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Distribution of medically important saprophytic Zygomycetes species in Bushenyi district, Uganda.

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Abstract

Background: In resource limited settings, the frequency of exposure of Zygomycetes from an environmental source, the influence of geographical location, and the minimum infective doses remains a public health challenge. Objective: To outline the environmental distribution of medically important Zygomycetes species in Bushenyi district of Uganda. Materials and Methods: Standard Mycological methods were used in the isolation and phenotypic identification of zygomycetes in 120 soil samples randomly collected from suspected zygomycetes habitable sites in different representative locations in Bushenyi district of Uganda. Results. We found 47% prevalence of zygomycetes species from the samples analyzed. Out of the 56 isolates identified using colonial morphology, slide cultures were performed to identify them further. 20 (35.7%) were *Rhizopus*, 16 (28.6%) *Rhizomucor*, 15 (26.8%) *Mucor*, 3 (5.4%) *Absidia*, 1 (1.8%) *Apophysomyces* and 1(1.8%) *Cunninghamella*. Conclusion: The soil samples analyzed contained a wide array of zygomycetes species comparable to the findings of other researches in global and African literature. The medical and public health importance of the isolated zygomycetes species are fully discussed.

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Introduction

The *Zygomycetes* represent two separate and uncommon types of fungi, the *Mucorales* and the *Entomophthorales*. *Mucorales* are divided into six families of medical and animal importance: *Syncephalastraceae*, *Mucoraceae*, *Saksenaea*, *Thamnidaceae*, *Cunninghamellaceae* and *Mortierellaceae*. Majority of human zygomycotic disease is caused by the members of the family *Mucoraceae*. *Mucoraceae/Absidiaceae*, contains *Rhizopus*, *Mucor*, *Absidia*, *Rhizomucor*, and *Apophysomyces* which have all been associated with human disease (1). *Rhizopus* species are the most common cause of zygomycosis in humans (2). *Cunninghamella bertholletiae* is the only species which infect humans (3).

Entomophthorales has two human pathogens, *Ancylistaceae* and *Basidiobolaceae* (4). *Entomophthorales* are identified by the production of coenocytic hyphae and by their production of sexually reproductive zygospores (4). They are distinguished from the *Mucorales* by their production of actively expelled asexual sporangioles and by their markedly compact and glabrous mycelial morphology. All cases of human disease are caused by *Basidiobolus ranarum* and *Conidiobolus coronatus*, *C. incongruus* (5).

The *Entomophthorales* represent much less common clinical isolates. Their possible role in human disease should be considered in cases of subcutaneous mycosis, sinusitis, and

even disseminated disease when a history of travel to a tropical climate is seen (6). Initial wrong designation of the diseases associated with the *Zygomycetes* as “mucormycosis” reflected the predominance of the *Mucorales* in causing disease in humans and ignored the role of the *Entomophthorales* in causing disease. Lumping the *Entomophthorales* together with the *Mucorales* as causes of “zygomycosis” does not adequately reflect the distinct morphologic and epidemiologic nature of these 2 fungi. In resource limited settings, the frequency of exposure from an environmental source, the influence of geographical location, and the minimum infective doses are not clear. The mechanism of mucociliary clearance, sinuses and what controls latency are not well understood.

Prevention of these infections may be difficult because little is known about risk factors, sources of infections and host-microbial interaction. Little is known about: how common zygomycetes are in the environment, which ecological niches these fungi are found in and how the fungi are transferred to man. Effective interventions centered on studies that will define the environmental source of these fungi welcome. In this study, we therefore outlined the environmental distribution of medically important Zygomycetes species in Bushenyi district of Uganda.

Materials and Methods

In this descriptive cross-sectional survey, one hundred and twenty soil samples were collected around Bushenyi-Ishaka Municipality following the method described by Oluoch *et al.*, (7) with slight modifications. The sites chosen for collection of samples were those that were highly suspected of containing Zygomycetes such as those covered with decomposing organic matter like leaves, fruits, and animal excreta. From each selected site, approximately two grams of top layer of soil was collected using an improvised sterile spoon and put into sterile bags. The bags were properly sealed and immediately transported to the lab for isolation. The collected 120 soil samples were air dried for six hours at room temperature. Approximately 0.5 grams of the dried soil was dispersed throughout the freshly prepared Sabouraud's dextrose agar (SDA) with chloromphenicol and incubated for three days at 30°C (8). The resulting colonies were examined and suspect Zygomycetes on the basis of colonial morphology were individually inoculated into freshly prepared SDA with chloromphenicol plates and incubated for another three days at 30°C.

