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MOLECULAR IDENTIFICATION OF FUNGAL SPECIES ASSOCIATED WITH MYCETOMA IN UGANDA

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DECLARATION

I **Luggya Tonny** hereby declare that this dissertation is original, developed by me, and has never been submitted or presented to any University /Institution. The work of others has been cited and referenced.

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DEDICATION

This piece of work is dedicated to my wonderful family notably my wife and children, my father Mr. Livingstone Kibuuka Sserunkuma and mother Madam Jesca Mulinde Namutebi, supervisors Dr. Beatrice Achan, Associate Professor David Bisagaya Meya, Associate Professor David Patrick Kateete, and Dr. Francis Ocheng with appreciation for all their academic and financial support that made this work a success, and above all greater thanks be given to the almighty God for all the ability and divine help.

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ABBREVIATIONS AND ACRONYMS

ATCC	American Type Culture Collection
Bp	Base pair
CAP	College of American Pathologists
DNA	Deoxyribonucleic Acid
FFPE	Formalin-Fixed, Paraffin- Embedded
Fw	Forward
H & E	Haematoxylin and Eosin
ITS	Internal Transcribed Spacer
KOH	Potassium Hydroxide
LPCB	Lacto Phenol Cotton Blue
MALDI-TOF MS	Matrix assisted laser desorption ionization-time of flight mass spectrometry
Ml	Millilitres
NTD	Neglected Tropical Disease
PCR	Polymerase Chain Reaction
PAS	Periodic Acid Schiff
Rev	Reverse
Spp	Species
Th	T helper
WHO	World Health Organization
µL	Microliters
rRNA	ribosomal RNA

OPERATIONS

Mycetoma: This is a chronic subcutaneous infectious condition with triad characteristics of forming tumor like-swellings, draining sinuses and grain formation.

Eumycetoma: This is a subcutaneous condition caused by more than 40 different fungal species

Actinomycetoma: This is a chronic subcutaneous infection caused by aerobic branching *actinomycetes*.

Internal transcribed spacer (ITS): This is a piece of non-functional DNA sequences located between structural ribosomal RNAs (rRNA) of a common precursor transcript, which is especially useful for elucidating relationships among congeneric species and closely related genera. ITS is not translated into proteins, but the coding regions have a critical role in the development of functional rRNA, with sequence variations among species, making, making them signature regions for molecular assays.

ABSTRACT

Background: Designated by the World Health Organization (WHO) as a neglected tropical disease (NTD), mycetoma is a chronic subcutaneous fungal and bacterial disease which causes profound morbidity. Mycetoma is mostly not a notifiable disease and thus, there is limited information on the global burden of the causative fungi and bacteria species. Because mycetoma is caused by either fungal or bacterial pathogens, its diagnosis is still a challenge particularly in developing countries such as Uganda where most methods are phenotypic. Therefore, this study was aimed to characterize the genotypes of fungal organisms associated with mycetoma.

Specific objectives: We aimed to determine: (i) The phenotypic identification of fungal and bacterial organisms associated with mycetoma in Uganda, (ii) The molecular identification of fungal species associated with mycetoma in Uganda, and (iii) The description of characteristics exhibited by mycetoma patients in Uganda **Methods:** This was a cross sectional study which was conducted using 14 archived biopsy samples which were originally diagnosed using histological Haematoxylin and Eosin, and positive mycetoma cultures. We used PCR, and aimed at fungal DNA targets such as: ITS[1] and ITS[2] fungal interspacer regions, *M. mycetomatis1*, *M. mycetomatis* 2, *S. boydii*, and *M. pseudomycetomatis* to determine the precise fungal species and strains. Microbiology assays, including, Twort Gram, Lacto Phenol Cotton Blue, 10% KOH-Calcoflour white with addition of a special stain for fungi (PAS) were performed, data on host risk factors and demographic information was collected using the study tool and the clinical notes from the medical records were captured and data was analyzed.

Results & their impact:

Using phenotypic stains, we demonstrated that of the n=14 biopsy samples, n=12 (85.7%) had fungal organisms and 2 (14.3%) were positive for bacterial organisms. PCR results were positive, using the ITA 1 target for biopsy numbers (UCIBS 731,MB 241 and B6546423). In the description of the characteritics exhibited by mycetoma positive patients in Uganda ,we included the age-were by mycetoma was detected in biopsies of participants of 18years and above compared to below 18 years at a frequency of n=11(78.6%) and n=3(21.4%), for sex-the results of mycetoma in these participants was more in males than in females at a ratio of n=9(64.3%) and n= 5(35.71%),for the tribe results showed that mycetoma was in Baganda at a frequency of n=4(28.6) for these participants,and the body part most affected by mycetoma was the leg at n=5(35.7%) frequency.

1.

CHAPTER ONE: INTRODUCTION

1.1 Background

Mycetoma is a subcutaneous chronic infection, designated by the World Health Organization (WHO) as a neglected tropical disease (NTD) (1). It is a granulomatous, localized, persistent, suppurative, and deforming disease (2). It causes profound morbidity (3) and is normally characterized by tumefaction, multiple draining sinuses, and grain formation (4).Mycetoma commonly occurs in low-income populations (5).

Madura foot was the name given to mycetoma infection (6). The First report on mycetoma was published at the turn of the eighteenth century(7) and one of the oldest descriptions ever noted dates to the ancient Indian Sanskrit text *Atharva Veda*(2). In 1842, the first clinical case of mycetoma was documented by Gill however, the overall number of cases globally as noted by Gokhale (1981) still remains uncertain. In the early 1950s, Abbott mapped the initial number of mycetoma cases. He studied 1,321 mycetoma cases in Sudan within a 2.5-year period and published in 1956(8) suggesting that the mycetoma burden was higher than previously estimated (9). Congo, Somalia, Argentina and Mexico(9), are known as the mycetoma belt, located between 15°S and 30°N (10). Several studies have shown the prevalence of mycetoma in certain regions or countries, but no published data exists on the estimated global prevalence of mycetoma (9). Sporadic cases have been reported in countries with temperate climate (11) and with a predominance among the rural regions of Africa, Latin America, and Asia (12).

Sudan has the highest burden of mycetoma in the World and Mexico is the second area of high endemicity. The predominant climate of the "*mycetoma belt*" is the subtropical or dry tropical with an annual average rainfall of about 500–1000 mm and temperatures range from 10–20°C to 20–40°C, respectively (12). Most cases occur in men aged 11-40 years, and diagnosis is often made up to a decade after initial inoculation (6).

In prior surveys, many different genera and species, both true fungi and bacteria cause mycetoma. Ahmed *et al* (8) reported 48 species (both fungal and bacterial) as causative pathogens. Some of these pathogens are considered common while others only rare.

In Uganda, no clear definition is given for which genera and species are the commonest pathogens of mycetoma and which are rarely implicated. The distribution of the mycetoma causative pathogens may not be the same around the globe (8). Actinomycetoma caused by bacteria is more commonly found in Middle and South-America whilst Eumycetoma caused by fungi is more commonly found in

Africa(8). Aerobic species of *Actinomycetes* that belong to genera *Streptomyces, Nocardia and Actinomadura* are known for causation of actinomycetoma with *Nocardia brasiliensis, Actinomadura madurae, Actinomadura pelletieri* and *Streptomyces somaliensis* being most common. Eumycetoma is caused by *Madurella mycetomati, Scedosporium boydii, Scedosporium apiospermum, Pseudallescheria boydii, Falciformispora senegalensis, Leptosphaeria senegalensis, Trematosphaeria grisea, Madurella grisea, Acremonium falciforme, Cephalosporium falciforme, Aspergillus fumigatus ,Exophiala jeanselmei , Geotrichum candidum, Neotestudina rosatii , Medicopsis romeroi or Biatriospora mackinnonii, Pyrenocha spp,Aspergillus flavus, Microsporum audouinii, Cochliobolus lunatus, Curvularia lunata, Rhinocladiella atrovirens, Aspergillus nidulans, Neoscytalidium dimidiatum, Fusarium spp, Cladosporium spp, Exophiala spp* and unidentified fungi (6).

Molecular techniques are used to identify the causative pathogens, with identification of the internal transcribed spacer located between the 18S and 28S genes for fungi. ITS regions are amplified, sequenced and identification is based on comparing the resulting sequence with sequences present in Gen Bank (13). Identification of *Actinomycetes*, the 16S rDNA region is analyzed (14). However, molecular identification is rarely used in endemic regions and thus the total number of the genera and species that cause mycetoma is un-known (8).

1.2 Problem Statement

As elsewhere in sub-Saharan Africa (5), there is a paucity of data on what precise genotype(s) of the fungal species are exactly associated with mycetoma in Uganda (6). Thus, this study aimed to bridge this knowledge gap by molecular identification of the fungal species associated with mycetoma, using histopathology and microbiology confirmed mycetoma positive biopsies from the period of 2010-2023.

Van de Sande *et al*, indicated how most mycetoma cases are in Mexico, Sudan,Senegal and India, with few reports from Uganda, Romania, Nigeria, Bulgaria and Thailand. Less than 20% of African countries so far have published mycetoma cases, yet it is known to be a significant health challenge in the tropical and sub-tropical regions (6). Determining the precise species of mycetoma associated fungal organisms, was beneficial in improving the management of the condition and preventing disfigurement. Culturing the grains was the gold standard for fungal species identification. However, the slow growth rate makes it time-consuming, tedious, fraught with contamination, and therefore requires expert microbiologists to identify the causative agents. Patients on medical treatment had non-viable grains, and hence it was difficult to identify the causative organism. Histopathology techniques could differentiate between actinomycetoma and eumycetoma but could not identify the fungal organisms to the species level. Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI- TOF MS) was a rapid technique for identification but it is costly and only available in a few reference centers such as the Clinical Microbiology Laboratory, Makerere University. Modalities such as histology, cytology, skin test, and serology lack specificity. With these aforementioned gaps therefore, molecular methods have tremendously improved mycetoma diagnostic capabilities and should be made more readily available in Uganda (15). Rapid and inexpensive species-specific PCR analyses can be used to identify new species.

1.3 Study Objectives

1.3.1 General Objective

To identify using molecular PCR fungal species associated with mycetoma in Uganda.

