Molecular Characterization and Antifungal Susceptibility Testing of *Aspergillus* spp Isolated from Sudanese Patients with Underlying Lung Diseases

A thesis Submitted for Fulfillment of Requirements for the PhD Degree in Medical Laboratory Science (Microbiology)

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DECLARATION

I declare that this thesis is submitted to Sudan University of Science and Technology, College of Medical Laboratory Science for the degree of doctor of philosophy and has not been previously submitted by me for this degree at this university or any other universities or institutes.

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بسم الله الرحمن الرحيم

الاءة

قَ الَّذِينَ آمَنُوا أَنَّ لَنَا مُنْتَجٍ مِّنَ الْخَيْرِ وَمَا نُعْطِينَ الْجِنّ مِثْلَ مَا نُعْطِي الْمُؤْمِنِينَ إِلَّا مَا رَأَى الْحَمِيزُ وَمَا نُعْطِينَ الْجِنّ مُجْرِمًا إِلَّا مَا رَأَى الْحَمِيزُ ﴿۱﴾

صدق الله العظيم

(سورة إبراهيم "1")
DEDICATION

To my father sole

To my lovely mother

My beloved brother and sisters; particularly my dearest brother, Ahmed, who stands by me
when things look bleak

My dearest husband, who leads me through the valley of darkness with light of hope and
support

To my lovely Kids

To my family

To my friends

To my colleagues

To anyone really know me

With infinitive love

Samar
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English Abstract

Pulmonary aspergillosis is lung infection caused by *Aspergillus* spp, most of which are harmless, but a few can cause serious illnesses when people with weakened immune systems, underlying lung disease or asthma inhale their spores. The incidence of this infection has increased, primarily due to the increasing number of immunosuppressed patients with the advent of solid organ and bone marrow transplantation, the increased use of corticosteroids and other immune-modulating drugs, and the infection with the human immunodeficiency virus (HIV).

This is descriptive cross sectional study conducted at different hospitals in Khartoum State during the period from 2016 to 2019, which aimed to detect *Aspergillus* spp from Sudanese patients with underlying pulmonary infections by conventional cultural method, to perform antifungal susceptibility of isolated *Aspergillus* spp using E test and to study molecular characterization by PCR and sequencing.

Three hundred and eighty four patients with pulmonary underlying diseases distributed as follows, chronic pulmonary infection 219 (57.0%), asthma 77 (20.1%), cystic fibrosis 34 (8.9%), pulmonary tuberculosis 29 (7.6%), pleural effusions 12 (3.1%), malignancy 9 (2.3%), emphysema, hemoptysis and lung abscess 4 (1.0%) were included in this study. Three hundred forty one sputum and forty three bronchoalveolar lavage samples were collected from them. The age of patients ranged from 9 to 90 years, with mean age of 42 ± 16.14 (SD) years distributed on the following level: 2 (0.5%) age group less than ten years, 258 (67.2%) age group (10-49 years) and 124 (32.3%) 50 years and older. Of whom 233 (61.0%) were males, while females were 151 (39.0%).

The sensitivity of the direct microscopy in this study was (28%) while the specificity was (99.4%). Twenty eight (7.4%) *Aspergillus* spp out of three hundred eighty four and 4/348 (1%) of *Candida* spp were isolated distributed as follows: 15 (46.9%) from patients with chronic pulmonary infections, 7 (29.1%) from asthmatic patients, 3 (9.4%) from patients with cystic fibrosis, 3 (9.4%) from pulmonary tuberculosis patients and the 4 (12.4%) *Candida* spp were isolated from patients with malignant diseases and chronic pulmonary infections, with a statistically significant relationship (*P* value = 0.003) between underlying lung disease and pulmonary aspergillosis. The isolated fungal organisms by conventional cultural techniques was
11(34.4%) A. *flavus*, 9 (28.1%) A. *fumigatus*, 7 (21.9%) A. *terreus*, 1 (3.1%) A. *niger* and 4 (12.5%) *Candida* spp.

The results of the E test for Itraconazole against *Aspergillus* spp showed: 3 (33.3%) A. *fumigatus* were sensitive, 2 (22.3%) intermediate and 4 (44.4%) resistant, While 6 (54.5%) A. *flavus* were sensitive, 4 (36.4%) intermediate and 1 (9.1%) was resistant, 5 (71.4%) A. *terreus* reflected sensitive, 1 (14.3%) intermediate and 1 (14.3%) resistant and A. *niger* was resistant to Itraconazole. E test for Voriconazole against *Aspergillus* spp: 7 (77.8%) A. *fumigatus* were sensitive, 1 (11.1%) intermediate and 1 (11.1%) was resistant, While 11 (100%) A. *flavus* were sensitive and 5 (83.3%) of A. *terreus* were sensitive, 1 (26.7%) was resistant. A. *niger* was intermediate for voriconazole.

Sequencing of interspace transcribed region was determined for 17 isolates of *Aspergillus* spp after performing conventional PCR, with gene product of 535-613 bp and the obtained sequences were aligned with reference strains in Gene bank. The sequence similarity was greater than 99% between reference *Aspergillus* strains from Gene bank and clinical isolates.

The Phylogenetic analysis was performed to compare the genetic distances and evolutionary lineage for 17 isolates with well-characterized reference isolates from Genebank and this study indicated that the isolated *Aspergillus* spp was related to several strains worldwide that are far from Sudan (China, Korea, Malaysia, Netherland, India, Egypt, Zimbabwe, South Africa, Brazil, Nigeria).

In conclusion: The significant association between pulmonary aspergillosis and underlying lung diseases indicate that these diseases might be the risk factors for infection by *Aspergillus*. Furthermore, the high activity of voriconazole against A. *flavus* which was the predominant isolate from Sudanese patients with pulmonary underlying diseases in this study may make it the treatment of choice.
المستخلص

داء الرشاشيات الرئوي هو عدوى الرئة التي تسببها الرشاشيات، ومعظمها غير ضار، ولكن القليل منها يمكن أن يسبب أمراض خطيرة عند الأشخاص الذين يعانون من ضعف في جهاز المناعة، أو أمراض الرئة الكامنة، أو الربو عن طريق استنشاق الأبواق.

ازدادت نسبة حدوث هذته العدوى، وذلك لزيادة عدد المرضى الذين يعانون ضعف المناعة مع ظهور زراعة الأعضاء لصدمة ونخاع العظام، وزيادة استخدام الستيرويدات القشرية وغيرها من الأدوية المثبطة للمناعة، وبائية العدوى بفيروس نقص المناعة البشرية.

أجرت هذه الدراسة الوصفية المقطعية في مستشفيات pars خليفة في ولاية الخرطوم في الفترة من 2016 إلى 2019، والتي هدفت إلى الكشف عن الرشاشيات في المرضى السودانيين المصابين بالالتهابات رئوية كامنة بطريقة التزرع التقليدي، وإجراء حساسية مضادات الفطريات من الرشاشيات المعزولة باستخدام اختبار E ودراسة التوصيف الجزيئي بواسطة تفاعل البصمة التسممي والتسمل الجيني.

جمعت العينات من ثلاثمائة وأربعة وثمانية وثمانين مريضا يعانون من أمراض رئوية كامنة موزعة عمى النحو التالي، التهاب رئوي مزمن 219 (57.0 %)، الربو 77 (20.1 %)، السل الرئوي 29 (7.6 %)، والأنصباب الجنيني 12 (3.1 %)، وارتفاع الرئة الخبيثة 9 (2.3 %)، وأنتفاخ الرئة ونفث الدم ونفث الدم والرئة 4 (1.0 %). تم جمع ثلاثة وأربعين عينة عينة وأربعون عينة من القصبات، تراوحت أعمار المرضى من 9 إلى 90 سنة بمتوسط عمر 64 ± 14 سنة (SD) الفئة العمرية أقل من عشرة ، 67.2% الفئة العمرية (10-49 سنة) و32.3% الفئة العمرية 50 سنة وأكبر. منهم 233 (61.0 %) ذكور، بينما كانت الإناث 151 (39.0 %).

بلغت حساسية الفحص المجهري المباشر في هذه الدراسة (28%) بينما كانت الأختصاصية (99.4%). تم عزل رشاشيات رشاشية و/4 (1%) من نوع الميبيسات موزعة على النحو التالي: 348/28 (7.4%) من الربو و32/15 (21.4%) من مرضى الربو، 32/7 (29.1%) من المرضى الذين يعانون من الالتهابات الرئوية المزمنة، 32/3 (9.4%) من
المريضين الذين يعانون من التليف الكيسي (3/32 %) من مرضى السل الرئوي و (3/4 %) مبيضات تم عزلها من المرضى الذين يعانون من الأمراض الخبيثة والالتهابات الرئوية المزمنة على التوالي. مع وجود علاقة ذات دلالة إحصائية (P value = 0.003) بين أمراض الرئة الكامنة وداء الرشاشيات الرئوي. كانت الكائنات الفطرية المعزولة بواسطة التقنيات الزراعية التقليدية (11 %) الرُّشَّاشِيَّةُ الصَّفْراء، (9.1 %) الرُّشَّاشِيَّةُ الأرضيَّة، (28.1 %) الرُّشَّاشِيَّةُ الدَّخْناء، (7.1 %) الرُّشَّاشِيَّةُ الأَرْضِيَّة، (3.1 %) الرُّشَّاشِيَّةُ السَّوداء و (12.5 %) من نوع المبيضات.

ضمن الرشاشيات كألاتي: الرُّشَّاشِيَّةُ الدَّخْناء (33.3 %) كانت حساسة، (22.3 %) حساسة تعتمد على الجرعة و (44.4 %) مستحيلة، بينما كانت الرُّشَّاشِيَّةُ الصَّفْراء (45.6 %) حساسة، (4 %) مستحيلة، (9.1 %) مستحيلة تعتمد على الجرعة و (4.1 %) مستحيلة تعتمد على الجرعة و (51 %) مستحيلة تعتمد على الجرعة و (43.4 %) مستحيلة تعتمد على الجرعة و (71 %) مستحيلة تعتمد على الجرعة.

Itraconazole: 2 % (33.3 %) كانت حساسة، (9.1 %) مستحيلة تعتمد على الجرعة و (11.1 %) مستحيلة، بينما كانت الرُّشَّاشِيَّةُ الصَّفْراء (54.5 %) مستحيلة، (14.3 %) مستحيلة تعتمد على الجرعة و (11.1 %) مستحيلة، (36.4 %) مستحيلة تعتمد على الجرعة و (22.3 %) مستحيلة تعتمد على الجرعة و (71.4 %) مستحيلة.

Voriconazole: 7 % (77.8 %) كانت حساسة، (11.1 %) مستحيلة تعتمد على الجرعة و (11.1 %) مستحيلة، بينما كانت الرُّشَّاشِيَّةُ الصَّفْراء (83.3 %) مستحيلة، (26.7 %) مستحيلة تعتمد على الجرعة و (11.1 %) مستحيلة تعتمد على الجرعة.

تم تحديد التسلسل الجيني من I TRAP17 عزلة من الرشاشيات بعد اجراء اختبار البلمرة التسلسلي interspace transcribed، وتمت مقارنة التسلسلات التي تم الحصول عليها مع سلالات مرجعية في bp من جينات متاحة من بيانات مرجعية في بنك الجينات. و كان التشابه التسلسلي الكلي بين سلالات الرشاشيات المشتركية والعينات الهيكلية من نفس النوع أكثر من 99 %.

من جهة أخرى، أجري تحليل الشجرة النسبية لمقارنة المسافات الوراثية و النسب التطوري لـ 17 عزلة رشاشية مع عزلات مرجعية ذات توصيفات جيدة في بنك الجينات. وأظهرت الشجرة النسبية في هذه الدراسة أن الرشاشيات المعزولة كانت مرتبطة بعدة سلالات في جميع أنحاء العالم بعيدة عن السودان (الصين، كوريا، ماليزيا، هولندا، الهند، مصر، زيمبابوي، جنوب أفريقيا، البرازيل، نيجيريا).
خلصت الدراسة إلى أن هناك دلالات إحصائية بين داء الرشاشيات الرئوي وأمراض الرئة الكامنة وأن هذه الأمراض قد تكون عوامل خطورة لعديد الرشاشيات. كما أن النشاط المرتفع من مضاد الفطر voriconazole ضد الرشاشية الصفراء وتعزى رشاشية ارتبطت بأمراض الرئة الكامنة لدى المرضى السودانيين مما يجعل العلاج الأمثل.
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### Chapter one

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<td>ABPA</td>
<td>allergic bronchopulmonary aspergillosis</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
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<tr>
<td>AMB</td>
<td>amphotericin B</td>
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<tr>
<td>BAL</td>
<td>Broncho Alveolar Lavage</td>
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<tr>
<td>CCPA</td>
<td>chronic Cavitary pulmonary aspergillosis</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic Fibrosis</td>
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<tr>
<td>CFPA</td>
<td>chronic Fibrosing pulmonary aspergillosis</td>
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<tr>
<td>CGD</td>
<td>Chronic Granulomatous Diseases</td>
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<tr>
<td>CLSI</td>
<td>Clinical Laboratory Standard Institute</td>
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<tr>
<td>CNPA</td>
<td>chronic necrotizing pulmonary aspergillosis</td>
</tr>
<tr>
<td>COPD</td>
<td>chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CPA</td>
<td>Chronic Pulmonary Aspergillosis</td>
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<tr>
<td>CT</td>
<td>computed tomography</td>
</tr>
<tr>
<td>CTAB</td>
<td>Cetyl trimethylammonium bromide</td>
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<tr>
<td>EORTC/MSG</td>
<td>European Organization for Research and Treatment of Cancer/Mycoses Study Group</td>
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<tr>
<td>EUCASTAFST</td>
<td>European Committee on Antimicrobial Susceptibility Testing Subcommittee on Antifungal Susceptibility Testing</td>
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<tr>
<td>GM</td>
<td>galactomannan</td>
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<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<tr>
<td>HSCT</td>
<td>hematopoietic stem cell transplant</td>
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<tr>
<td>IPA</td>
<td>invasive pulmonary aspergillosis</td>
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<tr>
<td>ITS</td>
<td>internal transcribed spacer</td>
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<tr>
<td>ITZ</td>
<td>itraconazole</td>
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<tr>
<td>KOH</td>
<td>Potassium Hydroxide</td>
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<tr>
<td>LPCB</td>
<td>Lacto Phenol Cotton Blue</td>
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<td>MCC</td>
<td>Mucociliary Clearance</td>
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<td>MH</td>
<td>Mueller–Hinton</td>
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<td>MIC</td>
<td>Minimum Inhibitory Concentration</td>
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<td>NK</td>
<td>Natural Killer</td>
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<tr>
<td>ODI</td>
<td>optical density index</td>
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<td>PA</td>
<td>Pulmonary Aspergillosis</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>PCZ</td>
<td>posaconazole</td>
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<tr>
<td>PDA</td>
<td>Potato Dextrose Agar</td>
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<td>rDNA</td>
<td>Ribosomal DNA</td>
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<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
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<tr>
<td>SA</td>
<td>simple aspergilloma</td>
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<tr>
<td>SDA</td>
<td>Sabouraud Dextrose Agar</td>
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<td>SOT</td>
<td>solid organ transplant</td>
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<td>TB</td>
<td>Tuberculosis</td>
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<td>VCZ</td>
<td>voriconazole</td>
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CHAPTER ONE
INTRODUCTION

1.1. Introduction

Aspergillus pulmonary infection causes a spectrum of diverse diseases according to host immunity. There are two major entities invasive pulmonary aspergillosis and chronic pulmonary Aspergillosis (CPA) (Gianella, 2014), and three syndromes; the first one is invasive aspergillosis (IA), which is now recognized to occur in patients with critical illness without neutropenia and in those with mild degrees of immunosuppression, including corticosteroid use in the setting of chronic obstructive pulmonary disease (COPD). The second syndrome is chronic pulmonary aspergillosis includes simple aspergilloma, which is occasionally complicated by life-threatening hemoptysis, and progressive destructive cavitary disease requiring antifungal therapy (Paterson and strek, 2014) and The third syndrome is Allergic Bronchopulmonary Aspergillosis (ABPA) is a hypersensitivity which is reaction to Aspergillus mycelia that colonize the bronchi, Asthma and cystic fibrosis are the common illnesses associated with ABPA with reported prevalence of 1-2% in asthmatics, 7-14% in steroid-dependent asthmatics, and 2-15% in cystic fibrosis (CF) (Gupta et al., 2012).

During the past decades, the incidence of invasive aspergillosis has increased worldwide. According to the WHO report in 2011, around 1.2 million people in the world have been estimated to have chronic pulmonary Aspergillosis (CPA) as a sequel to tuberculosis (TB) and most cases occur in South-East Asia, Western Pacific and African regions. Few data is available on CPA as a post-TB sequel and in structural lung diseases from developing countries (Iqbal et al., 2016).

Most cases of chronic and allergic Aspergillosis are misdiagnosed and treated as tuberculosis (Maturu and Agarwal, 2015).

The incidence of infection with Aspergillus has increased, primarily due to the increasing number of immunosuppressed patients encountered in clinical practice with the advent of solid organ and bone marrow transplantation, the increased use of corticosteroids and other immune-modulating drugs, and the epidemic of infection with the human immunodeficiency virus (HIV). Established infection in this group of patient has proven difficult to eradicate, and despite significant advances in antifungal therapy, overall mortality with invasive disease remains high, (George et al., 2008).
The clinical manifestations of the condition are largely determined by the integrity of the lung as well as the innate and adaptive immune responses of the individual to the inhalation of the fungal spores (Cendrine et al., 2014). Recurrent or massive hemoptysis is the most frequent manifestation of the disease, other symptoms include cough, dyspnea, malaise, weight loss, wheezing, chest pain, and/or fever. Many of the patients may remain asymptomatic for several years (Gupta et al., 2015).

*Aspergillus fumigatus* is the predominant etiological agent isolated from such patients and responsible for the majority (85 to 90%) of different clinical manifestations of severe mold infections followed by *Aspergillus flavus* and *Aspergillus niger* (Mandanas, 2005). It infects individuals with non-systemic or mildly systemic immunodepression or altered pulmonary integrity due to underlying disease (Cendrine et al., 2014).

