DEVELOPMENT OF A MULTIPLEX REAL-TIME PCR ASSAY FOR DETECTION OF FUNGAL PATHOGENS IN INVASIVE MYCOSES

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by

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LIST OF SYMBOLS

+	Plus
-	Minus
×	Multiplication
÷	Division
±	Plus-minus
~	Approximately
%	Percentage
<	Less than
>	More than
≤	Less than or equal
≥	More than or equal
°C	Degree Celcius
α	Alpha
β	Beta
Δ	Delta
μ	Micro sign
™	Trademark sign
®	Registered sign

LIST OF ABBREVIATIONS

ΔG	Delta G
A_{260}/A_{230}	Absorbance at 260 nm per absorbance at 230 nm
A_{260}/A_{280}	Absorbance at 260 nm per absorbance at 230 nm
ABPA	Allergic bronchoalveolar aspergillosis
AIDS	Acquired immunodeficiency syndrome
ATCC	American Type Culture Collection
BAL	Bronchoalveolar lavage
BDG	1,3-β-D-glucan
BHI	Brain-Heart infusion medium
BLAST	Basic Local Alignment Search Tool
CDC	Centers for Disease Control
CF	Complement fixation
CFU	Colony-forming unit
CIE	Counter-immunoelectrophoresis
CLSI	Clinical & Laboratory Standard Institute
COPD	Chronic obstructive pulmonary disease
COVID-19	Coronavirus disease 2019
Cq	Cycle threshold
CSF	Cerebral spinal fluid
CT	Computed tomography
DNA	Deoxyribose nucleic acid
EAPRI	European Aspergillosis PCR Initiative
ECV	Epidemiology cut-off
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
EUCAST	European Committee in Antimicrobial Susceptibility Testing
FDA	Food and Drug Administration
FFPE	Formalin-fixed paraffin-embedded
FISH	Fluorescence in situ hybridization
GM	Galactomannan
GMS	Gomori methenamine silver
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
HSCT	Hematopoietic stem cell transplantation
HUSM	Hospital Universiti Sains Malaysia

IA	Invasive aspergillosis
IAC	Internal amplification control
IC	Invasive candidiasis
ICU	Intensive care unit
IFI	Invasive fungal infection
IPA	Invasive pulmonary aspergillosis
ISHAM	International Society for Human and Animal Mycology
ITS	Internal transcribed spacer
IV	Intravenous
LOD	Limit of detection
MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiment
MSG	Mycoses Study Group
NCBI	National Center for Biotechnology Information
NGS	Next-generation sequencing
ODI	Optical Density Index
PAS	Periodic acid-Schiff
PCR	Polymerase chain reaction
PMN	Polymorphonuclear cell
PNA	Peptide nucleic acid
RFU	Relative fluorescence units
RIA	Radio-immunosorbent assay
RNA	Ribonucleic acid
ROI	Reactive oxygen intermediates
rRNA	ribosomal ribonucleic acid
SARS-CoV 2	Severe acute respiratory syndrome coronavirus 2
SDA	Sabouraud Dextrose Agar
SDS	Sodium dodecyl sulfate
Taq	Thermus aquaticus
Th	T-helper
UV	Ultraviolet
v/v	Volume per volume
WHO	World Health Organization
w/v	Weight per volume

LIST OF APPENDICES

PEMBANGUNAN UJIAN PCR MULTIPLEKS MASA NYATA BAGI PENGESANAN PATOGEN FUNGI DALAM MIKOSIS INVASIF

ABSTRAK

Mikosis invasif merupakan ancaman yang sangat mencabar kepada individu yang sistem imunnya terjejas dan sering kali menyebabkan keadaan morbiditi dan mortaliti yang teruk. Keberkesanan kaedah diagnostik semasa yang terbatas disebabkan oleh kelewatan dalam pengenalpastian jangkitan kulat yang turut mengakibatkan kelewatan dalam memulakan rawatan antikulat yang sesuai. Ini adalah berpunca daripada kelemahan kaedah konvensional. Kajian ini dijalankan untuk menyiasat mengenai protokol pengekstrakan DNA kulat dan pembangunan ujian tindak balas berantai polimerase (PCR) multipleks masa nyata untuk pengesanan patogen kulat invasif yang lebih cepat. Pembangunan ujian PCR multipleks masa nyata ini mampu mengenalpasti serentak pelbagai spesies kulat yang lazimnya dikaitkan dengan mikosis invasif secara serentak. Reka bentuk primer yang spesifik kepada spesies dan pengoptimuman asai menyasarkan *Aspergillus fumigatus*, *Aspergillus terreus*, *Candida albicans*, dan *Candida glabrata*. Keputusan kajian menunjukkan beberapa kaedah pengekstrakan DNA yang menghasilkan kepekaan dan kecekapan masa yang lebih unggul dengan implikasi besar bagi kegunaan klinikal. Ujian PCR multipleks masa nyata yang dibangunkan menunjukkan ketepatan yang tinggi dalam mengenal pasti patogen kulat. Sebanyak 65 isolat termasuk pelbagai strain kulat dan strain rujukan lain menunjukkan 100% spesifik bagi organisma sasaran untuk ujian spesifisiti analitik. Penilaian prestasi ujian PCR multipleks menggunakan sampel darah yang

diinokulasi dengan organisma (*spiked blood samples*) menunjukkan sensitiviti dan spesifisiti yang tinggi di mana kedua-duanya pada 100% dengan nilai ramalan positif (PPV) dan nilai ramalan negatif (NPV) juga pada 100%. Secara keseluruhan, penyelidikan ini menyumbang kepada peningkatan diagnosis makmal dan pengurusan jangkitan kulat yang invasif. Dengan menangani cabaran dalam pengekstrakan DNA kulat dan mencipta ujian PCR multipleks yang berkualiti tinggi, kajian ini menunjukkan pendekatan diagnosis yang lebih efisyen dan meningkatkan penjagaan pesakit dan hasil rawatan. Kajian penilaian lanjutan menggunakan sampel klinikal yang sebenar disyorkan untuk memperkukuhkan lagi nilai aplikasi kaedah ini dalam situasi klinikal masa kini.