1.3.2 Specific Objectives

1) To phenotypically identify fungal and bacterial organisms associated with mycetoma in Uganda.
2) To identify using molecular PCR fungal species associated with mycetoma in Uganda
3) To describe the characteristics exhibited by study participants with mycetoma in Uganda

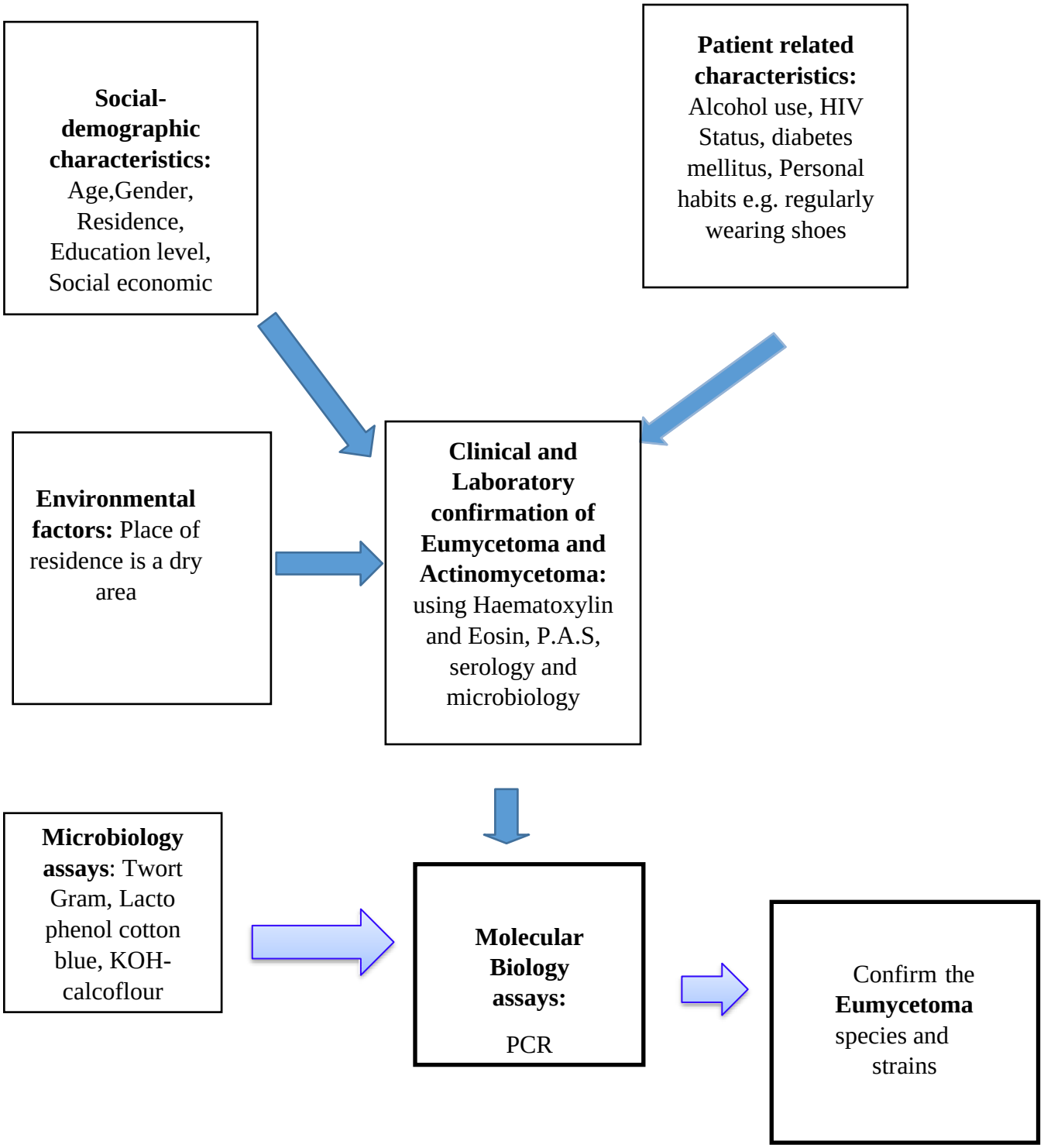
1.4 Research Question

1) Which of the fungal species is the most predominantly associated with mycetoma in Uganda and what characteristics are posed by individuals found of having a diagnosis of mycetoma?

1.5) Significance of the Study

Molecular identification of the fungal species associated with mycetoma in Uganda provided essential data which might contribute to policy on establishing routine laboratory diagnosis, management, and prevention of mycetoma in Uganda.

Figure 1: Conceptual framework



CHAPTER TWO: LITERATURE REVIEW

2.0 Definition

Mycetoma is a chronic infectious disease, which may have characteristics of forming tumor like-swellings, draining sinuses and the formation of grains. Mycetoma may begin with a minor trauma on the foot, which introduces the causative pathogen into the subcutaneous tissue (8). It is a devastating disease characterized by massive tissue involvement, which often leads to destruction, deformities, and disability (16).

Within the infected tissue, the causative agent will organize itself into small granules which may also be referred to as grains. Microbial granule, called "mycetoma" or "sclerotia," can be produced inside the host. It is very often spherical or ellipsoidal in shape, with a slow-growth of bacterial cells / fungal mycelium in the infected body site. These granules are most likely produced because of microbial growth in dense tissue (17). A small nodule may arise and gradually grow into a large subcutaneous mass with sinuses which will secrete pus and grains. Eventually, the bone may be invaded and a large mutilating lesion may be formed (8). Fungal pathogens form black or white grains, whilst bacterial pathogens have a more diverse color pallet ranging from red and yellow to white (18).

2.1 The mycetoma burden

The mycetoma global burden is unknown however, a rough estimation by performing a meta-analysis on the published literature from 1955 onwards (8) shows that only 50 papers could be found. A total number of 8,763 mycetoma cases have been documented since 1944. The globally reported number of cases is approximately 127 cases/year. The 8,763 mycetoma cases were reported from 23 different countries. Some countries only have data on 11 cases (for example Uganda), while others have data on 2,607 cases (Mexico) (8).

2.2 Geographical areas for mycetoma

Mycetoma is common between 15° and 30° south of the equator (19). Countries like India, Yemen, Saudi Arabia, Mexico, Venezuela, Argentina, Colombia, and Brazil are part of the mycetoma belt region. Sudan, Nigeria, Mauritania, Ethiopia, Chad, Kenya, Djibouti, Cameroon, Somalia, Tunisia, Niger, and Senegal are among the African countries where cases have been reported (9). Laos, Singapore, Malaysia, the Philippines, Indonesia, Cambodia, Thailand, and Vietnam have similarly reported cases. Mycetoma is occasionally reported in temperate European nations including Germany, Albania, Bulgaria, Greece, Italy, and Turkey and among African migrants in France (20). In the subtropical regions of the south-western United States, sporadic cases have been observed (20).

2.3 Groups Affected by mycetoma

No age is exempt from infections, but the majority of patients are young adults between 15–30 years (1) however, there are also reports of incidence in children and the elderly (18) with male predominance in most countries. Married individuals have a higher prevalence when compared to single individuals (21).

2.4 Prevalence and incidence of mycetoma in Uganda

The record of biopsy reports at the Pathology Reference Laboratory, Makerere University, Kampala, Uganda indicate that all throughout the 70-year period, 30 cases were identified, with 249 additional cases identified via review of biopsy reports (total of 279 cases). The prevalence of 8.32/100,000 persons per decade suggests an average incidence of 0.32/100,000 persons (6).

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2.5 Distribution of causative pathogens of mycetoma around the globe and in Uganda

Eumycetoma has been reported more in Africa, actinomycetoma more often in South and Central America (5). A study conducted at the Calcutta School of Tropical Medicine, India suggested a total of 197 cases wereactinomycetomas and 67 were eumycetomas (22). The distribution of causal pathogens was: *brasiliensis* (*n*=75), *N.caviae* (now called *N.otitidiscaviarum*) (*n*=38), *N.asteroides* (*n*=52), *Streptomyces* spp.(*n*=9), *Actinomadura* spp.(*n*=23), *Madurella grisea* (*n*=35), *M.mycetomatis* (*n*=8), *Pseudallescheria boydii* (*n*=5), *Acremonium* spp. (*n*=6), *Pyrenochaeta romeroi* (*n*=6), *Exophiala jeanselmei* (*n*=1) and unidentified eumycetoma agents (*n*=7) (23).

For another study in Venezuela,the most frequent pathogens were eumycetoma with a *eumycetoma*-to actinomycetoma ratio of 6:3. Subsequently, discrepancies with molecular identification were found with morphological identification for some strains. Case 1 was identified as *M. mycetomais* based on microscopic features. However, ITS and D1/D2 analysis matched case 1 to *Madurella pseudomycetomais*. Case 2, phenotypically identified as an *E. jeanselmei*, was reclassified as *Cyphellophora oxyspora* on the basis of molecular analysis.

Case 3, *Exophiala spp* were matched to *Exophiala oligosperma*. Case 8 previously classified as *N. asteroides*, was tagged as *N. wallacei* after analysis of the 16S rDNA region. In addition, Case 9, identified as *Nocardia sp*., was matched to *N. farcinica.* (14)**.** Data from France showed that 23 cases of eumycetoma were due to *Fusarium species*. The patients'origins were the tropics, Israel, and Italy or remained unspecified (24).

In West Africa, records showed that eumycetoma tends to be more predominant compared to

actinomycetoma. For actinomycetoma, *A. madurae, A. pelletieri* and *S. somaliensis* are the most prevalent and also *Actinomadura pelletieri* is very common more so in West African countries, except in Niger, where *S. somaliensis* was pointed out as the most isolated species. *Nocardia spp*. are rarely found in West African countries. From about 978 *eumycetoma* cases for the etiology determination, just about 669 were caused by *Madurella mycetomatis* (25). The second most predominant fungal causative *pathogen Falciformispora senegalensis*, was identified in 237 of cases (25). Fungi were the most common causative agents, up to 89% followed by *Nocardia* species (5%) and *Actinomycetes* (4%) in the study by Kwizera and colleagues in Uganda (6).

2.6 Pathogenesis of mycetoma

Antibodies against the pathogens are found in a number of individuals but only few develop the disease and this may be attributable to a complex interplay of factors between the host and pathogen (2). Among the host factors: The organism is usually innoculated after a penetrating injury. The high incidence in tropical regions may be due to decreased use of protective clothing. Some predisposing condition may be found such as poor general health, diabetes, and malnutrition, and this may lead to a more invasive and widespread infection (2).

Complement-dependent chemotaxis of polymorph nuclear leukocytes is induced by both fungal and actinomycotic antigens *in vitro*. Cells of the innate immune system attempt to engulf and inactivate these organisms. About the immune responses, three different types have been described in response to the grains of mycetoma. Neutrophil degranulation and adherence to the grain surface, leading to gradual disintegration of the grain. Outside the zone of neutrophils is a zone of granulation tissue containing macrophages, lymphocytes, and plasma cells, disappearance of neutrophils and arrival of macrophages to clear the grains and neutrophil debris, marked by the formation of epithelioid cell granulomas. T-cell responses also seem to play an important part in mycetoma progress (2). Th2-like responses (interleukin (IL)-4 and IL-10) were found in primary lesions and in draining lymph nodes in infections with *S. somaliensis*, but may also follow stimulation of peripheral blood mononuclear cells by *M. mycetomatis* antigens (2). Th1 responses present in the acute phase of disease and in healthy endemic controls.