*Aspergillus flavus* is the second leading cause of invasive and non-invasive Aspergillosis, however, in certain countries like India, Saudi Arabia, and Sudan *A. flavus* is the predominant, etiological agent in patients with fungal rhino sinusitis (Shivaprakash et al., 2011).

*Aspergillus* is a ubiquitous and hardy organism. It grows best in moist environments, although spore aerosolization and dispersion occur most effectively in dry climates. Spores survive harsh external conditions and adapt to a range of internal environments. Although there are hundreds of *Aspergillus* species, *A. fumigatus* is by far the most common pathogenic species in humans, where the small size and hydrophobicity of its spores confer a dispersion advantage, whereas less common, *A. flavus* and *A. niger* also contribute to the total burden of pulmonary Aspergillosis (Paterson and strek, 2014).

*Aspergillus* species enter the host most commonly through the lungs by the inhalation of conidia, however, infection has also been reported by exposure and inhalation of water aerosols contaminated with *Aspergillus* conidia. Invasive aspergillosis is a major cause of morbidity and mortality in immunosuppressed patients, without effective host defenses following pulmonary exposure, the conidia resting in alveoli begin to enlarge and germinate. Hyphal transformation with vascular invasion and dissemination of infection are potential sequel (George et al., 2008).

Diagnosis of pulmonary aspergillosis is usually missed as test of their detection cannot be undertaken in routine diagnostic laboratories (Shrimali et al., 2013). The diagnosis of pulmonary aspergillosis relies on a combination of criteria related to patient characteristics, thoracic CT scan findings and mycological analysis by detection of *Aspergillus*.
precipitins IgG in the serum and/or the isolation of *Aspergillus* spp. from respiratory samples. Radiologically, pulmonary aspergillosis (PA) usually presents as a single ball-like lesion or multiple ball-like lesions inside cavities, partially surrounded by a radiolucent crescent (Monod sign), but early disease may present with recent thickening of the cavity wall and/or pleural thickening. The diagnosis of PA is mainly based on the detection of anti-aspergillus antibodies (anti-Asp-Ab) in their serum due to most of these patients either do not expectorate or their sputa are negative for mycelia (Gupta *et al*., 2015).

*Aspergillus* species grow well on standard media; however, specimens should be plated on fungal media to allow optimal growth. False negative results may occur, typically as a consequence of previous or concurrent antifungal therapy (George *et al*., 2008). Conventional diagnosis of fungal infection relies on the identification of pathogens by means of morphological characters specific to the genus and species which is sometimes unsuccessful, however, because of the atypical features of some isolates, molecular biological identification systems for pathogenic aspergilli have been suggested as a solution to this problem: for example, a Polymerase chain reaction based diagnostic method for detecting the genus *Aspergillus* using 18S rDNA has been designed (Yamakami *et al*., 1996; Gaskell *et al*., 1997). Systems have also been described for specific detection of *A. fumigatus* with primers based on regions of the 28S rDNA or of the internal transcribed spacer (ITS) 1 and 2 regions of ribosomal DNA (rDNA) (Radford, 1998; Henry *et al*., 2000). These PCR systems up to date are useful only in identifying the genus *Aspergillus* as a whole or the single species *A. fumigatus*. The ITS region contains variable elements that allow for sequence-based identification of *Aspergillus* species (Iwen *et al*., 2002).

Primary therapy has been largely limited to amphotericin B and the triazole compounds itraconazole and voriconazole (Shawn *et al*., 2011). Itraconazole is a broad-spectrum triazole agent with potent activity against *Aspergillus* spp. (Denning and Hope, 2010). In Sudan itraconazole is the drug of choice for treatment of *Aspergillus* nasosinus Aspergillosis (Yagi *et al*., 1999), which is the first orally bioavailable antifungal agent with activity against *Aspergillus* spp. (Denning *et al*., 1994) also it has an established role for the prevention of *Aspergillus* infections (Al-Nakeeb *et al*., 2012).
The use of amphotericin B has been limited by its toxic side effects, and some studies showed that voriconazole was superior to amphotericin B, as a result, voriconazole is now first-line therapy for aspergillosis (Shawn et al., 2011)

1.2. Rationale

Recently; infections caused by *Aspergillus* species had been highlighted as one of major causative agents for diseases. This probably results from a higher number of patients being at risk, including transplant recipients, neutropenic individuals, allergic patients and those treated with corticosteroids or other immunosuppressive regimen (Pasqualtto, 2009). *Aspergillus* spp cause a wide range of disorders in immune-competent as well as immune-compromised hosts including allergic, colonizing and invasive diseases (Kousha et al., 2011).

To the best of my knowledge in Sudan there is little awareness about fungal infections generally as well as pulmonary aspergillosis and also there is a misdiagnosis of pulmonary Aspergillosis with other pulmonary infections such as pulmonary tuberculosis and also the effectiveness of itraconazole (drug of choice) or other antifungals against *Aspergillus* species. Therefore, it is essential for clinicians to be familiar with the clinical presentation, diagnostic methods and management of pulmonary aspergillosis.

Fungal infections in hospitals are much less frequent than other microbial infections, and susceptibility tests for fungi are not routinely performed; that’s why we attempted to do this study so as to establish a method that is sensitive, reliable and easy to be used in routine laboratory tests resulting in appropriate treatment for patients.
1.3. Objectives

1.3.1. General objective

General objective
To study the antifungal susceptibilities and molecular characterization of *Aspergillus* spp isolated from Sudanese patients with underlying pulmonary infections.

1.3.2. Specific objectives

1- To isolate and identify *Aspergillus* spp from sputum and bronchoalveolar lavage specimens using conventional methods.

2- To confirm the identified *Aspergillus* spp by PCR technique using universal specific primers.

3- To determine the MIC of Itraconazole and Voriconazole against the isolated *Aspergillus* spp by E- test.

4- To compare between the effectiveness of Itraconazle and Voriconazole against the isolated *Aspergillus* spp.

5- To compare the isolated *Aspergillus* isolates sequences with *Aspergillus* reference strains located in Gene bank.

6 - To determine phylogenetic relationship among the isolated *Aspergillus spp.* and other *Aspergillus* spp in Gene bank.

7 - To correlate between pulmonary aspergillosis and underlying diseases.
CHAPTER TWO

LITERATURE REVIEW

2.1. Aspergillosis

Aspergillosis is defined as a group of diseases caused by Aspergillus species which induce variable clinical manifestations. These diseases include allergic bronchopulmonary disease, mycotic keratitis, otomycosis, nasal sinusitis, and invasive infections (Henery et al., 2000). The spectrum of pulmonary disease ranges from noninvasive disease, such as colonization of the organism or the presence of a fungus ball (aspergilloma), or an allergic response responsible for the syndrome of allergic bronchopulmonary aspergillosis (ABPA), to semi-invasive or invasive infections such as chronic necrotizing pneumonia and invasive pulmonary aspergillosis (George et al., 2008).

Aspergillus species continue to be an important cause of life threatening infection in immunocompromised patients with prolonged neutropenia, allogeneic hematopoietic stem cell transplant (HSCT), solid organ transplant (SOT), inherited or acquired immunodeficiencies, corticosteroid use, and others (Patterson et al., 2016).

2.2. Etiologic agents

The genus Aspergillus belongs to the family Moniliaceae of class Hyphomycetes and phylum Deuteromycota. The teleomorphs of Aspergillus species are classified in 10 genera in the phylum Ascomycota (William et al., 2003).

2.2.1. Taxonomic history of Aspergillus

Aspergillus is a saprophytic, aerobic fungus that grows on dead or decaying organic matter and produces airborne spores that can be inhaled by man (Kaur and Sudan, 2014). It was first described in 1729 and received its name due to its resemblance to an aspergillum used to sprinkle holy water. It is closely related to another mold, Penicillium, but identification of the causative organism responsible for most infections is usually not difficult, with four species responsible for the majority of illnesses (George et al., 2008).

The genus Aspergillus was first known to produce conidiospores or conidia by mitosis, was classified as an asexual or anamorphic species. Later, the teleomorph state which produces ascospores through meiosis was discovered in 1854 and described. At that time, Aspergillus was referred to the anamorph (sexual) state and up to this decade is linked to approximately ten
different ‘teleomorph’ genera (Peterson, 2003; Geiser et al., 2007). Approximately one-third of these species have a known teleomorph stage (Geiser et al., 2007).

Asexual reproduction takes place in specialized structures, called conidiophores, which emerge from the vegetative mycelium as erect, aseptate, thick-walled and specialised hyphae that expand apically to form a vesicle, on which numerous conidiogenous cells known as phialides which are flask-shaped elements whose interior successively produces conidia.

Conidia are formed in interconnected chains, are strongly hydrophobic and generally hyaline or pale but in mass it may be of different colours that are reproduced in the colonies (Pasqualotto, 2010).

Phialides can be composed by a single element or formed by short branches (metulae), which are formed, conidiogenous cells are called biseriate. In the absence of metulae, conidiogenous cells are considered uniseriate (Pasqualotto, 2010).

Other features used for identification include cleistothecia, hülle cells and sclerotia. Cleistothecia are generally produced in the teleomorph state, and act as sexual reproductive structures that contain ascospores borne within asci. Hülle cells are usually found to be associated with cleistothecia, in which the cells are thick, and globose. Sclerotia are rounded masses of mycelium that resemble cleistothecia but the structure does not have sexual spores and it may serve as resting structures to allow the species to survive in harsh conditions, both cleistothecia and sclerotia generally have a rounded shape and may be scattered abundantly (Peterson, 2003).

Figure 2.1. Conidiophore of A. niger (biseriate) and A. fumigatus (uniseriate) (Peterson, 2003)
2.2.2. Aspergillus species

The genus *Aspergillus* contains many species and these are ubiquitous in the environment but not form part of the normal flora and their spores are regularly inhaled without harmful consequences. Some species, notably *A. fumigatus*, are able to cause a range of diseases (Al-Charrakh *et al.*, 2018). These species are responsible for more than 90% of invasive disease, with *A. flavus*, *A. terreus*, and *A. niger* responsible for the majority of remaining invasive aspergillosis cases (Patterson *et al.*, 2000). Other, less pathogenic, species may also cause invasive infection in those with more profound immunosuppression, including *A. amstelodami*, *A. avenaceus*, *A. caesiellus*, *A. candidus*, *A. carneus*, *A. chevalieri*, *A. clavatus*, *A. flavipes*, *A. glaucus*, *A. granulosus*, *A. lentulus*, *A. nidulans*, *A. ochraceus*, *A. oryzae*, *A. restrictus*, *A. sydowii*, *A. tetrazonus*, *A. ustus*, *A. versicolor*, and *A. wentii* (Torres *et al.*, 2003).

### 2.2.2.1. Aspergillus fumigatus (A. fumigatus)

*Aspergillus fumigatus* is a saprophytic fungus that plays an essential role in recycling environmental carbon and nitrogen. Its natural ecological niche is the soil, where it survives and grows on organic debris. Although it is not the most prevalent fungus in the world, it is considered one of the most ubiquitous of those with airborne conidia. It sporulates abundantly, with every conidial head producing thousands of small conidia released into the atmosphere (Latge, 1999).

*A. fumigatus* is the most common pathogenic species in humans, because of its small size and hydrophobicity spores that confer a dispersion advantage (Karen and Mary, 2014), and recently it became a major cause of death in immunocompromised patients, with the mortality rate in leukaemic patients approaching 90%, even when treated with antifungal therapy (Man *et al.*, 2003).

#### 2.2.2.1.1. Genes and molecules related to *A. fumigatus* virulence

The genes and molecules related to *A. fumigatus* virulence can be classified according to the process they are involved in, e.g., thermotolerance; cell wall composition and maintenance; resistance to the immune response; toxins; nutrient uptake during invasive growth; signaling, metabolism regulation, and response to stress conditions; and allergens (Abad *et al.*, 2010).

#### 2.2.2.1.1. Thermotolerance
Aspergillus fumigatus is thermophilic fungus able to grow at 55°C and survive at more than 75°C an essential ability to adapt in decaying organic matter and to infect mammalian hosts. Therefore, genes related to thermo tolerance may also contribute to the virulence of this mold. Until now, only four genes studied have been found to be necessary for thermotolerance, The tht A gene is essential for A. fumigatus growth at 48°C but does not contribute to the pathogenicity of the species and afpmt1 gene codes for an o-mannosyl transferase, necessary for growth over 37 °C, but is not involved in virulence (Abad et al., 2010 ). The genes that confer thermotolerance or facilitate fungal growth at different temperatures are not identified (Hohl and Feldmesser, 2007).

2.2.1.1.2. Cell wall

The cell wall is the main line of defense of the fungus against a hostile environment providing structural integrity and physical protection to the cell, responsible for the interaction with the host and their components and often it is the targets of the host immune system during fungal infections. In A. fumigatus, the cell wall is mainly composed of polysaccharides (at least 90%) and proteins. Among the polysaccharides there are linear β (1–3)-glucans (20–35%) branched with β (1–6) links (4%); linear β (1–3/1–4)-glucans (10%); α (1–3)-glucans (35–46%); chitins; and galactomannans (20–25%) (Abad et al., 2010).

Figure 2.2. Scheme of Aspergillus fumigatus cell wall (Abad et al., 2010).
2.2.2. *Aspergillus flavus*

*Aspergillus flavus* has a worldwide distribution that probably results from the production of numerous airborne conidia, which easily disperse by air movements and possibly by insects, and the atmosphere composition has a great impact on mould growth, with humidity being the most important variable (Gibson *et al*., 1994). The optimum temperature for *A. flavus* to grow is 37 °C, but fungal growth can be observed at temperatures ranging from 12 to 48 °C. This high optimum temperature contributes to its pathogenicity in humans (Hedayati *et al*., 2007).

*A. flavus* is particularly prevalent in the air of some tropical countries like Saudi Arabia and Sudan, with semi-arid and arid dry weather conditions and it is frequently described as a leading cause of invasive aspergillosis (Pasqualotto, 2009). It is the second leading cause of invasive and non-invasive aspergillosis and it is considered as the main *Aspergillus* species infecting insects. It is also able to cause diseases in economically important crops, such as maize and peanuts, cottonseed, almond, and pistachio, and produce potent mycotoxins which leads to substantial economic damage worldwide (Pasqualotto, 2009; Rudramurthy *et al*., 2011).

*A. flavus* is the predominant etiological agent in patients with fungal rhinosinusitis and endophthalmitis and it has been reported to cause outbreaks of mucocutaneous and subcutaneous aspergillosis, in immunosuppressed mice it was observed that much lower inocula of *A. flavus* spores could kill animals compared to *A. fumigatus* spores. The agent is also known to cause environmental aflatoxin contamination in crops like maize, (Rudramurthy *et al*., 2011).

Accurate species identification within *A. flavus* complex remains difficult due to overlapping morphological and biochemical characteristics. In general, it is known as a velvety, yellow to green or brown mould with a gold to red-brown reverse. It's conidiophores are variable in length, rough, pitted and spiny. They may be either uniseriate or biseriate covering the entire vesicle, and phialides point out in all directions. They produce globose to subglobose conidia, conspicuously echinulate, varying from 3.5 to 4.5 mm in diameter (Hedayati *et al*., 2007).

2.2.2.1. Mycotoxins

Mycotoxins are fungal secondary metabolites that are potentially harmful to animals or humans and the word ‘aflatoxin’ came from ‘Aspergillus flavus toxin’, since *A. flavus* and *A. parasiticus* are the predominant species responsible for aflatoxin contamination of crops prior to harvest or
during storage (Yu et al., 2004). The aflatoxins B1, B2, G1 and G2 are the major four toxins amongst at least 16 structurally related toxins (Goldblatt, 1969). Aflatoxin B1 is particularly important, due to its toxic and potent hepatocarcinogenic natural compound (Bennett and Klich, 2003).

2.2.3. Aspergillus terreus

Aspergillus terreus (A terreus) is a common soil saprophyte which produces globose, heavy-walled hyaline cells laterally on the hyphae, that are called accessory conidia or sometimes referred to as aleuroconidia that produces only by A. terreus (Amutha and Godavari, 2014). It is considered as an important human pathogen, and often causes disseminated infection with increased lethality compared to other Aspergillus spp. Recent data indicate that the accessory conidia produced by A. terreus can induce elevated inflammatory responses in a pulmonary model of aspergillosis (Deak et al., 2009).

Although the majority of IPA cases are caused by A. fumigatus, A. terreus has also emerged recently as a significant respiratory pathogen (Mokaddas et al., 2010). Colonies are buff to beige to cinnamon, conidial heads are biseriate and columnar. Conidiophores are smooth-walled and hyaline. Globose, sessile accessory small conidia are produced on submerged hyphae. Colonies color and fruiting structures are characteristic for this species, molecular characterization has shown that A. terreus is also a species complex (Patterson et al., 2011).

It causes infections ranging from superficial infections to allergic bronchopulmonary aspergillosis, aspergilloma, and invasive disease in severely immunocompromised hosts. Previous studies demonstrated that A. terreus infections were associated with dissemination, resulting in higher patient mortality, compared with other Aspergillus species (Lass Florl, 2012). A. terreus in particular is an amphotericin B (AMB) resistant mold that has been recognized as a cause of frequently lethal infections (Blum et al., 2008).

2.2.4. Aspergillus niger

Aspergillus niger (A. niger) is a filamentous ascomycete fungus that is ubiquitous in the environment which has been implicated in opportunistic infections of humans, in addition, A. niger is economically important as a fermenter organism used for the production of citric acid which represents one of the most efficient, highest yield bioprocesses used currently by industries (Scott, 2006).
*A. niger* is widespread in soil and on plants and is common in foods such as pepper. Colonies are initially white but quickly become black with the production of the fruiting structures. It grows rapidly with a pale yellow reverse side. Like other *Aspergillus* species, hyphae are hyaline and septate, conidial heads are biseriate and cover the entire vesicle, brown to black and are very rough (4–5 mm). It is commonly associated with colonization and otic infections, produces oxalate crystals in clinical specimens and also contains several related species (Patterson, 2011).

