MOLECULAR IDENTIFICATION OF *Curvularia* SPECIES AND THE CHARACTERISTICS OF THEIR INFECTIONS

by

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Dissertation submitted in partial fulfillment of the requirements for the degree of Bachelor of Health Science (Honours) (Biomedicine)

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CERTIFICATE

This is to certify that the dissertation entitled "Molecular Identification of Curvularia Species and The Characteristics of Their Infections" is the bona fide record of research work done by Ms Nur Shahira binti Kassim during the period from July 2018 to December 2018 under my supervision. I have read this dissertation and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation to be submitted in partial fulfillment for the degree of Bachelor of Health Science (Honours) (Biomedicine).

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DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated and duly acknowledged. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at Universiti Sains Malaysia or other institutions. I grant Universiti Sains Malaysia the right to use the dissertation for teaching, research and promotional purposes.

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Nur Shahira Binti Kassim

Date: 8/1/2019

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LIST OF ABBREVIATIONS

AFS	Allergic fungal sinusitis
AIDS	Acquired Immune Deficiency Syndrome
ALL	Acute Lymphoblastic Leukemia
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
CCB	Center for Chemical Biology
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
EDTA	Ethylene Diamine Triacetic Acid
EFI-α	Translation elongation factor 1-α
GPDH	Glyceraldegyde 3-phosphate dehydrogenase
H&E	Hematoxylin and Eosin
HIV	Human immunodeficiency virus
ITS	Internal Transcribed Spacer
LPCB	Lacto-Phenol Cotton Blue
LSU	Large SubUnit-coding sequence
$MgCl_2$	Magnesium chloride
NCBI	National Center for Biotechnology Information
PAS	Perodic acid-Schiff
PCR	Polymerase Chain Reaction
rRNA	Ribosomal Ribonucleic Acid

SDA	Sabouraud Dextrose Agar
Spp.	Species
SrRNA	Small ribosomal subunit RNA
SSU	Small SubUnit-coding sequence
Taq	Thermus aquaticus
TBE	Tris/Borate/EDTA
TE	Tris-EDTA
UK	United Kingdom
USA	United State of America
USM	Universiti Sains Malaysia
UV	Ultraviolet

LIST OF SYMBOLS

%	Percentage
°C	Degree celcius
µL	Microlitre
µM	Micromolar
Cm	Centimeter
et al.	*et alia*-'And others'
G	Grams
Min	Minute
mL	Mililitre
ng/µL	Nanogram per microlitre
Rpm	Revolution per minute

ABSTRAK

Kejadian jangkitan kulat telah meningkat secara dramatik pada tahun-tahun kebelakangan ini. Diagnosis awal dan intervensi segera adalah elemen kritikal untuk menentukan keberkesanan rawatan jangkitan kulat. Diagnosis makmal dari jangkitan *Curvularia* di Hospital USM masa kini bergantung kepada kaedah konvensional mikroskopik dan kultur yang memakan masa, dan kepekaannya tidak diketahui. Dalam kajian ini, identifikasi *Curvularia* secara molekular dijalankan dengan menggunakan tindak balas rantai polimerase -*Internal Transcribed Spacer* (ITS-PCR) dan penentuan jujukan DNA. Ciri-ciri jangkitan *Curvularia* di Hospital USM juga dihuraikan. Sejumlah 19 Curvularia spp. telah diasingkan dari semua spesimen dari tahun 2001 hingga 2018. Jujukan DNA ITS dapat memberikan identifikasi 11 (57.8%) *C. lunata*, tiga *Curvularia geniculata*, dua *C. brachyspora,* and satu *C. crepinii*. Dari segi jantina, pesakit lelaki adalah lebih ramai dari pesakit perempuan. Lebih dari 50% pesakit berada dalam julat umur melebihi 40 tahun. Sebahagian besar Curvularia dipencilkan daripada spesimen kornea. (74%, n=14). Lain-lain spesimen termasuk darah, cecair bronko-alveolar dan tisu. Ulser kornea merupakan manifestasi klinikal yang didapati dalam sebahagian besar pesakit. Sejumlah 16 pesakit (84%) mendapat rawatan antikulat dan majoriti pesakit (79%) sembuh secara klinikal.