DEVELOPMENT OF A MULTIPLEX REAL-TIME PCR ASSAY FOR DETECTION OF FUNGAL PATHOGENS IN INVASIVE MYCOSES

ABSTRACT

Invasive mycoses pose significant challenges to immunocompromised individuals, often resulting in severe morbidity and mortality. Current diagnostic methods are hindered by delays in identification and initiation of appropriate antifungal therapy, stemming from the limitations of conventional techniques. This study undertakes a thorough investigation into fungal DNA extraction protocols and the development of a multiplex real-time PCR assay for rapid identification of invasive fungal pathogens. This research aims to compare fungal DNA extraction methods and develop a multiplex real-time PCR assay capable of simultaneously identifying multiple fungal species commonly associated with invasive mycoses. Species-specific primer design and assay optimization target *Aspergillus fumigatus*, *Aspergillus terreus*, *Candida albicans*, and *Candida glabrata*. Results reveal certain extraction methods exhibiting superior sensitivity and time efficiency, with implications for clinical use. The developed multiplex real-time PCR assay demonstrates promising accuracy in identifying fungal pathogens. Analytical specificity testing, which involved detecting 65 isolates including various fungal strains alongside other reference strains, yielded a 100% identification rate for target organisms. Performance evaluation of the multiplex PCR assay using spiked blood samples showed high specificity and sensitivity, both at 100%, with positive predictive value (PPV) and negative predictive value (NPV) also at 100%. Overall, this research contributes to advancing laboratory diagnosis and management of

invasive fungal infections. By addressing challenges in fungal DNA extraction and creating a high-performance multiplex PCR assay, this study lays the groundwork for more efficient and reliable diagnostic approaches, thereby enhancing patient care and treatment outcomes. Further validation studies on real clinical samples are recommended to confirm the applicability of these methodologies in the current clinical settings.

CHAPTER ONE

INTRODUCTION

1.1 Problem statement

Invasive mycoses represent a formidable threat to individuals with compromised immune systems, often leading to severe morbidity and mortality. The current diagnostic landscape is fraught with challenges, characterized by delayed identification and subsequent start of suitable antifungal treatment due to the limitations of conventional methods. This study seeks to address this critical gap by focusing on the development of a molecular diagnostic assay capable of simultaneously detecting various invasive fungal pathogens. The lack of a comprehensive and rapid diagnostic tool has profound clinical implications, resulting in delayed interventions and compromised patient outcomes.

The urgency of this study is underscored by the need to revolutionize diagnostic approaches, providing clinicians with a swift and accurate means of identifying invasive mycoses. The anticipated assay aims to improve the precision of diagnosis, enabling early therapeutic interventions tailored to the specific fungal pathogens involved. Furthermore, the study recognizes the potential economic impact of such a diagnostic advancement, as it could contribute to more cost-effective and efficient healthcare delivery by streamlining diagnostic processes and reducing unnecessary treatments. Through this investigation, the aim is to bridge the existing diagnostic gap, enhance patient care, and ultimately contribute to a substantial reduction in the burden posed by invasive mycoses on both healthcare systems and affected individuals.

1.2 Rationale of the study

Increased incidence of invasive mycoses has prompted various investigations which include finding ways to overcome diagnostics and management problems in the susceptible cohort of patients. Detection of invasive fungal infections have been a diagnostic dilemma for both clinicians and medical microbiology laboratory technologists which lies in the need for accurate and timely identification of these fungal infections. In immunocompromised patients, invasive mycoses like invasive aspergillosis and candidemia pose life-threatening risks. An early detection and appropriate treatment are crucial for improving the patient outcomes.

Current diagnostics depend on conventional culture methods with aid of serological tests and limited molecular diagnostic methods, which may be available in reference centres (Kidd et al., 2019; Mendonca et al., 2022). They have limitations in terms of sensitivity, specificity and turnaround time. Protocols for DNA extraction ranged from simple commercialized kit-based method (which may not give good yield) to laborious chloroform-based methods (which may not be feasible in a routine diagnostic laboratory). Nevertheless, the currently published protocols still did not provide the best result, optimum DNA extraction, particularly from clinical samples.

It is of paramount importance to accurately identify fungal pathogens such as *Aspergillus* and *Candida* species, as these are the most frequently encountered pathogens causing invasive fungal infections and specific treatment must be initiated promptly. In clinical setting, once a susceptible patient is suspected to have invasive aspergillosis, an effective pre-emptive therapy must be commenced

immediately. Unfortunately, the occurrence of *Aspergillus* species detection remains uncertain until results can be produced by the laboratory which may take up to one week (Cadena et al., 2016). Therefore, it would be very beneficial if it can be detected directly from the clinical specimen for example blood in timely manner.

As the currently available assays could not be standardized due to poor reliability, further studies need to be embarked to provide the solutions i.e. optimization of DNA extraction from clinical samples and timely detection of fungal pathogens in multiplex real-time PCR assay format. This assay could provide several benefits, including the ability to identify infections caused by different fungal pathogens in a single test, enabling prompt initiation of targeted antifungal therapy. This study is focused to develop a sensitive and specific multiplex real-time PCR for simultaneously detecting invasive *Aspergillus fumigatus*, *Aspergillus terreus*, *Candida albicans* and *Candida glabrata*.