Although the species *Aspergillus niger* is infrequently encountered, the endobronchial visualization of black necrotic debris or a fungus ball or the finding of black acidic sputum or pleural fluid suggests the presence of *A niger* and the destructive by-product of its fermentation, oxalic acid (Kimmerling *et al*., 1992).

### 2.2.3. Sources of *Aspergillus* Spp

#### 2.2.3.1. Air and Ventilation Systems

Most patients appear to develop aspergillosis following inhalation of conidia into the alveoli or the upper airways. Sampling of outside air, hospitals and other buildings routinely reveals *Aspergillus* species (Perdelli *et al*., 2006; Schmitt *et al*., 1990). In some instances, variations in the number of colony forming units in air samples inside hospitals have been linked to corresponding changes in infection rates in immune compromised patients (Arnow *et al*., 1991), other studies could not verify this correlation (Hospenthal *et al*., 1998). Outbreaks have been ascribed to aerosolisation of conidia from insulation, fire-proofing materials, and carpeting (Hahn *et al*., 2002).

Faulty ventilation systems have allowed increased numbers of conidia to be dispersed, and has been associated with contaminated air filters and defects intrinsic to air handling systems (Pasquallato, 2009).

#### 2.2.3.2. Water

Samples taken from showerheads, faucets, and shower drains yielded *Aspergillus* species, but whether water is a major source of infection is still debated (Anaissie and Costa, 2001). One case have been documented, in which molecular typing showed similarity between a strain found on the shower wall in the patient’s hospital room and the strain causing the patient’s infection (Anaissie *et al*., 2002).
2.2.3.3. Other Sources of *Aspergillus* spp

Routinely, pepper is heavily contaminated with *Aspergillus* conidia, and other foodstuffs, including tea in bags, freeze-dried soups, and various fruits with “fuzzy” surfaces may carry *Aspergillus* spp (Brenier et al., 2006).

An unusual source of infection with *Aspergillus* spp. is Cannabis, marijuana cigarettes routinely yield *Aspergillus* species on culture, but infections linked to smoking marijuana have been rare and only in immunosuppressed hosts (Marks et al., 1996).

Amongst children, aspergillosis has been linked to contaminated adhesive tape arm boards used to secure intravenous lines (James et al., 2000).

Also aerosolisation that took place during dressing changes of a heavily colonized abdominal wound in a patient who had undergone liver transplantation was shown by molecular typing studies to be responsible for subsequent IA in a second liver transplant recipient who was cared for in the same open unit (Pegues et al., 2002).

2.3. Epidemiology of pulmonary aspergillosis

2.3.1. Transmission of *Aspergillus*

Air plays a big role in spreading of *Aspergillus* spp. environment and transmission to patients. *Aspergillus* spp. release large amounts of conidia in the air from conidiophores that arise from the mycelial mass. When conidia gradually settle out resulting in contamination of anything in contact with air and remain viable for months (Warris and Verweij, 2005).

Inhalation of fungal conidia which is relatively small in sizes ranging from 2–5 µm, depositing in the upper and lower respiratory tracts is the primary acquisition route for aspergillosis (Einsele et al., 1998) *Aspergillus* spores are released from different reservoirs and often remain in the air for prolonged periods due to their tiny size (Warris and Verweij, 2005).

Recent investigations have not found correlations between the number of airborne *Aspergillus* conidia and the seasons, and there is no consensus on safe concentrations of airborne conidia; their levels are probably correlated to the severity of immunosuppression (Marie-Christine et al., 2011).

In most individuals, inhaled conidia will be cleared by the alveolar macrophages, without affecting the individuals’ health. In contrast Immunocompromised patients are extremely susceptible to local invasion of respiratory tissue by deposited conidia, resulting in invasive
growth of hyphae (Denning, 1998). Therefore, it has been hypothesized that the inhalation of airborne Aspergillus conidia, either directly or through intermediate nasopharyngeal colonization, is a direct cause of pulmonary infection in immunocompromised patients (Warris and Verweij, 2005).

2.3.2. Prevalence of pulmonary aspergillosis

Prospective surveillance among transplant recipients performed during 2001-2006 found that invasive aspergillosis was the most common type of fungal infection among stem cell transplant recipient (Kontoyiannis et al., 2010) and was the second-most common type of fungal infection among solid organ transplant recipients (Pappas et al., 2010). During 1992-1993 the suggested annually rate was 1 to 2 cases of aspergillosis per 100,000 population (Rees et al., 1998).

Although most cases of aspergillosis are sporadic, outbreaks of invasive aspergillosis occasionally occur in hospitalized patients. Invasive aspergillosis outbreaks are often found to be associated with hospital construction or renovation, which can increase the amount of airborne Aspergillus, resulting in respiratory infections or surgical site infections in high-risk patients (Vonberg and Gastmeier, 2006; Weber et al., 2009). The incubation period for aspergillosis is unclear and likely varies depending on the dose of Aspergillus and the host immune response (Kontoyiannis et al., 2010).

Allergic bronchopulmonary aspergillosis (ABPA) likely affects between 1 and 15% of cystic fibrosis patients. One study calculated that 2.5% of adults who have asthma also have ABPA, which are approximately 4.8 million people worldwide. Of these 4.8 million people who have ABPA, an estimated 400,000 also have chronic pulmonary aspergillosis (CPA). Another 1.2 million people are estimated to have CPA as a sequel to tuberculosis, and over 70,000 people are estimated to have CPA as a complication of sarcoidosis (Denning et al., 2013). Allergic forms of aspergillosis such as allergic bronchopulmonary aspergillosis (ABPA) and allergic Aspergillus sinusitis are generally not life-threatening. In contrast, invasive aspergillosis although uncommon, is a serious infection and can be a major cause of mortality in immunocompromised patients. A large prospective study found that the one-year survival for people who had invasive aspergillosis was 59% among solid organ transplant recipients and 25% among stem cell transplant recipients (Kontoyiannis et al., 2010).
2.4. Immunity to aspergillosis

The development of pulmonary aspergillosis requires host’s predisposing factors such as allergic status (asthma), airways diseases (bronchiectasis, cystic fibrosis), chronic lung cavities (tuberculosis, sarcoidosis) or immune deficiency (Chabi et al., 2015).

Host defence mechanisms against fungi are numerous, and range from protective mechanisms that were present early in the evolution of multicellular organisms (innate immunity) to complicated adaptive mechanisms that are induced specifically during infection and disease (adaptive immunity) (Romani, 2004). Two mechanisms of immune responses act to generate the most effective form of immunity for protection against Aspergillus species. Primarily the interactions between the fungus and cells of the innate immune system, followed by the actions of T cells will feedback into this dynamic equilibrium to regulate antifungal effector functions and the balance between pro inflammatory and anti-inflammatory signals (Romani, 2010).

Host defence against Aspergillus comprises recognition of the pathogen; , a rapidly deployed and highly effective innate effector phase ; and a delayed but robust adaptive effector phase characterized by immunologic memory (Phadke and Mehrad, 2005), and there are three different mechanisms of defence: (a) physical barriers (b) phagocytosis; and (c) humoral compounds (Blanco and Garcia, 2008).

2.4.1. Physical barriers

Physical barriers include mucous membranes, mucociliary clearance and local secretion of inflammatory mediators by innate immunity cells (Crameri and Blaser, 2002), and the airway mucus constitutes a physical, chemical and biological barrier of secretory products from the mucous membrane that facilitates the elimination of inspired particles, including fungal conidia. This fluid contains glycoproteins, proteoglycans, lipids, lysozyme and surfactants (McCormack and Whitsett, 2002). Therefore, any defect in these innate immune molecules might contribute to increased susceptibility to Aspergillus infections. These collectings are not primitive, innate molecules but are highly specialized and able to modulate responses to foreign agents (Madan et al., 2005).

2.4.2. Phagocytosis

The role of phagocytes in the defence against Aspergillus is essential to avoid the development of the disease. The attachment of conidia with macrophages via non-specific receptors, such as mannosylfucosyl, does not rely on the presence of opsonization factors such as complement or
immunoglobulin, the conidia are internalized by the macrophages and prevented from growth for several hours until the macrophage begins to destroy them. In spite of the enormous capacity of the macrophages to kill conidia, their effectiveness is not 100% (Latge, 1999). This mechanism greatly reduces pathogenicity by blocking germination and development of the fungus (Romani, 2004). Polymorphonuclear neutrophils are responsible for the destruction of the hyphae of *A. fumigatus*, and they are able to kill the conidia that escape destruction by the macrophages (Duong *et al*., 1998; Romani, 2004). The hyphae are too large to be engulfed and the neutrophils bind to the surface without the need for complement or immunoglobulin. This contact between neutrophils and hyphae triggers secretion of reactive oxidative intermediary agents that rapidly damage the hyphae, 50% of hyphae are destroyed in 2 hrs (Latge, 1999). Natural Killer (NK) cells are considered as an important effector cell in aspergillosis (Walsh *et al*., 2005) especially in neutropenic patients (Morrison *et al*., 2003).

**2.4.3. Humoral compounds**

Cytokines are important tools in control, immune regulation and activation of pulmonary host defences (Walsh *et al*., 2005). Pathogen recognition via soluble and cell-bound microbial pattern recognition receptors is quickly followed by the afferent limb of pathogen recognition, which consists of the elaboration of an initial group of cytokines including TNF and members of the IL-1 family among which, IL-1β is induced in alveolar macrophages in response to *Aspergillus* antigens and in peripheral blood monocytes in response to *Aspergillus* conidia and hyphae (Park and mehrad, 2009).

Direct activation of the alternative complement pathway by *A. fumigatus* has been demonstrated, although the mechanism that begins the cascade of the complement resting conidia, germinated conidia and hyphae seems to be different (Feldmesser, 2005).

In general, the immune response against an *A. fumigatus* infection is usually a mixed response that is as much humoral as cellular, but it is effective only if it is associated with a cellular response, with increase of CD4 lymphocytes, and elevation of the levels of IL-2, IFN-γ and IL-12. If the response is largely humoral, with an increase in the production of antibodies, IL-4, IL-5 and IL-10, it is usually associated with progression of the disease (Mehrad and Standiford, 1999; Roilides *et al*., 1998, 1999).
2.5. Clinical presentations

The manifestations of aspergillosis are myriad and dependent upon both the site and the severity of involvement and host immune status. Although infection with *Aspergillus* has been reported involving virtually all organ sites, the upper airways, lungs, and surrounding structures are those most frequently involved (George et al., 2008).

The spectrum of this disease begins with two non-invasive pulmonary diseases, allergic Bronchopulmonary Aspergillosis (ABPA) and the Aspergilloma as a saprophytic form, semi-invasive forms as chronic necrotizing aspergillosis (CAN) and the airway-invasive aspergillosis (AIA) and finally the invasive pulmonary aspergillosis (IA) which is a major cause of mortality in severely immunocompromised patients (Walsh et al., 2008).

![Diagram of clinical presentations](image)

*ICH = immunocompromised host; IPA = invasive pulmonary aspergillosis; ABPA = allergic bronchopulmonary aspergillosis*

The clinical spectrum of conditions resulting from the inhalation of Aspergillus spores (Soubani and Chandrasekar, 2002)

2.5.1. Allergic Bronchopulmonary Aspergillosis (APBA)

ABPA was first described by Hinson and colleagues in 1952, it is a hypersensitivity disorder induced by a fungus *Aspergillus* and affects non-immunocompromised patients (Gupta et al., 2012).
ABPA occurs in patients with asthma and cystic fibrosis patients (Al-Malaky et al., 2015). The prevalence of ABPA is reported to be 1-2% in asthmatics, 7-14% in steroid-dependent asthmatics, and 2-15% in cystic fibrosis (CF) (Gupta et al., 2012). This complex hypersensitivity reaction (type I and III) to Aspergillus organisms that proliferate in the airway lumen, results in a constant supply of antigen with excessive mucus production and abnormal ciliary function and The fungal hyphae were commonly seen in mucoid plugs without evidence of tissue invasion findings as the mucoid impaction. Bronchial wall damage and bronchiectasis are typical of this disease with involving the segmental and sub segmental bronchi (Ruiz et al., 2013). Almost all patients have clinical asthma, and patients usually present with episodic wheezing, expectoration of sputum containing brown plugs, pleuritic chest pain, and fever (Ruiz et al., 2013). Patients with chronic allergic bronchopulmonary aspergillosis may also have a history of recurrent pneumonia (Franquet et al., 2001). Chest radiograph findings may be normal in the early stages of the disease, and during acute exacerbations, pulmonary infiltrates are characteristic feature of the disease that tends to be in the upper lobe and central in location. There may be transient areas of opacification due to mucoid impaction of the airways, which may present as band-like opacities emanating from the hilum with rounded distal margin (gloved finger appearance) The ’ring sign’ and ’tram lines’ are radiological signs that represent the thickened and inflamed bronchi may be seen on chest radiographs. Central bronchiectasis and pulmonary fibrosis may develop at later stages (Zmeili and Soubani, 2007). Acute clinical symptoms include recurrent wheezing, malaise with low-grade fever, cough, sputum production, and chest pain. ABPA is most frequently associated with A. fumigatus; however, other Aspergillus species have been implicated including A niger, A. flavus, A. nidulans, A. oryzae and A. glaucus (Agbetile et al., 2012).

### 2.5.2. Chronic pulmonary Aspergillosis CPA

The definition of chronic pulmonary aspergillosis (CPA) in non-severely immunocompromised patients remains vague, and a wide range of clinical, radiologic, and anatomic pathologic entities have been described with a variety of names ie, simple aspergilloma (SA), semi/chronic invasive aspergillosis, chronic necrotizing pulmonary aspergillosis (CNPA), complex aspergilloma, chronic cavitary and fibrosing pulmonary and pleural aspergillosis, and pseudomembranous tracheobronchitis caused by Aspergillus (Camuset et al., 2007). All these diseases share...
common characteristics, suggesting that they belong to the same group of CPA disorders. These characteristics include firstly a specific cause (e.g., alcohol, tobacco abuse, or diabetes) that is responsible for the deterioration in local or systemic defenses against infection (Saraceno et al., 1997); secondly an underlying bronchopulmonary disease (e.g., active tuberculosis or tuberculosis sequelae, bronchial dilatation, sarcoidosis, or COPD) (Camuset et al., 2007), thirdly generally, the prolonged use of low-dose oral or inhaled corticosteroids, and finally the absence of or presence of very little vascular invasion, a granulomatous reaction, and a low tendency for metastasis (Denning et al., 2003).

Chronic pulmonary aspergillosis (CPA) is estimated to affect 3 million persons worldwide. It usually occurs in patients with underlying pulmonary disease, and the lesion usually progresses latently (Kosmidis et al., 2015). Therefore, it is not uncommon for patients to present with hemoptysis and/or respiratory failure, it is considered to be one of the most refractory pulmonary infectious diseases; the estimated 5-year survival for CPA ranges from 50–85%, which is similar to that of idiopathic pulmonary fibrosis (Page et al., 2016).

Diagnosis of CPA relies on a combination of criteria related to patient clinical findings, thoracic CT scan findings and mycological analysis, i.e. the detection of Aspergillus precipitins (IgG) in the serum and/or the isolation of Aspergillus spp. from respiratory samples. Each can be observed individually in cases of lung cancer or other chronic respiratory infections such as tuberculosis (TB) or non-TB mycobacterial infection but also in a typically complete form of CPA, so the isolation of Aspergillus spp. may often only be the evidence of colonisation of the respiratory tract, especially as the sensitivity/specificity of galactomannan (GM) antigen testing in the serum and respiratory secretions for the diagnosis of CPA has still to be elucidated (Cendrine et al., 2014). It is is slowly progressive fungal disease over months to years, and may require life-long therapy (Crosdale et al., 2001).

2.5.2.1. Aspergilloma

Aspergilloma (fungus ball) is a mass composed of fungal hyphae, inflammatory cells, fibrin, mucus and tissue debris that develops in a pre-existing cavity in the lung, without invading the surrounding lung parenchyma or blood vessels, although exceptions have been noted and the lesion remains stable in the majority of cases, but it may decrease in size or resolve spontaneously without treatment in 10% of cases. Rarely, the aspergilloma may increase in size (Kousha et al., 2011). There are many cavitary lung diseases can be complicated by
aspergilloma, including tuberculosis, which is the most common condition, sarcoidosis, bronchiectasis, bronchial cysts, bulla, pneumatoceles, ankylosing spondylitis, neoplasm, and pulmonary infarction (Franquet. et al., 2001; Ruiz et al. 2013).

It often misdiagnosed as tuberculosis (TB) in developing countries where the prevalence of TB is high, hemoptysis is often equated with TB, and most patients are diagnosed clinically (Afua et al., 2016).

It may occur in isolation, where they are termed “single pulmonary aspergillomas”, or may coexist in the context of either Chronic Cavitary Pulmonary Aspergillosis (CCPA) or chronic fibrosing pulmonary aspergillosis (CFPA). The term single (or simple) pulmonary aspergilloma describes a single fungal ball in a single pulmonary cavity in the absence of any other signs of CCPA and usually describes those found after infection with *M. tuberculosis*. In contrast with other forms of CPA, simple aspergillomas run an indolent course and are only very slowly progressive, with many being detected incidentally and the most commonly reported feature is haemoptysis, arising from stimulation of additional surrounding bronchial vasculature (Hayes and Novak-Frazer, 2016).

### 2.5.2.2. Chronic Necrotizing Aspergillosis

Also called semi-invasive or subacute invasive aspergillosis, was first described in 1981. It is an inactive, cavitary and infectious process of the lung parenchyma secondary to local invasion by *Aspergillus* species, usually *A. fumigatus*. In contrast to IPA, CNA runs a slowly progressive course over weeks to months, and vascular invasion or dissemination to other organs is unusual (Koush et al., 2011).