Morphological identification of the isolates was done first using the cellotape method and confirmed using the slide culture technique as described by Ellis *et al.*, (9) and De Hoog & Guarro (10) and observed under a compound microscope (x40) and results recorded. The parts of the fungi such as the sporangiophore, collumellae, sporangium and the location of the rhizoids were helpful in the identification of the isolates Ellis *et al.*, (9). The isolates were sub cultured regularly and stored at 4°C during the entire study period while on completion of the study the isolates were preserved, using 10% glycerol at -20°C (11). Quality control was enhanced by ensuring that Standard operating procedures were established in the lab and strictly adhered to and a laboratory journal was prepared in which all the results were recorded. In the identification of the isolates the supervisors confirmed them while during the assaying of the extracts positive and negative controls were used. Also, aseptic conditions were observed with all the tests being conducted under a laminar flow hood.

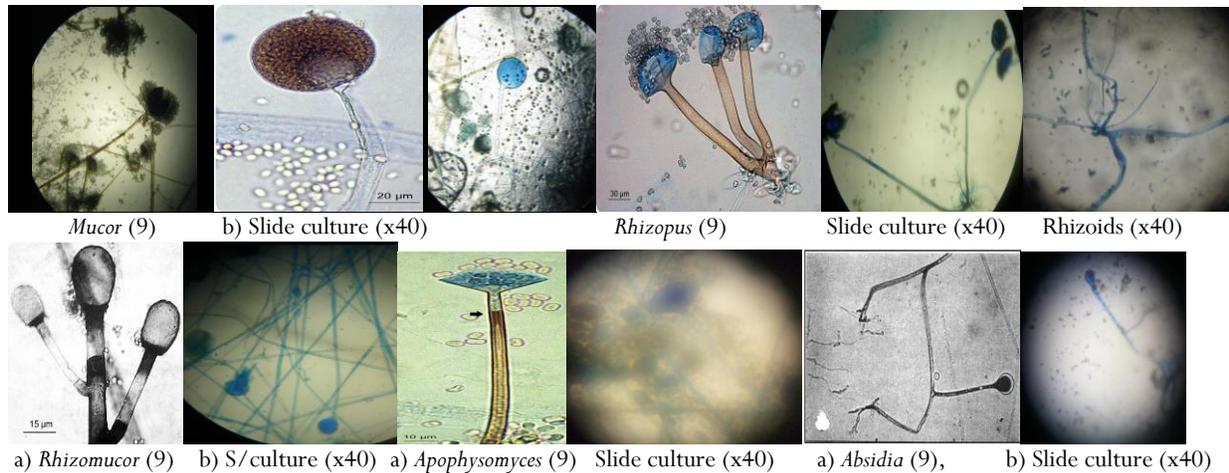
Results

We found 47% prevalence of zygomycetes species from the soil samples analyzed. One hundred and twenty (120) Soil samples randomly and aseptically collected from suspect around Ishaka-Bushenyi Municipality were used for the isolation of the fungi. SDA with chloramphenicol was used for inoculation while slide cultures were used for the identification of the isolates. When the soil was inoculated on the media, based on colonial characteristics 56 (46.7%) were suspected to be Zygomycetes and therefore were sub-

cultured on freshly prepared SDA with chloromphenicol and slide culture technique done to affirm the results. Sixty four (64) (53.3%) did not yield any significant results.

Out of the 56 isolates identified using colonial morphology, slide cultures were performed to identify them further and 20 (35.7%) were *Rhizopus*, 16 (28.6%) *Rhizomucor*, 15 (26.8%) *Mucor*, 3 (5.4%) *Absidia*, 1 (1.8%) *Apophysomyces* and 1(1.8%) *Cunninghamella*. SDA with chloromphenicol, colonies of *Mucor* were very fast growing, cottony white and later become dark-grey. Microscopically, *Mucor* has sporangiophore which is erect, simple or branched and the sporangium is large, globose to spherical. Apophyses are absent and there is well-developed columella as illustrated by the diagrams below. On SDA with chloromphenicol, colonies of *Rhizopus* were fast growing and cover the agar surface with a dense cottony growth that is at first white and later become grey or brown. Microscopically, *Rhizopus* is characterized by the presence of stolons and rhizoids, formation of sporangiophores directly above the rhizoids, globose to oval sporangia, apophysate and columellate. After spore release the apophyses and collumellae often collapse to form an umbrella-like structure as shown by the diagrams below.