Humoral antibodies also have a role in pathogenesis; in immunocompetent BALB/c mice, IgM antibodies induced specific protection in experimental *N. brasiliensis* infection. Men may be due to progesterone inhibiting the growth of organisms (2). Amongthe factors related to pathogen, certain

species are more commonly found in the immunocompetent individuals such as *N. brasiliensis*, whereas others such as *Nocardia farcinica*, *Nocardia nova*, and *Nocardia cyriacigeorgica* mostly affect immunosuppressed individuals, and this may be due to ability of *N. brasiliensis* to survive the first-line innate immune response by phagocytes (2). The persistence after an initial inoculation appears to be related to its ability to evade host defenses through a variety of adaptations such as cell wall thickening and melanin production, which protect microorganisms against ultraviolet radiation and destruction by alveolar macrophages, enzymatic lysis, and oxidants and might protect against antifungal drugs (2). The incubation period is unknown, disease symptoms present months to years after traumatic inoculation, depending on the inoculum size, strain virulence, and the host immune response (12).

2.7 Mode of transmission

The soil is thought to be the principal reservoir for mycetoma causative pathogens (26). Among the *Actinomycetes, A. madurae, A. pelletieri, N. asteroides*, *N. brasiliensis, S. somalienis,* and amongthe fungi, *Falciformispora senegalensis* (previously known as *Leptosphaeria senegalensis*), *M. mycetomatis, Neotestudina rosatii* and *Scedosporium boydii* have been cultured from soil (26). Recent attempts to grow the fungus *M. mycetomatis* from soil have not been sucessful, although the nucleic acid material of the organism has been detected in 17 out of 74 soil samples and also one out of 22 thorn samples. There is clear evidence basing on phylogenetic analyses that *M. mycetomatis* may well be related to dung-inhabiting fungi, suggesting that its primary reservoir could overlap with the natural niche of these fungi (27). Another Mycetoma-causative agent, *N. asteroides*, has been isolated from cow dungin India (26).

More studies are needed to establish the environmental niche of the mycetomacausative pathogens and their mode of transmission to patients. The causative pathogen must somehow enter the subcutaneous tissue in order to cause mycetoma (28). This has been linked to milddamage produced by thorn pricks, stones, or snake and insect bites. Research has focused on the grain's components, but the formation itself is not fully understood. Discovery of thorn fragments inside mycetoma patients' lesions has confirmed the role of thorns, especially because thorns are plentifulin mycetoma-endemic areas.

2.8 Immunity against mycetoma

The immunological response of the human host to mycetoma-causative pathogens has been studied (9). The significance of neutrophils in the early defense against mycetoma has been proven by research that found substantial numbers of neutrophils in the mycetoma lesion, innate immune responses are a

significant factor in mycetoma (9). Macrophages and monocytes appear at the site of infection when activated by cytokines, interferon-γ, and tumor necrosis factor-α, these cells enhance their microbicidal activity (29). Neutrophil adhesion and degranulation results in grain breakdown; macrophage replacement to ingest grain and neutrophil debris; and epithelioid granuloma development are the three host tissue reactions to mycetoma. Immunity in mycetoma is dependent on cell-mediated immunity, with T cells playing a key role (9).

Protective immunity from mycetoma is provided by T helper (Th) type 1 lymphocyte responses, but disease progression is linked to the Th2 immune response (9). Innate and adaptive immunity may play a role in host resistance to *N. brasiliensis* infection in murine models and humans (30). Further evidence suggesting a genetic predisposition to mycetoma includes familial clustering of mycetoma patients (9).

2.9The clinical features of mycetoma

The triad including a painless subcutaneous swelling, multiple draining sinuses and discharge that presents with grains is very typical manifestation of mycetoma (1). The swelling may appear firm and rounded, but at other times may be soft,lobulated and, rarely, cystic and it is often mobile. Multiple secondary nodules then evolve, the nodules may or may not suppurate and drain through multiple sinus tracts, and these sinuses may close transiently after discharge during the active phase of the mycetoma (1). Fresh adjacent sinuses may open while some of the old ones may heal completely. The discharge normally presents as serous, serosanguinous or purulent.

The sinuses discharge grains, consisting of the microorganisms which are encapsulated in a melanin (cement-like) and other materials. The grains are thought to play a protective role for these microorganisms. The grain color is dependent on the organism responsible. The grains can be black, yellow, white or red and are of varying sizes and consistency. In several mycetoma cases, the grains are visible to the naked eye; the exception being on actinomycetoma, which is caused by *Nocardia* species, in which the grains are too small and only visible under the microscope (1). Black grains are also commonly due to infections by *M. mycetomatis* and other related organisms , the red ones are caused by *A. pelletierii*, the yellow are caused by *Streptomyces somaliensis* and the white grains may be caused by *A. madurae*. However, black fungi may generate grains of pale color (1).

2.10 Predisposing factors to mycetoma infections

The susceptibility can be due to environmental, genetic or immunogenic factors (16). Children and adolescents are the common affected group reported in some studies, previous reports showed farmers and workers are affected most (16). The fact that mycetoma is common in this age group, and children are commonly in contact with the soil during playing or helping their families in farming activities,

this may explain why. The soil can harbor the mycetoma causative agents and they are more prone to minor injuries which enhances inoculation of mycetoma pathogens (16).

2.11 Specimen collection

The discharging fluid or scrapings of sinus walls, and tissue biopsy should be investigated for the presence of grains. Saline dressings as used overnight on the swelling or aspiration of grains directly from an unopened sinus tract can as well be useful (31). Evaluation of spontaneously extruded grains may not necessarily allow diagnosis because these grains may be composed of non-variable organisms except for molecular assay (2). The extracted grains are investigated in different ways to rule out the diagnosis: direct clinical examination, microscopy, culture and PCR(2).

2.12.0 Clinical and direct diagnosis of mycetoma

The clinical triad of a subcutaneous mass, sinuses, and granular discharge, when seen in a patient from an endemic area, is diagnostic (32). The differential diagnosis of actinomycetoma may include sporotrichosis, tuberculosis, osteomyelitis, coccidiomycosis, phaeohyphomycosis, botryomycosis, other fungal infections, and neoplasias of the bone and soft tissues (29).

Further diagnosis of the causative pathogen can be made by microscopic observation of a grain. Various serological, cultural and molecular assays have also found place in diagnosis. Direct diagnosis involves evaluating the variation in size, color, and consistency of the grains, which can be helpful in rapid but provisional identification of the etiological pathogen (2).

2.12.1 Direct microscopy

A Gram stained preparation is of considerable value in distinguishing between actinomycetomaand eumycetoma(2). Granules can be ground on the slides and stained, most notably lacto phenol cotton blue, which would allow differentiation between the thin actinomycetoma filaments and the thicker hyphae of eumycetoma.

2.12.2 Histology

It is usually needed when the drainage material cannot be obtained, and in these cases, ideally a deep punch biopsy should be taken to include the subcutaneous tissue. Hematoxylin and eosin (Hand E) stain shows suppurative granulomas (composed of neutrophils), surrounding characteristic grains which are present in the subcutaneous tissue (2). Grains are aggregates of septate and branched, radially arranged broad hyphae, sometimes with vacuole formation. They are seen as broad, pink- stained

hyphae surrounded by a sharp basophilic strand (2).

The neutrophilic infiltrate is, in turn, surrounded by palisading histiocytes, beyond which also is mixed inflammatory infiltrate which comprise of, eosinophils, lymphocytes, plasma cells and macrophages. For some cases, fibrosis may also be appreciated in the outermost layer. In eumycetomas apart from Haematoxylins and Eosin, periodic acid–Schiff and Grocott–Gomori staining may be performed for finer details. When an actinomycetoma is suspected, an additional Gram staining should be performed (33).

2.12.3 Culture

Grains of many species have overlapping morphological features, and therefore, culture in special media such as Sabouraud dextrose agar, or Kimmig's agar is capable of providing an accurate identification of the causative pathogens (2). Cultivation of the grains or from biopsy sample can be performed, and incubation at 37°C is sufficient to isolates the pathogens because they are from human and probably will be cultured at 37°C. Fungal cultures should be kept for a longer time, approximately 4–6 weeks, in order not to lose out on the slow growing fungi. *M. mycetomatis*, an important causative pathogen of eumycetomas, grows very slowly. First, growth of colonies is usually not seen before10–15 days of cultivating (25). Cultivation of *Actinomycetes* requires special media and a longer incubation time (10 days). Recommended culture media for Actinomycetes are Lowenstein Jensen media, thioglycollate broth, Columbia agar, and brain heart infusion agar.Incubation time should be roughly 48–72 hours and longer at 35°C–37°C (2).

2.12.4 Serology

Serological tests can prove useful in the early stages of the disease, even before granule formation (2). However, several serological assays have been used, including immunoblots, indirect hemagglutination assays, immunodiffusion, counterimmunoelectrophoresis, and ELISA (2). However, ELISA appears to be a sensitive test for the detection of circulating antibodies. Serological diagnosis has been used in few studies for *N. brasiliensis*, *M. mycetomatis*, and *P. boydii*; however, their sensitivity and specificity are low and may be positive in healthy endemic controls.

2.12.5 Imaging techniques

Radiology and ultrasonography will enable the prognosis and bony involvement if any (2).
Standard X-ray studies can reveal bony involvement such as periosteal erosion secondary to invasion, osteoporosis, and changes consistent with osteomyelitis, osteolysis, and osteosclerosis. However, it can pick up only fairly advanced disease. Ultrasonography successfully differentiates the mycetoma from osteomyelitis or tumor (2). Subtle differences between actinomycetoma and eumycetoma can

also be picked up. The use of helical computerized tomography has recently been shown to provide detailed assessments of soft tissue and visceral involvement and appears to be more sensitive for detecting early changes. Magnetic resonance imaging provides the most comprehensive method for assessment of the bone and soft tissue involvement and may also be useful in evaluating the differential diagnosis of the swelling. A "dot-in-circle sign" has been described as a potentially specific diagnostic finding seen with MRI. The dots are tiny hypo intensefoci (believed to be grains) within spherical, high-intensity lesions (the circle) surrounded by low-intensity matrix on T2-weighted imaging, which represent granulomas scattered in areas of fibrosis. T1-weighted, fat-saturated, post gadolinium images may also produce this appearance (2).

