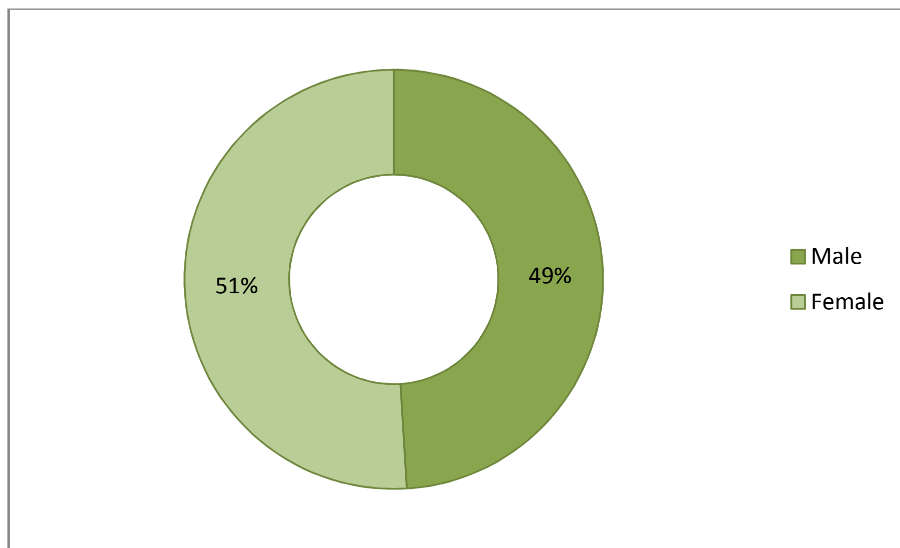

Figure1. Distribution of hospitalized respiratory tract diseases patients according to gender (N = 100).

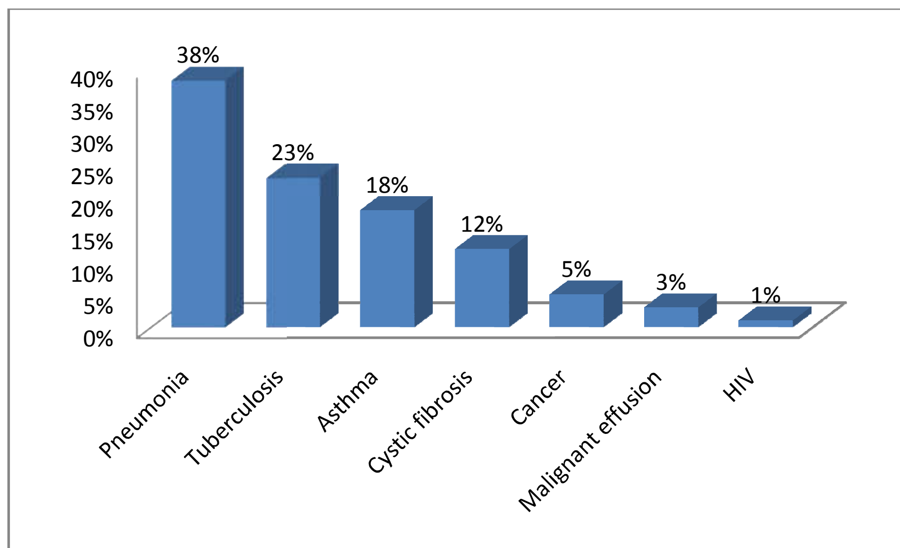

Figure 2.Clinical history of hospitalized respiratory diseases patients

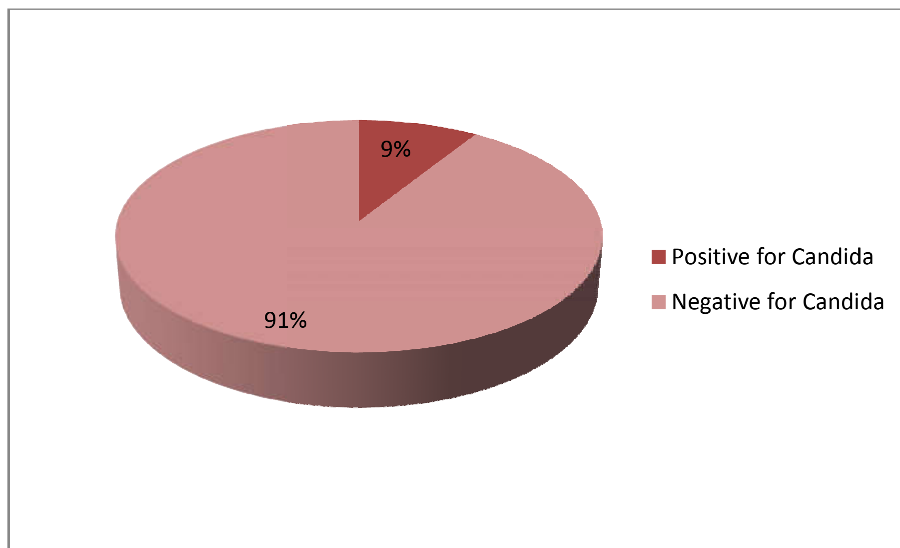

Figure 3.Frequency of Pulmonary Candidiasis among hospitalized patients with respiratory diseases (N= 100).