On SDA with chloromphenicol, colonies of *Rhizomucor* were dirty grey. Microscopically it has stolons and poorly developed rhizoids at the base of the sporangiophores. The sporangiospores are hyaline, smooth-walled, globose to subglobose, and the sporangia are globose each possessing an oval or pear-shaped columella as illustrated by the diagrams below. On SDA with chloromphenicol, colonies of *Apophysomyces* were fast growing, white, becoming creamy white to buff with age. The sporangiophores arise at right angles from the aerial hyphae and often have a septate basal segment resembling the "foot cell". Rhizoids are predominantly unbranched while the Sporangia are typically pyriform in shape, sepia coloured when mature, columellate and strongly apophysate. Columellae are hemispherical in shape and the apophyses are distinctively funnel or bell-shaped. Sporangiospores are smooth-walled, mostly oblong but occasionally subglobose and sub-hyaline to sepia in mass as illustrated by the diagrams below. On SDA with chloromphenicol, colonies of *Absidia* were gray with a coarse, woolly texture. Microscopically, rhizoids are present, but the Sporangiophore arises between the nodes of the stolon. The Sporangium contains a Columella and the sporangium is relatively small, globose, pyriform or pear-shaped as illustrated by the diagrams below.



Discussion

The spread of diseases to susceptible host depends on many factors which underlie the host microbial interactions. Successful elimination or reduction to barest minimum of disease agents depends on a clear understanding of the factors underlying disease spread and possible source of the disease agents. The soil is a reservoir of fungal disease agents and many factors mediate the transmission of these agents from the soil to the susceptible human host. The Zygomycotic agents found in the soil and the underlying factors associated with their transmission to cause human disease are briefly summarized below. First, Zygomycetes found in dust, composting vegetation and on rotting fruit are faced with temperatures which are selective for thermophilic species, such as some species of *Absidia*, *Mucor*, *Rhizopus*, and *Rhizomucor* (12). Zygomycetes are transmitted through inhalation of spores from dust (13), and in outbreaks of rhino cerebral or pulmonary zygomycosis linked to excavation, construction, or contaminated air conditioning filters (14). Second, needle-stick exposures have been implicated in zygomycotic infections occurring at the site of medicine injection, catheter insertion sites, injection sites (15), and tattooing (16). Third, the development of wound zygomycosis has been seen with a variety of adhesive products used in the hospital setting (17).

Fourth, diabetes mellitus remain a major risk factor for developing rhinocerebral zygomycosis due to two well-known main processes: failure to suppress the germination of spores and subsequent failure to kill proliferating hyphal elements. In normal hosts, macrophages prevent the initiation of infection by phagocytosis and oxidative killing of the spores. In hosts with diabetes, the monocytes/macrophages are dysfunctional and fail to suppress this spore germination process (18). Fifth, the mechanism by which the corticosteroids enhance susceptibility to developing zygomycosis may be that steroids suppress the normal inflammatory cell response that would otherwise occur, and also, they may induce a

diabetic state (19). Sixth protein calorie malnutrition (20), diarrhea, typhoid fever, and gastric ulcers, and amebic colitis have all been associated with the development of gastrointestinal disease by the zygomycetes.

We isolated and identified 20 (35.7%) of *Rhizopus* out of the soil samples analyzed. *Rhizopus (oryzae) arrhizus* is the most common environmental *Rhizopus* species seen and has been identified in India, Pakistan, New Guinea, Taiwan, Central and South America (21), Africa, Iraq, Somalia, Egypt, Libya, Israel, Turkey, Spain, Italy, Hungary, Czechoslovakia, Germany, Ukraine, the British Isles, and the United States (22). It has been found in soils from cultivated grassland and forest locations and also been cultured from volcanic mud (23). This organism has been isolated from hay (24), decaying grass and leaf mold, and a variety of food stuffs including barley, sorghum, wheat, corn (25), oat, rice, onions, cotton, groundnuts, sweet potatoes, pecans, Brazil nuts, and tomatoes (22). Strains of this mold are responsible for the fermentation of various oriental foods and Indonesian alcoholic drinks (26).