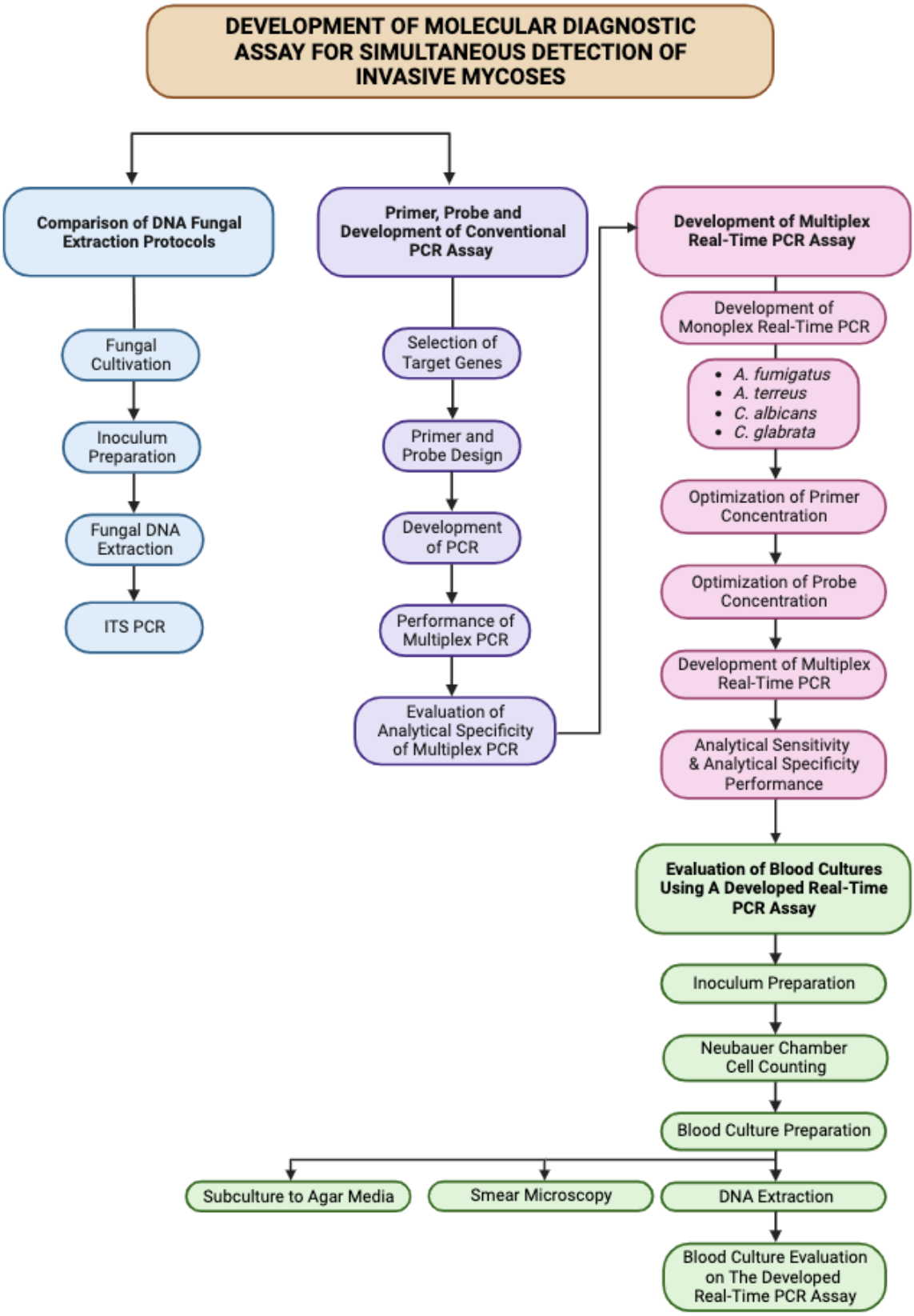
1.3 Research questions of the study

1. What are the most effective DNA extraction methods for detecting fungal DNA in clinical samples using PCR assay?
2. How can specific primers be designed to specifically target invasive mycoses (*A. fumigatus*, *A. terreus*, *C. albicans* and *C. glabrata*)?
3. How can a multiplex real-time PCR assay be developed and optimized for the simultaneous detection of pathogen in invasive mycoses?
4. What are the key parameters to consider for the analytical validation of the developed multiplex real-time PCR assay?
5. How does the developed multiplex PCR assay perform in terms of sensitivity, specificity and accuracy when tested on clinical samples?

1.4 Objectives of the study

1. To optimize fungal DNA extraction methods for its effective detection in clinical samples by PCR assay.
2. To design specific primers targeting *Aspergillus fumigatus*, *Aspergillus terreus*, *Candida albicans,* and *Candida glabrata* (monoplex PCR).
3. To optimize and develop the multiplex real-time PCR for simultaneous detection of *Aspergillus* spp. and *Candida* spp. and an internal control gene.
4. To incorporate internal control gene detection in the multiplex real-time PCR assay developed in the third objective.
5. To perform analytical validation (sensitivity & specificity) of the developed multiplex real-time PCR assay.
6. To evaluate the performance of the developed multiplex real-time PCR assay in clinical samples and spiked samples.

1.5 Flow chart of the study



CHAPTER TWO

LITERATURE REVIEW

2.1 Invasive fungal infections

Invasive fungal infections (IFIs) account for 1.5 million death and up to three million people are infected annually worldwide (Fernandes, C. M. et al., 2022; Shaw, 2022; Zhang et al., 2022). These fungal infections are potentially life threatening in numerous types of patients, including solid organ transplantation, chemotherapy and extended duration in the intensive care unit (ICU) (Kim, D. Y. et al., 2022). In public health, IFIs are among the biggest concerns which lack of implementation of definitive diagnosis (Osman et al., 2020). These IFIs are the primary cause of death for immunocompromised hosts, with invasive aspergillosis being the primary cause of death in these individuals (Fidler et al., 2022).

Since coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) hit the world at the end of 2019, IFIs represent with a significant increase in mortality, morbidity and length of hospital stay in severely ill patients at their middle and latter stages (Cattaneo et al., 2023). Besides, the World Health Organization (WHO) published in October 2022 an outline of the first list of priority fungal pathogens (Figure 2.1) that present the treat to public health (Fisher & Denning, 2023). The primary aim of is combatting the IFIs associated with these pathogens. The formal acknowledgment by the WHO spotlights a category of infections that have long been overlooked in terms of both awareness and the research funding required to effectively address the escalating impact of these diseases (Fisher & Denning, 2023).

Of utmost significance on this list were *Aspergillus fumigatus*, *Candida*

albicans, *Candida auris* and *Cryptococcus neoformans*, followed by *Candida glabrata*, *H. capsulatum*, and various fungi responsible for mucormycosis or mycetoma. *Fusarium* species and *Candida tropicalis* were classified as highly important. This comprehensive assessment generated a range of recommendations, emphasizing three key priority areas which are research and development, public health interventions, and surveillance (Fisher & Denning, 2023).