CNA usually affects middle-aged and elderly patients with altered local defenses, associated with underlying chronic lung diseases such as COPD, previous pulmonary tuberculosis, thoracic surgery, radiation therapy, pneumoconiosis, cystic fibrosis, lung infarction, or sarcoidosis, also it may occur in patients who are mildly immunocompromised due to diabetes mellitus, alcoholism, chronic liver disease, low-dose corticosteroid therapy, malnutrition, and connective tissue diseases such as rheumatoid arthritis and ankylosing spondylitis (Zmeili and Soubani, 2007). Mannose-binding lectin polymorphisms may be susceptibility factors for CNPA (Crosdale, 2001).
2.5.3. Invasive pulmonary aspergillosis (IPA)

The IPA was first described in 1953, due to widespread use of chemotherapy and immunosuppressive agents. Its incidence has increased over the past decades. It increased from 17% to 60% of all mycoses, the mortality rate of IPA exceeds 50% in neutropenic patients and reaches 90% in haematopoietic stem-cell transplantation (HSCT) recipients (Kousha et al., 2011).

Acute invasive aspergillosis is a devastating opportunistic infection in severely immunocompromised and patients at risk including those with prolonged neutropenia, (HSCT) recipients, solid organ transplant recipients (particularly lung transplant recipients), advanced AIDS and CGD (Segal, 2009; Segal and Walsh, 2006). Mortality from invasive aspergillosis has increased by several-fold in the 1980s and 1990s in the U.S. and Europe (Sheriff and Segal, 2010). There is also a growing appreciation of invasive aspergillosis in persons with less severe levels of immunocompromise, for example, chronic necrotizing pulmonary aspergillosis (CNPA), or modest immune impairment, such as occurs with diabetes, poor nutrition, chronic obstructive pulmonary disease, or low-dose corticosteroids (Sheriff and Segal, 2010).

Invasive pulmonary aspergillosis has also been reported in critically ill patients without a documented systemic immune disease (Meersseman et al., 2007). It has become a leading cause of death, mainly among hematology patients, the average incidence of IA is estimated to be 5 to 25% in patients with acute leukemia, 5 to 10% after allogeneic BMT, and 0.5 to 5% after cytotoxic treatment of blood diseases or autologous BMT and solid-organ transplantation (Latge, 1999).

The IPA principally involves the sinopulmonary tract, a reflection of inhalation being the most common route of entry of Aspergillus spores with rarely other sites of entry, such as the gastrointestinal tract or skin. Fever, cough, and dyspnea are frequent, although non-specific, findings of pulmonary aspergillosis. The most common site of invasive Aspergillosis is vascular invasion may manifest as pleuritic chest pain due to pulmonary infarction or hemoptysis. Central nervous system involvement is a devastating consequence of disseminated aspergillosis, and may manifest with seizures or focal neurological signs from mass effect or stroke. Premature neonates can also develop aspergillosis, and skin being the most common site of disease (Singer et al., 1998; Horri and Nopper, 2007).
2.6. Pathogenicity

Infections caused by *Aspergillus* species have grown in recent years. This probably results from a higher number of patients being at risk, including transplant recipients, neutropenic individuals, allergic patients and those treated with corticosteroids or other immunosuppressive regimens, there are several virulence factors which have a big role in *Aspergillus* pathogenicity (Pasquallotto, 2009).

2.6.1. Conidial size, surface and pigments

*Aspergillus* species produce conidia (asexual spores) that can easily be dispersed in the soil and air which uptake by a susceptible host. This is usually the initial event in *Aspergillus* diseases with alveolar macrophages acting as first-line defence, the difference in conidia size of *Aspergillus* spp has a great in importance in pathogenicity for example, *A. fumigatus* conidia to reach the pulmonary alveoli much easier than those of *A. flavus* because of it’s smaller size, and this probably explains why *A. fumigatus* is the main agent of invasive pulmonary aspergillosis, while *A. flavus* is an important aetiology of *Aspergillus* sinusitis and a frequent cause of cutaneous and wound aspergillosis (Hedayati et al., 2007).

In addition to conidial size, the outermost cell wall layer of *Aspergillus* conidia which contains rodlets that are associated with hydrophobic properties may also be of importance because of its role in resisting extreme atmospheric conditions and facilitate airborne dispersion of *Aspergillus* conidia (Hohl et al., 2007).

Pigment seems to give protection to the conidia against environmental damage from UV radiation and in addition, it seems to protect against phagocytosis in vitro and in vivo (Jahn et al., 1997). Melanin may also reduce complement opsonization by ‘camouflaging’ binding sites, which for instance can reduce C3 ability to bind conidia (Brakhage et al., 2005). It could be more important as a facilitating factor for fungal survival in the external environment than for virulence in the host, since several non-pathogenic fungi are also known to produce melanin, this pigment is probably not essential for the occurrence of invasive fungal diseases in humans (Brakhage and Liebmann, 2005).

2.6.2. Adhesion of *Aspergillus* conidia to the lung epithelia

The adhesion of *Aspergillus* conidia to proteins present in the lung cell basal lamina is considered an important initial step in the development of invasive aspergillosis, these Important proteins include fibronectin, laminin, type IV collagen, fibrinogen, complement, albumin, and
surfactant proteins. In a comparison involving several Aspergillus species, conidia of A. niger, A. fumigatus and A. flavus were found to bind significantly better to fibrinogen than A. terreus conidia (Pasquallotto, 2009).

2.6.3. Germination rate and thermotolerance

The germination rates at 37°C differed significantly for the most common pathogenic Aspergillus species. When using the same inoculum of aspergillus spores in RPMI 1640 medium, A. fumigatus germinated faster than A. flavus, which in turn germinated faster than A. niger. The percentage of germination markedly increased 3- to 10-fold for both A. fumigatus and A. flavus when temperature was increased from 20°C to 30°C, and again 2- to 3-fold from 30°C to 37°C. However at 41°C germination of A. fumigatus was still enhanced, while germination of A. flavus decreased by 45% (as compared with 37°C). So temperature plays a crucial role in selecting and promoting pathogenic species of Aspergillus, with A. fumigatus being the species most able to adapt to extreme changes in environmental conditions (Manavathu et al., 1999; Araujo and Rodrigues, 2004).

2.6.4. The role of albumin

Although albumin was shown to significantly promote germination of A. fumigatus, the germination of both A. flavus and A. niger was reduced in presence of albumin. A. flavus germination was reduced by 20 and 25% in the presence of 2 and 4% of human albumin, respectively and similar effects were obtained with the use of bovine albumin, the formation of conidiophores and maturation of A. fumigatus conidia were also faster in the presence of human albumin (Pasquallotto, 2009).

2.6.5. Secondary metabolites and toxins

Aspergillus species produce several secondary metabolites during invasive hyphal growth in tissues (Hohl et al., 2007). Many of these substances which include fungal enzymes and toxins have been identified as being important in the process of fungal assimilation of nutrients from the host. It remains however a subject of debate whether any of these metabolites actually represent a virulence factor (Cox et al., 2001).

Filamentous fungi require the activity of extracellular enzymes to degrade the structural barriers in the host (Shibuya et al., 2006; Mellon et al., 2007). These enzymes include nucleases, oxidases, catalases, phosphatases, peptidases and proteases that are produced to degrade complex macromolecules in order to provide nutrients for the fungus. Fungal proteases may also induce
local airway inflammation by activating inflammatory pathways via epithelial cells (Tomee and Kaufmann, 2000).

Other proteases have been detected during Aspergillus infection, including the alkaline serine protease, the metalloprotease and an aspartic protease (Ibrahim et al., 2008).

Amongst the several secondary metabolites produced by A. flavus are aflatoxins, which are known as the most toxic and potent carcinogenic natural compounds ever characterized (Hedayati et al., 2007). These aflatoxins may impair mucociliary clearance (MCC) and other innate defense pathways, enhancing the pathogenicity of A. flavus and possibly other co-infecting pathogens as well (Lee et al., 2016).

A. fumigatus during invasive hyphal growth produce Gliotoxin which is one of the most abundant metabolites that exerts a broad spectrum of immunosuppressive effects in vitro, including inhibition of cytokine production, antigen presentation and production of reactive oxygen species by macrophages, and reduced cytotoxicity in T-cells (Kupfahl et al., 2007). Also it was found to be produced much faster under certain culture conditions, such as at 37°C and under high oxygen content, which is close to the environment in the host. It could be detectable in the sera of aspergillosis mice and of aspergillosis patients, so it is becoming evident that gliotoxin is produced in the infected organs of patients of aspergillosis at a significant level (Kamei and Watanabe, 2005).

2.7. Diagnosis

Diagnosis of different forms of aspergillosis presents a major challenge in medicine because of non-specific nature of their clinical presentation, lack of a sensitive and accurate diagnostic assay to ensure an early diagnosis, and finally the fact that pathogenic aspergilli can only be rarely isolated from infected persons (Thornton, 2010; Lackner and Lass-Florl, 2013).

Furthermore, diagnosis of pulmonary Aspergillosis is usually missed as test of their detection cannot be undertaken in routine diagnostic laboratories (Shahid et al., 2001). Molecular and immunologic tests promise better, faster laboratory diagnosis of aspergillosis, but microscopy and culture remain commonly used as essential tools (Mcclenny, 2005).

Conventional diagnosis of fungal infection relies on the identification of pathogens by means of morphological characters specific to the genus and species, but sometimes unsuccessful, because of the atypical features of some isolates. Molecular biological identification systems for pathogenic aspergilli have been suggested as a solution to this problem: for example, a PCR
based diagnostic method for detecting the genus *Aspergillus* using 18S rDNA has been designed, and systems have also been described for specific detection of *Aspergillus fumigatus* with primers based on regions of the 28S rDNA or of the internal transcribed spacer (ITS) 1 and 2 regions of ribosomal DNA (rDNA) (Singh *et al.*, 2016).

### 2.7.1. Radiographic Studies

Radiographic findings may be useful to suggest a diagnosis of *Aspergillus* infection. Plain chest radiographs are too insensitive to make the diagnosis, as extensive pulmonary disease may be present with few findings on chest films. In neutropenic and hematopoietic stem cell transplant patients with invasive aspergillosis and other angioinvasive moulds, chest CT scans often demonstrate lesions that are not visible on plain radiographs, “halo” of low attenuation surrounding a nodular lung lesion in a high-risk patient has been associated with an early diagnosis of infection (Caillot *et al.*, 2001).

A nodular lesion may subsequently undergo cavitation to form an “air crescent” sign that is associated with aspergillosis; this radiographic sign occurs later in the course of illness. Usually after recovery of neutrophils, the presence of a CT “halo” sign as a trigger to begin presumptive therapy in high-risk patients resulted in favorable responses, particularly when combined with detection of circulating levels of serum galactomannan (Maertens *et al.*, 2005).

### 2.7.2. Laboratory Diagnosis

#### 2.7.2.1. Microscopy

Both microscopy and culture should be attempted on appropriate specimens from patients at risk for IA with a priority for culture in most cases where insufficient material is available, demonstrating tissue invasion by hyphae through microscopic examination of biopsy or autopsy material provides a diagnosis of proven invasive fungal infection, however, the sensitivity of microscopy for invasive aspergillosiosis IA is 50% at best (Rüchel and Schaffrinski, 1999).

Specimens may be examined as a wet mount preparation with or without the addition of 10% potassium hydroxide, fluorescent dyes such as calcofluor white or blancophor have the advantages of increased sensitivity, but are not specific for *Aspergillus*. Gomori’s methenamine silver stain (GMS) and periodic acid-Schiff (PAS) can be applied to histological sections and smears and should be conducted in all cases in which IA is considered. Microscopic methods, such as wet mounts, Gram stains, and conventional histopathology, provide clues that suggest the presence of *Aspergillus* spp. in tissue, *Aspergillus* Phenotypic markers detected by
histopathologic stains, as well as by Gram stain or wet mounts, provide valuable information for clinically important fungi, especially in the absence of culture (Mcclenny, 2005).

2.7.2 Isolation

The significance of isolating *Aspergillus* spp in sputum samples depends on the immune status of the host. In immunocompetent patients, isolation of *Aspergillus* spp. from the sputum almost always represents colonization with no clinical consequences (Soubani *et al*., 2004). Respiratory secretions from patients with suspected aspergillosis must be processed rapidly for culture to prevent overgrowth by bacteria and yeasts, to achieve optimal recovery of aspergillus from BAL fluid. Centrifugation of the sample and examination of the sediment. It is recommended that cultures of big volume of untreated sputum and BAL should be performed as opposed to culturing small volumes of digested, liquefied samples (Fraczek *et al*., 2014). Using of specific media to support fungal growth are recommended and species identification to the complex level should be carried out for clinically relevant isolates from patients who need antifungal treatment, and for epidemiological purposes (Ullmann *et al*., 2018).

In the immunocompetent patient with *Aspergillus* isolated from the sputum, antifungal therapy is generally not indicated, but appropriate diagnostic studies should be considered to exclude IPA. On the other hand, isolation of an *Aspergillus* species from sputum is highly predictive of invasive disease in immunocompromised patients. Studies have shown that sputum samples that are positive for *Aspergillus* in patients with leukaemia, or in those who have undergone HSCT, have a positive predictive value of 80–90% (Horvath and Dummer, 1996; Soubani *et al*., 2004). However, sputum samples that are negative do not rule out IPA; negative sputum studies have been noted in 70% of patients with confirmed IPA. Blood cultures are rarely positive in patients with confirmed IPA (Zmeili and Soubani, 2007).

*Aspergillus* spp grow well on standard media and can be identified to species level in most laboratories. Culture confirmation, is important to differentiate aspergillosis from other filamentous fungal infections, such as fusariosis and scedosporiosis. Blood cultures are of limited utility, because the results are often not positive even in disseminated infection. Bronchoalveolar lavage, transthoracic percutaneous needle aspiration, or video assisted thoracoscopic biopsy are standard procedures for establishing a diagnosis of invasive pulmonary Aspergillosis. Fluid and tissue specimens from these procedures may reveal characteristic angular dichotomously
branching septate hyphae on direct microscopic examination and/or *Aspergillus* species on culture (Horvath *et al*., 1996; Munoz *et al*., 2003).

In patients with high rates of infection, such as patients with neutropenia or those undergoing stem cell transplantation, the presence of *Aspergillus* in a respiratory sample, particularly if obtained from BAL fluid, is highly suggestive of the diagnosis of invasive aspergillosis. The joint European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) panel to propose that positive BAL cultures in conjunction with clinical illness and pulmonary infiltrates in neutropenic or allogeneic hematopoietic stem cell transplant patients constitute adequate criteria for a diagnosis of probable invasive pulmonary aspergillosis (Walsh *et al*., 2008).

Determining the significance of *Aspergillus* spp. growing in culture is often a challenge when microscopic examination of the specimen is negative, Brown *et al*., found that the presence of more than two colonies in a culture and infection in more than one site predicted significant infection (Brown *et al*., 1996). In granulocytopenic patients with acute leukemia, a single isolation from a lower respiratory specimen must be considered significant (Mcclenny, 2005).

### 2.7.2.3. Serological diagnosis

All serological tests have their limitations and the reliability of individual sero diagnostic tests as indicators of disease varies appreciably. This due to the large numbers of antigens produced by the fungal cell, the cross-reactions between related and, unrelated pathogenic species and the rarity of accepted reference reagents and standard procedures. Also antibody formation may be delayed, reduced or absent in patients with defective immune systems. In acute or early stages of infection, soluble antigens rather than antibodies may be present in serum and other body fluids, and tests for circulating antigen can therefore provide valuable supportive evidence for infections with opportunistic mycoses (Page *et al*., 2016). A variety of immunological tests are available that can be used to diagnose the disease (Arvanitis and Mylonakis, 2015), assays based on antibody detection have been successful to diagnose allergic aspergillosis and aspergilloma, while assays for fungal antigen detection showed great potential in diagnosing invasive aspergillosis (Richardson and Hope, 2003; Lackner and Lass Florl, 2013).

Serological tests can be of great value as diagnostic aids in many of the clinical manifestations of aspergillosis (Persat, 2012).
Aspergillus-specific IgG is a key component in CPA diagnosis, serum detection of IgG antibodies to *Aspergillus* is considered to be the most reliable method for diagnosing CPA (Denning *et al.*, 2016; Page *et al.*, 2016). Immunodiffusion analysis is widely used to detect the anti-*Aspergillus* antibody precipitin; however, it takes up to 1 week to obtain results, fluorescent immunoenzyme assays for quantifying IgGs to *Aspergillus* seemed to be more sensitive than conventional anti-*Aspergillus* antibody detection (Baxter *et al.*, 2013; Denning *et al.*, 2003). Many cellular and extracellular products of *Aspergillus* are immunogenic, and antibodies are readily formed by immunocompetent patients infected with *aspergillus* (Page *et al.*, 2016).

Galactomannan (GM) detection in fluids (especially BAL) is more sensitive than culture for diagnosis of IA, It is reported as optical density index (ODI). Serial screening for serum GM in prolonged neutropenia and in allogeneic stem cell transplantation recipients during the early engraftment phase has a high sensitivity and negative predictive value for IA (Maertens *et al.*, 2007). Sensitivity of serum GM testing is significantly lower in nonneutropenic versus neutropenic patients (Teering *et al.*, 2014), and decrease of the ODI during the first 2 weeks of antifungal therapy is a reliable predictor of a satisfactory response in cancer patients (Nou_er *et al.*, 2011).

GM detection in BAL specimens has an excellent performance with evidence that ODI of 0.5-1.0 has decreased predictive values compared with results of >1.0 (D'Haese *et al.*, 2012). The test also has diagnostic value in patients undergoing lung transplantation or who are in intensive care, a sensitivity of 100% and a specificity of 90.4% was defined at cut-off of 1.5 (Pasqualotto *et al.*, 2010). (1-3)-β-D-glucan is another component that can be examined. Even though positive results from these two assays are not definite indications of the fungus. (Denning *et al.*, 2016).

### 2.7.3. Histopathological diagnosis

Histopathological diagnosis, by examining lung tissue obtained by thoracoscopic or open-lung biopsy, remains the 'gold standard' in the diagnosis of IPA (Ruhnke *et al.*, 2003). The presence of septate, acute, branching hyphae invading the lung tissue samples, along with a culture that is positive for *Aspergillus* from the same site, is diagnostic of IPA. Histopathological examination also allows for the exclusion of other diagnoses, such as malignancy or non-fungal infectious diseases. Histopathological findings associated with IPA have been recently shown to differ
according to the underlying host. In patients with allogeneic HSCT and GVHD, there is intense inflammation with neutrophilic infiltration, minimal coagulation necrosis, and low fungal burden while, IPA in neutropenic patients is characterized by scant inflammation, extensive coagulation necrosis associated with hyphal angio-invasion, and high fungal burden. Dissemination to other organs is equally high in both groups (Chamilos, 2006).