ABSTRACT

The incidence of fungal infection has dramatically increased in recent years. Early diagnosis and rapid intervention is a critical element to determine the effective treatment in fungal infection. The laboratory diagnosis of the *Curvularia* infection in Hospital USM currently depends on the conventional methods of microscopic and culture which is time-consuming, and the sensitivity is not known. In this study, molecular identification of *Curvularia* species was done using Internal Transcribed Spacer – Polymerase Chain Reaction (ITS-PCR) and sequencing. The characteristics of *Curvularia* infections in Hospital USM were also described. A total of 19 Curvularia spp. were isolated from all specimen from the year 2001 to 2018. ITS sequencing was able to identify 11 (57.8%) *C. lunata*, three *Curvularia geniculata*, two *C. brachyspora,* and one *C. crepinii*. Males were noted to be more affected than females. More than 50% of the patients belonged to age group of 40 years and above. *Curvularia* spp. were isolated mainly from corneal scraping (74%, n=14). Other specimens included blood, bronchoalveolar lavage and tissue. Most of the patients presented with corneal ulcers. A total of 16 patients (84%) received antifungal therapy and majority of the patients (79%) had clinical cure.

CHAPTER ONE: INTRODUCTION

1.1 Background of the study

In the recent years the incidence of fungal infection has increased in frequency and severity due to a few factors such as the onset of acquired immunodeficiency syndrome (AIDS) and medical techniques progress for example the use of antibiotic, immunosuppressive therapy and organ transplantation has led to the increase of the fungal infection cases (Bagyalakshmi et al., 2007).

Early diagnosis and rapid intervention is a critical element to determine the effective treatment in fungal infection. Most of the hospital in Malaysia relied traditionally on conventional method such as direct microscopic examination, histology examination and culture. However, microscopic and culture examination for the diagnostic performance extremely depend on the quality of clinical specimens and the experience of the laboratory staff (Wagner et al., 2018). Conventional method was shown to have low sensitivity in detection of the fungal species compared to the molecular method such as Polymerase Chain Reaction (PCR).

In many instances, species identification is necessary to determine definitive antifungal therapy. Morphological identification is often unable to identify fungal pathogen to its species level. Some clinical fungal species do not fully express their morphological characteristics when identified in the laboratory as most of them exist in the laboratory culture in asexual state (anamorph), thus making diagnosis difficult. As molecular method identify organism based on specific DNA sequences, there is a great advantage of PCR techniques in improving the diagnosis of fungal infection.

This study focused on the specific identification of *Curvularia* species that infect human in Hospital USM using ITS-PCR. This PCR technique using a primer that amplify conserved region of the 28SrRNA, 18SrRNA with further separation of genus and species targeting internal transcribe spacer (ITS) region (Bagyalakshmi et al., 2007). The Internal Transcribed Spacer (ITS) region can be found in the fungal ribosomal DNA (rDNA) is the highly variable sequences to differentiate the fungal species by PCR analysis (Martin and Rygiewicz, 2005). Though many target genes for the purpose of fungal identification have been previously published, ribosomal RNA gene including ITS region is the most commonly used gene with tremendous sequence data in publicly accessible database such as the GenBank.

1.2 Problem statement

In the recent years a significant increase in the severity and frequency of fungal infection has been observed. *Curvularia* species are able to cause a spectrum of diseases from superficial to deep disseminated diseases. Clearly emerging pattern of *Curvularia* infections which usually involve patients with mycotic keratitis followed by respiratory tract infections. Disseminated *Curvularia* infection possessed a very high mortality rate despite surgical and antifungal therapies.

The laboratory diagnosis of *Curvularia* infection in Hospital USM currently depends on conventional methods of microscopy and culture. The culture and microscopic technique are still considered as gold standard, but are known for low sensitivity, laborious and time-consuming with poor accuracy.

The difficulty in identification of *Curvularia* spp. on morphological basis alone poses a diagnostic dilemma because high degree of similarity with other species and sometimes the morphological patterns are not clear due to factors such as absence of spore, prior antifungal treatment and slow growth of the organism (Alex et al., 2013). These fungi frequently produce the reduction of conidial size and decrease in sporulation during strain maintenance and culturing which make the identification more difficult (Kumar and Shukla, 2005; Pimentel et al., 2005; Alex et al., 2013; Krizsan et al., 2016).

1.3 Research Objective

1.3.1 General objective

To describe specific *Curvularia* spp. from clinical specimens, their proportion and characteristics of their infections in Hospital USM

1.3.2 Specific objective

1. To describe the morphology of *Curvularia* spp. by culture and microscopy technique.

2. To identify specific species of Curvularia using ITS-PCR and sequencing.

3. To determine the proportion of different *Curvularia* species in Hospital USM

4. To describe the characteristics of *Curvularia* infections in Hospital USM

1.4 Significance of Study

This study will provide preliminary data on Curvularia infection in Malaysia. There has been very limited data on specific Curvularia infections worldwide, particularly in low-cost clinical settings due to poorly accessible molecular techniques. The ability to specify the identity of Curvularia species in the current setting enables contribution in improving the diagnosis of the disease related to the *Curvularia* infections which subsequently lead to the better patients' management. As with other fungal infections, accurate and timely diagnosis of the infection is critically important to provide insights on preemptive antifungal therapy for the *Curvularia* since specific antifungal susceptibility testing for this organism is not routinely available.