IFIs can be broadly categorized into two categories based on their characteristics and clinical significance. The first category consists endemic mycoses, which are caused by specific fungal pathogens and are typically associated with specific geographic regions. These endemic mycoses include blastomycosis, coccidioidomyosis, histoplasmosis, paracoccidioidomycosis, penicilliosis, and sporotrichosis (Osman et al., 2020). Each of these infections is caused by particular fungal species and is often found in specific areas around the world.

The second category encompasses opportunistic mycoses, which are caused by a diverse group of fungi and tend to affect individuals with underlying health conditions or compromised immune systems. These infections include invasive aspergillosis, invasive candidiasis, cryptococcosis, hyalohyphomycosis, phaeohyphomycosis and zygomycosis (Osman et al., 2020), can be pose significant challenges in healthcare settings. These infections are frequently linked to individuals with weakened immune systems, such as cancer patients, individuals with HIV, organ transplant recipients, and individuals on immunosuppressive therapies. It is imperative to grasp the attributes and risk factors of opportunistic mycoses to effectively control and prevent these infections among susceptible populations.

In accordance with the WHO's recommendations, strategies aimed at reducing the worldwide impact of fungal diseases which include enhanced surveillance of fungal infections, ensuring cost-effective availability of diagnostic tools near patients. Moreover, there is a demand for focused support for research and development to expedite the deployment of novel antifungal medications and improved diagnostic methods (Fisher & Denning, 2023).

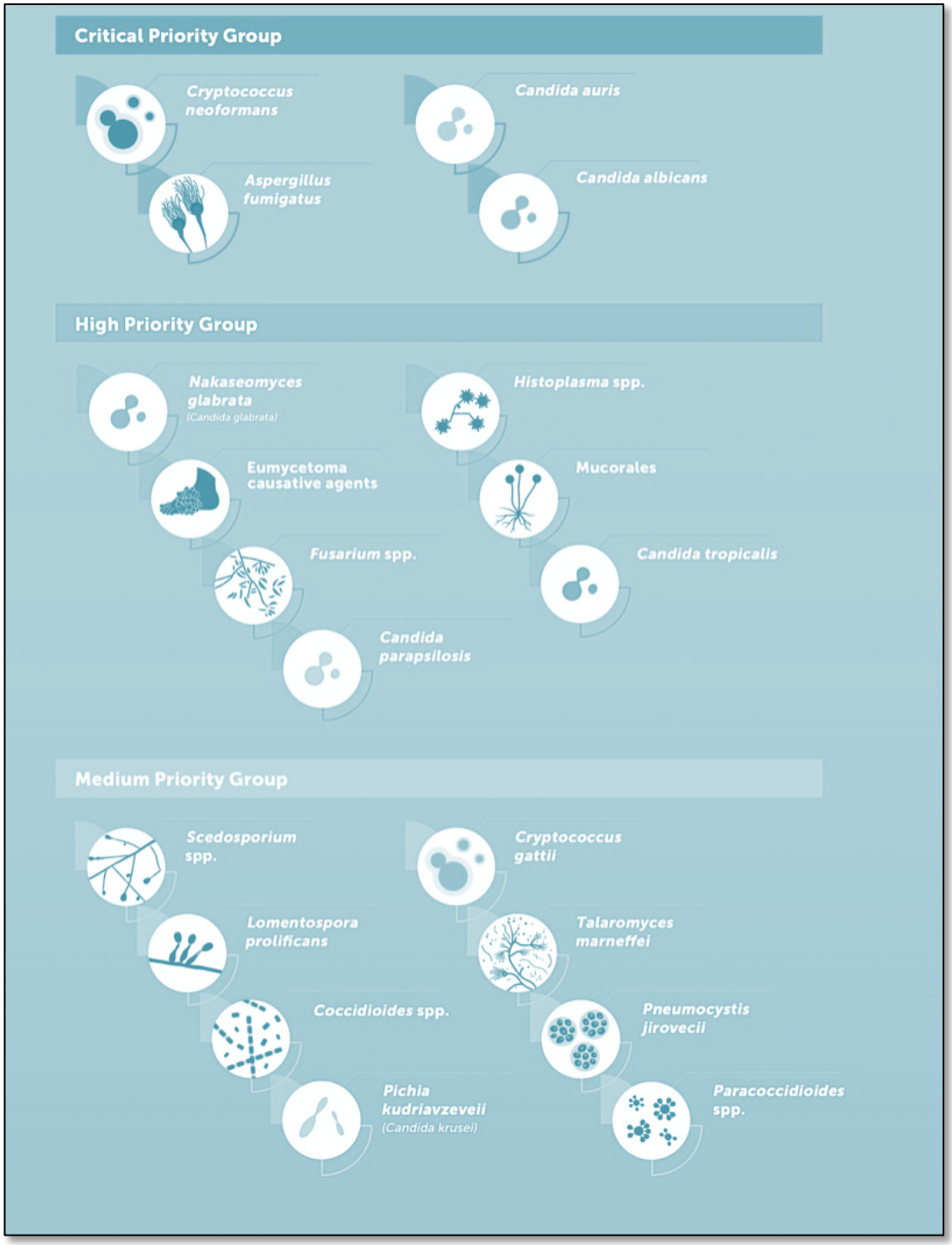


Figure 2.1 List of fungal pathogens prioritized by the World Health Organization (WHO). Adopted from (CDC, 2022).

2.2 Invasive aspergillosis

Invasive aspergillosis (IA) is an opportunistic, potentially fatal and systemic infectious disease causes of mortality and morbidity which is usually encountered in immunocompromised patients (Badiee et al., 2022; Lian et al., 2022). IA is severe form of IFIs in immunocompromised hosts caused by *Aspergillus* species (Sato et al., 2022) and mainly found in the respiratory tract system and it spreads from pulmonary locations to the brain, skin, kidney, liver, and gut (Badiee et al., 2022). Besides, the prevalence is on the rise among non-neutropenic patients with significant underlying conditions, including patients in ICU, patients who undergone solid organ transplantation, patients with viral infections caused by SARS-CoV-2, patients with chronic obstructive pulmonary disorder (COPD) and other respiratory complications (Jenks et al., 2021).