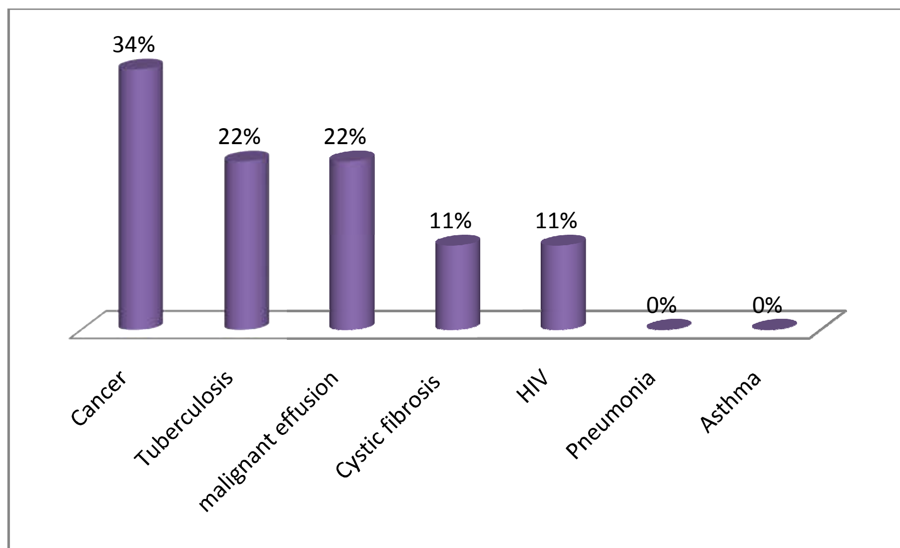

Figure 4.Percentage of Pulmonary Candidiasis and clinical history of patients

Table 1. Association between Pulmonary Candidiasis and clinical history of hospitalized patients with respiratory diseases.

Clinical history	Culture		Total
	Positive for *Candida*	Negative for *Candida*	
Cancer	3 (34%)	2 (2%)	5
Malignant effusion	2 (22%)	1 (1%)	3
Tuberculosis	2 (22%)	21 (23%)	23
HIV	1 (11%)	0 (0%)	1
Pneumonia	0 (0%)	38 (42%)	38
Cystic fibrosis	1 (11%)	11 (12%)	12
Asthma	0 (0%)	18 (20%)	18
Total	9	91	100

P value = 0.00

Table 2. Association between Pulmonary Candidiasis and age groups among hospitalized patients with respiratory diseases.

Culture	Age group in years			Total
	20-39	40-59	60-80	
Positive for *Candida*	1(11%)	2 (22%)	6 (67%)	9
Negative for *Candida*	30 (33%)	37 (41%)	24 (26%)	91
Total	31	39	30	100

P value = 0.001

Table 3.Association between Pulmonary Candidiasis and gender among hospitalized patients with respiratory diseases.

Gender	Culture		Total
	Positive for *Candida*	Negative for *Candida*	
Male	4 (44%)	45 (49%)	49
Female	5 (56%)	46 (51%)	51
Total	9	91	100

P value = 0.774

CHAPTER FIVE

DISCUSSION

5. DISCUSSION

5.1 Discussion

Candida is recognized as an important microorganism in healthcare-related infections, which have a rising incidence due to increases in extensive antibiotic administration, invasive treatments, and immunosuppressant use. *Candida* infections are associated with a higher risk of mortality than non-fungal infections; the mortality rate can be as high as ninety percent, depending on associated risk factors (Choi *et al*., 2017).

In this study, the overall frequency of *pulmonary candidiasis* among hundred respiratory tract disease patients was nine percent, *Candida albicans* represent all isolates.

The frequency of *Candida* in patients with respiratory tract disease in this result nine percent and this was agreement with other study conducted in 1997, Ten percent of the isolates represented fungi and yeasts: *Candida* species represent nine percent (El-ebiary,1997).

Another study performed in 2001, the most frequently encountered fungi were Aspergillus species fifty seven percent, followed by Cryptococcus species twenty one percent and Candida species fourteen percent (Chen *et al*., 2001).