2.12.6 Molecular Laboratory diagnosis

PCR is done directly on the biopsy specimen, and sequencing of gene regions, for example, internal transcribed spacer 1 (ITS1), ITS2 is usually sufficient in most isolated fungi. In distinct cases, to identify the mold species, however it will be necessary to use other gene regions as a multilocus sequence analysis, for example, the large subunit, small subunit 18S nrDNA, β-tubulin, and chitin synthase 1 regions (2). For filamentous fungi identification, internal transcribed spacer and DI/D2 domain of the 28S ribosomal DNA (rDNA) are evaluated. Due to the large number of bacterial causative pathogens implied, there are no species-specific PCRs for all causative pathogens (34). However, in these cases there are PCRs developed to allow identification to the genus level. A pan-*Nocardia* PCR, a Pan-*Streptomyces* PCR and a pan-*Actinomadura* PCRs are available. These PCRs are based on the genus conserved regions of the 16S rRNA gene. For the pan-*Nocardia* primers, this results in a 590 bp PCR product. Although more than 15 different bacteria can cause actinomycetoma, only *Nocardia farcinica* species-specific PCRs have been developed. DNA from mycetoma cultures (fungi and *Actinomycetes*) can be extracted. The extracted DNA is amplified by PCR with different primer sets. Amplicons can be purified and subjected to sequencing in a Genetic analyzer and compared to Gen Bank sequences (35).

2.12.7 Treatment and prognosis

The treatment of choice will depend mainly from distinguishing between eumycetoma and actinomycetoma. Surgical debridement or antibiotics or antifungal use are the cornerstones of mycetoma treatment (36). However, recurrence rate is high, probably due to poor compliance and poor response to the drugs. Generally, the prognosis of actinomycetoma is better than eumycetoma (37).

CHAPTER THREE: METHODOLOGY

3.1 Study Design

This study was a cross sectional study. The study utilized archived biopsies originally diagnosed by either histology or microbiology and reported as mycetoma positive from the year 2010-2023.

3.2 Study Site and Setting

This study was analyzing mycetoma positive biopsies archived at the Pathology Department, College of Health Sciences, Makerere University. The Pathology Department was founded in 1937with the goal of providing high-quality medical education, research, and diagnostic services, Basic sciences, histology, autopsy pathology, cancer epidemiology, and clinical research are all part of the department's interdisciplinary and multi-institutional research. It provided diagnostic pathology services to Mulago National Referral Hospital (6). The specimens included biopsies that contained grains and reported to be mycetoma positive by either Haematoxylins and Eosin, Periodic Acid Schiff (special stain) or microbiology. Biopsies were washed several times in sterile physiological saline and maintained at −20°C until use (35). Another facility from which specimens were collected is the Uganda Cancer Institute Pathology Laboratory.

The Department of Immunology and Molecular Biology is located on the 3rd floor pathology/Microbiology Building, Old Mulago Hill Road. The Department operated under the School of Biomedical Sciences, Makerere University College of Health Science-Kampala, Uganda. The Genomics and Molecular Laboratory was started in 2003, and most of the departmental basic and applied research and training was being done in this laboratory. The Genomics, Molecular Laboratory is equipped with PCR equipment, Nanodrops, Vortex mixers, thermomixers, Water baths, Freezers, Refrigerators, Hybridization oven, gel electrophoresis machine, trans eluminator among other equipment. Genotyping of the mycetoma fungal pathogens was performed at this facility.

The clinical Microbiology Laboratory/ Department of Medical Microbiology is located on Mulago hill, on the 2nd floor of the Pathology & Microbiology building. The Department of Medical Microbiology has one of the oldest teaching Clinical Microbiology Laboratory in Uganda and the laboratory is CAP accredited. The laboratory was equipped with light microscopes, florescent microscopes, staining area, stains and culture facility. The phenotypic identification was done at this Laboratory.

3.3 Study Population

The study's accessible population comprised of archived biopsies, which were reported to be mycetoma positive using Haematoxylin and Eosin, P.A.S or microbiology.

3.4 Sampling Strategy

Sample Size Calculation

The sample size for this study was determined using the Kish Leslie formula (38).

$$N = \frac{Z\alpha^2 P (1-P)}{D^2}$$

Where N = sample size required, Zα = Standard normal value corresponding to the set level of confidence =95%=1.96, P = was the estimated prevalence in Sudan by a previous study conducted by Wendy W. J. van deSande *et al* 2014 (13) = 1.81 per 100,000 inhabitants.

D = was the total width of confidence interval (95%) =0.05.Therefore,

$$N = \frac{1.96^2 \text{ X}1.81/100{,}000\,(1-1.81/100{,}000)}{0.05^2}$$

N =0.027812

N=0.027812*1000=27.812/ 1000 people.

Therefore, N=28/1000 people.

3.5 Sampling Technique

This study used a simple random sampling technique.

3.6 Eligibility Criteria

3.6.1Inclusion Criteria

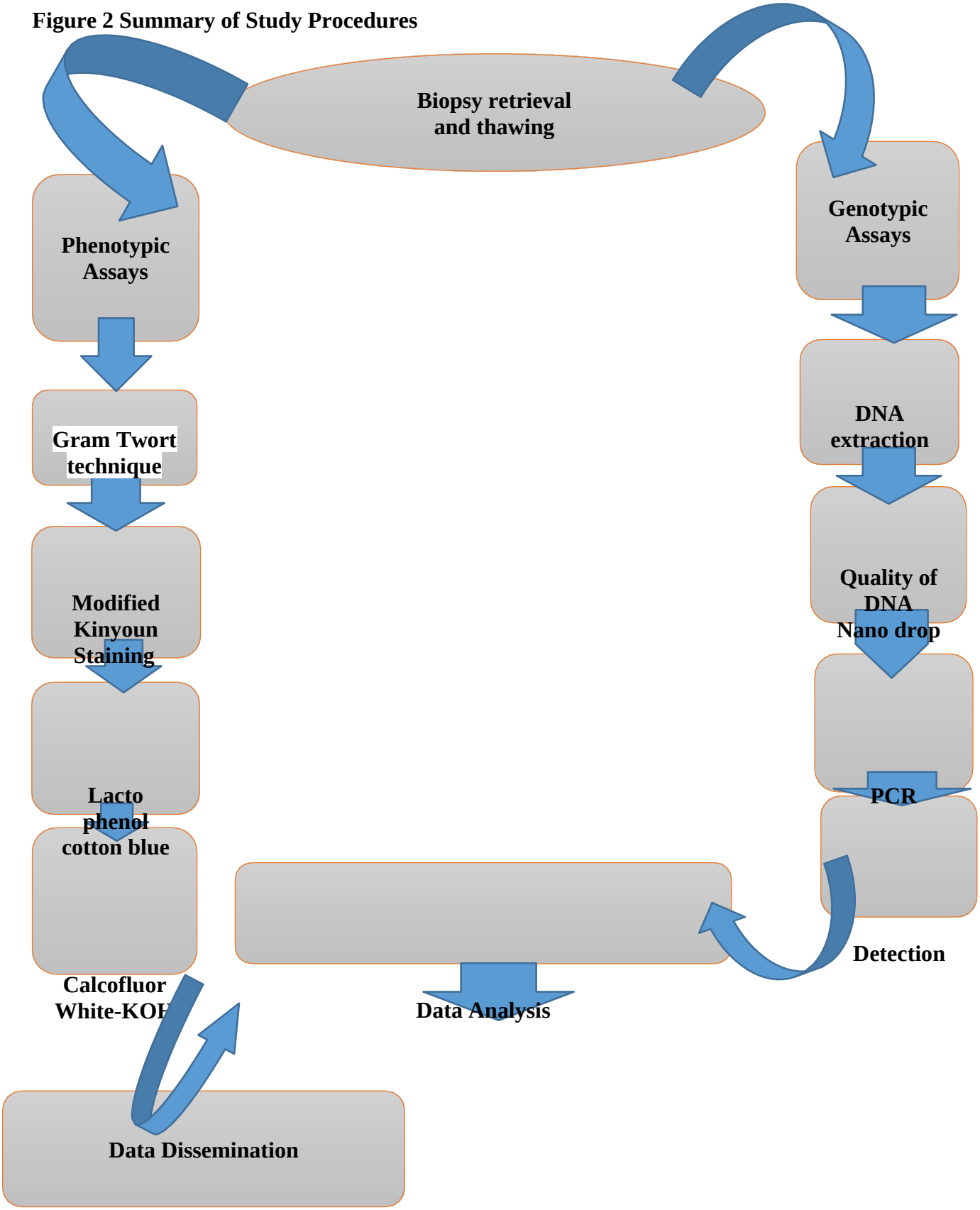
Well labeled, archived biopsies originally reported to be mycetoma positive by either Histopathology (Haematoxylin and Eosin, P.A.S) or Microbiology and having the accompanying patient's demographic data and other relevant information were included.

3.6.2 Exclusion Criteria

Biopsies with just clinical diagnosis and no Histopathology (Haematoxylin and Eosin, PAS), or Microbiology mycetoma positive report.

Biopsies whose patients' accompanying demographic or relevant data as was required by the study was missing or had erased or damaged labels were excluded.

Figure 2 Summary of Study Procedures



3.7 Laboratory Methods

Phenotypic assays were performed to identify the mycetoma associated organisms. Twort-Gram modification was used and bacterial causative organisms were identified as Gram-positive, while fungal causative organisms were Gram-positive and larger in size. The *Actinomycetes* consisted of fine, branching filaments, about 1 micron thick, whereas the *Eumycetes* grains composed of septate hyphae 4–5 microns thick. Modified Kinyoun Acid Fast staining technique was superior in discriminating between actinomycetoma organisms that is *Nocardia spp*. which were positive,whereas *A. madurae* and *S. somaliensis* were negative (13). Lacto phenol cotton blue wet mounts and Calcofluor white –KOH preparations were performed. Lacto Phenol Cotton Blue staining was done to look for fungi and 10% potassium hydroxide (KOH) technique mixed with calcoflor white was performed to look out for fungal hyphae and elements.

3.7.1 The Gram –Twort modification technique for bacteria in sections

This modification was used because it can be differentiated easily with better color contrast. Preparation: Modified Tworts stain- Stock solution of 0.2 % absolute alcoholic neutral red and 0.2 % absolute alcoholic fast green. For use, diluted 1 volume of stock solution with 3 volumes of distilled water. **Procedure:** Stained in 1% aniline- crystals violet (4 minutes), washed in distilled water, treated with Grams iodine (3 minutes), washed in distilled water and blotted dry, decolorized with 50% acetone –alcohol untilno more color was coming away (5 seconds), Washed, counter stained with the modified Tworts stain (5 minutes), decolorized in 2% acetic acid in absolute alcohol until no more red color came away. **Results:** Gram positive bacteria were dark blue, Gram negative bacteria were pink, Nuclei-red, Cytoplasm-Light green, Red blood cells -green (39).

3.7.2 Modified Kinyoun Acid-Fast Staining

The fungus like bacteria *Nocardia spp* was partially acid-fast and appeared red against a blue back ground (13). Reagents- Preparation-Kinyouns Carbolfuchsin: Basic fushsin 4g,95% ethanol 20ml, distilled water 100ml Dissolved the basic fuchsin in ethanol, carefully added phenol and water. Mixed well.50% ethanol: Concentrated ethyl alcohol 50 ml, distilled water 50ml, Mixed the aboveingredients together.1% sulfuric Acid-Concentrated sulfuric acid 1ml, distilled water 99ml. Carefully added acid to water and mixed well, Methylene blue- 2.5g,95% ethanol – 100ml, Dissolved methylene blue in alcohol and mixed well.