2.7.4. Molecular Identification

PCR-based assays have been developed and it can improve early diagnosis of aspergillosis with advantages of high sensitivity, ability to establish diagnosis at the species level and capacity to detect genes that confer antifungal resistance (Segal, 2009). PCR is fast, inexpensive and can be applied to diverse types of sample, such as blood, sputum and tissue (Arvanitis and Mylonakis, 2015). The European Aspergillus PCR Initiative has made significant progress in developing a standard real-time quantitative PCR protocol, but its clinical utility has to be established in formal and extensive clinical trials (Gomez, 2014). Aspergillus PCR has been applied mostly to blood and BAL fluid, the performance of serum PCR is not significantly different from that of whole blood. Prospective screening of high-risk haematological patients by a combination of GM and PCR improves the diagnostic accuracy and associated with an earlier diagnosis (Ullmann et al., 2018).

The target for genus level detection of Aspergillus have included the 18S rRNA gene, mitochondrial DNA, the intergenic spacer region, and the internal transcribed spacer (ITS) regions. The latter are located between the 18S and 28S rRNA genes and offer distinct advantages over other molecular targets including increased sensitivity due to the existence of approximately 100 copies per genome, the rRNA gene for 5.8S RNA separates the two ITS regions, sequence variation of ITS regions has led to their use in phylogenetic studies of many different organisms. (Henry et al., 2000).

The internal transcribed spacer rDNA region (ITS1-5.8S-ITS2) is the official DNA barcode for fungi, because it is the most frequently sequenced marker in fungi and has primers that work universally (Schoch et al., 2012). This region can be used as the initial step of identification including unknown fungal isolates to be categorized into appropriate genus, subgenus and section up to species level (Balajee et al., 2009). Although not translated into proteins, the ITS coding regions have a critical role in the development of functional rRNA, with sequence
variations among species showing promise as signature regions for molecular assays (Iwen et al., 2002).

Schematic diagram of the internal transcribed spacer (ITS) region between small subunit (SSU) rDNA and large subunit (LSU) rDNA, which includes ITS I, ITS II, and 5.8S rDNA (Horisawa et al., 2009).

2.8. Antifungal susceptibility testing against Aspergillus spp.

Several different methods exists for determining in vitro susceptibilities for Aspergillus spp. such as broth (macro- and micro-dilution) and agar diffusion-based methods, followed by commercial test kits. One of the most widely used assays is the M38-A2 reference method for filamentous fungi, published by the Clinical Laboratory Standard Institute (CLSI) (CLSI, 2008), and the European guidelines for in vitro susceptibility testing of antifungals against aspergillus spp. and molds (Lass Florl et al., 2006).

The advantage for performing in vitro susceptibility testing in Aspergillus spp is providing an early insight into drug activity and therefore a proactive patient management also, monitoring resistance is of great importance for empirical treatment (Lass Florl et al., 2008).

2.8.1. Broth Methods

Both reference (CLSI and EUCAST) include a microtiter format, 48 h incubation at 35–37°C and a no growth visual end point. The main differences between the two standards are inoculum preparation by spectrophotometer versus hemocytometer, inocula of $0.4–5 \times 10^{4}$ versus $2–5 \times 10^{5}$ CFU/ml and 0.2% glucose versus 2% glucose for CLSI and EUCAST, respectively. Both methods allow detection of itraconazole resistance in Aspergillus spp. but lack discrimination of amphotericin B-resistant isolates from susceptible ones, with the exception of some isolates of A. terreus. MIC reading is carried out after 48 h incubation; at 24 h. In general, broth reference methods are not an ideal method for routine testing or for testing multiple isolates because they
are labor-intensive and technically demanding, however they have a high inter- and intra-laboratory agreement (Lass Florl, 2010).

### 2.8.2. Agar based methods

#### 2.8.2.1. E test

E test is a commercially available method and directly quantifies antifungal susceptibility in terms of discrete MIC values. For *Aspergillus* spp., good correlations with amphotericin B and itraconazole E test and M38-A method have been demonstrated (Pfaller *et al*., 2000; Rex *et al*., 2001).

#### 2.8.2.2. Disk Diffusion

Disk-based susceptibility testing is convenient and economical and several commercial disk systems are available. However, the results are somewhat contradictory and the choice of growth medium appears critical. Some investigators use RPMI-1640 agar supplemented with 0.2% glucose while others apply Mueller–Hinton (MH) agar supplemented with 2% glucose and 0.5 µg/ml methylene blue so the zone sizes are easy to read (Lass Flor, 2010).

#### 2.8.2.3. Commercial Kits

The Sensititre YeastOne™ colorimetric antifungal panel has been favorable compared with CLSI methodology with amphotericin B, itraconazole (Guinea *et al*., 2006), voriconazole and posaconazole for *Aspergillus* spp., In general, agreement was 82% for voriconazole, ranging from 100% for *A. niger* and *A. terreus* to 62% for *A. flavus* (Castro *et al*., 2004). Discrepancies were due to higher Sensititre MICs, Overall, the Sensititre YeastOne method could have potential value for susceptibility testing of *Aspergillus* spp. to voriconazole and is able to detect resistance to itraconazole, with the advantage of easy to performing, but breakpoints are not available (Martin *et al*., 2003).

#### 2.9. Treatment

The treatment of aspergillosis is based on the use of azole antifungal drugs, such as voriconazole (VCZ), which is the treatment of choice, itraconazole (ITZ), posaconazole (PCZ), and more recently, isavuconazole (ISZ) (Patterson *et al*., 2016 ; Walsh *et al*., 2008). Nevertheless, many studies have reported resistance of *A. fumigatus* to the azole antifungal drugs that is often due to the cross-resistance to the agricultural triazoles. Resistance rates vary widely across medical centers around the world, with some studies showing high resistance rates, and others with rates
even lower than 1%. Lipid formulations of amphotericin B (AMB) and echinocandins are also being used nowadays in the treatment of aspergillosis as an alternative to the use of azoles (Denardi et al., 2018).

2.9.1. Chronic cavitary pulmonary aspergillosis CCPA

Treatment of CCPA has to be individualised, depending on the severity of symptoms, extent and location of the pulmonary involvement, relationship to other diseases, and the patient’s general condition. In consequence treatment of fungal ball must be adapted specifically to each patient, with the entire clinical presentation dictating the appropriate management (Pasqualotto, 2010). There are no codified treatment guidelines for CPA, bronchial artery embolization may stop hemoptysis in certain cases. Surgery is generally impossible because of impaired respiratory function or the severity of the comorbidity, and when it is possible that morbidity and mortality are very high (Camuset et al., 2007).

Resection should only be undertaken if there is sufficient respiratory reserve and the resection removes minimal functioning pulmonary tissue (Pasqualotto, 2010). It is the mainstay of management for patients with simple aspergilloma and it's results are excellent. However, there are many patients with extensive multicavity CPA who fail medical therapy and in whom surgery is speculated and the results of surgical treatment for this group are not so good (Farid et al., 2013).

2.9.1.1. Antifungal Therapy

The CCPA is regarded as an incurable disease requiring long-term antifungal therapy, since many patients relapse after this is stopped (Denning et al., 2003). Oral itraconazole treatment was superior to conservative treatment in stabilizing the clinical and radiological manifestations in patients with CCPA (Agarwal et al., 2013). It has been adopted widely as first line treatment for CPA given its availability and modest cost. It has been reported an overall improvement of 77% after 6 months of Itraconazole treatment when compared to standard supportive care, which showed a response of 37% (Agarwal et al., 2013).

However, Itraconazole is not free from side effects and can cause considerable toxicity. The adverse effects include gastro intestinal upset, hair loss, peripheral neuropathy, hypertension and ankle oedema which may or may not be an early sign of congestive heart failure (Maghrabi and Denning, 2017). Another antifungal is voriconazole which due to its costs is reserved to patients failing therapy with oral itraconazole due to toxicity (Howard et al., 2009). The limited
options for long-term oral treatment of CCPA are one of the major problems in treating this disease. Some patients failing therapy with voriconazole may still be treated with posaconazole, although a high risk for cross-resistance exists (Pasqualotto, 2010). Amphotericin B deoxycholate has poor penetration in fungal balls and intravenous treatment with intravenous amphotericin B has been regarded as ineffective, however short-lived responses to IV amphotericin B occurred in 80% of patients failing itraconazole therapy has been reported (Denning et al., 2003).

2.9.2. Allergic bronchopulmonary aspergillosis ABPA
The goals of treatment of allergic bronchopulmonary aspergillosis (ABPA) are to preserve lung function through suppression of the immunologic response to the Aspergillus antigens and the inflammatory response of asthma (Walsh et al., 2008).

2.9.2.1. Steroid therapy
The mainstay of therapy for ABPA is oral corticosteroids to suppress the immunologic response to Aspergillus antigens and the secondary inflammatory reaction. The duration of therapy should be individualized according to the patient's clinical condition but most of them require prolonged therapy to control their symptoms and minimize relapses (Ruiz et al., 2013).

2.9.2.2. Antifungal therapy
Eradication of Aspergillus species from the airways is another approach to the treatment of ABPA, although Aspergillus species are ubiquitous and are inhaled in large quantities by humans on a daily basis (Mezzari et al., 2002). Antifungal therapy may lower the fungal antigen load in airways. Azoles (e.g., ketoconazole, itraconazole, voriconazole, and posaconazole) have known efficacy against A. fumigatus through inhibition of ergosterol synthesis in the fungal cell membrane, thereby inhibiting fungal growth. Ketoconazole and itraconazole have also been shown to reduce eosinophilic airway inflammation. The severe side effects of ketoconazole, including hepatic toxicity, suppression of testosterone synthesis, and suppression of adrenal steroid synthesis limit its usefulness (Gilley et al., 2010).

The newer azole antifungal agents, voriconazole and posaconazole have potent in vitro activity against Aspergillus and have a more favourable safety profile than itraconazole (Hilliard et al., 2005).

The role of amphotericin B in ABPA is limited by its toxicity, cost, and its limited ability to reach the airway in high concentrations (Gilley et al., 2010).
2.9.3. Invasive pulmonary aspergillosis (IPA)

Voriconazole was more effective than amphotericin B deoxycholate (AmB-D) as initial therapy for invasive aspergillosis and was associated with significantly improved survival (71% vs. 58%, respectively) in a randomized trial (Herbrecht et al., 2002). The rate of successful outcomes was superior in voriconazole compared to AmB-D recipients (53% versus 32% respectively). The poorest prognosis occurred in extrapulmonary aspergillosis and in allogeneic HSCT recipients (Sherif and Segal, 2010).

Echinocandin derivatives such as caspofungin, micafungin and anidulafungin are also effective agents in the treatment of IPA refractory to standard treatment, or if the patient cannot tolerate first-line agents (Spanakis et al., 2006; Cohen et al., 2006). While polyenes and azoles target the fungal cell membrane, echinocandins inhibit the (13)-b-D-glucan constituent of the fungal cell wall (Kousha et al., 2011).

Surgical resection has generally a limited role in the management of patients with IPA, but it becomes important in cases with invasion of bone, burn wounds, epidural abscesses and vitreal disease (Limper et al., 2011). It should also be considered in cases of massive haemoptysis, pulmonary lesions close to the great blood vessels or pericardium, or residual localised pulmonary lesions in patients with continuing immunosuppression or those who are expected to have immunosuppressive therapy in the future (Kousha et al., 2011).

2.10. Antifungal resistance

Azole resistance is increasingly recognized as a problem in Aspergillus diseases (Snelders et al., 2008), depending on location, up to 12% of Aspergillus infections are estimated to be resistant to antifungal medications, antifungal resistance was identified in up to 7% of Aspergillus specimens from patients with stem cell and organ transplants (Baddley et al., 2009; Kontoyiannis et al., 2010; Pappas et al., 2010).

Resistant Aspergillus infections can develop in people who have taken certain antifungal medication, however, resistant infections are also found in people who have not taken antifungal medications, this demonstrates that antifungal resistance in Aspergillus is likely acquired before entering the healthcare setting and is partially driven by environmental sources (Mortensen et al., 2010; Verweij et al., 2009). There is a potential resistant infections if people with weak immune systems breathe in spores (Verweij et al., 2009).
Resistance to active triazoles, itraconazole, posaconazole, voriconazole, and isavuconazole can develop with prolonged clinical exposure (Wiederhold, 2017).

2.10.1. Intrinsic resistance
The wild-type isolates of Aspergillus are susceptible to all the licensed mould active azoles and echinocandins (Hope et al., 2013), however, A. fumigatus species complex includes more than 30 sibling species which cannot be differentiated morphologically from one another or from A. fumigatus. Several of these have been isolates from humans and been shown to be intrinsically resistant to one or more antifungals (Varga, 2008). Intrinsic amphotericin B resistance has been recognized in A. terreus for many decades, but also A. flavus and other less common species have reduced susceptibility to amphotericin B, Importantly, a number of these rare Aspergillus species are also resistant to azoles and in some cases also to echinocandins posing obvious challenges for patient management (Arendrup, 2012).

2.10.2. Acquired resistance
The acquired resistance in A. fumigatus is caused by point mutations in the CYP51A gene, which encodes the Cyp51 enzyme responsible for the conversion of lanosterol to ergosterol. Different mutations can differentially affect the azoles, with some causing resistance to voriconazole and isavuconazole, some causing resistance to posaconazole and itraconazole, and others causing pan-azole resistance. In addition, it is now known that environmental exposure to the azoles, which are used in a variety of means, including agriculture to protect plants and crops, can also lead to the development of azole resistance (Wiederhold, 2017).

The majority of isolates harbour mutations in the target gene CYP51A, but other mechanisms are important as well, including efflux pumps and target gene up regulation encoded by a mutation in the hapE gene. Acquired azole resistance has also been reported in A. terreus and A. flavus, which poses obvious challenges regarding clinical management of related infections as these two species have intrinsically reduced susceptibility to amphotericin B (Liu et al., 2012; Arendrup et al., 2012).

Susceptibility testing of Aspergillus and echinocandins is, however, even more challenging than susceptibility testing of azoles and amphotericin B due to the lack of total inhibition in vitro and therefore no clear endpoint, hence, acquired resistance to echinocandins (Arendrup et al., 2009; Madureira et al., 2007).
2.11. Prognosis

The strongest prognostic factor for IPA is successful therapy of the underlying disease, in patients with acute leukaemia and IPA. The complete remission of the haematological disease was the main prognostic factor associated with a significantly better outcome (Reichenberger et al., 2002). After surgery for IPA, the survival of patients was limited due to relapse or uncontrolled malignancy, but not by complications of the surgical procedure (Reichenberger et al., 1998).

Some patients with ABPA progress to end-stage pulmonary fibrosis with cor pulmonale while others maintain almost stable pulmonary function tests for years. It has been reported that asthmatic patients with ABPA have more compromised pulmonary physiologic tests than did asthmatic patients without ABPA. And Both groups had features of asthma (decreased flow rates and increased lung volumes), but asthmatics with ABPA tended to exhibit decreased diffusing capacity and total lung capacity. This is in accordance with superimposition of a restrictive defect pulmonary fibrosis with bronchiectasis on a pre-existing obstructive defect (Gupta et al., 2012).

2.12. Prevention

Prevention of IA plays a central role in the clinical management of chronic granulomatous disease (CGD) patients, it consists of reducing environmental exposure to moulds and the prophylactic use of antifungals. Exposure to mulch, hay, wood chips and rotting plants (compost heaps), visiting caves, stables, sheds and areas of construction and/or renovation, and activities such as gardening should be avoided (Warris and Henriet, 2014).

2.12.1. Prophylactic antifungal therapy indications in high-risk patients

Although fluconazole is not effective against Aspergillus, it significantly decreases the incidence of fungal infections after bone marrow transplantation and is the most frequently used oral prophylactic antifungal therapy (Boyle et al., 2000).

Oral itraconazole has been found to be less effective than fluconazole, probably because of poor bioavailability of the drug in capsule form. Voriconazole is effective as secondary prophylaxis in patients who have been successfully treated for Aspergillus infection and who again require chemotherapy for consolidation or relapse (Cordonnier et al., 2004).
The FDA has approved an intravenous formulation of posaconazole (Noxafil), which is indicated for the prophylaxis of invasive Aspergillus and Candida infections in severely immunocompromised adults who are at high risk of developing these infections (Brooks, 2014).
3. MATERIALS AND METHODS

3.1. Study design
This study was descriptive, cross sectional hospital based study.

3.2. Study area
Different centers and Hospitals located in Khartoum State were included in the study which include: Mycology reference lab in National Health Laboratory, Alshaab Teaching Hospital, Omdurman Chest Hospital and Omer Sawy Hospital.

3.3. Study duration
This study was conducted in period from 2016 to 2019.

3.4. Study population
The study population was Sudanese hospitalized or referral patients with different age groups suffering from chronic respiratory diseases who attended the above mentioned study areas.

3.5. Inclusion criteria
Patients experienced pulmonary symptoms, such as bronchiectasis, recurrent infections, chronic obstructive pulmonary diseases, cystic fibrosis, uncontrolled asthma, pulmonary tuberculosis, or a suspected case of fungus ball were included in this study.

3.6. Exclusion Criteria
Patients with acute pulmonary infections or known bacterial pneumonia and patients who refused to participate in this study were excluded.

3.7. Ethical consideration
The ethical approval was obtained from Sudan University of Science and Technology, Medical Laboratory College Research Board, Ministry of Health Research Committee. After explaining the purpose of the research with simple and clear words to the participants, they told that they have rights to voluntary and they signed inform consent and they can withdraw at any time without any deprivation. All participants have rights to no harm (privacy and confidentiality) by using coded questionnaire and the remaining samples were not to be reused for other research and the data will be secured. All participants’ has rights to benefit from the researcher knowledge and skills about pulmonary asprgillosis and the investigations results will be received by treating doctor immediately.