The agreement of results although sample size and the methods used for detection and isolation of *Candida* species are different and the similarities in result due to same environmental factors around patients and personal hygiene's and habit or due to any causes that let patients susceptible to Pulmonary *Candida* infection.

The frequency of *Candida* in patients with respiratory tract disease in this result nine percent and was disagreement and lower than the frequency of *Candida* in study conducted in 2018, The *Candida* species growth was observed in the culture of twenty nine percent(Badiee *et al*.,2018).

Another study performed in 2017, twenty nine isolated yeasts; including fifty nine percent of *C. albicans/dubliniensis* complex and fourty one percent of non albicans isolates(Kianipour *et al*., 2017).

Also lower than result of study conducted in 2006, respiratory *Candida* colonization twenty six percent (Azoulay, 2006).

The difference in result may be due to difference in sample size because the researchers have large sample of patients in comparison with me and may be due to difference in the methods used for isolation and identification of *Candida* species from specimens, may be due to different personal hygiene's and habit of patients and environmental factors around patients.

5.2 Conclusion

Pulmonary Candidiasis a disease of considerable importance especially in immunocomporomised patients and elderly patients with respiratory diseases.

Candida albicans was the most prevalent pulmonary *Candida* species especially in elderly patients.

There was significant association between *Pulmonary Candidiasis* and age of patients, also there was significant association between *Pulmonary Candidiasis* and clinical history of patients. While there was no significant association between *Pulmonary Candidiasis* and gender of patients.

5.3Recommendations

1. Specimens must be collected from deep couph and early morning.
2. Collection of specimens befor antimicrobial therapies were started.
3. Increase sample size.
4. Collect more than one type of specimens from every patient (sputum and BAL) and diagnosis must be done by chromogenic media and more advanced method.
5. PCR and histopathological method must be done for more idtentification.

6. It is therefore recommended that further study be carried out to more research about pulmonary candidiasis especially immunocomporomised and elderly patient.

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APPENDCIES

APPENDICES

Appendix [1]:

Sudan University of Science and Technology
College of Medical Laboratory Science
Questionnaire

Frequency of Pulmonary Candidiasis among hospitalized patients with respiratory tract disease attending El-Shaab Teaching Hospital -Khartoum state

تردد داء فطر المبيضات الرئوي بين المرضى المصابين بامراض الجهاز التنفسي في مستشفى الشعب التعليمي - ولايه الخرطوم.

Demographic data: Name: …………………………………… Age: ……

Gender: Male :( ) Female :( )

Clinical data

- Respiratory disease: Yes ( ) No ( ) if yes: specifying ………..
- History of cancer: Yes ( ) No ( ) if yes: specifying ………….
- History of TB: Yes ( ) No ( ) if yes: specifying ………….
- History of Asthma: Yes ( ) No ( )
- History of cystic fibrosis: Yes ( ) No ( )
- History of HIV infection: Yes ( ) No ( )
- History of malignant pleural effusion: Yes ( ) No ( )
- Signs and symptoms: cough ( ) dyspnea ( )

Laboratory investigation:

- Macroscopic examination: Color ( ) Consistency ( )
- Microscopic examination:
- Wet preparation: 10% KOH……………… Gram's stain……….……
- Culture on SDA …………………………………………………...
- Identification………………………………………………………

Appendix [2]:

Potassium hydroxide, 200 g/l (20% w/v): To make 50 ml dissolve Potassium hydroxide (KOH) 10 g in 50 ml Distilled water.

Weigh the potassium hydroxide pellets. Transfer the chemical to a screw cap bottle. Add the water, and mix until the chemical completely dissolved. Store it at room temperature. The reagent is stable for up to 2 years.

Appendix [3]:

Gram stain reagents

1- Crystal violet:

To make 1 litre:

Crystal violet 20 g

Ammonium oxalate 9 g

Ethanol or methanol, absolute 95 ml

Distilled water 1 liter

Weigh the crystal violet on a piece of clean paper (pre-weighed). Transfer to a brown bottle pre marked to hold 1 liter. Add the absolute ethanol or methanol and mix until the dye is completely dissolved. Weigh the ammonium oxalate and dissolve in about 200 ml of distilled water. Add to the stain. Make up to the 1 litre mark with distilled water, and mix well. Store at room temperature.

2- Lugol's iodine solution

To make 1 litre:

Potassium iodide 20 g

Iodine 10 g

Distilled water 1 liter

Weigh the potassium iodide, and transfer to abrown bottle premarked to hold 1 liter. Add about a quarter of the volume of water, and mix until the potassium iodide is completely dissolved. Weigh the iodine, and add to the potassium iodide solution. Mix until the iodine is dissolved. Make up to the 1 litre mark with distilled water, and mix well. Label the bottle, and store it in a dark place.