CHAPTER TWO: LITERITURE REVIEW

2.1 Fungal infections

Fungi are ubiquitous in nature and exist as free-living organism. Most of the fungi are widespread in nature and often can be found on diseased body surface. Fungal infections and fungal diseases are often accidental. A few fungal species can be found as microflora such as *Candida albicans* and *Malassezia furfur*. These fungi may also serve as opportunistic pathogens.

Fungal infection is a global health problem and it can affect anyone especially person with weakened immune systems, such as patients with malignancy or HIV/AIDS patient. Fungal diseases are often caused by common fungi that can be found in environment. There are a million of fungal species on earth, but only about 300 of those are pathogenic (Centers for Disease Control and Prevention, 2017).

Fungal infections contribute to significant rates of morbidity and mortality worldwide ranging from mild to invasive fungal infection in human, plants and animals. Individuals with dysfunctional immune system such as immunocompromised patient with underlying HIV infection, elderly and very young children, are more vulnerable to fungal infections (Velayuthan et al., 2018).

2.2 Curvularia species

Curvularia is dematiaceous fungus that can be found ubiquitously in soil. It was first described by Wakker and Went as *Acrothecium lunata* in 1989. In 1959, *Curvularia* was documented as a human pathogen when it was isolated from a case of mycetoma (Carter and Boudreaux, 2004). This genus is comprised of approximately 40 species. The most frequently reported clinical species is *Curvularia lunata*, however other species such a *C. americana, C. brachyspora, C. chlamydospora, C. clavata, C. hominis, C. inaequalis, C. muehlenbeckiae, C. pallescens, C. psedolunata, C. senegalensis, C. geniculata, C. crepinii and C. verruculosa* have also been reported by a few clinical cases (Revankar and Sutton, 2010; Da Cunha et al., 2013).

2.2.1 Taxonomic Classification

The taxonomic classification of *Curvularia* is as the following:

Kingdom: Fungi

Division: Ascomycota

Class: Dothideomycetes

Order: Pleosporales

Family: Pleosporaceae

Genus: *Curvularia*

Curvularia belongs to the family Pleosporaceae, which also includes other medically-important fungi such as *Alternaria*, *Drechslera, Exserohilum* and *Bipolaris.*

2.2.2 Epidemiology

Most *Curvularia* species can be found as saprobic soil agent and animate on dead plant or grain material as weak pathogens. It can cause plant disease, ranging from seed failure to leaf blight and grass "fade-out' during hot and humid weather (Liu, 2011). Human also have a risk to get infected when dealing with soil during outdoor recreation or occupation activities. Infection cause by *Curvularia* spp. is known as "curvulariosis" which is also a type of pheohyphomycoses. In the infected tissue, these fungi may appear yeast-like form, hyphae, or both. Diseases caused by *Curvularia* spp. in human including eye infection, respiratory tract infection, skin and nail infections, infection complicating peritoneal dialysis as well as deep and disseminated infection. (Krizsan et al., 2016).

2.2.3 Pathogenicity

Curvularia was thought to be plant pathogen but in the last centuries it has been encountered in human infection (Shinde et al., 2015). *Curvularia* spp. and other dematiaceous fungi not only cause disease in immunocompromised patient but are also able to infect immunocompetent individual, mainly in tropical and subtropical areas. *Curvularia* is able to produce several mycotoxins such as curvularins, brefeldins, and radicinin that act as cytotoxin and antivirals. It also metabolizes certain steroids and able to produce other toxin and enzymes, including cellulases, chloroperoxidase, endoglucanase, glucosidase, galactosidase, lipid phosphatase, zaragozic acid, pectinases, spirostaphylotrichins, pyrenocenes, triticones, neocoprogen, cytochalasins, curvapallides, and anthroquinones. The roles all of these enzymes have not been not fully understood and explained (Wilhelmus and Jones, 2001).

Most of *Curvularia* spp. can cause different types of infection such as keratitis, sinusitis, cutaneous and subcutaneous infections, peritonitis, onychomycosis, endocarditis, endophthalmitis, cerebral phaeohyphomycosis, and allergic bronchopulmonary as well as disseminated disease (Moody et al., 2012). Mycotic keratitis is the most common disease cause by *Curvularia* other than another ocular diseases (Kaushik et al., 2001). *Curvularia* spp. was the fourth most common fungal isolate identified from cases of keratitis, following *Candida*, *Fusarium* and *Aspergillus*. The eye infection is usually related to the injury or manipulation of the eye. The use of the contact lens provides the greater risk for fungal keratitis, which also remains as a common cause of corneal ulcer in the southern United States (Iyer et al., 2006). This infection often presents as a slowly progressing, feathery, superficial corneal infection, and endophthalmitis.