Procedure: Made smears, flooded the slides with Kinyouns carbolfuchsin, Stained (5 minutes), washed, flooded the slides with 50%ethanol until all excess dye was removed, then washed, decolorized

with 1% sulfuric acid (3 minutes), washed, counter stained with methylene blue for 1 minute, washed and allowed the slides to air dry (40). **Results:** *Nocardia* stained red , other bacteria and fungus blue (41).

3.1.1 Lacto phenol cotton blue (LPCB) wet mount

Placed a portion of biopsy on a slide, and added one drop of LPCB stain, mixed well and added a cover slip, observed under the microscope for fungal elements (42).

3.1.2 Calcofluor White-KOH Preparation

Mixed 1 drop of calcofluor white and 1 drop 10% KOH on a clean microscope slide, mixed the biopsy with solution, added a coverslip, and examined under the ultraviolet microscope for fungal elements, which showed fluorescence (43).

3.1.1 Molecular Biology Methods

25 μm thick section was obtained from each paraffin block, included blocks with the mycetoma negative controls. The negative and positive controls were distributed among the tissue blocks. Deparaffinization (44) was done by placing the FFPE tissue into 1.5 mL cryal vail and exposed them to 1200 μL xylene for 20 minutes and to 1200 μL 100% ethanol for 30 minutes which was done under gentle constant agitation. Following each 10-minute step, the supernatant was discarded after centrifugation at 13 000 ×g for 10 minutes. The tissue was then air dried (45).

3.1.1.1 Extraction procedure using the QIAamp DNA FFPE advanced kit

Following the manufactures procedure, each FFPE section was placed in a 1.5 mL micro centrifuge tube, then 300 µL deparaffinization solution, vortexed vigorously for 10 seconds, and centrifuged briefly to bring the sample to the bottom of the tube. Incubated at 56 ^{o} C for 3 minutes, allowed to cool to room temperature, added 25 µL buffer FTB,55 µL RNase-free water, and 20 µL Proteinase, centrifuged to spin down any FFPE tissue that was stuck to the tube wall or under the cap of the tube after vortexing, incubated for 1 hour at 56 ^{o} C and 1000 rpm, incubated again for 1 hour at 90 ^{o}C, discarded the upper blue phase, and kept the lower aqueous lysate, proceeded by adding 150 µL RNase free water, added 2 µL RNase A, and incubated for 2 minutes at room temperature, added 20 uL Proteinase K, and incubated for 15 minutes at 65 ^{o} C and 450 rpm, added uL buffer AL and 250 µL ethanol 96% to each sample and mixed thoroughly by vortexing, transfered 450 µL lysate to the minElute column and centrifuged at 15000 X g for 30 seconds, transfered the residual lysate to the same minElute column, centrifuged at 15000 X g for 1 minute. Discarded the flow-through, added 500 µL buffer AW1 and centrifuged at 15000 X g for 30 seconds, discarded the flow-through , added 500 µL buffer AW2, and centrifuged at 15000 X g for 30 seconds, added 250 µL ethanol 96% to the spin

column, and centrifuged at 15000 X g for 30 seconds, then placed the spin column into a new 2mL collection tube and centrifuged for 3 minutes at full speed which removed any residual liquid , having placed the MinElute column into a clean 1.5 mL micro centrifuge tube, opened the lid of the Min Elute column and applied 50 µL buffer ATE to the center of the membraned , and closed the lid incubated at room temperature for 1 minute, then, centrifuged at full speed for 1 minute to elute the DNA(46). DNA quantification was performed using Nano drop 1, which quantified eluted DNA and determined its purity at 260/280nm absorbance. DNA was stored at -20°C for short term (35).

To genotype the fungal species associated with mycetoma, the study mainly based on the internal transcribed spacer (16). Identification of eumycetoma pathogens up to the species and strain level was by amplification of ITS since ITS sequence is a universal DNA barcode marker for fungi. Amplification was done with sets of fungal primers on the conserved regions of the 18S, 5.8S and 28S ribosomal genes. The length(size) of the product was detected and this was dependent on the fungal species and strain associated with eumycetoma infections for the specific study biopsy specimen. This was used for the species and strain identification. Another target that was used in this study for the identification of fungal species and strains associated with eumycetoma infection included the SSU gene (21) *Madurella species*, based on the ITS region, PCR generated a product of 370 bp in size with *M. mycetomatis, M. pseudomycetomatis, M. fahalii* and M. tropicana and no amplification was obtained with *T.grisea, N. mackinnonii, M. romeroi, Pyrenochaeta unguis-homini*, and *Chaetosphaeronema larense.* This study used species-specific PCRs for *M. mycetomatis, S. boydii. M. pseudomycetomatis*. Two sets of primers were designed, sets a and b which generated PCR products of 490 bp and 370 bp, respectively. With primer set a, a 490 bp product was obtained with *M. mycetomatis* and a 325 bp product was generated with *M. pseudomycetomatis*. No product was obtained for *M. fahallis* and *M. tropicana*. The internal transcribed spacer regions with species-dependent variable fragment lengths(sizes) was recorded on an excel sheet.

Figure 3: Fungal species and strain specific primers for the genotyping of the fungal mycetoma associated organisms

Species	Target	Forward(Fw) primer(5'-3')	Reverse(Rev) primer(5'-3')	Annealing Temp	Size(bp)
M.myecetoma [1]	ITS	AATGAGTTGGGCTTTAACGG	TCCCGGTAGTGTAGTGTCCCT	55^0C	490
M.myecetoma [2]	ITS	GCAACACGCCCTGGGCGA	TCCGCGGGGCGTCCGCCGGA	55^0C	370
S.boydii	SSU	GAGGCAATAACAGGTCTGTGATGC	TTACTACGCAGAAGGCAA	52^0C	800
M.pseudomycetomatis		GCGTGAAGAGTCTGCTGTTG	TAGCCTGAATCCCACAAACC		325
ITS [1]		TCCGTAGGTGAACCTGCGG	GCTGCGTTCTTCATCGATGC		
ITS [2]		GCATCGATGAAGAACGCAGC	TCCTCCGCTTATTGATATGC		

KEY: [1] and [2] refers to *M.myecetoma* Strain one and strain two respective(15)

Study PCR protocols were adapted to the cycler which was used prior to the analysis. Respective adaptations were cycler specific and comprised of reaction mixes, MgCl2 contents, and cycling protocols (45).

3.7 Data Management

Quality Control of data

The data was collected on to the laboratory data collection sheets, reviewed for completeness and consistency, was then double entered and cleaned before using STATA version 15 for analysis.

3.8 Ethical Considerations

Ethical approval for this study was obtained from the School of Biomedical Sciences, College of Health Sciences, Makerere University, Research and Ethics committee with reference application SBS-2023-293S.

The study obtained a waiver of consent to use the Biopsy specimens from the confirmed mycetoma positive cases. Confidentiality was maintained at all times and the research dissertation met the key research ethical principles namely;

Value: the research aimed at enhancing health and/or knowledge. Scientific validity: the research was methodologically sound,

Favorable risk-benefit ratio: risks to the research participants were minimal,

Informed consent: Approval for a waiver of consent to use the patient's specimens for research was obtained from the ethics committee.

Respect for enrolled subjects: Participants privacy was protected and no names was entered in the data collection sheet but rather numbers were assigned. Study files and databases were only accessible to the principal investigator and the Makerere University supervisors. The data was availed to the Department on request.

CHAPTER FOUR: RESULTS

This study determined the molecular identification of fungal species associated with mycetoma, phenotypic identification of fungal and bacterial organisms associated with mycetoma and the description of the characteritics exhibited by mycetoma positive participants from the year 2010-2023. Mycetoma positive biopsies (n=14) confirmed using histology and microbiology techniques 'were retrieved from the Pathology Department, College of Health Sciences, Makerere University and the Uganda Cancer Institute-Pathology Laboratory, they were processed and analyzed phenotypically to identify bacteria and fungi, and genotypically identified the genotypes of four fungal species which were associated with mycetoma.

4.0 Phenotypic identification of the fungal and bacterial organisms associated with mycetoma in Uganda

Phenotypic identification of the fungal and bacterial organisms associated with mycetoma in Uganda was performed using histological and microbiological stains that is Haematoxylin and Eosin, Periodic Acid Schiff (special stain), Twort Gram, Lacto phenol cotton blue, Modified Kinyoun Acid Fast stain and 10% Potassium Hydroxide with Calcofluor white respectively.

Haematoxylin and Eosin results.

All the n=14 biopsies had features histologically suggestive of mycetoma, and some, recommendation was made to perform a special stain (PAS) in order to ascertain the surety of the presence of fungi. Periodic Acid Schiff was performed on the study biopsies.

Periodic Acid Schiff results.

For the n=14 study biopies, n=6 (42.9%) were PAS positive and n=8 (57.1%) exhibited PAS negative results.

Twort Gram results

The n=14 biopsies, n=12 (85.7%) were positive for fungal hyphae, fungal spores, and Splendore Hoepli reaction, with all having inflammatory cells infiltrates and the n=2 (14.3%) having bacterial organisms with inflammatory cells.

The Kinyoun Acid Fast stain

n= 2 (14.3%) of the n=14 study biopsies, had Acid Fast bacteria and the remaining n=12 (85.7%) had no acid-fast bacteria.

10% Potassium Hydroxide and Calcofluor white,

Biopsies were positive at a frequency of n=12 (85.7%) for fungal cells and n=2 (14.3%) were negative.