A. fumigatus has an extensive environmental presence that is part of its infectious life cycle. It produces conidia that are airborne by asexual reproduction (Figure 2.2). When inhaled by specific patient groups with weakened immune systems, these conidia establish themselves in the lungs. Subsequently, they undergo germination, leading to two possible outcomes: either controlled fungal growth with pronounced inflammation mediated with corticosteroid therapy, or uncontrolled hyphal growth without polymorphonuclear cell (PMN) infiltration especially in cases of corticosteroid therapy. In severe instances, this uncontrolled growth can result in dissemination, particularly in cases of neutropenia (Dagenais & Keller, 2009).

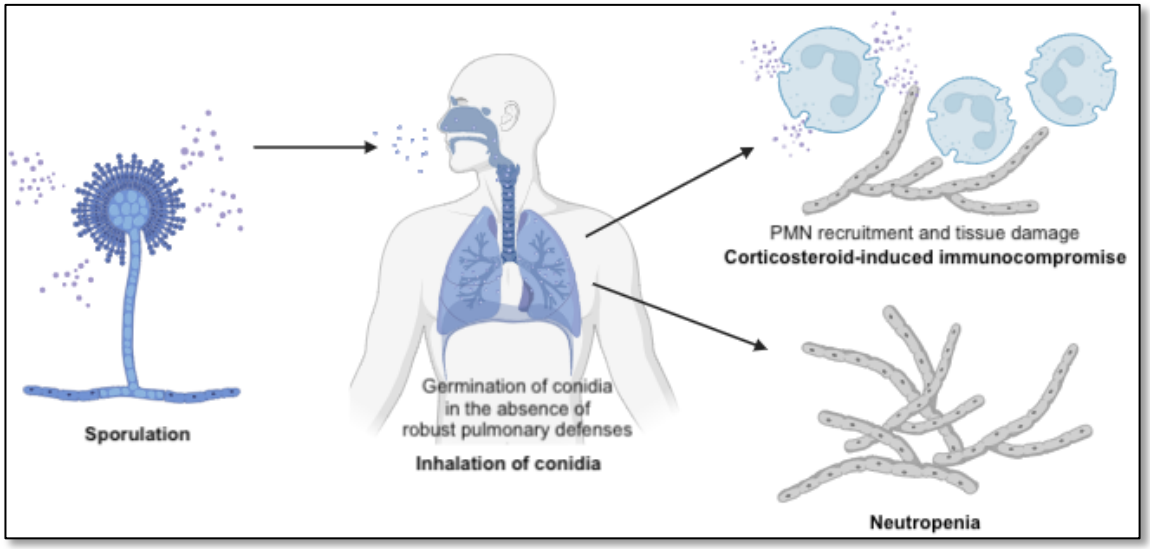


Figure 2.2 Infectious life cycle of *Aspergillus fumigatus*.

2.2.1 Burden of disease and epidemiology

Due to changes in epidemiological patterns, numerous emerging fungi are becoming increasingly significant in causing infections among immunocompromised individuals. Recent published data provides evidence that the incidence of invasive aspergillosis (IA) is on the in several countries, including France and a significant number of cases go undetected and are only diagnosed during autopsy (Danion et al., 2019).

After candidemia and *Pneumocystis* pneumonia, IA is the third most common cause of fungal infection disease in France. In a number of countries, including France, the incidence of IA has been rising. From 1.1 cases per 100,000 people in 2001 to 1.8 cases per 100,000 people in 2010, this incidence increased by +4.4% annually. Recent data indicates that a significant number of cases go undetected and are only diagnosed post-mortem (Danion et al., 2019). A published study based on autopsies conducted in Japan from 1989 to 2009 revealed that the fungal infections were found in 4.5% of the 13,787 cases with (49%) 299 cases involving *Aspergillus* species (Suzuki et al., 2017). While the percentage of cases of severe aspergillosis has declined since 2001, *Aspergillus* species infections have become more common over time (Danion et al., 2019).

Besides, a multicentric study by Chakrabarti et al. found that *Aspergillus* species were the most frequently isolated fungi in patients with non-classical risk factors. The study, which examined invasive mold infections in Indian intensive care units with a total of 142 cases of IA were found (63.5%) (Chakrabarti et al., 2019; Rudramurthy et al., 2019). Furthermore, more recent observations indicate that IA can occur simultaneously with severe even in individuals who appear to have a normal immune response (Rudramurthy et al., 2019).

Aspergillus species were responsible for most invasive mold infections. *A. fumigatus* stood out as the most frequently isolated species, although in India, *A. flavus* was equally prevalent. This observation aligns with prior findings (Slavin & Chakrabarti, 2012) and may be linked to the hot climate conditions (Rotjanapan et al., 2018). In fact, an aerobiological survey carried out in Delhi revealed that *A. flavus* was the most commonly occurring *Aspergillus* (Rotjanapan et al., 2018).

However, the impact of climate is less straightforward, as shown by the persistence of *A. fumigatus* as the predominant mold in Singapore and Thailand, two countries with consistently hot climates, like those found in temperate regions. Interestingly, under temperatures between 20 to 30°C, the germination rates of various *Aspergillus* species were found to be similar. Yet, the germination rate of *A. fumigatus* rose to 41°C, while that of *A. flavus* decreased by 45% (Araujo & Rodrigues, 2004; Rotjanapan et al., 2018). This phenomenon can be attributed to the fact that the mold concentrations in the air were lowest during the dry and hot summer months, rising during periods of precipitation (Park et al., 2010).

The epidemiology of IA is an intricate and evolving field, with data that can differ among regions and evolve over time. Hence, it is advisable to refer to local surveillance data for precise insights into IA within a particular population or setting. This approach can lead to more effective measures for mitigating the impact and burden of fungal infections.

2.2.2 Mycology and taxonomy

Over 250 distinct species of *Aspergillus* are arranged into numerous subgenera and sections that were once referred to as groupings. *A. fumigatus* is the most commonly found species in cases of invasive disease, followed by, followed by *A. flavus*, *A. terreus*, and *A. niger* (Arastehfar et al., 2021). Other species have also been associated with infections in severely immunocompromised patients. While it is generally not challenging to identify these fungi at the genus level, difficulties may arise, especially with poorly sporulating isolates, making identification even at the species complex level a daunting task (Cadena et al., 2016).