R. stolonifer occurs in a similar environmental distribution to *R. arrhizus*, with preference for the tropical and subtropical climates. It is found in soil samples from forests, deserts, salt marshes, grasslands, and cultivated fields and has been isolated from hay (24), decaying vegetable matter, peat, garden compost, municipal waste, sewage (22), lumber, sawdust, and wood pulp (13). It has been cultured from foodstuffs including barley, corn, sorghum, wheat (27), soybeans (28), rice, beans, tomatoes, groundnuts, pecans, brazilnuts, bananas, and cotton (22). *R. stolonifer* has also been implicated in causing soft rot in both stored sweet potatoes and strawberries (4). They have been isolated from soil collected in forests and garbage (29) and from skin scrapings from otherwise healthy fowl. *4R. microsporus* has also been isolated from moldy lumber in Norwegian sawmills (30) and other wood products (31).

In the home environment, the use of air conditioning, rugs, pillows, drapes and other cloth, and wooden furniture provides suitable environments for the proliferation and

sequestration of fungal spores. *Rhizopus* spores have been identified both in house dust and from air plates placed in kitchen sites. Spores from *R. arrhizus* (14), *R. stolonifer* (32), and *R. microsporus* (32) have all been collected from air samples or dust collected from air conditioning systems. *Rhizopus* spp. have also been found as part of the microbiota of dog hair, peaking in occurrence during the summer months (33). *R. stolonifer* has been found associated with healthy fingernails and toenails of Egyptian students (34). Infection by *Rhizopus* spp. has been transmitted by the respiratory, percutaneous, and gastrointestinal or oral routes. Most infections caused by *Rhizopus* involve the rhinocerebral and pulmonary sites. Bandages used to hold dressings in place over surgical wounds, became infected with *R. (oryzae) arrhizus* (16). *R. microsporus* var. *oligosporus* has been seen in cutaneous infection following surgery (35), and *R. microsporus* var. *rhizopodiformis* has been seen in a surgical wound covered by an adhesive ostomy bag (36), both representing iatrogenic transmission. Sporadic cases of transmission by an insect bite (37), intramuscular injection (38), and catheter insertion site infections (16) have all been described. Intravenous drug use has also been linked to *Rhizopus* zygomycosis (39).

About 15 (26.8%) *Mucor*, were isolated and characterized from our studied population. Members of the genus *Mucor* are saprophytes that are ubiquitous in nature. They are found in soil and environmental samples worldwide, from the Arctic to the tropics (40). Spores have been demonstrated in air or dust samples obtained from both the home and hospital settings (41). *Mucor* species have also been seen as part of the microbiota of dog fur, showing seasonal fluctuations during the wet and temperate fall and spring months. Spores of *Mucor* species have also been demonstrated in a variety of food and medicinal products. *M. circinelloides* and *M. hiemalis* have been isolated from cereals, nuts, and flour. *M. racemosus* is the most common *Mucor* species found in wheat grains (25), and it has also been found in stored Egyptian soybean seeds (28). Both *M. circinelloides* and *M. rouxianus* have been identified in herbal or naturopathic remedies (42). *M. hiemalis* has been identified in stored white cabbage (43), while *M. circinelloides* has been isolated from oranges associated with an outbreak of onychomycosis (44).

Cases of rhinocerebral and pulmonary disease have been attributed to inhalation of spores. Infection of the alimentary tract is most probably acquired through ingestion of fungal spores in food products or herbal preparations (45). Cutaneous disease has resulted from percutaneous exposures due to injections, insect bites (46); and other forms of traumatic implantation of spores. Nosocomial infections have resulted from implantation of spores into an erosive wound caused by the removal of an adhesive bandage (47) or introduction of spores subcutaneously by placement of an intravenous catheter (48). An outbreak of fungal nail infections occurred in workers who handled *M. circinelloides*-infected oranges.

Workers contracted the nail infection while squeezing cull oranges with their bare hands (49).

Exactly 16 (28.6%) *Rhizomucor*, were also isolated and identified from samples analyzed, *Rhizomucor* species are found worldwide in eastern Europe, the British Isles, North America, Japan, Indonesia, India, and Africa. It has been isolated from decaying or composting garden and municipal wastes, composted wheat straw, self-heated hay and corn, cultivated mushroom beds, manure, guano, leaf mold, and grass. *Rhizomucor pusillus* is the most common species seen and has been detected in a variety of food items including grains, seeds, nuts, and beans. It has been cited as the most common thermophilic fungus to be isolated from soybeans in Egypt (36). Spores from *Rhizomucor* are easily airborne due to their small size and have been isolated from air samples collected outdoors and in hospitals (50).