3.8. Sample size
\[ n = (1.96^2 \times 0.5 \times (1 - 0.5))/0.05^2 \]
Where Z = value from standard normal distribution corresponding to desired confidence level (Z=1.96 for 95% CI)
P is expected true proportion
e is desired precision (half desired CI width).
For small populations n can be adjusted so that n(adj) = (Nxn)/(N+n)
According to above equation 384 BAL and sputum specimens were collected.

3.9. Sampling technique
This study is based on non-probability convenience sampling technique.

3.10. Method of data collection
Data were collected through self and non self-administrated questionnaire from patients. Some information was taken from patient’s clinical reports.

3.11. Laboratory Methods
3.11.1. Collection of Specimens
Early morning deep cough sputum specimens were collected in sterile clean dry containers for direct microscopy and culture while bronchoalveolar lavages (BAL) were collected in sterile containers by physicians.

3.11.2. Direct Microscopy
3.11.2.1. Potassium Hydroxide (KOH) preparation
All sputa BAL samples were also subjected to Potassium hydroxide preparations for the detection of fungal elements. Briefly, purulent portion of the specimen was mixed with 10% KOH left to stand for 20 minutes, and examined at X40 magnification for fungal elements. The purpose was to provide descriptive morphological information of these pathogens to aid in the identification (Mwaura et al., 2013).

3.11.2.2. Gram's staining technique
All sputum and BAL samples were also subjected to Gram staining procedures to detect the presence fungal elements. Heat fixed smears were prepared on glass slides. The slides were put on a staining rack, and then flooded will crystal violet stain for one minute. The excess dye were poured off and washed gently in tap water. The smears were exposed to Grams iodine for one minute. Then the iodine was washed carefully with tap water. The smears were washed with acetone as a decolorizer for 30 seconds. Smears were washed with tap water to stop the decolorization. The smears were counter stained with neutral red for 30 seconds. The smears were scored for the presence or absence of bacterial, fungal elements and yeast cells (Chakaya et al., 2003).
3.11.3. Isolation
Sputum and BAL specimens were inoculated on two tubes of Sabouraud’s Dextrose Agar with chloramphenicol using direct method of inoculation and incubated at 25°C and 37°C up to 1 week with daily examination.

3.11.4. Identification

3.11.4.1. Macroscopic Examinations
The isolates were identified by macroscopic appearance (Surface topography, texture and pigment). Colony features including diameter after 7 days, color of conidia, mycelia, exudates and reverse, colony texture and shape (Diba et al., 2007).

3.11.4.2. Microscopic Examinations
The cultures were identified microscopically by lactophenol cotton blue preparation and slide culture as per standard recommended procedures. The Microscopic characteristics for the identification were: conidial heads, stipes, color and length, vesicles shape and seriation, metula covering, conidia size, shape and roughness (Diba et al., 2007).

3.11.5. Antifungal susceptibility testing

3.11.5.1. Preparation of Inoculum
The inoculum was prepared according to M51-A and M38A2 Clinical and Laboratory Standards Institute (CLSI) guidelines. To induce sporulation, the isolates were grown on potato dextrose agar (PDA) slants at 35°C for 2–7 days before testing. The conidia of the isolates were mixed in sterile saline with two drops of 0.05% tween 20. The turbidity of the mixture was adjusted spectrophotometrically to optical density range of 0.09 to 0.11 at 530 nm (equivalent to 10^6 spores/mL) (Hassan et al., 2018).

3.11.5.2. Seeding
The plates were inoculated with conidial suspensions by swabbing the Roswell Park Memorial Institute (RPMI) 1640 medium containing L-glutamine and buffered to pH 7.0 with 0.165 M MOPS in three directions. After excess moisture was absorbed into the agar and the surface was completely dry, E test strips (Itraconazole and voriconazole concentrations ranging from 0.002 to 32 g/ml) were applied to the RPMI Dextrose agar (CLSI, 2008).

3.11.5.3. Reading and interpretation of results
E test MICs were determined after 24 and 48 h of incubation at 35°C and was read as the drug concentration at which the border of the elliptical inhibition zone intersected the scale on the antifungal test strip. The MICs for itraconazole and voriconazole were interpreted according to European Committee on Antimicrobial Susceptibility Testing (EUSAT) interpretation criteria. Appendix I.

3.11.6. Molecular Technique

3.11.6.1. Culture preparation and DNA extraction method

The fungal isolates were cultured on Potato Dextrose Agar medium and then incubated at 28°C for 72 h. The mycelium of pure culture colonies was used for DNA extraction. Fresh fungal mycelium firstly were freezed with liquid nitrogen and then ground using sterile mortar and pestle and DNA was extracted by using quick Cetyl trimethylammonium bromide (CTAB) method (Zhang et al., 2010) as follows:

- Screw cap tubes (2ml capacity) were filled with 490 μl CTAB-buffer 2x and 6-10 acid washed glass beads. Some fungal material (1-10 mm³) were added followed by addition of 10 μl Proteinase K stock solution.
- This mixture was mixed by vortexing for 10 minutes and placed on water bath for 60 minutes at 60°C until the tissue was completely dissolved. Then 500 μl (chloroform:isoamylalcohol 24:1) were added and shaken for 2 min. to form an emulsion and was spun in a microfuge at 14000 rpm for 10 min. (long enough to produce a clear supernatant). The aqueous (upper) layer were collected in a clean, labeled tube. 2/3 volume of ice cold iso-propanol were added to the collected layer and incubated at -20°C overnight. Next day the tubes were spun in a microfuge at 14,000 rpm for 10 min, and the alcohol supernatant were poured off. One ml of ice-cold 70% ethanol was added to each tube and mixed gently. The content was spun again at 14000 rpm for 2 min. and as much as possible of alcohol was poured off. The samples were dried by air and the dried pellets were re-suspended in 50 μl TE-buffer and were stored frozen at -20 °C. Quality of DNA was checked by running 2-3 μl on a 0.8% agarose gel.

3.11.6.2. PCR

2.11.6.2.1. PCR amplification.

The PCR assay was performed with 5 μ of test sample in a total reaction volume of 25μl ml consisting of PCR buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl; 0.1 mM (each) dATP, dGTP,
dCTP, and dTTP; 1.5 mM MgCl2; 0.3 mM (each) primer; and 1.5 U of PlatinumTaq high-fidelity DNA polymerase (Intron).

Forty cycles of amplification were performed in a Stratagene Robocycler model 96 thermocycler (China) after initial denaturation of DNA at 95°C for 4.5 min. Each cycle consisted of a denaturation step at 95°C for 30 s, an annealing step at 50°C for 30 s, and an extension step at 72°C for 1 min, with a final extension at 72°C for 3 min following the last cycle. After amplification, the products were stored at 4°C until used.

Primers: Two oligonucleotide fungal primers described by White et al. (1990) were used for amplification. The ITS region primers (ITS 1, 5'-TCC GTA GGT GAA CCT GCG G-3; ITS 4, 5'-TCC TCC GCT TAT TGA TAT G-3) make use of conserved regions of the 18S (ITS 1) and the 28S (ITS 4) rRNA genes to amplify the intervening 5.8S gene and the ITS 1 and ITS 2 noncoding regions.

The PCR products were analyzed on 2% agarose gel stained with ethidium bromide (10ng/100ml) and visualized under a UV transilluminator apparatus.

3.11.6.3. DNA Sequencing

PCR products of 19 isolates were purified and commercially sequenced using forward primers ITS1 and backward ITS4 by Macrogen Company (Seol, South Korea).

3.11.6.4. Bioinformatics analysis

3.11.6.4.1. Sequences similarity and alignment

The sequences obtained in this study were identified by searching data bases using BLAST sequence analysis tools (http://www.ncbi.nlm.nih.gov/BLAST/). The sequences were compared using nucleotide-nucleotide BLAST (blastn) with default setting except the sequences were not filtered for low complexity. Species were identified based on the highest similarity score (100%) with reference database sequence. Highly similar sequences were retrieved from NCBI and subjected to multiple sequence alignment using the BioEdit software.

3.11.6.4.2. Phylogenetic tree

Phylogenetic tree of Aspergillus spp genes and their evolutionary relationship with well-characterized reference strains obtained from NCBI database (NR_131276.1, NR_121481.1, NR_111041.1, AY373852.1 and EF661186.1) was constructed by the neighbor-joining method with the Bootstrap test of phylogeny in Molecular Evolutionary Genetics Analysis (MEGA).
program, version 7. Bootstrap resembling strategy and reconstruction were carried out 100 times to confirm the reliability of the phylogene.

3.11.7. Data analysis
Data were analyzed using statistical package for social science software (SPSS version .20) Data were presented in form of tables and figures. Frequencies mean and standard deviation were calculated. Chi square test was performed between qualitative variables. Independent T test were performed to quantitative and quantitative variables. A P.value of < 0.05 was considered as significant for all statistical tests in the present study.
4. Results

A total of 384 patients with pulmonary diseases were participated in this study, their age ranged from 9 to 90 years and mean age of 42 years ±16.14 (SD). Out of them, 233 (61.0%) were males while 151 (39.0%) were females as shown in figure (4.1).

Patients enrolled in the study were divided into three age groups: less than 10 yrs 2 (0.5%), 11-49 yrs 258 (67.2%), and equal or more than 50 yrs 124 (32.2%) as shown on table (4.2).

A total of (341) (89%) sputum specimens and 43 (11%) Bronchoalveolar Lavage specimen shown on figure 4.2 were collected from patients attended with (chronic pulmonary infections) 219 (57.0%) Asthma 77 (20.1%), Cystic Fibrosis 34 (8.9%) ,Pulmonary Tuberculosis 29 (7.6%), Pleural effusions 12 (3.1%) , Malignancy 9 (2.3%) , Emphysema , Hemoptysis and lung Abscess 4 (1.0%) as shown on Table( 4.1 ).These specimens collected from different hospitals in Khartoum State : Alshaab Hospital , Omer Sawy Hospital  and Omdurman chest hospital and Mycology reference Laboratory. Among the study population the frequency of the specimens according to gender was 205 (53%) sputum specimen and 28 (7%) BAL specimens collected from males while 136 (36%) sputum specimens and 15 (4%) BA1 specimens were collected from females, as represented in figure (4.3) Also other data were registered in the submitted questionnaire (Appendix 1V).

All sputum and BAL specimens were examined directly with 10% potassium hydroxide wet mount and Gram's staining, 11 (2.9%) sputum specimens were positive for fungal elements and 330 (85.9 %) were negative while all BA1 specimens were negative for fungi on direct microscopic examination as shown on table (4.3) and figure (4.4).the sensitivity of the direct microscopy in this study was 28% while the specificity was 99.4%.

After primary isolation out of 341 sputum specimens 27 (7%) showed Aspergillus growth and 4 (1 %) showed yeast growth While 1(0.4%) BAL specimen grown as Aspergillus as shown on figure (4.5.)

The isolates were identified by macroscopic and microscopic characteristics for the identification and the results were:11 (34.4%) A. flavus, 9 (28.1%) A. fumigatus, 7 (21.9%) A. terrus, 1 (3.13%) A. niger and 4 (12.5%) Candida spp as shown on table (4.4) and figure (4.6).

The association between underlying diseases and positive culture was statistically significant (P value=0.003) as revealed on table (4.5).
Out of 32 fungal isolates 15 (46.9%) Aspergillus spp were isolated from patients with chronic pulmonary infections, 7 (29.1%) from asthmatic patients, 3(9.4%) from patients with cystic fibrosis .3 (9.4%) from pulmonary tuberculosis patients and the 4 (12.4%) candida spp were isolated from patients with malignancy and chronic pulomonary infections respectively with significant relation between the underlying diseases and different fungal isolates (P.value=0.015) as displayed on table (4.6).

The MICs of E-test for Aspergillus spp was 1.6 ±1.8 for Itraconazole while the MICs for Voriconazole was 0.6±0.93 SD.

The susceptibility testing for all Aspergillus isolates against itraconazole and voriconazole was 14(50%) were sensitive, 7(25%) SDD and 7(25%) were resistant to itraconazole while 23 (85.2%) were sensitive, 2 (7.4%) susceptible dose dependent and 2 (7.4%) were resistant as dislayed on figures (4.7) and (4.8).

The results of the E test for Itraconazole against different Aspergillus spp showed: 3(33.3%) A. fumigatus were sensitive,2 (22.3%) intermediate and 4 (44.4%) resistant, While 6 (54.5%) A.flavus were sensitive, 4 (36.4%) intermediate and 1(9.1 %) resistant, A.terrus reflected 5 (71.4%) sensitive, 1 (14.3%) intermediate and 1 (14.3%) resistant and A.niger was resistant to Itraconazole as shown on figure (4.9).

Figure (4.10) displayed results of E test for Voriconazole against Aspergillus spp: 7 (77.8%) A.fumigats were sensitive, 1(11.1%) intermediate and 1 (11.1%) was resistant, While 11(100%) A. flavus were sensitive and 5(83.3%) of A.terrus were sensitive, 1(16.7%) was resistant. A.niger was intermediate to voriconazole as showed on figure (4.10).

The MICs of Itraconazole and Voriconazole for A. flavus showed statistically significant differences between the MICs of two antifungals (P value=.003 ), while :A. terrus and A. fumigatus showed no statistically significant differences between the two drugs MICs (P value=.08 , 0.6) respectively Table (4.7).

Amplification of the ITS 1–5.8S–ITS 2 regions from the Isolated Aspergillus strains generated PCR products ranging in size from 565 to 613 bp (Figure 4.11). The PCR products of the IT’S region of 17 isolates were sequenced and aligned with references in the NCBI database.

Direct sequencing of PCR products were performed and the obtained sequences aligned with reference strains on Gene bank. Figure (4.10) and (4.12).
Successful sequencing of interspace transcribed was determined for 17 isolates of Aspergillus spp after performing conventional PCR, with gene product of 535-613 bp as shown in figure (4.13).

Figure (4.14) displayed nucleotide sequence alignment of all clinical isolates and reference strains showing the species specific nucleotides.

The results of comparisons between clinical isolates and referenced strain sequences of the same Aspergillus species are shown showed that the greatest intraspecies variation was seen among isolates of A. flavus and isolates of A. terrus. For A.terrus species, one nucleotide base differences between the sequences of one clinical isolate and that of the referenced strain existed figure (4.15) while for A.flavus one nucleotide base differences between the sequences of one clinical isolate and that of the referenced strain Figure(4.16) and two nucleotides base difference between the isolates figure(4.17). Considering the length of the ITS region amplified, the overall sequence similarity between the referenced Aspergillus strains and clinical isolates of the same species was greater than 99%.

The Phylogenetic tree analysis was performed to compare the genetic distances and evolutionary lineage for 17 isolates with well-characterized reference isolates from Genbank as shown in figures (4.18) and (4.19).
Figure (4.1): Frequency of sex among study population
Figure (4.2): Distribution of specimens among study population

- Sputum: 341 (89%)
- BAL: 43 (11%)
Figure(4.3) : Frequency of specimens among gender
### Tabel (4.1): Distribuion of diseases among study population

<table>
<thead>
<tr>
<th>Disease</th>
<th>Frequency and percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic pulmonary infection</td>
<td>219 (57.0%)</td>
</tr>
<tr>
<td>Asthma</td>
<td>77 (20.1%)</td>
</tr>
<tr>
<td>Cystic fibrosis</td>
<td>34 (8.9%)</td>
</tr>
<tr>
<td>Pulmonary tuberculosis</td>
<td>29 (7.6%)</td>
</tr>
<tr>
<td>Pleural effusions</td>
<td>12 (3.1%)</td>
</tr>
<tr>
<td>Malignancy</td>
<td>9 (2.3%)</td>
</tr>
<tr>
<td>Emphysema +hemoptysis + lung abscess</td>
<td>4 (1.0%)</td>
</tr>
<tr>
<td>Total</td>
<td>384 (100%)</td>
</tr>
</tbody>
</table>
Table 4.2: Frequency and percentages of Age groups

<table>
<thead>
<tr>
<th>Age groups</th>
<th>Frequency</th>
<th>Percent (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>less than 10</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td>10-49</td>
<td>258</td>
<td>67.2</td>
</tr>
<tr>
<td>≥50</td>
<td>124</td>
<td>32.3</td>
</tr>
<tr>
<td>Total</td>
<td>384</td>
<td>100.0</td>
</tr>
</tbody>
</table>
Table (4.3) Results of direct microscopy for sputum and BAL specimens

<table>
<thead>
<tr>
<th>Specimens</th>
<th>-ve</th>
<th>Pseudohyphae</th>
<th>True hyphae</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum</td>
<td>330 (85.9%)</td>
<td>4 (1.1%)</td>
<td>7 (1.8%)</td>
<td>341 (88.8%)</td>
</tr>
<tr>
<td>BAL</td>
<td>43 (11.2%)</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>43 (11.2%)</td>
</tr>
<tr>
<td>Total</td>
<td>373 (97.1%)</td>
<td>4 (1.0%)</td>
<td>7 (1.8%)</td>
<td>384 (100%)</td>
</tr>
</tbody>
</table>
Figure 4.4  (A) Pseudohyphae of *Candida* SPP by Gram's stain with X100 objectives
(B) Dichotomous hyphae of *Aspergillus* SPP by Gram's stain with X100 objectives  
(C) Fungal hyphae with 10% KOH wet preparation with X40 objectives
Figure (4.5): Results of primary isolation from specimens
### Table 4.4 Frequency of *Aspergillus* spp and *Candida* spp among study population

<table>
<thead>
<tr>
<th>Organism</th>
<th>Frequency</th>
<th>Percent (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. flavus</em></td>
<td>11</td>
<td>34.4</td>
</tr>
<tr>
<td><em>A. fumigatus</em></td>
<td>9</td>
<td>28.1</td>
</tr>
<tr>
<td><em>A. terrus</em></td>
<td>7</td>
<td>21.9</td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>1</td>
<td>3.1</td>
</tr>
<tr>
<td><em>Candida spp</em></td>
<td>4</td>
<td>12.5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>32</strong></td>
<td><strong>100.0</strong></td>
</tr>
</tbody>
</table>
Figure (4.6) *Aspergillus* spp by tease mount using Lactic acid and Lactophenol cotton blue with 40X objectives. (A) *A. fumigatus* (B) *A. niger* (C) *A. flavus* (D) *A. terreus*
### Table 4.5. Correlation between positive culture result and underlying lung diseases