Figure 4: Showing the Identification of fungal and bacterial organisms associated with mycetoma in Uganda

STUDY NO	BIOPSY	H&E AND MICRO	Periodic acid schiff	Twort Gram	LPCB	Kinyoun acid fast stain	Calcoflour white stain	Deduction
1	BM2408	Mild inflammation,fibromascular tissue	Negative	2+ fungal hyphae,pseudohyphae,> 45inflammatory cells see	Fungal hyphae	no acid fast organism	2+hyphae	Fungal organism
2	B610	Granulomatous inflammation(mycetoma)	Negative	2+ fungal hyphae,>45 inlammatory cells	Arthoconidia	no acid fast organism	2+hyphae	Fungal organism
3	U457	Statis dermatitis	Negative	Hyphae and fungal spores	Fungal hyphae	no acid fast organism	2+hyphae	Fungal organism
4	B731	Skin fungal infection	Positive	Hyphae and fungal spores	Fungal hyphae	no acid fast organism	2+hyphae	Fungal organism
5	MB 241	Chronic non-specific inflammation-Maduramycosis	Positive	Fungal elements	Fungal hyphae	no acid fast organism	2+hyphae	Fungal organism
6	MB 580	Granulomatous inflammation-aseptate hyphae	Negative	2+septate hyphae	Fungal hyphae	no acid fast organism	2+ aseptate hyphae	Fungal organism
7	MB 3173	Chronic inflammation with Actinomycosis organisms	Negative	2+ filamentous bacteria and >45 Inflammatory cells	Hyphae	acid fast organism present	Hyphae	Bacterial organism
8	MB 2293	Mycetoma	Positive	2+ hyphae and >45 Inflammatory cells	Hyphae	no acid fast organism	Fungal hyphae	Fungal organism
9	MC 251	Madurella mycetomatis-acute inflammation and granulama	Positive	2+ septate hyphae,sypriondore Hoeppli,> 45 inflammatory	Fungal hyphae	no acid fast organism	2+hyphae	Fungal organism
10	U 3258	Fungal infection	Positive	2+ fungal hyphae	Hyphae	no acid fast organism	2+hyphae	Fungal organism
11	U1108	Mucormycosis	Negative	None branching hyphae	Hyphae	no acid fast organism	2+hyphae	Fungal organism
12	B6546423	Aspergillus infection-Mycetoma	Positive	3+ long septate hyphae seen	Hyphae	no acid fast organism	2+hyphae	Fungal organism
13	6522 L	No bacterial or fungal growth	Negative	Filamentous bacteria seen	Hyphae	acid fast organism present	Hyphae	Bacterial organism
14	3477L	Trichophyton interdigitale and Aspergillus terreus	Negative	2+ fungal hyphae	Hyphae	no acid fast organism	2+hyphae	Fungal organism
15	Positive control	ATCC 10231 *C.albicans*	Positive	3+ pseudohy,budding yeasts	Yeast cells	3+ AFB	2+ hyphae	
16	Negative control		Negative	no microorganism seen	No organisms	no acid fast organism	No organism seen	

4.2 The molecular identification of the fungal species associated with mycetoma in Uganda

The polymerase chain reaction results were generated after extracting the FFPE biopsies using the QIAamp DNA FFPE advanced kit (46), DNA quantification was performed using a Nano drop 1, and on average the Nano drop 1 result showed DNA quantification for all the 14 biopsies was averagely approximated to have been 16.26 ng/µL. Having optimized the PCR, the conventional PCR was run mainly focusing on ITS and SSU. Six primers were used that is *M. mycetomatis* [1], *M. mycetomatis*[2], ITS [1], ITS [2], *S. boydii* and *M. pseudomycetomatis* respectively. The 14 study biopsies plus the positive and negative controls were exhaustively analyzed for the presence of the above-mentioned specific targets. The PCR results are displayed in the table below and in a narrative: n=3 (21.4%) biopsies were PCR positive with ITS[1] primers (particularly Biopsy numbers, UCIBS 731,MB 241 and B6546423) and the PCR results were negative for primers ITS[2], *M. mycetomatis*[1].*M. mycetomatis*[2] and *S. boydi* , *M. pseudomycetomatis*, and thus the interpretation is 3 PCR positive results with ITS [1] meant that there was presence of fungal DNA targets, which were detected using ITS[1] primers in the 3 PCR positive biopsies and this is because ITS[1] primers are known universal fungal barcode (47), but from the same biopsies when analyzed with ITS[2], *M.mycetomatis* [1], *Mmycetomatis* [2] , *S.boydi, M.pseudomycetomatis,* the PCR was negative because the specific DNA target was not present.

Figure 5: Showing the quantification of nucleic acid in the 14 study biopsies

STUDY NO.	Sample Name	Nucleic Acid(ng/uL)	A260/A280	A260/A230
1	BM 2408	9.563	4.622	0.058
2	UCIB610	9.87	5.589	0.058
3	UCI 457	18.3	2.422	0.101
4	UCIB731	67.028	1.176	0.296
5	MB 241	8.181	4.363	0.051
6	MB 580	14.974	2.755	0.089
7	MB 3173	10.133	4.611	0.057
8	MB 2293	12.818	3.281	0.075
9	MC 251	13.541	2.854	0.084
10	UCI 3258	11.71	3.735	0.066
11	UCIB 1108	8.131	4.743	0.051
12	B6546423	15.71	3.008	0.093
13	6522 L	17.666	2.747	0.09
14	3477 L	10.049	4.622	0.059

Figure 6: Showing the genotypic identification of fungal species that are associated with mycetoma in Uganda using Polymerase Chain Reaction.

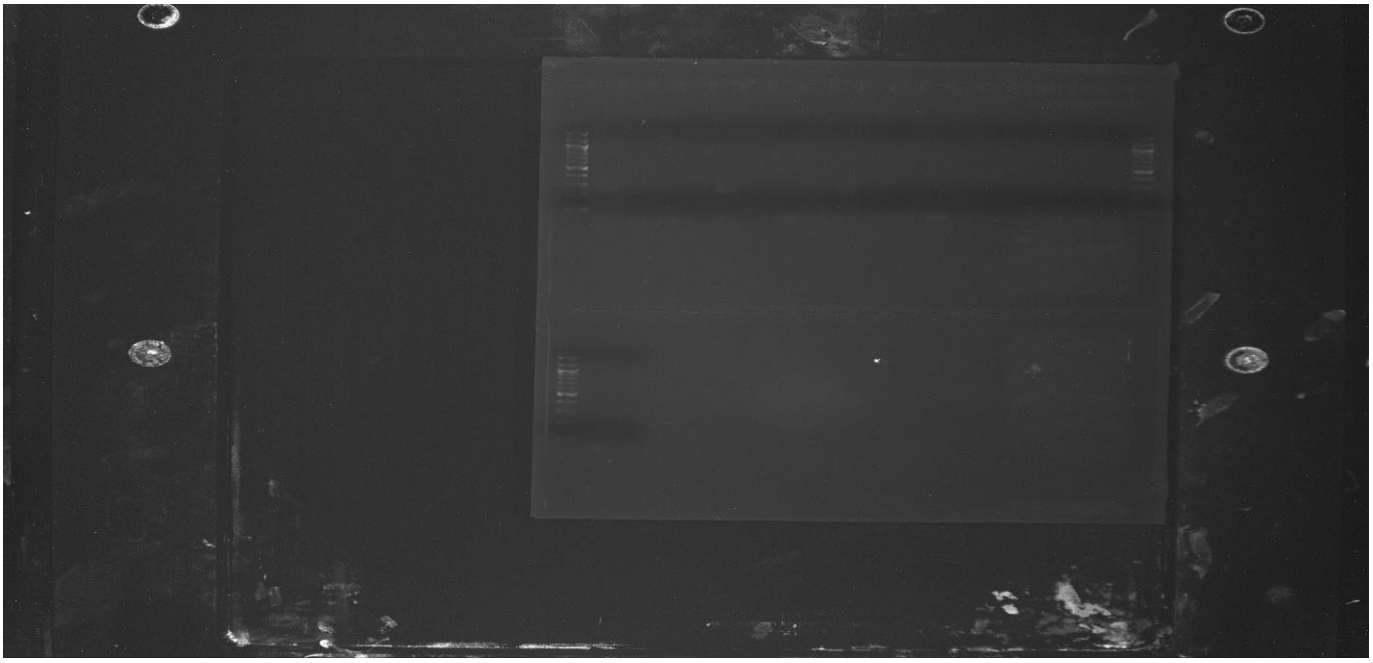
Patient Identification	***M.mycetomatis*[1]**	***M.mycetomatis*[2]**	***S. boydii***	***M.pseudomycetomatis***	**ITS[1]**	**ITS[2]**
BM2408	-	-	-	-	-	-
UCIB610	-	-	-	-	-	-
UCI457	-	-	-	-	-	-
UCIB731	-	-	-	-	+	-
MB241	-	-	-	-	+	-
MB580	-	-	-	-	-	-
MB3173	-	-	-	-	-	-
MB2293	-	-	-	-	-	-
MC251	-	-	-	-	-	-
UCI3258	-	-	-	-	-	-
UCIB1108	-	-	-	-	-	-
AK	-	-	-	-	+	-
6522L	-	-	-	-	-	-
3477L	-	-	-	-	-	-
Positive control					+	+
Negative control					–	–

KEY

- \- Negative PCR result
- \+ Positive PCR result

Figure 7: showing Lane 1 and 20 contains a 100bp ladder

1 2 3 4 5 6 7 8 910 11 12 13 14 15 16 17 18 19 20



For the lanes 2,6 and 12 shows PCR product for 3 study biopsies which were PCR positive for ITS [1]. Lanes 3,4,5,7,8,9,10,11,13,14,15,16,17,18,19 shows the PCR products for15 study biopsies which PCR negative for ITS [1]

4.3 The description of the characteristics exhibited by study participants who had mycetoma in Uganda

The baseline characteristics of the n=14(100.0%) individuals whose biopsies were included in this study showed that mycetoma associated organisms were detected more in males than in females at a frequency of n=9 (64.3%) and n=5 (35.71%) respectively and out of the total percentage of the gender 2 (14.3%) cases who were female had bacterial organisms and the remaining n=12 (85.7%) who had fungal organisms n = 9 (75.0%) were males and n=3 (25.0%) were females.

The mycetoma associated organisms were detected more in individuals who were 18 years and above compare to individuals below 18 years at a frequency of n=11 (78.6%) and n=3 (21.4%) respectively. Of the total number, those who had causal bacterial organisms, were 18 years and above and the remaining cases that had fungal a causal organism, n=9 (75.0%) were 18 years and above and n=3 (25.0%) below the age of 18 years.

The health care facilities in which the biopsy samples were received from included hospitals, private health facilities and this was at a frequency of n=13 (92.9%) and n=1 (7.1%) respectively. On analysis of the results further, biopsies with bacterial causal organisms revealed that the n=2 (14.3%) were obtained from patients in hospital while the remaining n=12 (85.7%) with fungal causal organisms,n=11 (91.7%) were received from hospital and n=1 (8.3%) had been received from a medical center.

The marital status of individuals who were diagnosed with mycetoma was as the proceeding frequencies, n=1 (7.1%) individual was married, n=2 (14.0) individuals were single and n=11 (78.6%) individuals never had marital status documented.

The individuals among the mycetoma positive cases who wore shoes presented at the following frequencies, n=2 (14.3%) individuals wore shoes and n=12 (85.7%) had no record of wearing shoes and all the cases that were positive for bacterial causal organisms had insufficient documentation about wearing of shoes, at a frequency of n=2(14.3%) the individuals who were found to have fungal causal organisms wore shoes, the other n=10(83.3%) were lacking documentation about the wearing of shoes and only n=2(16.7%) were recorded to have been wearing shoes.

All the n=14 study biopsies were collected from the black race, and the results showed that for both cases of bacterial causal organisms and fungal causal organisms, only the black race was affected.