After mycotic keratitis, the second most common human infection cause by *Curvularia* spp. is sinusitis. Allergic fungal sinusitis (AFS) is a most common sinusitis caused by *Curvularia* spp. based on previous study (Krizsan et al., 2016). AFS is caused by the presence of fungal hyphae in the sinuses. The infected patient may demonstrate facial pain, headache, nasal stuffiness, nasal obstruction, difficulty of breathing through nose, mucosal thickening, purulent discharge and mass in the sinus. In certain cases, it led to obstruction of lacrimal duct with enlargement of the lacrimal sac, painful swelling of the eyelid as well as decrease or loss in visual acuity (Krizsan et al., 2016).

Other than keratitis and sinusitis, a lot of study also reported *Curvularia*-related infections such as skin and nail infection, infection complicating peritoneal dialysis as well as deep and disseminated infection. In skin infection, *Curvularia* produces characteristics of black granule on the skin surface and are composed of dense, interwoven mass of septate

mycelium and thick-walled, chlymydospore-like cells embedded in a cement-like substance (Garg et al., 2008). In peritonitis, the possible causes of the infection are environmental contamination such as soil contact. The causative agents of the most peritonitis cases were usually isolated from peritoneal fluid as well as the catheter and dialysis bag. *C. hawaiiensis* and *C. spicifera* strains have been reported to cause sinusitis infections (Krizsan et al., 2016). *Curvularia* has also been reported to cause series of deep infections that affect the central nervous system including cerebral abscesses, cerebral mycetoma, encephalitis, meningitis and meningoencephalitis. Some of the cases were disseminated which affect other organs like lungs, sinuses and lower limbs (Krizsan et al., 2016).

2.2.4 Lab Diagnosis and Treatment

2.2.4a Culture and microscopic examination

Laboratory diagnosis of *Curvularia* spp. is usually done by conventional methods which are by culture and microscopic examination. *Curvularia* often grow rapidly on routine culture plates at 28°C or 30°C for 5 days for the colonies to reach maturity. The common fungal media used in *Curvularia* culture are Sabouraud dextrose agar (SDA), Czapek Dox agar, brain heart infusion broth, blood agar and potato dextrose agar (Wilhelmus and Jones, 2001). The colonies display brownish-black to dark olive-green surface and dark-colored to black reverse. The filamentous and expanding colonies can be observed (Liu, 2011).

Microscopically, a Lactophenol Cotton Blue Preparation (LPCB) or other preparation will demonstrate conidiophores which are erect, straight to flexuous, septate, often geniculate and sometimes nodulose. Conidia present in ellipsoidal shape, often curved or lunate,

tapering at the base with rounded at the end. The conidia color can be observed in pale brown, medium reddish brown to dark brown. The septate usually present in 3 to 5 septa with smooth conidia wall to verrucose. Hilum protuberant also can be observe in some species (Kidd et al., 2016). Other than optical microscope, confocal microscopy and scanning electron microscopy also can be used to identify this melanized fungus. Other than LPCB slide examination, other staining preparation and histopathology examination can also be done. The most used techniques include 10% or 20% potassium hydroxide preparation, Gram, Giemsa and fluorescent calcofluor white stains, Fontana-Mansson, Gomori methenamine silver, hematoxylin and eosin (H&E) and Perodic acid-Schiff (PAS) staining. The staining used will highlight the particular features of interest for the clinical diagnosis.

Recent studies have shown that the morphological identification does not correlate with the molecular identification (Da Cunha et al., 2013). The morphology-based identification is often difficult and uncertain because of the high variability of the discriminative character of the isolate culture. *Curvularia* spp. and *Bipolaris* spp. are common saprophytes on the plant material and present in soil. Both develop dark colonies and pigmented multicelled spores. However, the spores are not routinely found on the culture or smear by direct examination. The differentiation only relies on microscopy examination of sporulating structure. The other key feature of the *Curvularia* spp. is the presence of true septate conidia while *Bipolaris* are compartmentalized by distoseptate (Alex et al., 2013).

However, some study have found there is no clear morphological boundary between these two genera of *Curvularia* and *Bipolaris* and some species show intermediate morphology (Manamgoda et al., 2012). This diagnostic dilemma of *Curvularia* identification can be resolved only by molecular identification.