While certain species of *Aspergillus* may have a sexual form, most species reproduce asexually. The standard procedure is to refer to all fungi under one name, "Aspergillus," even if it is possible for both anamorph and teleomorph stages to occur. An Italian priest compared *Aspergillus* to the holy water sprinkler, or "aspergillum," based on the physical characteristics of the conidial head of the fungus (Rudramurthy et al., 2019). Researchers have uncovered cryptic *Aspergillus* species that share morphological characteristics with the primary *Aspergillus* sections. However, it has been discovered that a large number of these cryptic species have greater triazole minimum inhibitory concentrations (MICs) (Cadena et al., 2016).

In order to determine the relationships within the genus *Aspergillus* at the subgeneric level, identification of these organisms depends on molecular techniques, such as sequencing of particular genes like the internal transcribed spacer region, actin, beta-tubulin, RNA polymerase II, and calmodulin genes (Cadena et al., 2016; Sun et al., 2022). The recognition of cryptic species commenced with *A. lentulus*, which was identified as a subset of *A. fumigatus* characterized by poor sporulation and elevated triazole MICs. Subsequently, *Neosartorya pseudofischeri* was identified, followed by

the discovery of *A. udagawae*, *A. viridinutans*, *A. fumigatiaffinis*, and *A. novofumigatus* within the Fumigati section. Other examples of cryptic aspergillosis encompass *A. alliaceous* (Flavi section), *A. carneau*, and *A. alabamensis* (Terrei section); *A. tubingensis*, *A. awamori*, and *A. acidus* (Nigri section); and *A. calidoustus*, *A. insuetus*, and *A. kevei* (Usti section), among others (Cadena et al., 2016).

Phylogenetic method has played a role in addressing challenges pertaining to taxonomy and naming conventions (Figure 2.3) (Kocsube et al., 2016). Nonetheless, investigations employing multigene phylogenetic methods have revealed that *Aspergillus* forms a broadly monophyletic group, distinct from its closely related genus Penicillium (Kidd et al., 2023; Kocsube et al., 2016). This monophyletic nature of *Aspergillus* facilitated the retention of its name for the majority of species within the genus, ensuring the preservation of its clinical significance. Species traditionally recognized by their teleomorphs were consequently reclassified under the *Aspergillus* genus (Kidd et al., 2023).

Inter-laboratory variances have been raised by the difficulties in definitively identifying these organisms due to the lack of uniform identification protocols. There is ongoing discussion over the need of precise identification in treatment options and patient outcomes. This is because the majority of clinical trial participants are typically diagnosed with probable disease based on clinical symptoms, radiographic findings, specific blood tests, or bronchoalveolar lavage (BAL) results, such as the detection of galactomannan (GM), without the recovery of a definitive isolate. This is due to most clinical trials have not studied isolates to this level of detail (Johnson et al., 2012; Kidd et al., 2019).

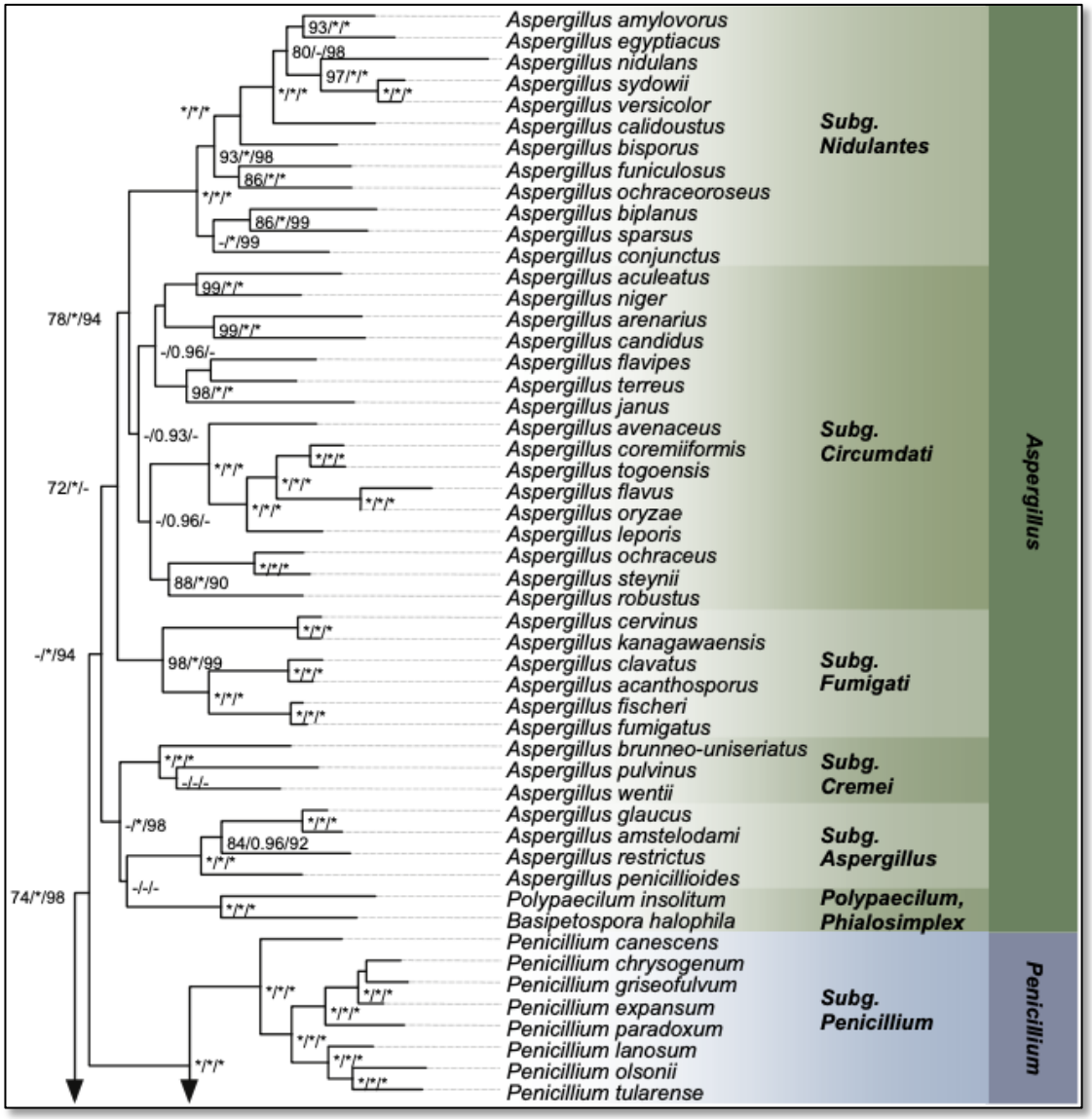


Figure 2.3 Phylogenetic tree based on six genes consisted of 3395 base pairs by maximum likelihood method. Adopted from (Kocsube et al., 2016).

