The Pathogenicity of Aspergillus Fumigatus and Aspergillus Niger Isolated from Accumulated Poultry and Goat Droppings in Immuno-Competent Albino Mice

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ABSTRACT

The pathogenicity of Aspergillus fumigatus and Aspergillus niger isolated from poultry and goat droppings in immuno competent albino mice was investigated. A total of 12 soil samples of both poultry and goat droppings were collected and screened for the presence of Aspergillus fumigatus and Aspergillus niger using microbiological standard method. The pathogenicity of the isolates on immuno-competent albino mice were investigated by challenging the mice subcutaneously using 0.5 ml of the inoculum. All mice were kept under complete observation for four (4) weeks for pathological signs and symptoms, mortalities and gross morphological lesions of the internal organs were observed and recorded. The study revealed that the total mean plate count of Aspergillus fumigatus was higher than that of Aspergillus niger and also, there were significant pathological lesions among some organs of the infected mice after a pathological cross examination. Thus, the result of the examination shows that congestion, haemorrhage, air sacculitis, pericarditis, perihepatitis and hypertrophy were observed from the immuno-competent mice under study. The obvious pathological features of these fungi on immuno-competent albino mice shows invariably that they can pose a high level life-threatening infections in immuno-compromised individuals particularly farmers handling the animals. Thus, there is need for good healthy hygiene practices, maintenance of environmental sanitation and managing the environmental factors that supports the multiplication of these fungal infections.

Keys words: Pathogenicity, immuno-competent, albino mice, Aspergillus fumigatus, Aspergillus niger, poultry and Goat droppings.

INTRODUCTION

Aspergillus fumigatus and Aspergillus niger are keratinophilic and ecologically important group of fungi which could be found in the soil. They include a variety of filamentous fungi mainly comprising hyphomycetes and several other taxonomic groups. Hyphomycetes include dermatophytes and a great variety of non-dermatophytic filamentous fungi. These fungi occurred abundantly in the superficial soil layer of landfills and surroundings and distributed worldwide (Deshmukh and Verekar, 2006). Aspergillus fumigatus and Aspergillus niger are present in the environment with variable distribution patterns that depends on different factors, such as humans or animal presence, which are of fundamental importance. They occur on cornfield debris in the soil and degrade hard keratin and keratinous material. Therefore, they play an important ecological role in decomposing such residue. They are generally considered as soil saprophytes. Soil that is rich in keratinous material is most conducive for the growth and occurrence of these fungi (Ali-Shtayeh and Jamous, 2000).

Aspergillus fumigatus is an opportunistic fungal pathogen that causes life-threatening infections especially in immuno-compromised individuals. Aspergillus fumigatus is widely spread in nature, and is typically found in soil and decaying organic matter, such as compost heaps, where it plays an essential role in carbon and nitrogen recycling. Aspergillus niger is a mold commonly found throughout the environment within soil and water, on vegetation, in fecal matter, and suspended in air. It is ubiquitous in soil and is commonly found in

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indoor environments, it is also one of the most extensively studied groups of fungi. Isolation of Aspergillus fumigatus and Aspergillus niger from soil dates back to the middle of the century when a hair baiting technique was described. However, the existence of their relatives is of ancient origin. The prevalence of these fungi depends on different factors, such as the presence of creatinine in soil, pH, and geographical location. Some of these fungi such as dermatophytes are well known to cause tinea infections which could be transmitted from soil to humans. In general, soil could be considered as a reservoir for human infection. Forests, farmyards, park soils, and sediments of the rivers and ocean's containing humus and organic materials are the best candidates for growth of these fungi (Gupta et al., 2005)

The vast majority of people with fungal infection of skin, hair and nails are otherwise healthy, but a small group is immuno-compromised. Most of the Aspergillus fumigatus and Aspergillus niger are not dermatophytes but soil inhabitants. Some groups of these fungi are causative agents of cutaneous fungal infections named dermatophytosis, and other saprophyte fungi mainly represent hyalohyphomycosis (Ali-Shtayeh and Jamous, 2000).

During the past years, many researchers reported about isolation of Aspergillus fumigatus and Aspergillus niger around the world. Also, a lot of reports were available about the isolation of geophilic dermatophytes and keratinophilic fungi from the soils of many parts of the world and also dermatophytosis due to the geophilic fungi during the last decade. Nowadays, most people spend their time with their children in the parks for fun and are potentially at risk for direct contact with soil and being exposed to these fungal diseases. (Gupta et al., 2005). Several incidences of fungi diseases caused by Aspergillus fumigatus and Aspergillus niger have been reported which were traced back to poor personal hygiene, direct contact with soil contaminated with pig, goat, and poultry droppings (Hilda et al., 2004). Previous studies on these fungi focused on the isolation and characterization of the organisms from different animal waste soil samples (Ali-Shtayeh and Jamous, 2000), but there are still reported cases of Aspergillus fumigatus and Aspergillus niger diseases worldwide and recent years (Gupta et al., 2005). This shows that there is still paucity in determining the pathogenic features of these organisms particularly in some vital human organs. This study aimed at evaluating the potential pathogenic features of Aspergillus fumigatus and Aspergillus niger isolated from poultry and goat droppings in immuno-competent albino mice.