2.2.4b Antifungal Treatment

The optimal antifungal therapy for *Curvularia* infection is not known but several reported cases showed infected patient response to treatment with different antifungal drugs such as amphotericin B, natamycin, azoles, miconazole, ketoconazole, terbinafine and itraconazole (Moody et al., 2012). In cases of fungal sinusitis, the treatment usually consists of surgery and administration of steroids, and itraconazole or amphotericin B. For allergic bronchopulmonary mycosis, patients were often treated with steroids and itraconazole. In ocular infection, the suggested treatment has been natamycin and azoles, such as itraconazole, fluconazole, posaconazole, or voriconazole (Revankar and Sutton, 2010). Other than that, patients with cutaneous infections have been treated successfully with voriconazole, itraconazole and ketoconazole. However, studies have also reported treatment failure with amphotericin B, voriconazole and flucytosine in central nervous system and disseminated infections (Carter and Boudreaux, 2004).

2.3 Molecular identification

2.3.1 ITS-PCR

Diagnostic tests using molecular markers enable rapid and accurate identification of fungi. It provides great assistance in differentiation of closely related species. PCR amplification and sequencing of the ITS of ribosomal RNA gene cluster is the common procedure in identification of ascomycetous species because ITS is able to resolve interspecific and sometimes intraspecific difference in fungi species (Krizsan et al., 2016). ITS region is located within ribosomal DNA (rDNA), superior to the 18S rRNA gene and it allows higher phylogenetic resolution regarding species identification of some genera (Wagner et al., 2018). Although the ITS-coding regions are not translated into functional protein, they possess critical role in the development of functional rRNA and due to the sequence variation of this regions among different species, this region provides potentials for the development of molecular biology-based assays (Kumar and Shukla, 2005). The PCR primers that gain wide acceptance for fungal ITS is "ITS1" and "ITS4" which amplify the highest variable of ITS1 and ITS2 sequence that surrounding 5.8S-coding sequence and located between the Small SubUnit-coding sequence (SSU) and Large SubUnit-coding sequence (LSU) of the ribosomal operon (Martin and Rygiewicz, 2005).

2.3.2 Other target genes

The other target marker genes used in previous studies are GPDH (glyceraldegyde 3-phosphate dehydrogenase), LSU (large subunit) and EFI-α (translation elongation factor 1-α). Study by Manamgoda *et al.* (2012) reported that a comparison of single and combine gene analyses of the generic complex show that the combine tree of ITS and GPDH best resolved for the phylogenetic and taxonomic re-evaluation of Group 1 (*Bipolaris*) and Group 2 (*Curvularia*) (Manamgoda et al., 2012). In cases of disseminated infections where the morphology identification failed to identify the causal agent, the isolate could be identified by LSU-PCR (Kobayashi et al., 2008).

CHAPTER THREE: MATERIALS AND METHODS

3.1 Material

3.1.1 *Curvularia* isolates

Curvularia spp. isolates were obtained from the Mycology Laboratory, Department of Medical Microbiology and Parasitology, School of Medical Sciences, Universiti Sains Malaysia. The isolates were subcultured onto Sabouraud dextrose agar (SDA) and incubated at 30°C until satisfactory pure growth were obtained.

3.1.2 Reagents and Chemicals

The reagents, chemicals and media used in this study are shown in Table 3.1

Table 3.1: List of reagents, chemical and media used in this study

Reagent	Manufacturer/Supplier
Undenatured absolute ethanol	Merck, Germany
Ethanol 70%	Merck, Germany
Ethylenediaminetetraacetic acid (EDTA)	Thermo Scientific, USA
Natrium chloride (NaCl)	Thermo Scientific, USA
Tris	Thermo Scientific, USA
2-mercaptoethanol	Sigma, USA
Phenol: chloroform: isoamyl alcohol	Sigma, USA
Isopropanol v:v:v 25:24:1	Thermo Scientific, USA
Agarose	1st BASE, Singapore
Sabaroud Dextrose Agar	Oxoid Ltd., UK
Cornmeal Agar	Oxoid Ltd., UK
DNA ladder	Thermo Scientific, USA
Primer	Intergrated DNA Technologies, Singapore
10x TBE Buffer	1st BASE, Singapore

Table 3.1, continued

Reagent	Manufacturer/Supplier
PCR water	Bioline Ltd., UK
FluoroSafe DNA stain	1st BASE, Singapore
MyTaq™ Red DNA Polymerase	Bioline Ltd., UK
5X MyTaq Red Reaction Buffer	Bioline Ltd., UK

3.1.3 Laboratory Equipment's and Consumables

The equipment and consumables used in this study were listed in Table 3.2 and Table 3.3.