<table>
<thead>
<tr>
<th>Lung disease</th>
<th>Aspergillosis</th>
<th>Candidiasis</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPI</td>
<td>15 (46.9%)</td>
<td>2 (6.2%)</td>
<td>17 (53.1%)</td>
</tr>
<tr>
<td>Asthma</td>
<td>7 (21.9%)</td>
<td>0 (0.0%)</td>
<td>7 (21.9%)</td>
</tr>
<tr>
<td>CF</td>
<td>3 (9.4%)</td>
<td>0 (0.0%)</td>
<td>3 (9.4%)</td>
</tr>
<tr>
<td>PTB</td>
<td>3 (9.4%)</td>
<td>0 (0.0%)</td>
<td>3 (9.4%)</td>
</tr>
<tr>
<td>Malignancy + pleural effusions</td>
<td>0 (0.0%)</td>
<td>2 (6.2%)</td>
<td>2 (6.2%)</td>
</tr>
<tr>
<td>Total</td>
<td>28 (87.5%)</td>
<td>4 (12.5%)</td>
<td>32 (100.0%)</td>
</tr>
</tbody>
</table>

Sig=0.003

CPI: Chronic pulmonary Infection, CF: Cystic Fibrosis, PTB: Pulmonary Tuberculosis
Table (4.6) Correlation between the underlying diseases and isolated organisms

<table>
<thead>
<tr>
<th>Disease</th>
<th>A. fumigatus</th>
<th>A. flavus</th>
<th>A. terrus</th>
<th>A. niger</th>
<th>Candida spp</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPI</td>
<td>6 (18.8%)</td>
<td>6 (18.8%)</td>
<td>3 (9.4%)</td>
<td>0 (0.0%)</td>
<td>2 (6.2%)</td>
<td>17 (53.1%)</td>
</tr>
<tr>
<td>Asthma</td>
<td>3 (9.4%)</td>
<td>2 (6.2%)</td>
<td>2 (6.2%)</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>7 (21.9%)</td>
</tr>
<tr>
<td>CF</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>2 (6.2%)</td>
<td>1 (3.1%)</td>
<td>0 (0.0%)</td>
<td>3 (9.4%)</td>
</tr>
<tr>
<td>PTB</td>
<td>0 (0.0%)</td>
<td>3 (9.4%)</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>3 (9.4%)</td>
</tr>
<tr>
<td>Malignancy + pleural effusions</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>2 (6.2%)</td>
<td>2 (6.2%)</td>
</tr>
<tr>
<td>Total</td>
<td>9 (28.1%)</td>
<td>11 (34.4%)</td>
<td>7 (21.9%)</td>
<td>1 (3.1%)</td>
<td>4 (12.5%)</td>
<td>32 (100.0%)</td>
</tr>
</tbody>
</table>

\[ P \text{ value} = 0.015 \]
Figure (4.7) E-test gradient strips of Itraconazole and Voriconazole antifungal agents showing susceptibility of *A. fumigatus*, *A. flavus*, *A. terreus*
Figure (4.8) Comparison between Itraconazole and Voriconazole susceptibilities against *Aspergillus* spp by E test
Figure (4.9) susceptibility testing of Itraconazole against different *Aspergillus* isolates by E test
Figure (4.10) susceptibility testing of Voriconazole against different *Aspergillus* isolates by E test
Table (4.7) Correlation between Itraconazole and Voriconazole minimum inhibitory concentrations of *Aspergillus* spp

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Itraconazole MIC mean ±SD</th>
<th>Voriconazole MIC mean ±SD</th>
<th><em>P</em>.value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. fumigatus</em></td>
<td>2.2 ± 1.8</td>
<td>0.78 ± 1.3</td>
<td>.08</td>
</tr>
<tr>
<td><em>A. flavus</em></td>
<td>1.28 ± 0.88</td>
<td>0.36 ± 0.23</td>
<td>0.003</td>
</tr>
<tr>
<td><em>A. terrus</em></td>
<td>0.88 ± 0.79</td>
<td>0.6 ± 1.05</td>
<td>0.06</td>
</tr>
</tbody>
</table>
Figure (4.11): Gel electrophoresis of ITS region of *Aspergillus spp* (565 -613 bp)
lane 1:DNA marker (100bp), lane 2, 3, 4, 5, 6, 9, 10, 12 are +ve samples, lane 7, 8, 11 are -ve samples.
Figure (4.12): Nucleotide sequence alignment of *A. terrus* isolate with reference *A. terreus* ATCC 1012
Figure (4.13): Nucleotide sequence alignment of *Aspergillus fumigatus* isolate with reference *Aspergillus fumigatus* ATCC 1022
Nucleotide sequence alignment of Aspergillus isolates and reference strains in gene bank; A. flavus (ATCC 16883), A. fumigatus (ATCC 1022), A. niger (ATCC 16888), A. terreus (ATCC1012). The alignment consists of the 3' end of the 18S ribosomal DNA (rDNA) gene (which contains the ITS 1 primer site), the complete ITS 1 region, the complete ITS 2 region, and the 5' end of the 28S rDNA gene (which contains the ITS 4 primer site). Black boxes indicated species specific nucleotides.
Figure (4.15) difference of single nucleotide base between *A. terrus* clinical isolate and reference strain. Analysis was done by BioEdit alignment editor v7.2.5.
Figure (4.16) difference of single nucleotide base between *A. flavus* clinical isolates and reference strain. Analyses was done by BioEdit alignment editor v7.2.5.
Figure (4.17) difference of two nucleotide base between *A. flavus* clinical isolates. Analysis was done by BioEdit alignment editor v7.2.5
Figure (4.18): Phylogenetic tree based on interspaced gene sequences of 17 isolates from pulmonary Aspergillosis patients. The phylogenetic tree analysis was constructed using the neighbor-joining method in MEGA 7.
Figure (4.19): Phylogenetic tree based on interspaced gene sequences *Aspergillus* isolates from pulmonary Aspergillosis patients compared to most similar *Aspergillus* spp from NCBI. The phylogenetic tree analysis was constructed using phylogeny.fr website (http://www.phylogeny.fr/index.cgi).
CHAPTER FIVE

DISCUSSION

5.1. Discussion

Fungal infections have recently emerged as a world-wide health care problem, due to extensive use of broad spectrum antibiotics, immunosuppressive agents and increasing debilitated patients. Aspergillus infection is the commonest invasive fungal infection which involves respiratory tract (Chawla et al., 2013).

Aspergillus-related lung diseases are traditionally dependent on the immunologic status of the host and the existence of an underlying lung disease. Allergic broncho-pulmonary Aspergillosis (ABPA) affects patients with asthma or cystic fibrosis, (Aspergilloma) occurs in patients with abnormal airways or chronic lung cavities, Chronic necrotizing Aspergillosis occurs in patients with chronic lung pathology or mild immunodeficiency and finally invasive Aspergillosis occurs in severely immuno-compromised patients (Chabi et al., 2015).

Although the diagnostic significance of isolating Aspergillus spp. from respiratory cultures has been studied in immunocompromised hosts with invasive pulmonary Aspergillosis (IPA), little is known about these infections in immunocompetent patients with other forms of Aspergillosis (Tashiro et al., 2011). In this study, the frequency of Aspergillus spp. and antifungal susceptibility was investigated among Sudanese patients with underlying pulmonary diseases.

Three hundred and eighty four patients suffering from chronic pulmonary diseases most of them were male, with male/female ratio about 1.5:1. This finding in agreement with Chawla and his colleagues (2013) in Southern India, who reported that male /female ratio about 1.2.:1. Similar results were observed by Hisano et al (2012) in Japan, his finding showed that most of populations under study were males with 2.8:1 ratio.

The present study showed that high frequency of Aspergillus had been isolated from age group between 11 and 49 years followed by age group (≥50).

Chronic pulmonary infections were the most common underlying lung disease in this study followed by asthma, cystic fibrosis, Pulmonary tuberculosis, Pleural effusions, Malignancy while Hemoptysis, lung abscess and emphysema were the lowest one, these findings were in agreement with previous study conducted in Canada by Al.Alawy et al.(2005) who found that
individuals with pre-existing structural lung disease, atopy, occupational exposure or impaired immunity are susceptible.

Examination by direct microscopy failed to detect 21 samples which were later found to be positive with culture, therefore direct microscopy is 28% sensitive in detecting *Aspergillus* spp in sputum smears. This could be attributed fungal load or population differences. These similar with that reported by Paugam *et al* (2010) in France (29%) and lower than those detected by Njunda *et al* (2012) who found that the sensitivity of direct microscopy is 90%, while Khodavaisy *et al* (2015) study showed that direct microscopy and culture remain negative in invasive pulmonary aspergillosis patients.

This study showed that patients with pulmonary diseases and patients with lower immune status are mainly at risk of infection by pathogenic *Aspergillus* spp, and there was a significant association between isolated species and pulmonary related diseases (*P value*=0.003). These findings in consistent with several studies; Kosmidis and Denning (2015) who reported invasive Aspergillosis (IA) predominantly affects patients with profound defects in immune function and CPA present in patients with underlying lung disease, but with no or only obvious generalized immune compromise. Another study was done by Ader and his colleagues (2006) reviewed that chronic lung disease, in particular chronic obstructive pulmonary disease (COPD), is a third important predisposing factor for acute invasive pulmonary aspergillosis.

Higher frequency of isolates was found in chronic pulmonary patients, followed by asthmatic patients and equally in cystic fibrosis and pulmonary tuberculosis. These results were in agreement with study obtained by Al-Malaky and his colleagues (2015) who found *Aspergillus* spp was the predominant fungal pathogen isolated from patients with pulmonary diseases, notably from patients with chronic obstructive pulmonary disease and pulmonary TB.

Phenotypic results in this study revealed that the dominant species were *A. flavus* (34.4%) followed by *A. fumigatus* (28.1%), *A. terrus* (21.9%) and *A. niger* (3.1%). Similar results obtained by Mahgoub and Elhassan (1972) in Sudan who found six cases of pulmonary aspergillosis from Sudan were due to *A. flavus*, and to our knowledge this study was the only one done in Sudan.

On the other hand, these results disagree with a study done in India by Kurhade *et al* (2002) who found *A. fumigatus* was the commonest species isolated followed by *A. niger* and *A. flavus*. 
In the present study *A. fumigatus* was the most predominant isolate in both chronic pulmonary infection and asthmatic patients followed by *A. flavus* and *A. terreus*. In contrast Tashiro and his colleagues (2011) reported that *A. niger* was the most frequent isolate (40%) followed by *A. fumigatus* (30%), *A. versicolor* (20%), and *A. terreus* (10%) from patients with allergic bronchopulmonary aspergillosis. *A. flavus* in this study was isolated from patients with pulmonary tuberculosis, different results was obtained by Mwaura and his colleagues (2013) in Kenya who reported that *A. niger* (2.3%), was the most frequent filamentous fungus isolated from PTB patients while *A. flavus* frequency was (1.2%).

*A. terreus* in the present study was distributed in all clinical forms with high frequency in chronic pulmonary infections. This in consistency with a study done by Agbetile *et al* (2012) who isolated *A. terreus* from asthmatic patients, and Baddley *et al* (2003) who isolate *A. terreus* from patients with different lung diseases. Moreover, the study observed that the percentage of *A. terreus* isolates in comparision to other *Aspergillus* species were significantly increased which consider an important pathogen because of relative amphotericin B resistance and cause rapidly potentially progressive invasive infections in immunocompromised patients.

*A. niger* was isolated from cystic fibrosis patient. This in consistency to study done by Tashiro and his colleagues (2011) in which *A. niger* was most frequently associated with patients diagnosed with ABPA (40%).

All *Aspergillus* spp were tested against itraconazole and voriconazole by E.test. The minimum inhibitory concentration results demonstrated that voriconazole was more activity against *Aspergillus* spp (0.6±0.93 SD.) in comparison with itraconazole (1.5 ±1.8 SD).

Voriconazole MICs were low even for isolates for which itraconazole MICs were high and were similar to those for isolates for which itraconazole MICs were low. This is in accordance with the study results of Verweij *et al* (2002) in Netherland that evaluated *In vitro* Activities of Itraconazole and voriconazole against *A. fumigatus* who reported the resistance to itraconazole was seen in (25%) of isolates, where’s (7.14%) of isolates were resistant to voriconazole.

The MIC results obtained by E-test methods revealed that voriconazole had a good activity against all *A. flavus* spp., similar results obtained by Badiee and his colleagues (2012).

Another study done by Lalitha *et al* (2007) who reported that voriconazole and itraconazole had the lowest MICs against *Aspergillus* spp in comparison with other fungi included in his study.
In present study (50%) of Aspergillus were susceptible, (25%) resistant and (25%) was susceptible dose dependent to itraconazole. This was different from results reported by Badiee et al (2012) who found that (86.1%) of species were susceptible to itraconazole and (13.9%) were resistant.

From our analysis there were no statistically significant differences between the susceptibilities of A. terrus and A. fumigatus against itraconazole and voriconazole (P.value=0.6, 0.08) respectively which is relatively similar to study done by Baddley et al (2003) who reported that both itraconazole and voriconazole were highly active against A. terreus, in contrast A. flavus revealed statistically significant difference between Itraconazole and voriconazole activities (P value=0.003), and both antifungal agents showed low activity against A. niger. Relatively different results obtained by Tokarzewski, et al (2012) who revealed that A. niger strains tested exhibited high susceptibility to voriconazole, and resistance to itraconazole. And in consistent to study done by Gheith and his colleagues (2014) in Tunisia who found that itraconazole have been active on all tested Aspergillus species except for A. niger which showed a reduced susceptibility to itraconazole.

The present study demonstrates the excellent efficacy of voriconazole against A. flavus and A. fumigatus species and suggests that voriconazole may be the treatment of choice in pulmonary aspergillosis caused by A. fumigatus and A. flavus. Similar result was reported by Diekema et al (2003) who documented the new triazoles posaconazole, ravuconazole, and voriconazole have excellent in vitro activity against Aspergillus spp.

In this study fragments of the ITS1-5.8S-ITS2 were amplified by the use of the primers ITS1 and ITS4 universal primers for identification of Aspergillus species, 22/28 (78.6%) Aspergillus species were confirmed by using the primers ITS1 and ITS4, 6 (21.4%) isolates were negative. The negative results may be attributed to the isolate species not genetically the same that can be detected by ITS1 and ITS4 or a mutation has occurred with the isolated species so changed the nucleotide sequence and not associated with the mentioned primer.

Many similar studies were done for the identification and detection of fungi, by using internal transcribed spacer (ITS) and were published by several scientist (Haughland et al., 2004; Druzhinina et al., 2005). More recent similarity, Leema et al. (2010) confirmed the species A. flavus using the molecular methods by amplification of the internally transcribed spacer regions. Shalini et al. (2014) in his study proved that the genomic DNA containing 18s rRNA
based PCR is suitable for probing large range of medically significant fungi owing to its higher level of analytical sensitivity and specificity.

In the present study the fragments of ITS1- 5.8S-ITS2 amplified by PCR were sequenced, and aligned with references in the NCBI database. Improved DNA sequence information, in conjunction with other identification methods, will enable the establishment of a standardized, objective taxonomic structure for the classification of fungi and therefore, lead to improved diagnosis, therapy, and management of fungal diseases, including invasive aspergillosis (Hinrikson et al., 2005).

In our study the comparison of obtained ITS 1–5.8S–ITS 2 region sequences between referenced sequences deposited in the NCBI database and clinical isolates of 17 Aspergillus species revealed that sequences had 99-100% identity. Some variations in different sequence were detected between some isolates and pathogenic referenced Aspergillus strains. All A. fumigatus and A.niger were identical to reference sequences from gene bank A. fumigatus (ATCC 1022), A niger (ATCC 16888), while two isolates of A.flavus and A.terreus showed a variation in a single nucleotide from that of reference sequences A. flavus (ATCC 16883), A. terreus (ATCC1012), also there was intra species variation in Aspergillus A.flavus, these variations may be due to sub species of A. terreus and A. flavus.

Findings of phylogenetic analysis in this study indicated that the isolated Aspergillus spp was related to several strains worldwide that are far from Sudan (China, Korea, Malaysia, Netherland, India, Egypt, Zimbabwe, South Africa, Brazil, Nigeria). This is believed that their presence reflects the wider circulation of these strains in our geographical area and worldwide for both immunosuppressed as well as immunocompetent with pulmonary underlying diseases.

In conclusion isolation of an Aspergillus spp. from respiratory samples does not confirm it as the etiologic pathogen because airway colonized by Aspergillus spp. is a common feature in several chronic lung diseases. Repeated isolation of the identical Aspergillus species and detection of anti-Aspergillus antibodies and/or Aspergillus antigens in sera are needed to determine the isolate represents the etiologic agent of disease in immunocompetent or mildly immunocompromised individuals.
5.2. Conclusion

This study concluded that there is a significant correlation between frequency of *Aspergillus* spp in patients with chronic pulmonary underlying diseases. *A. flavus* was the most dominant *Aspergillus* spp isolates. Voriconazole antifungal was more active than itraconazole especially against *A. flavus*. E test method is a sensitive, reliable and easy to use in routine laboratory tests. The comparison of obtained ITS 1–5.8S–ITS 2 region sequences between referenced sequences deposited in the NCBI database and clinical isolates of *Aspergillus* species revealed 99-100% identity so the amplification of this region is suitable for identification of *Aspergillus* spp. Isolated *Aspergillus* spp was related to several strains worldwide that are far from Sudan (China, Korea, Malaysia, Netherland, India, Egypt, Zimbabwe, South Africa, Brazil, Nigeria).
5.3. Recommendations

Based on this study we recommended that:

Pulmonary tuberculosis patients, particularly relapse and treatment failures should be subjected to fungal investigation to reduce the disease burden, and for better clinical management of tuberculosis.

Fungi isolated from tuberculosis media should be considered to be of clinical significance, and not merely as contaminants.