The place of residence reflected on the individual's records showed that at a frequency of n= 4 (28.6%) mycetoma cases were from rural areas, n=5(35.7%) were discovered from urban places and n=5 (35.7%) cases had no clear documentation about their places of residence. For individuals who were positive for bacterial causal organisms that is n=2(14.3%), 1 individual originated from a rural area in Uganda and the other individual, the place of residence was not documented as per say, and following up on individuals who came out fungal causal organisms ,n=4 (33.3%) came from urban areas in Uganda ,n=2(16.7%) individuals originated from rural parts of Uganda and 6 (50.0%) individual had no clear documentation of their residence.

About the tribes, in which mycetoma positive individuals belonged, the frequencies were as stipulated: Muganda at a frequency of n=4 (28.6), then Munyankole, Musoga, Mufumbira, Kumum, Alur, and Langi at a frequency of n=1 (8.3%) for all respectively and n=4 (28.5%) had no clear documentation of tribal record. Of the individuals with bacterial etiology, n=1(50.0%) were Baganda while of the individuals with fungal etiology, n=1 (8.3%) individual each was a Munyankole, Musoga, Mufumbira, Kumum, Alur, Langi, and n=4 (33.3%) were Baganda and n=3(25.0%) individuals had no clear documentation of tribe.

Apart from the individuals who presented with the site of mycetoma infection to be the leg at a frequency of n=5 (35.7%), the undocumented sites of mycetoma in this study were at a frequency of n=3 (21.43%), then the other body parts particularly the eye, lower limb, knee joint, forearm, oropharynx, the head, neck and back, all presented at a frequency of n=1 (7.14%) each respectively. The individuals who had bacterial etiology presented with a frequency of n=1 (50%) when having the leg as site of mycetoma and other individuals at n=1 (50%) who had no clear documentation of the site of mycetoma. For the results of individuals who had fungal causal organisms, n=4(33.3%) of the individuals had the leg as the site of eumycetoma, the frequency of the site in conditions of eumycetoma being the eye, fore arm, oropharynx, head, neck and back was 1(8.3%) for each respectively and the frequency of the eumycetoma affected individuals whose site of condition was not documented stood at n=2 (16.7%).

Results for the clinical history revealed that most mycetoma positive individuals had swellings, lesions, pus and grains and of which all these are typical characteristics of the condition and this well coincided with the findings by Vineet *et al* (2) of which Over 75% of patients had a lesion of lower extremity, most commonly the foot (70%) followed by hand involvement. Other sites included the head, neck, chest, shoulder, and arms.

The results for the clinical history of the n=14 (100.0%) are detailed in the table below.

Figure 8: Showing the clinical History of the study participants

STUDY ID	BIOPSY ID	Clinical history
1	BM2408	Swollen leg
2	UCIB610	Loss of right eye
3	UCI 457	non tender fungating+pus
4	UCIB 731	ulcerated mass lesions
5	MB241	Swoolen and woody
6	MB 580	Swollen left forearm
7	MB3173	Synovioma
8	MB2293	Multiple lessions
9	MC 251	
10	UCI 3258	Sysphyic
11	UCIB 1108	Spontaneous cellulitis
12	B6546423	Swelling
13	6522L	
14	3477L	chronic ulcer simplex

Figure 9: Showing risk factors associated with mycetoma

Variable	N=14	%
Sex		
Male	9	64.29
Female	5	35.71
Marital status		
Married	1	7.14
Not married	2	14
Unknown	11	78.57
Wearing shoes		
Yes	2	14.3
No	12	85.7
Hospital reported		
Kadic hospital	1	8.33
Jinja hospital	1	8.33
Angal hospital	1	8.33
General Military	1	8.33
Ultra care med.services	1	8.33
Mulago National Referal Hospital	5	41.67
Race		
Black	14	100
Residence		
Rural	4	28.6
Urban	5	35.7
Not provided	5	35.7
Tribe of the individual		
Alur	1	7.14
Kumam	1	7.14
Mufumbira	1	7.14
Musoga	1	7.14
Langi	1	7.14
Muganda	3	21.43
Munyankole	3	21.43
Not provided	3	21.43
Age		
Below 18	3	21.4
Above 18	11	78.6
Body part affected		
Leg	1	7.14
Eye	1	7.14
Lower limb	1	7.14
Knee joint	1	7.14
Left fore arm	1	7.14
Left foot	2	14.29
Foot	2	14.29
Oropharynx	1	7.14
Head,neck & back	1	7.14
Not provided	3	21.43

Figure 10: The table below shows a description of the mycetoma positive patients.

Patients ID	Gender	Age	Wearing shoes	Marital status	Time to diagnosis	Health facility	Place of residence	Comorbidities	Tribe
BM2408	M	38	Not provided	Unknown	2 years	Mulago National Referral Hospital	Mulago	Not documented	Munyankole
UCIB610	F	21	Not provided	Unknown	1.5 years	Mulago National Referral Hospital	Kamwokya (Kampala)	not documented	Mufumbira
UCI 457	M	68	Not provided	Unknown	-	General Military	Kawempe (Kampala)	no known illness	Muganda
UCIB 731	F	12	Not provided	not married	Long standing	Mulago National Referral Hospital	-	not documented	Muganda
MB241	M	17	Not provided	Unknown	1.2 years	Kadic Hospital	Katabi (Wakiso)	not documented	Kumam
MB 580	F	8	Not provided	not married	0.5 years	Jinja Hospital	Nawampiti (Kaliro)	Loss of weight and appetite	Musoga
MB3173	F	24	Not provided	Unknown	-	Kumi	Mubende (Mubende)	not documented	Muganda
MB2293	M	36	Not provided	Unknown	2 years	Angal Hospital	Kaseta Kaboya	not documented	Alur
MC 251	M	21	Not provided	Unknown	-	Mulago National Referral Hospital	-	not documented	-
UCI 3258	M	31	Not provided	Unknown	-	-	-	not documented	-
UCIB 1108	M	71	Wears boots	Married	4 years	Mulago National Referral Hospital	Mubende	Hypertensive	Munyankole
B6546423	M	24	yes	Unknown	3 years	Mulago National Referral Hospital	Luzira Prison	H.I.V-negative	-
6522L	F	22	Not provided	Unknown	-	-	-	not documented	-
3477L	M	50	Not provided	Unknown	20 years	Ultracare medical services	-	?Atypical cutaneous mycobacterial disease	Munyankole

CHAPTER FIVE: DISCUSSION, CONCLUSION AND RECOMMENDATION

5.0 Discussion

5.1 Discussion for the phenotypic identification of the fungal and bacterial organisms associated with mycetoma in Uganda

Our results showed that fungi are the most common etiologic agents of mycetoma, and therefore most of the cases are eumycetoma.

Our results are more less similar to a study conducted by Kwizera *et al* in Uganda (6), in which fungi were the most common causative organisms at a frequency of (88.8%) of mycetoma, followed by bacteria. However, our findings showed that out of the n=14 mycetoma-positive biopsies, n=12 (85.7%) had fungal causal organisms and n=2 (14.3%) were of bacterial etiology.

Wendy. W.J.Van desande *et a l*(8), had suggested that the distribution of the mycetoma causative organisms is not equal around the globe such that for overall, bacterial causative organisms were more commonly found in Middle and South-America whilst fungal causal organisms were more commonly found in Africa.

Data from the study carried out in South India (48) showed that the prevalence of actinomycetoma was slightly higher than eumycetomas and this was similar to other studies in India, in which actinomycetomas outnumbered eumycetomas in South India, whereas the reverse was true in North India. In our study, more fungi were detected as opposed to the number of bacterial organisms and the explanation for this could be what Francisca Hernanande *et al* discussed (49) that although there is as yet no evidence about how mycetoma cases have increased due to global warming, but it is likely that with climate change and rising temperatures (subsequent to deforestation and environmental degradation), the environmental fungi that cause mycetoma are more likely to find their optimum growth temperature and increase their population, thus representing a greater risk of infection for the vulnerable hosts.

Secondly, as stated in the results from a survey by Natalia Hounsome *et al* (50) it was revealed that poor sanitation and limited access to clean water, lack of qualified medical help and substantial healthcare costs are important factors, which might have contributed to the progression of mycetoma. In this survey, 80% of people with suspected mycetoma did not seek professional help and there are several reasons for this say due to the lack of pain at the onset of mycetoma, affected individuals presented themselves at later stages of the condition, but also the abundance of thorny trees in endemic areas (51). As reflected by Hanninghton *et al*, all this could have contributed to skin injuries through which mycetoma-associated organisms penetrated, and then the poor adherence to the wearing

of protective shoes by affected individuals could also have been a contributing factor. Other factors could have been as stipulated in a study conducted by Rayan S Ali *et al* (9) the explanation of the observation which could have risen from the point of host genetic factors that may also play a vital role in the affected individuals who present with the mycetoma.

5.2 Discussion of the molecular identification of the fungal species associated with mycetoma in Uganda

Madurella mycetomatis is the most common causative fungi of human eumycetoma worldwide, as evidenced in a review and meta-analysis by Sarah *et al* (35) but referring to our study results, the PCR assays that we ran and the results which were generated revealed that out of the n=14 study biopsies, their extracted DNA was run against the following primers, that is *M.mycetomatis* [2], *S.boydii*, *M.pseudomycetomatis,* and the results for the PCR were negative this implying that for the afore mentioned DNA targets which had to be amplified by the primers, they were not found in our study biopsies and all the n=14 study biopsies were negative for these DNA targets, this being synonymous with the absence of the strains of *M.mycetomatis, S.boydii, M.pseudomycetomatis* which carry these targets. Only *M.mycetomatis*[1] primer had 3 positive PCR results.

In a study by Darcy Emery and David W. Denning (5), their results revealed that in the whole of North Africa, West Africa, East Africa, West Asia, South East Asia *M.mycetomatis* was the most predominant fungal causal organism of mycetoma and for the Middle Africa and North America *Scedosporium spp* was the most common cause of eumycetoma in these areas respectively. There are differences between our results and these data because neither the DNA target of *M. mycetomatis* two strains nor that of *Scedosporium boydii* strain that we targeted was positive for the n=14 study biopsies and to be specific, because PCR is a highly very specific assay as documented by Jens *et a l*(52)., The *M. mycetomatis* strains and *Scedosporium boydii* that we did not detect was specifically for the strains that our study primer sequences could capture and not any other stains or variants of these organisms. With this explanation, it is clear that not capturing any of the 3 specific organism's DNA targets in our study biopsy was a merit rather than a limitation for the following reasons: As noted by Mahmoud (44), much less is known about the virulence factors of *Madurella mycetomatis,* but when they suggest that *M.mycetomatis* proteins were potential virulence factors by having a relative difference in the average alignment scores of pathogens vs. controls, 44 different proteins, 31 being functionally annotated. Besides the myosin identified in this list included orthologues of known virulence factors from other species, as well as a surprising number of enzymes involved in nucleic acid synthesis or degradation. Whilst the initial lesions caused by *M. mycetomatis* affected subcutaneous tissues, they

would invasively progress to deeper tissues and bone and the fact that there is no vaccine for eumycetoma, no satisfactory chemotherapy, and there were no adequate diagnostic methods this made us summarize that *M. mycetomatis* is a very virulent pathogen to manage more so when it is causing mycetoma condition.