3- Acetone-alcohol decolorizer

To make 1 liter:

Acetone 500 ml

Ethanol or methanol, absolute 475 ml

Distilled water 25 ml

Mix the distilled water with the absolute ethanol (ethyl alcohol) or methanol (methyl alcohol). Transfer the solution to a screw-cap bottle of 1 liter capacity. Measure the acetone, and add immediately to the alcohol solution. Mix well. Store in a safe place at room temperature. The reagent is stable indefinitely.

4- Safranin

For prepare safranin, 10g of safranin was weigh and transferred to a clean brown bottle. Then absolute ethanol (200ml) was added and mixed until the dye was dissolved, stored at room temperature.To prepare the working solution, 100ml of safranin stock solution was taken to clean bottle, then 400 ml of distilled water was added and mixed.

Appendix [4]:

Preparation of culture media:

1- Sabouraud's dextrose agar (SDA) (Hi Media Laboratories Pvt. Ltd. Mumbai, India)

Oxoid dehydrate medium formula (CN41)

Ingredients Gms/ Litre

Mycological peptone 10 g

Dextrose 40g

Agar No.1 15 g

Final pH (at 25ċ) 7.0 +/- 0.2

Preparation

The medium is used at concentration of 6.5 gram in every 100 ml of DW. Prepared and sterilize the medium as structured by manufacture. Allow to cool to 50-55Ċ, mix well and dispense aseptically in 15-20 ml amount in sterile petri dishes.

2- Corn Meal Agar medium (Hi Media Laboratories Pvt. Ltd. Mumbai, India)

Corn Meal infusion provides nitrogen, vitamins, minerals and amino acids essential for growth. Bacteriological agar is the solidifying agent.

Corn Meal infusion 50g

Agar 15g

Final pH 6.0 ± 0.2 at 25ºC.

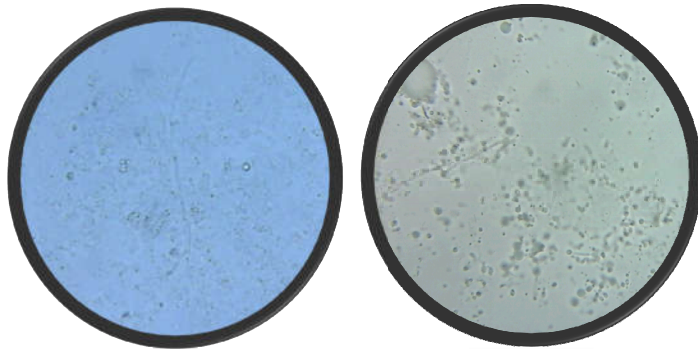
Preparation

Suspend 17 grams of the medium in one liter of distilled water. Mix well and dissolve by heating with frequent agitation. Boil for one minute until complete dissolution. Sterilize in autoclave at 121ºC for 15 minutes. Cool to 50ºC, mix well and dispense into Petri dishes.

Appendix [5]:

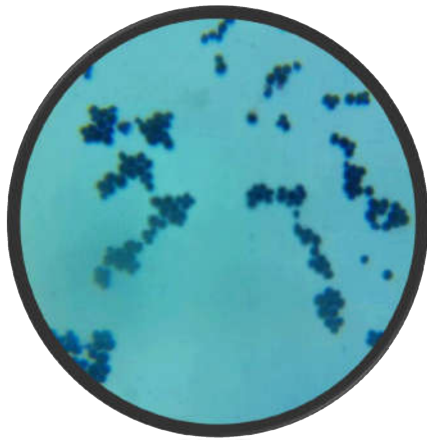
Candida grow as white, pasty colony on SDA

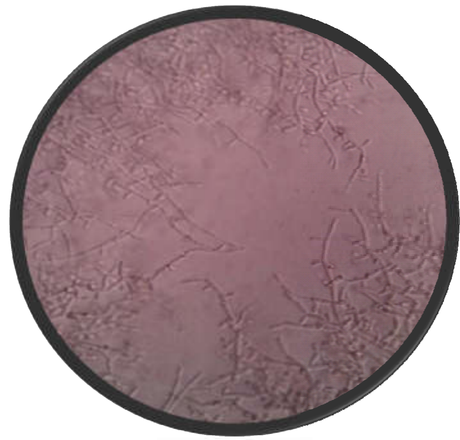
Direct KOH preparation for pseudohyphae and budding yeast of *Candida*



Gram's stain of *Candida*

Chlamydospore of *Candida*





Germ tube test for *Candida*