Laboratory personnel should be trained on mycological procedures of isolation and identification fungal pathogens of clinical significance.

Further studies should be done using more sensitive methods, such as polymerase chain reaction (PCR), to compare and make a logical conclusion in the diagnosis of the fungal infection in Patients at risk.

Awareness ought to be made to clinicians and pulmonary patients on fungal infection as possible pathogen which can contribute to the complication of pulmonary infections.

Voriconazole is more potent than itraconazole and with regard to reports based on presence of itraconazole resistant isolates, voriconazole should be the drug of choice for pulmonary Aspergillosis especially the invasive forms.
REFERENCES


Clinical Mycology Laboratory: Where Are We and Where Should We Go from Here?" Journal of Clinical Microbiology.47(4): 877-884.


Karen, CP., Mary E.S. (2014). Diagnosis and Treatment of Pulmonary Aspergillosis Syndromes, J Recent Advances in Chest Medicine, Chest, **146** (5): P 1358-1368.


Mann, P.A., Raulo M. Parmegiani, Shui-Qing Wei, Cara A. Mendrick, Xin Li, David Loebenberg et al. (2003). Mutations in Aspergillus fumigatus Resulting in Reduced Susceptibility to Posaconazole Appear To Be Restricted to a Single Amino Acid in the Cytochrome P450 14α-Demethylase. Antimicrobial Agents and Chemotherapy. 47 (2): 577-581.


Appendix I

Reagents and Stains

Gram Stain (Cheebsbrough, 2007)

The Gram reaction base on differences in the cell wall structure into Gram positive which after being stained dark purple with crystal violet are not decolorized by acetone or ethanol and Gram negative which after being stained with crystal violet lose their color when treated with acetone or ethanol and stain red with Saffranin.

Requirements

Crystal violet Gram stain (HiMedia)

To make 1 liter:

Crystal violet .........................................................20 g
Ammonium oxalate ...........................................9 g
Ethanol or methanol, absolute .................................95 g
Distilled water.................................................... to 1 liter

Lugol’s iodine (HiMedia)

To make 1 liter:

Potassium iodide ..................................................20 g
Iodine .................................................................10 g
Distilled water............................................. To 10 liter

70% alcohol

Absolute alcohol ..................................................70 ml
Distilled water ...................................................30 ml
Saffranin (HiMedia)

Method of Preparation

• The dried smear was fixed by heat.
• The fixed smear was covered with crystal violet for 30-60 minutes.
• The stain was washed off with clean water.
• All water was tipped and the smear covered with lugol’s iodine for 30-60 minutes.
• The stain was washed off with clean water.
• 70% alcohol was rapidly applied for 10-20 seconds for decolourization and then washed rapidly with clean water.
• The smear then covered with Saffranin stain for 2 minutes.
• The stain was washed off with clean water, back of slide was cleaned.
• After air-dry, smear was examined microscopically by using X 100 lens.

Results

Fungal elements appear as Gram positive.

20% Potassium Hydroxide

KOH is a strong alkali. When specimen such as skin, hair, nails or sputum is mixed with 20% w/v KOH, it softens, digests and clears the tissues (e.g., keratin present in skins) surrounding the fungi so that the hyphae and conidia (spores) of fungi can be seen under microscope.

Procedure to make 100 ml of KOH 20% w/v solution:

1. Weigh 20 g potassium hydroxide (KOH) pellets.
2. Transfer the chemical to a screw-cap bottle.
3. Add 50 ml distilled water, and mix until the chemical is completely dissolved, add remaining distilled water and make the volume 100 ml.

4. Label the bottle and **mark it corrosive**. Store it at room temperature. The reagent is stable for up to 2 years.

**Lactophenol Cotton Blue** (Himedia)

Lactophenol Cotton Blue is used as staining solution for fungi. Fungi are eukaryotic organism and they are classified into two main groups that is yeast and molds. Its cell wall is made up of chitin. Lactophenol Cotton Blue reagent is used for staining as well as for wet mounting of fungi. Lactic acid preserves the fungal structure and clears the tissue while phenol acts as a disinfectant and cotton blue imparts blue colouration to the fungal spores and hyphae.

**Ingredients:**

- Phenol crystals 20.000
- Cotton blue 0.050
- Lactic acid 20.000
- Glycerol 20.000
- Distilled water 20.000

**Directions**

1) Place a drop of Lactophenol Cotton Blue reagent on a clean and dry slide. The stain imparts a blue colouration on hyphae.

2) By using a nichrome inoculating wire, carefully tease the fungal culture, into a thin preparation.

3) Place a coverslip on the preparation. Wait for about 5 minutes.

4) Observe first under microscope with low power for screening in low intensity.
**Culture media**

**RPMI 1640 glucose agar**

---

### RPMI 1640 Agar w/ MOPS & 2% Glucose w/o Sodium bicarbonate  M1972

(Twin Pack)

**Intended use**

RPMI 1640 Agar w/ MOPS & 2% Glucose w/o Sodium bicarbonate is used for determination of susceptibility of microorganisms to antifungal agents.

**Composition**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Gram / Litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Part A</td>
<td></td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>0.050</td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>0.020</td>
</tr>
<tr>
<td>L-Cystine dihydrochloride</td>
<td>0.0652</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>0.020</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>0.300</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.010</td>
</tr>
<tr>
<td>L-Histidine hydrochloride monohydrate</td>
<td>0.02096</td>
</tr>
<tr>
<td>L-Hydroxyproline</td>
<td>0.020</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>0.050</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>0.050</td>
</tr>
<tr>
<td>L-Lysine hydrochloride</td>
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</tr>
<tr>
<td>L-Methionine</td>
<td>0.015</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
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</tr>
<tr>
<td>L-Proline</td>
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</tr>
<tr>
<td>L-Serine</td>
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</tr>
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<tr>
<td>L-Tryptophan</td>
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<tr>
<td>L-Tyrosine disodium salt</td>
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<tr>
<td>L-Valine</td>
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<tr>
<td>D-Biotin</td>
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<tr>
<td>D-Calcium Pantothenate</td>
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<tr>
<td>Choline chloride</td>
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<tr>
<td>Folic acid</td>
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<tr>
<td>Inositol</td>
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<tr>
<td>Niacinamide</td>
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</tr>
<tr>
<td>p-Amino benzoic acid (PABA)</td>
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</tr>
<tr>
<td>Riboflavin</td>
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</tr>
<tr>
<td>Pyridoxine hydrochloride</td>
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</tr>
<tr>
<td>Thiamine hydrochloride</td>
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<tr>
<td>Vitamin B12</td>
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<tr>
<td>Calcium nitrate tetrahydrate</td>
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</tr>
<tr>
<td>Potassium chloride</td>
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</tr>
<tr>
<td>Magnesium sulphate anhydrous</td>
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<tr>
<td>Sodium chloride</td>
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<tr>
<td>Sodium phosphate dibasic anhydrous</td>
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</tr>
<tr>
<td>Glutathione reduced</td>
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</tr>
<tr>
<td>Phenol red sodium salt</td>
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<tr>
<td>MOPS Buffer, Free acid</td>
<td>34.500</td>
</tr>
<tr>
<td>L-Arginine hydrochloride</td>
<td>0.241</td>
</tr>
<tr>
<td>Part B</td>
<td></td>
</tr>
<tr>
<td>D-Glucose</td>
<td>20.000</td>
</tr>
<tr>
<td>Agar</td>
<td>15.000</td>
</tr>
<tr>
<td>Final pH (at 25°C)</td>
<td>7.0±0.1</td>
</tr>
</tbody>
</table>

**Formula adjusted, standardized to suit performance parameters**
Directions
Part A: Suspend 42.91 grams of Part A in 500 ml distilled water. Stir gently until the medium is completely dissolved. Do not heat. Filter sterilise the medium using sterile membrane filter of 0.22 micron or less.
Part B: Suspend 35 grams of Part B in 500 ml distilled water. Mix well and heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 mins. Cool to 45-50°C.
Aseptically add filter sterilized Part A to Part B. Mix well before pouring into sterile Petri plates.
Note: The performance of this batch has been tested and standardised as per the current CLSI (formerly NCCLS) document.

Principle And Interpretation
RPPI-1640 medium developed by Moore et al., at Roswell Park Memorial Institute is well known media used for cell culturing. The formulation is based on the RPMI-1630 series of media utilizing a bicarbonate buffering system and alterations in the amounts of amino acids and vitamins.
Invasive fungal infections have been increased over the past two decades. Due to the life threatening nature of these infections and reports of drug resistance, susceptibility testing of yeast pathogens has become very important. The CLSI have published a reference method for broth dilution antifungal susceptibility testing of Yeast. Also for use with the gradient-strip method when testing *Candida* spp. directly from colonies grown on nonselective media(1). RPMI-1640 Agar can be used to determine MIC values for various antifungal agents. Amino acids, vitamins and salts provide essential nutrients. Glucose is the carbohydrate source. MOPS buffers the media. Agar acts as solidifying agent.

Type of specimen
Clinical samples: Pure cultures isolated from urine, stool, blood etc.
Antifungal Strips

MIC Test Strip

**DESCRIPTION**

Quantitative assay for determining the Minimum Inhibitory Concentration (MIC) of antifungal agents against microorganisms and for detecting the resistance mechanisms.

**MIC Test Strip** are paper strips with special features that are impregnated with a predefined concentration gradient of antibiotic, across 15 two-fold dilutions of a conventional MIC method.

On one side of the strip is indicated a MIC scale in μg/ml, and a code that identifies the antifungal agent.

For ESB (Extended Spectrum Beta-Lactamase) and MBL (Metallo Beta-Lactamase) detection, the double-sided gradient carries the appropriate diagnostic markers.

**MIC Test Strip** are available in a large variety of configurations. Each configuration is available in packages of 10, 30, and 100 tests.

**CONTENT OF THE PACKAGES**

The 10-test box contains 10 strips individually packed in desiccant envelopes and an instruction sheet.

The 30-test box contains 30 strips individually packed in desiccant envelopes and an instruction sheet.

The 100-test box contains 10 desiccant envelopes, each containing 10 strips, and an instruction sheet. The 100-test pack also contains a storage tube.

**METHOD PRINCIPLE**

When the MIC Test Strip is applied onto an inoculated agar surface, the performed exponential gradient of antifungal agent is transferred to the agar matrix.

After incubation for 24 to 48 hours, a symmetrical inhibition ellipse centered along the strip is formed. The MIC is read directly from the scale in terms of μg/ml, at the point where the edge of the inhibition ellipse intersects the strip.

**COMPOSITION**

The strips are made of high-quality paper and each strip is impregnated with a predefined concentration gradient across 15 two-fold dilutions of antibiotic.

**GATHERING AND KEEPING SAMPLES**

The colonized samples that are to be subjected to the evaluation of Minimum Inhibition Concentration (MIC) are taken up by culture media that have been previously swabbed with the sample under examination. The growth of the microorganisms in the solid culture media must be performed before inoculation.

**TEST PROCEDURE**

1. Allow unopened envelope to come to room temperature before opening it, for minimizing condensation on the strip.
2. Swab 4 to 5 well isolated and morphologically similar colonies with a culture medium and suspend them in 5 ml of a suitable suspension medium. Fastidious microorganisms should be suspended in broth and used within 15 minutes.
3. Compare the turbidity to the appropriate McFarland standard.
4. Dip a sterile swab in the broth culture or in a diluted form thereof and suspend it on the wall of the test tube to eliminate excess liquid.
5. Drag it along the surface of the medium contained on the plate so as to produce even growth; allow excess moisture to be absorbed and ensure that the surface is completely dry before applying strips.
6. Apply the strip to the agar surface with the MIC scale facing upwards and end of the strip to the outside of the plate, pressing it with a sterile forceps on the surface of the agar and ensure that whole length of the antibiotic gradient is in complete contact with the agar surface. Once applied, do not move the strip.
7. Incubate plates at an inverted position under conditions appropriate for the microorganisms.
8. Put the not used strips onto the tube contained in the package.
## MIC breakpoints

<table>
<thead>
<tr>
<th>Antifungal agent</th>
<th>A. flavus</th>
<th>A. fumigatus</th>
<th>A. nidulans</th>
<th>A. niger</th>
<th>A. terreus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S ≤</td>
<td>R &gt;</td>
<td>S ≤</td>
<td>R &gt;</td>
<td>S ≤</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Posaconazole</td>
<td>-</td>
<td>-</td>
<td>0.125</td>
<td>0.256</td>
<td>-</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>2</td>
<td>-</td>
</tr>
</tbody>
</table>
**Molecular reagents**

**10 X TBE buffer**

Formula in grams per liter

Tris base…………………………………….. 108 gm

Boric acid……………………………………55gm

EDTA…………………………………………40 ml of 0.5M

Deionized water……………………………1 liter

**Preparation**

Amount of 108 gm. Tris base were weighed and added to 55gm of boric acid and 40 ml of 0.5M EDTA then dissolved into 1 liter deionized water pH 8.0.

**1X TBE buffer**

Formula in ml per liter

10 X TBE………………………………………10 ml

Deionized water………………………………90 ml

Ten ml of 10 X TBE buffer was added to 90 ml deionized water and heated until completely dissolved.
**Ethidium bromide solution**

**Formula in grams per 1ml**

Ethidium bromide……………………………10 mg

Deionized water………………………………1 ml

**Preparation**

Twenty milligrams of ethidium bromide powder were dissolved into 1000 µl deionized water, and kept into brown bottle.

**Agarose gel**

**Preparation**

Amount of 2 gm of agarose powder dissolved by boiling in 100 ml 1X TBE buffer, then was cooled to 55°C in water bath, then, 1.5 µl of Ethidium bromides stock (10 mg/ml) per 100 ml gel solution for a final concentration of 0.5 ug/ml were added, mixed well and poured on to the casting tray that has been taped up appropriately and was equipped with suitable comb to form well in place. Any bubbles were removed and the gel was allowed to set at room temperature. After solidification, the comb was gently removed and the spacer from the opened sides was removed.
APPENDIX II

Sigma 1-14 Germany Microcentrifuge Device

CLASSIC K960 China Thermocycle Device
Gel Electrophoresis and Power Supply Device

UV Light Transilluminater Device
SiZer™ DNA Markers

DESCRIPTION
INTRON supplies a wide range of products for accurate size and mass estimations (quantitation) of nucleic acid fragments. Nucleic Acid Markers are available for sizing linear, or supercoiled DNA and single-stranded DNA fragments. A variety of these markers are available in the ready-to-use SiZer™ formats.

SiZer™ DNA Markers are ideal for determining the size of large DNA fragments from 60–10,000 bp base pairs. The SiZer™ DNA Markers consist of 7–15 linear double-stranded DNA fragments. Several fragments are present at increased intensity to allow easy identification. All fragments are precisely quantified and mixed during the production.

For 5 µl loading, all fragments except typical band DNA fragments are 40 ng. The typical band of DNA fragments is 100 ng. These ladders are pre-mixed with loading dye and are ready to use.

All DNA Markers can be stained with RedSafe™ Nucleic Acid Staining Solution, ethidium bromide (EB) or other DNA stains.

CHARACTERISTICS
- Ideal for determining the size of DNA
- Stable for more than 12 months at -20°C
- Ready to use without any handling.

KIT CONTENTS

<table>
<thead>
<tr>
<th>Product</th>
<th>Contents</th>
<th>Cat. No.</th>
</tr>
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<tr>
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<tr>
<td>SiZer™–50 plus DNA Marker</td>
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<tr>
<td>SiZer™–ADNA/HindIII DNA Marker</td>
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<td>24077</td>
</tr>
</tbody>
</table>

STORAGE
- Store at 4°C and stable for more than 5 months. For more stable storage, should be eluted and then stored at -20°C. (stable for more than 12 months)

GENERAL USE
- No DNase and RNase detected.
- Load 5 µl per well of agarose gel.

QUALITY CONTROL
Well-defined bands are formed during agarose gel electrophoresis. The DNA concentration is determined spectrophotometrically.

The absence of nucleic acid is confirmed by a direct nucleic acid activity assay.

ELECTROPHORESIS
- The 5 µl of ladder DNA was loaded, and then electrophoresed for 1 hr at appropriate concentration of gel.

PRODUCT USE LIMITATION
This product is developed, designed and sold exclusively for research purposes and in vitro use only. The product was not tested for use in diagnostics or for drug development, nor is it suitable for administration to humans or animals.

NOTICE BEFORE USE
- Do not heat before loading
- For quantification, adjust the concentration of the sample to equalize it approximately with the amount of DNA in the nearest band of the ladder.
- Visualize DNA by staining RedSafe™, ethidium bromide (EB) or other DNA stains.

DETAIL INFORMATION

<table>
<thead>
<tr>
<th>Size range (bp)</th>
<th>Conc. (ng/µl)</th>
<th>Typical bands</th>
<th>Other bands</th>
<th>Loading Vol. (µl)</th>
<th>Band number</th>
<th>Contents</th>
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<tr>
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<td>60–300</td>
<td>128</td>
<td>100g/µl</td>
<td>40g/µl</td>
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RELATED PRODUCTS

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<th>Cat. No.</th>
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<td>21411</td>
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<tr>
<td>DNA-spin™ Plasmid DNA Extraction Kit</td>
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<td>MEGAgap-spin™ Total Fragment DNA Purification Kit</td>
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<td>Maxime™ PCR Premix (3-Startaq)</td>
<td>25165</td>
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<tr>
<td>Maxime™ PCR Premix (5-pfu)</td>
<td>25165</td>
</tr>
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</table>
APPENDIX III

Color Plates
APPENDIX IV

Sudan University of Science and Technology
College of Graduate Studies
Questionnaire for requirement of PhD degree

ID number ……………………… Date of collection ………………………………………

Age………………………………………………………

Sex:   male                  female

Occupation:

………………………………………………………………………………………………………………

Residence………………………………………………………………………………………………

Place of sample collection

………………………………………………………………………………………………………………

Symptoms………………………………………………………………………………………………

………………………………………………………………………………………………………………

Diagnosis

………………………………………………………………………………………………………………

Other diseases:

………………………………………………………………………………………………………………

Laboratory diagnosis:
Results

KOH

Gram's stain

Culture

E test

PCR

Sequencing