Two modes of transmission have been suggested for *R. pusillus*: inhalation of spores and percutaneous introduction of spores into a susceptible host. Several cases have resulted from probable nosocomial exposures (51), with one series of iatrogenic cases being linked to *R. pusillus* spore-positive air and facility surface cultures (51). Cases of disseminated disease and endocarditis probably arose from primary pulmonary infections (52). Cutaneous disease caused by this fungus is acquired by two mechanisms. Disease may result from direct traumatic implantation of spores into subcutaneous tissues (51) or may occur as a manifestation of disseminated infection.

We isolated 3 (5.4%) *Absidia*, species out of the samples analyzed. Due to poor facilities we could not further identify the species of the organism we isolated. *Absidia corymbifera* is a saprophytic organism that is isolated primarily from soil and decaying vegetation (53). This fungus is distributed worldwide, with environmental isolates having been collected from Europe, the Middle East, Indonesia, North America, Africa (53), the British Isles (53), South America and India (46). *A. corymbifera* is seen as a colonizer of wheat straw compost and has been isolated from many different grains, nuts, seeds, and cotton (53). It has also been cultured from dust samples collected in British homes (54). It grows well under moist, humid conditions, having been cultured from waterlogged grasslands, swamps, mangrove mud, human sewage, animal dung, and bird and bat guano (53). *A. corymbifera* has been identified in hay and animal fodder in British farms (24). It is noteworthy that *A. corymbifera* has also been recovered from dried grass and the right boot that accompanied the frozen, well-preserved prehistoric corpse, Ice Man, with an age of approximately 5,300 years (55)!

Inhalation of spores, ingestion of food contaminated with fungal spores, and traumatic implantation of spores probably underlie most cases of *A. corymbifera* infection. *A. corymbifera* infection has been reported to occur as a result of injuries with spore-contaminated farm equipment (56), of corneal laceration with a galvanized nail (57), and in a patient with meningitis that resulted from a strike to the

left frontal region of the head with the sharp end of a pickaxe (58). Inoculation of spores into disrupted skin probably occurred in one patient who handled animal material or possibly moldy hay or feedstuffs (59). Intravenous inoculation of fungi contaminating drugs, skin, or inoculating needles is thought to be the route of infection in intravenous drug users (60).

Exactly 1 (1.8%) *Apophysomyces* were isolated and identified from our study. The genus *Apophysomyces* was first described from soil samples (61). *Apophysomyces elegans* was subsequently identified from soil and air filter dust samples association with human infections (62). Its distribution in tropical and subtropical climates is further suggested by the occurrence of human infections with this agent.

Infection with *A. elegans* is predominantly the result of the introduction of spore-containing soil or vegetation into traumatic wounds (63). In the two cases from Australia, the origin of infection was confirmed by culturing *A. elegans* from soil or dust samples at the site of exposure (64). Percutaneous routes of infection are also suggested by several other cases including postoperative surgical wound infections (65), an infection following an injection (15). Several patients were immunocompromised: one with severe burns (64).

Exactly 1(1.8%) *Cunninghamella* species were isolated and identified from our study. *Cunninghamella* spp. are well-known environmental organisms, having been isolated from soil, peat, sewage, water, air, seeds, nuts, flowers, and other vegetation worldwide, predominantly in more temperate climates (36). They have been isolated from sewage in Egypt and camel dung in French Sudan. They have been found in dried or decaying flowers in China, Venezuela, and Puerto Rico and in dead wood in France. Fungi of this family have been isolated from air samples collected in some hospitals in the developed world. Although there are five species of *Cunninghamella*, only *C. bertholletiae* (28) has been definitively shown to cause disease in humans. Evidence that *Cunninghamella* infections are acquired predominantly by inhalation of sporangioles into the upper respiratory tract is two-fold. First, most cases of *Cunninghamella* involve the lungs or sinuses as the primary sites of infection (19 of 23 cases).

Transmission by direct percutaneous implantation of the sporangioles is suggested by two additional cases of primary cutaneous and subcutaneous disease. Although *C. bertholletiae* was not recovered from the padding material, it is thought that either the padding or environmental sporangioles implanted at the time of original injury were the most likely sources of infection.

In conclusion, the soil samples analyzed contained a wide array of zygomycetes species comparable to the findings of other researches in global and African literature. *Rhizopus* is the most predominant Zygomycete isolated from soil samples collected from Ishaka-Bushenyi municipality with a percentage of 35.7%.

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