2.2.3 Virulence factors

In invasive aspergillosis (IA), various virulence factors play a role, including biofilm, haemolysin, α-amylase, lipase, proteinase, pectinase, and phospholipase. The formation of biofilm actively prevents the phagocytosis of *Aspergillus* species and allows exponential growth (Raksha et al., 2017). For example, haemolysin is produced by *A. fumigatus* isolates from diverse sources, including clinical and environmental samples. Its toxicity may stem from the ability of the haemolysin to induce DNA damage and mutations in animal models and cell cultures (Zarrin et al., 2017). These toxins can contribute to the pathogenesis *A. fumigatus* by directly invading host tissues. Various haemolysin can induce genotoxicity of dietary carcinogens *in vitro*, with the level of induction strongly dependent on the species (Zarrin et al., 2017). Additionally, haemolysin is responsible for the lysis of red blood cells (Raksha et al., 2017).

Enzyme production is a contributing factor in the virulence process. The positivity observed in environmental samples is lower than in patient samples. Virulence factors appears to be naturally present in environmental samples and persist in a small percentage to ensure survival in that environment (Ali et al., 2019). However, upon entry into the human tissue, their activity intensifies as a response to unfavourable conditions and the defensive mechanisms of the human body, which attempt to eliminate microorganisms (Ali et al., 2019).

Virulence factors of IA can confirm the pathogenicity and invasive nature of fungi. Detecting these factors not only aids in distinguishing pathogenic from non-pathogenic *Aspergillus* species but also assists physicians in initiating antifungal treatment when necessary (Raksha et al., 2017).

2.2.4 Risk factors

In immunocompromised patients, invasive aspergillosis (IA) constitutes a significant source of morbidity and mortality, with persistently high fatality rates despite advancements in antifungal treatment. Consequently, there is considerable interest in developing preventive measures and identifying key risk factors to guide prevention strategies (Baddley, 2011).

The risk factors for IA encompass various aspects, including the underlying health condition of the host, comorbidities, medications, and environmental factors. Extensive research has delved into these risk factors in specific populations, such as those with hematologic malignancies and transplant recipients. Additionally, emerging groups, including intensive care unit (ICU) patients, individuals with chronic obstructive pulmonary disease (COPD), HIV patients, and solid organ transplant recipients, have been studied (Baddley, 2011). Notably, the latest widespread occurrence of IA is observed in SARS-CoV-2 patients, characterized by immune dysregulation affecting both Th1 and Th2 responses (Montrucchio et al., 2021).

For COVID-19 patients, host-related comorbidities like COPD and asthma in the ICU are identified as risk factors for IA. Intriguingly, the data concerning the time elapsed between diagnosis presents notable considerations (Montrucchio et al., 2021). Significant variations in diagnostic latency may arise from the implementation of diverse screening strategies, such as GM testing, and the use of invasive BAL in intubated patients (Montrucchio et al., 2021).

2.2.5 Pathogenesis

Aspergillus species are widespread in nature, commonly found in natural environments like soil and decaying vegetation where they thrive as saprophytes, prolifically sporulating and dispersing conidia into the environment (Challa, 2018; Pathakumari et al., 2020). However, the risk of exposure to these conidia varies significantly, contingent upon climatic factors such as humidity, temperature and wind patterns (Challa, 2018). Generally, a healthy individual with intact host defence mechanisms rarely succumbs to disease after inhaling *Aspergillus* spp. conidia.

On the other hand, infections caused by Aspergillus species may lead to invasive infections in individuals with weakened immune systems, resulting in increased rates of morbidity and mortality (Challa, 2018). In high-risk populations such as individuals with HIV, neutropenia, cancer chemotherapy and bone marrow transplants, the mortality rate for IA can range from 40 to 90% (Pathakumari et al., 2020). Normally, the human host does not mount a response to *Aspergillus* spp. conidia even when exposed. This is due in part to the presence of abundant antimicrobial factors within the mucus layer of the sinopulmonary tract, which neutralize the conidia, and the ciliated epithelium, which actively clears them (Challa, 2018). Disease risk arises when the spore concentration remains consistently high, often exacerbated by hot and humid conditions, thus posing a threat to the host (Challa, 2018).

In such cases, the host initiates a robust inflammatory response, involving the recruitment of neutrophils and macrophages to eliminate the conidia (Challa, 2018). However, this response is carefully modulated and downregulated to prevent excessive tissue damage (Challa, 2018). Moreover, the mucus layer creates a nutrient-limited environment that inhibits conidia germination. Therefore, the intact ciliated epithelium, coupled with the mucus layer and the presence of neutrophils and alveolar

macrophages, holds an essential part to the host's defense systems. (Challa, 2018).

Aspergillus species employ various strategies to elude the immune system, such as concealing crucial pathogen-associated molecular patterns, inhibiting the fusion of phagosomes with lysosomes, and producing antioxidants like catalase and mannitol (Pathakumari et al., 2020). Furthermore, certain *Aspergillus* species produce specific secondary metabolites with various immunosuppressive effects, such as fumagillin, actibind, gliotoxin, and cytochalasin E (Pathakumari et al., 2020). The capacity of Aspergillus to produce melanin pigment, serving various purposes such as shielding conidia from adverse external conditions outside their mammalian hosts and scavenging reactive oxygen intermediates (ROI) within host cells, is another distinguishing characteristic that sets them apart from other fungi (Pathakumari et al., 2020). Melanin also plays a role in interfering with the intracellular trafficking of phagocytosed conidia and masking β-glucans (Dagenais & Keller, 2009; Pathakumari et al., 2020).