Table 3.2: List of equipment used in this study

Laboratory Equipment	Manufacturer/Supplier
Bio-safety cabinet class ii	NuAire, Inc, USA
Centrifuge machine (5424)	Eppendorf, Germany
Centrifuge mini spin	Eppendorf, Germany
Freezer (-20°C)	Medfrez, China
Biomedical freezer (-80°C)	Panasonic, Taiwan
Dry block thermostat	BioSan, Europe
Vortex mixer	Labinet, USA
Electrophoresis system	NYXTECHNIK, USA
Gel image analyzer	G.Box, USA
Microwave oven	ELBA, Japan
Mastercycler PCR machine	Eppendorf, Germany
Pipette	Transferpette, Germany
Laminar air flow	NuAire, Inc, USA
Autoclave	Nerima-KU, Japan
Cryobox	Helix biotech, Malaysia
CO_2 incubator	Forma Scientific, USA
Bunsen burner	INTEGRA Biosciences, Switzerland
Analytical balance	Sartorius, Germany

Table 3.3: List of consumables used in this study

Consumables	Manufacturer/Supplier
Aluminum foil	Diamond, USA
Falcon tubes (15 ml)	Axygen, USA
Filter pipette tips (10 µL, 20 µL, 100 µL, 200 µL, 1000 µL)	Axygen, USA
Latex glove (medium)	ScienceValley, Malaysia
Microcentrifuge tubes (1.5 ml)	Axygen, USA
PCR tubes (0.2 ml)	Axygen, USA
Micropipette tips (10 µL, 20 µL, 100 µL, 200 µL, 1000 µL)	Axygen, USA
Duran bottles (100 ml, 500 ml)	Schott Duran, Germany
Sterile disposable Pasteur pipette	Fisher Scientific, UK

3.1.4 Culture media

3.1.4a Sabouraud Dextrose Agar (SDA)

SDA agar used for *Curvularia* culture in this study was ready-to-use media plates available in the laboratory. The SDA agar was supplied in lyophilized powder form and was prepared according to the manufacturer's instruction. The method for its preparation is included in Appendix 1

3.1.4b Cornmeal agar

Cornmeal agar used for preparation of slide culture and maintenance of fungal stock culture was ready-to-use plates available in the laboratory. Supplied from the manufacturer as lyophilized powder form, the Cornmeal agar was prepared according to the manufacturer's instruction. The detail of its preparation is included in Appendix 2.

3.1.5 Preparation for reagent for Polymerase Chain Reaction (PCR)

3.1.5a Primers

The primers used for this study is the fungus-specific universal primers ITS-1 (5'TCCGTAGGTGAACCTGCGG3') and ITS-4 (5'TCCTCCGCTTATTGATATGC3'). The primers were supplied in lyophilized powder form and were reconstituted prior of usage. The primers vials were spun for 3 minutes at 12,000 rpm to ensure the powder accumulated at the bottom of the tubes. The primers were dissolved and well-mixed in TE buffer provided by manufacturer (First BASE Sdn. Bhd., Malaysia) to prepare a stock concentration of 100μM each. The stock solutions were store at -20°C.

3.1.5b PCR reagents

The PCR reagent used in this study was master mix MyTaq™ Red DNA Polymerase (Bioline, UK), in accordance to the manufacturer's instruction. A total volume of 25 μL per reaction was prepared which consisted of 20 ng/μl of DNA template, 20 μM of each forward and reverse primers, PCR water, MyTaq™ Red DNA Polymerase and 5X MyTaq™ Red Buffer which includes 5mM of deoxynucleotide triphosphates (dNTPs), 15Mm of $MgCl_2$, stabilizers and enhancers. The concentration of each component has been

extensively optimized, reducing the need for further optimization. The volumes of each component were stated in Table 3.5 and the primer sequences used for the PCR were listed in Table 3.6

Table 3.4: Components of PCR Master Mix

Components	**One reaction (µL)**
PCR water	15.75
5X MyTaq™ Red Reaction Buffer	5
Forward primer (20 µM)	1
Reverse primer (20 µM)	1
MyTaq™ Red DNA Polymerase (5U/µL)	0.25
DNA template (20 ng/µL)	2
TOTAL VOLUME	**25**

Table 3.5: Primers used for PCR amplification for ITS1 and ITS4

Primer	**Primer sequences (5'-3')**	**Basepair**	**References**
F ITS1	TCCGTAGGTGAACCTGCGG	500	(Taira et al., 2014)
R ITS4	TCCTCCGCTTATTGATATG		

3.1.6 Preparation for agarose gel electrophoresis

3.1.6a Tris Borate EDTA (TBE) buffer (10X)

The buffer was commercially purchased (First BASE Pte. Ltd., Singapore) and stored at room temperature.