As also discussed by Rollin *et al* (53) in their write-up that *Scedosporium* cell wall contains glycosylated molecules involved in important biological events related to virulence and pathogenicity and represents a significant source of antigens and as well polysaccharides, peptidopolysaccharides, *O*-linked oligosaccharides and glycosphingolipids which have been identified on the *Scedosporium* surface indeed which suggests that this organism adapts to difficult conditions and more so in terms of its ability to cause eumycetoma. In summary, if the DNA targets for these organisms was not detected amongst all the n=14(100.0%) biopsies as it is now, then it is to the advantage of the patients.

Furthermore (54) B. Nyuykonge *et al* showed that *M. pseudomycetomatis* causes eumycetoma in Latin-America and elsewhere and not commonly in Uganda, and similarly our results from the PCR specific primers we used showed how we never had a positive PCR, because the DNA target for the specific *M. pseudomycetomatis* strains was not there and thus the particular organisms strain *(M. pseudomycetomatis*) which is known to have this DNA target was not present. The explanation for this is well discussed by Zeinab Borjian Boroujeni *et al* (55) because of lack of proper prevalence data, currently the true burden of this disease is not known as it being caused by *M.psuedomycetomatis* and the reflection as from our data is that this organism is not present in our n=14(100.0%) biopsies probably because it is not endemic in this area and also the genetic composition of the n=14 mycetoma individuals might not have favored the patients susceptibility to the organisms invasiveness and being able to proliferate and establish a clinically significant condition.

The reason for the occurrence as per our study data (no *M.mycetomatis* and *S.boydii*) could be explained in line to what Sarah.A *et al*(56) shared that lower number of cases in Egypt compared with Sudan might have been due to the presence of certain genetic predispositions toward *M. mycetomatis* infection in the Sudanese population or better construction of villages in Egypt and the use of footwear, the same explanation can be picked up because it might be true that the genetic composition of mycetoma positive individuals is probably not the same with what we have in the population of mycetoma positive individuals who are in Uganda and second to this also ,amongst the mycetoma positive individuals ,who were diagnosed with *M.mycetomatis* and *S.boydii*,majority have a poor adherence to the wearing of shoes and they often get injuired with contaminated sharp objects such as thorns which might not exactly be the same case for our individuals and probably also the endemicity

of these species in Uganda is not high or even not there.

In another write-up (57) they high lightened ITS1 and ITS2 to be able on a large extent to yield similar results when used as DNA meta barcodes for fungi but still analyses demonstrated the limitations of ITS as a marker, as ITS sequences from different species often cluster together and many species split into several clusters thus connecting this information to this study's data which reflected 3 biopsies (specifically Biopsy numbers, UCIBS 731,MB 241 and B6546423) which yielded positive PCR results when they were run against ITS 1 target and no amplification was observed when the biopsies were run against the ITS ^{2} primers, this implied that only the primer pairs for ITS ^{1} had their specific target present as it is conserved in these particular fungi. Therefore we are reporting the presence of fungal DNA targets, more specifically having the sequences which were amplifiable by the ITS ^{1} primers but no amplification was observed for the ITS ^{2}. The ultimate outcome of the PCR results that were run looking for these specific targets in this study indicated no specific species target was present because their specific primer designs had no target to amplify and specifically the DNA targets were for *M. mycetomatis* ^{1}, *M. mycetomatis*^{2}, ITS ^{2}, *S. boydii* and *M. pseudomycetomatis* ,but only ITS ^{1}which is a universal fungal barcode and in comparison to another study by Hamza.M *et al* (58) if the ITS ^{1} was able to capture and detect among the 14 biopsy sample, 3 had the target sequence for ITS ^{1} and none of the 14 mycetoma positive biopsies had the ITS^{2} target. According to their study results (58) differential abundance analysis demonstrated that some taxa were exclusively detected only by ITS2, and vice-versa for ITS1. However, the shotgun metagenomic approach showed a taxonomic profile with a closer resemblance to ITS1 than ITS2 and so basing on their study results, neither of the barcodes evaluated is perfect in terms of distinguishing all species but using both barcodes offers a broader view of the fungal population and from our study results. This could explain why ITS ^{2} had negative PCR results as oppose to the 3 positive PCR results, all amplified by ITS 1 just in terms of the targets. On the other hand, since a lot of fungi can cause eumycetoma as discussed by Wilson L *et al* (59), and he states that the condition can be caused by more than 40 different fungal causative agents and the reality is none of the primer pairs we used could capture all the targets for these 40 or many organisms as explained and thus this can explain our results, that even the 3 positive biopsies with ITS ^{1} are possibly due to other possible eumycetoma causative fungi but not specifically the specific species primers we used and thus they were not captured for the above reasons.

The discordance between the phenotypic histopathologic stain results versus the genotypic PCR findings could have been since there are many fungi which are known to be associated with eumycetoma and we used species specific primers and even the ITS 1 and ITS 2 primers

sequnces we used may not have been able to capture all the ese fungal targets and thus the PCR results.

5.3 Discussion of the characteristics exhibited by the study participants with mycetoma in Uganda

Develoux *et al*, (60) showed the predominance of mycetoma in males: 4,060 versus 1,175 in females and the difference could not be explained by a difference of exposition to the risk of contamination according to sex but rather a possible role of hormonal influence in mycetoma susceptibility is suspected but as per for our study's results, detection was mostly in males n=9 (64.29%) than females (35.71%) for which the reason could be attributed to the type of work men engage in for their social-economic activities such as farming, brick laying and the mycetoma causative organisms such as the eumycetes and actinomycetes are commonly found in the soil, so men get exposed more. Since the majority are bread winners and they tend to interface a lot with soil plus probably the hormonal factor, this might have contributed to the raised number of male mycetoma positive individual than females. In another study which was conducted in Mexico by Alexandro Bonifaz *et al* (61), they observed that mycetoma primarily affects men, with a male: female ratio of almost 3:1 similar to a previous report about mycetoma incidence in Mexico thus this particular factor is almost constantly within that ratio for those various reasons spelled out.

According to the study by Michel (60), most of their mycetoma patients were aged between 11 and 40: 70% of 5,240 cases but from our study's data, biopsies of individuals who were 18 years and above were more positive for mycetoma as compared to individuals who were below 18 years for which these were less positive at a frequency of n=11 (78.6%) and n=3 (21.4%) respectively. In another study by Rowa *et al* conducted in Sudan, (16) the majority of patients (84%) were young adults below the age of 40 years old at presentation which almost coincides with our study results that reflected most of the positive mycetoma cases are from individuals who are 18 years and above. The reason for this could be that it is the adults or individuals who are above 18 years that mostly work to sustain their families and at the end since they interface with contaminated soils, they might easily end up having mycetoma. As regards to the tribe of the patients, from a study which was carried out in Uganda, the data showed (6) the majority of cases were from the tribes of Baganda (34.1%, n = 85), Basoga (12.5% n = 31), Banyankole (9.2% n = 23), Acholi (6.8% n = 17) and Karamojong (4.0% n = 10) but in this study, the results showed that Muganda presented at a frequency of n= 3 (21.43), Munyankole 3 (21.43), then Musoga, Mufumbira, Kumum, Alur, Langi all each had 1 (7.14%) and those whose tribe was not documented were at 3 (21.4%), and the explanation for this could be that for most of the tribes in which

mycetoma was found to present at a high frequency, these are typically people who practiced agriculture as a major economic activity and since they get into contact with contaminated soils very often, chances of exposal to the mycetoma causative organisms are very high and thus they often get infected and may be another factor to reflect about is the genetic susceptibility of some tribes as compared to others.

In the study by Ahmed, school children n=11 (33%) were affected most, followed by farmers n=8 (24%). One patient (3%) was unemployed because of the prolonged illness and disability. Housewives constituted 21% of the affected patient population. Most patients were born and lived in the village; those who lived outside the village were living in nearby villages in the same state. When we compare with our study data, the information pertaining the occupation of the mycetoma positive individual was not well captured and thus comparison may not be very informative.

As from our study data the site of condition presented as follows: the leg at a frequency of n=5 (35.7%), the undocumented sites of mycetoma in this study were at a frequency of n=3 (21.43%), all other body parts that is eye, lower limb, knee joint, fore arm, oropharynx, the head, neck and back, with a frequency of n=1(7.14%) but reflecting on some results which were discussed by Kwiezera *et al* (62) carried out in Uganda, they stated that the foot was the most involved site (72.3%) followed by the leg and knee (13.3%), thigh and buttocks (3.2%), arm and shoulder (3.2%), trunk (1.6%) then head and neck (0.4%) and this almost agree with our study data in that the foot is normally the most affected site of the body in mycetoma condition and the explanation for this could be that since the feet are near and close to the ground in the same line, the mycetoma causing agents are also known to be found in the soil and if the foot are not well protected, and they become traumatized say by contaminated thorns, then easily will the tendency be of contaminating the foot and thus they end becoming infected. But still in another study by Shahindakht *et al* the stated the foot to have been the most affected in 62.8% of the cases, followed by hands (25.7%), face (2.9%), and other affected sites were the trunk (2.9%), neck (2.9%), and buttocks (2.9%).

5.1 Conclusion

The study was conducted by analyzing n=14 archived biopsy tissues. We phenotypically identified the fungal and bacterial organisms, and identified using molecular PCR fungal species the fungal species associated with mycetoma in Uganda, and described the characteristic of study participants with mycetoma in Uganda.

The study findings showed that out of the n=14 biopsy samples, n=12 (85.7%) had fungal organisms and 2 (14.3%) had bacterial organisms as the likely etiological agents of mycetoma. Using PCR, the molecular identification of fungal species revealed that out of the n=14 included in the study only 3 biopies had positive fungal targets that were non-specific. Age, sex, tribe of individual, body part affected, not wearing of shoes were among the study characteristics described for the mycetoma positive participants from whom the tissues were sampled.

5.2 Recommendations and take home message

We recommend that further genomics studies work such as sequencing may be conducted to ascertain the exact species of fungi and bacteria that cause mycetoma in Uganda and this is because our study findings, none of the fungal species-specific genotypes was captured but there was evidently a clear presence of fungal DNA targets in the three biopsy samples that came out to be positive with the ITS 1, which is a universal fungal primer. Furthermore, also because we used biopsy tissues fixed in formalin, thus we recommend usage of fresh samples.

Appendix I

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