Nonetheless, disease can still occur in specific scenarios, such as when the immune response is weakened, as seen in IA, or when it becomes overly exuberant, as observed in conditions like allergic fungal rhinosinusitis (AFRS) or allergic bronchoalveolar aspergillosis (ABPA) (Challa, 2018). Additionally, *Aspergillus* colonization and the formation of mycetomas may result from chronic inflammation or the presence of cavity lesions (Challa, 2018).

2.2.6 Clinical manifestations

The clinical manifestation of aspergillosis varies widely, contingent upon both the specific site of infection and the host’s capacity to mount an effective and coordinated immune response. The upper respiratory tract, including the trachea,

bronchi, lung tissue, and adjacent structures, is commonly affected, although dissemination or infection can potentially affect any organ system (Cadena et al., 2016). The clinical syndromes associated with aspergillosis can be categorized into allergic presentations, non-invasive saprophytic infections, semi-invasive conditions, and invasive diseases (Cadena et al., 2016).

For non-invasive infections, there are two diseases: (1) aspergilloma and (2) allergic bronchopulmonary aspergillosis (ABPA). As aspergilloma, also known as an *Aspergillus* fungus ball, develops within an existing pulmonary cavity that is typically the result of conditions like emphysema, malignancy, or pulmonary tuberculosis. When observed on medical imaging, it appears as a solid mass that can move freely within the cavity. Clinical symptoms associated with aspergilloma vary, ranging from asymptomatic cases with radiographic findings discovered incidentally to sever cases with life-threatening haemoptysis necessitating immediate intervention (Denning, 2003). For individuals with symptomatic aspergilloma, surgical resection is a viable treatment option. However, surgical intervention is not feasible for several patients due to the presence of underlying structural lung disease and poor pulmonary function. In such cases, effective illness stabilization and management require a combination of embolization and antifungal medication (Cadena et al., 2016; Denning, 2003).

ABPA is a clinical condition characterized by ongoing immunological activation, lung infiltrates, and asthma (Shah & Panjabi, 2016), which may eventually lead to the development of bronchiectasis. Diagnostic criteria include various characteristics, such as the co-occurrence of eosinophilia, central bronchiectasis, pulmonary infiltrates, acute skin response to *A. fumigatus*, elevated serum immunoglobulin E levels, and the presence of anti-IgG precipitins against *Aspergillus*. It is important to note that none of these findings alone constitutes a definitive

diagnosis for this condition (Cadena et al., 2016; Greenberger et al., 2014). While radiological patterns may vary, a high-resolution computed tomography (CT) scan of the chest can often reveal the characteristic central bronchiectasis associated with the condition. Cavities and fibrosis are frequently observed in later stages of the illness (Greenberger et al., 2014). Following diagnosis, many patients are initiated on a combination of corticosteroids and itraconazole to expedite symptom improvement. However, previous research has demonstrated itraconazole's ability to reduce the need for corticosteroids during ABPA treatment. As such, attempts to gradually decrease or discontinue corticosteroid treatment ought to be done after the initial improvement. Patients who do not react to medication or who become intolerant to itraconazole can sometimes find success with other triazoles, such as posaconazole or voriconazole. However, there is a lack of information regarding the use of additional azoles and other medicines, such as omalizumab. (Cadena et al., 2016; Greenberger et al., 2014).

As for invasive diseases, invasive pulmonary aspergillosis (IPA) is one of them. IPA progresses relatively quick, typically within days to a few weeks, and has a high mortality rate, often exceeding 50% (Kanj et al., 2018). In most cases, patients were observed to exhibit symptoms within 10 to 21 days after hematopoietic stem cell transplantation (HSCT), particularly in the context of severe granulocytopenia. The most common clinical signs of IPA are haemoptysis, shortness of breath, fever, chest discomfort, or pleuritic pain. However, because they are unable to properly develop an inflammatory response, people with weakened immune systems, such as those suffering from neutropenia, may exhibit more subdued symptoms (Cadena et al., 2016). Fever may not be present in this patient group, and other symptoms, such as haemoptysis and pleuritic chest pain, which are frequently caused by angioinvasion, may be more noticeable (Cadena et al., 2016). A characteristic radiological finding

linked to IPA is the halo sign, which is a region of reduced attenuation encircling a pulmonary nodule. Nevertheless, imaging findings in high-risk patients usually lack specificity and closely resemble those of other pulmonary infections. Initial CT scan results followed by a progressive increase in lesion volume within the initial 7 days (Cadena et al., 2016; Caillot et al., 2001).

To confirm the diagnosis, histopathological features specific to *Aspergillus* are valuable. However, obtaining tissue for analysis can often be challenging due to underlying host characteristics such as thrombocytopenia or hemodynamic instability that preclude invasive diagnostic testing. Angioinvasion is a common hallmark of the condition when tissue samples are available. One morphological feature that may suggest the presence of invasive pulmonary aspergillosis (IPA) is the presence of dichotomous branching hyphae (Challa, 2018). However, these physical characteristics are not unique to *Aspergillus*, and a definitive diagnosis requires culture confirmation (Cadena et al., 2016).

The second one is tracheobronchitis. The significance of *Aspergillus* species in respiratory cultures can vary widely, encompassing colonization through to infection. Tracheobronchitis is characterized by an infection that is predominantly confined to the tracheobronchial tree (Cadena et al., 2016; Kanj et al., 2018). Clinical features of tracheobronchitis consist of non-specific symptoms, including cough, chest pain, fever, and haemoptysis, which can contribute to diagnostic delays. Bronchoscopy often reveals pseudo-membranes and ulcerative lesions. Since sputum cultures have limited sensitivity and specificity, additional diagnostic methods are often necessary for an accurate diagnosis. Dehiscence may occur in lung transplant recipients if tracheobronchitis affects the anastomotic site (Cadena et al., 2016). The management and treatment will be further explained in the next point.