3.1.6b Tris Borate EDTA (TBE) buffer (0.5X)

The 0.5X working buffer was prepared based on the manufacturer's instruction. Fifty ml of 10X TBE buffer (First BASE Pte. Ltd., Singapore) was diluted with 950 ml of sterile distilled water. The buffer solution was mixed well and stored at room temperature.

3.1.6c 1.0% agarose gel

The 1% agarose gel was prepared by adding 1.0 g of agarose powder to 100 ml of 0.5X TBE buffer in 500 ml Duran bottle. The solution was mixed and heated using microwave oven for 3 to 5 minutes until the agarose gel was completely dissolved. The gel solution was cooled by swirling the bottle in a basin of water. Then, 2.5 µl of Florescence dye (FluoroSafe DNA stain, 1st BASE, Singapore) was added to the warm agarose solution and mixed well. Then, the molten agarose was poured into the gel tray in the casting chamber without producing bubbles in the gel. The gel was left to solidify for 30 minutes at room temperature.

3.2 Methods

3.2.1 Study Design and Sampling

This is a cross-sectional descriptive study. The actual sample size was not calculated and purposive sampling was done due to the limited number of *Curvularia* isolation. All non-repetitive *Curvularia* spp. isolated from all clinical specimens from the year 2001 to 2018 were included.

3.2.2 Flow Chart of the Study

The flow chart of the study is illustrated in Figure 3.1

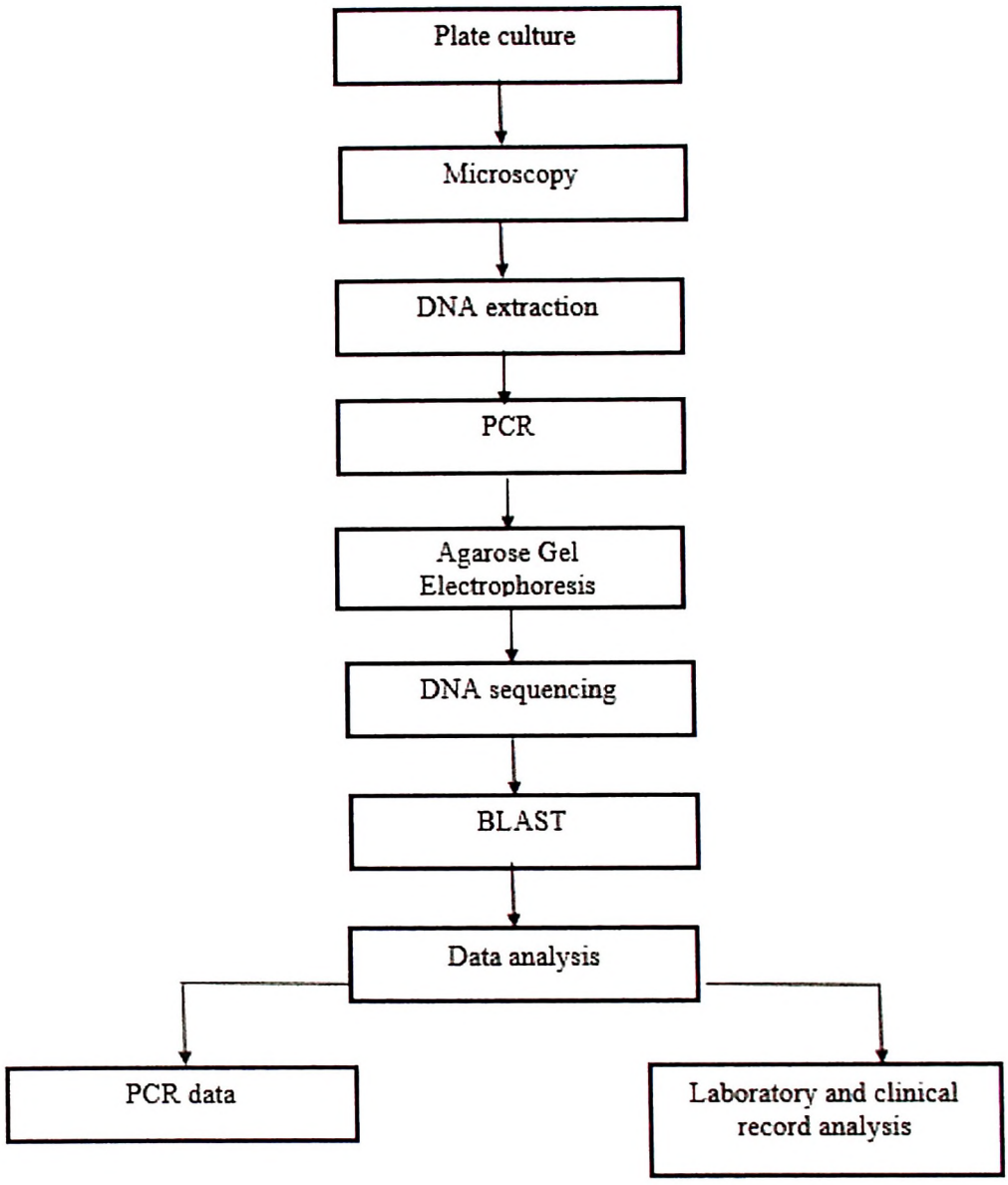

Figure 3.1: Overall flow chart of the study

3.2.3 Sterilization of Materials and Instruments

All glassware, 1.5 ml microcentrifuge tubes, 0.2 ml PCR tubes, 10 µl, 200 µl and 1000 µl unfiltered micropipette tips were autoclaved for sterilization at 121°C for 15 minutes. All these items were then dried in 50°C oven overnight before storage.

3.2.4 Isolation of *Curvularia* spp.

As the *Curvularia* spp. isolates were subculture from archived stock culture, it is important to make sure that the isolates are viable, with absence of contamination. Archived isolates were stored as water culture. Under aseptic technique, a fragment of fungal material was removed from the stock bottle and inoculated on Sabouraud dextrose agar (SDA). This first subculture was examined for fungal viability and presence of contamination.

3.2.5 Culture

3.2.5a Plate culture

Curvularia isolate was subcultured on SDA agar (Oxoid Ltd., UK) and incubated at 30°C for 72 hours. The colony morphology and purity of the culture were observed.

3.2.5b Slide culture

A pair of applicator stick was placed in a sterile Petri dish to serve as a support for a glass microscope slide. Blocks of cornmeal agar were cut into 1cm x 1cm in size and the agar block was transferred to the surface of the glass slide. Using a straight inoculating wire, the margins of the agar block was inoculated (stab) in four places with a small portion of the colony. A sterile coverslip was placed over the agar block. A piece of sterile gauze was

placed beside the glass slide in the Petri dish. A small amount of distilled water was poured to wet the gauze and then the culture was incubated at 30°C for 24 to 48 hours. Colony growth under the surface of the cover slip was observed. At the end of the incubation period, the cover slip was examined microscopically using LPCB preparation. The presence of characteristic morphology was observed to make a presumptive identification.

3.2.6 Microscopy

Microscopy examination for fungal identification was performed using scotch tape method followed by staining using lactophenol cotton blue (LPCB) preparation. The transparency tape method of preparing cultures for microscopic examination is often helpful because the spore arrangement of the more delicate filamentous mold is better preserved. The sticky side of unfrosted cellophane tape was pressed gently but firmly to the surface of the colony. A portion of the aerial mycelium was picked up. The prepared cellophane tape was placed on a drop of LPCB stain on a glass microscope slide. One end of the tape was stuck to the surface of the slide adjacent to the drop of stain. The tape was stretch over the stain, gently lowering it so that the mycelium becomes permeated with stain. The opposite end of the tape was stretched to the other end of the glass, avoiding as much as possible the trapping of air bubbles. The preparation was examined under microscopic and the morphology of the fungus was observed.

3.2.7 Molecular identification method

3.2.7a DNA Extraction

DNA extraction was performed using phenol:chloroform extraction method. The isolates were subcultured on SDA plates and incubated at 30°C for 48 hours. One loopful of *Curvularia* colonies were placed in 1.5ml microcentrifuge tube and stored in freezer (-20°C) overnight. A volume of 500 µL lysis buffer and 5µL β-mercaptoethanol were added to frozen young colonies and incubated at 65°C on block heater for one hour. Then, 500µL phenol chloroform: isoamyl alcohol (v:v:v: 25:24:1) (Sigma, USA) was added and mixed thoroughly for 2 minutes, then centrifuged at 10000 rpm for 10 minutes (Figure 2). The upper aqueous layer was transferred into a new 1.5 ml microcentrifuge tube. An equal amount of isopropanol was added before incubating at -20°C for at least two hours or overnight to improve the DNA yield. Upon completing incubation, the tube was centrifuged at 10000 rpm for 10 minutes at 4°C. The resulting supernatant was discarded and 500 µL ethanol 70% was added to the pellet. The tube was centrifuged again at 10000rpm for 10 minutes and this step was repeated twice. Supernatants were discarded, and the pellet was dried for at least two hours before reconstitution with 100 µL sterile water.