MATERIALS AND METHOD

Study Area

This study was conducted at the Microbiology Laboratory (Chukwuemeka Odimegwu Ojukwu University, Uli, Anambra State- Nigeria). Poultry and goat droppings were harvested from a polluted animal farm in Umu-aku village Uli in Ihiala Local Government Area. Uli is a town located between latitudes 5.47 N and 5.783°N and longitudes 6.52°E and 6.87'E on the eastern part of Nigeria. The samples were analyzed immediately upon reaching the laboratory for the presence of Aspergillus fumigatus and Aspergillus niger following the procedures in the fungal analytical manual of the FDA- USA (Feng et al., 2017; Andrews et al., 2018).

Collection of Samples

Six (6) samples each were randomly collected from the poultry and goat farms, totaling 12 samples. The samples were collected from the superficial layer of soil at a depth not exceeding 3-5cm with sterile stainless spoon in sterile polyethylene bags, brought to the laboratory and processed within 4h of collection to ensure maximum recovery of the organism.

Sterilization of glass wares and Media preparation

The glass wares used were sterilized using an autoclave at 121°C for 15 minutes. They were then washed with detergent and rinsed with distilled water. These were air dried and placed in an inverted position inside the staircase of the hot air oven and set the thermostat at 160°C for 3h, while the media used were prepared in applied microbiology laboratory of Chukwuemeka Odimegwu Ojukwu University, Uli. Sterilization was done by the use of an autoclave, which provided moist heat to kill the organisms present. Sterilization of most

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glasswares and media used were done at a temperature of 121°C for 15 minutes. Work benches were also cleaned using ethanol to ensure adequate sterility.

Sample Processing and Isolation of the Organisms

One gram of each of the samples was weighed out and transferred into sterile capped test tubes containing ten millilitres (10 ml) of sterile water. The test tubes were shaked for 30 minutes and allowed to stand for 15 minutes, of which 1ml of the supernatant was collected and subjected to serial dilution technique. One milliliter (1.0 ml) each was pipetted from the test tube containing 10^{-3}, 10^{-4}, and 10^{-5} dilutions into sterile Petri dishes respectively. Twenty millilitres (20 ml) of molten Sabouraud Dextrose Agar (Biotech) supplemented with chloramphenicol (50mg/l) and cycloheximide (500 mg/1) was poured each into the plates containing the samples. The plates were swirled gently to ensure homogeneity and to prevent formation of air bubbles (pour plate technique). The plates were inverted and incubated at room temperature for 7-12 days. Macroscopic features of colonies were noted. Grown colonies were then subcultured on sterile agar plate preparation and slide culture was performed prior to identification following the method of St-Germain and Summerbell, (1996).

Hair Baiting Technique

The petri dishes were half filled with the animal waste samples and sterile defatted human or animal hair (not exceeding 20 years) was spread over the surface of the samples. Sterile distilled water was then added to the samples and incubated at room temperature for 4 weeks in the dark. Sterilization of the hair used for this study was achieved by washing the hair several times, first with detergent (hair shampoo), followed by water, and then with diethyl ether, and finally autoclave at 121°C, 15psi for 15 minutes. The colonies generated from the plates were subcultured on SDA (Biotech) supplemented with Chloramphenicol (50 mg/l) and cycloheximide (500 mg/l), and incubated at room temperature for 7 days (Aziz and Seema, 2015).

Preparation and microscopic examination of needle mount

A drop of Lactophenol cotton blue (LCB) solution was placed on the center of a clean grease free slide. A fragment of the colony was placed in the drop and teased with two straight needles.

A coverslip was placed over the preparation and pressed by tapping gently with a blunt ended object (pencil eraser). Excess fluid from the outside from the coverslip was wiped with tissue paper and the slide was passed through a flame to warm it (care was taken not to overheat the set-up). This treatment also removed the air bubbles that were trapped underneath the coverslip and facilitated staining of fungal elements. The slide was examined under compound light digital microscope, using low power objective (x10) followed by high power objective (x40). This revealed the nature of the hyphae, conidia and other diagnostic features (St-Germain and Summerbell, 1996). Fungal atlas aided the quick confirmation of the suspected fungal organisms (Aziz and Seema, 2015).

Slide Culture Technique

Riddel's method as described by St-Germain and Summerbell (1996) was used. A filter paper was cut and placed on the bottom of petri-dish. Two slides were crossed over each other on top of the filter paper and the filter paper was moistened. The set-up was sterilized by autoclaving at 121°C for 15 minutes. Approximately one-centimeter square agar block was cut from already prepared Potato Dextrose Agar (PDA) and placed on the intersection of the two slides. The four edges of the agar block were inoculated with the test organisms. It was then covered with sterile coverslip and incubated at room temperature for 7-10 days. After 10 days of growth, the coverslip was removed and inverted over a slide containing a drop of lactophenol cotton blue (LCB). The agar block was removed and discarded. A drop of LCB was also placed on top of the adherent colony on the slide and covered with sterile coverslip.

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The edges of the coverslip were sealed with nail polish to prevent evaporation of the stain. The slides were examined under the microscope. The isolates were identified using standard descriptions given by St-Germain and Summerbell, (1996) and Aziz and Seema (2015).

Procurement of Albino Mice

A total of 17 mice of mixed sex obtained from an animal keeping house in Nnewi, Anambra State were used for the study. They were housed in thoroughly cleaned and disinfected metal cages and provided with feeds and water prior to infection.

Inoculum Preparation

All isolates were first cultured on Potato Dextrose Agar (PDA) for 3-5 days at room temperature. The inoculum was prepared by flooding the surface of the agar plate with sterile normal saline (0.85% Nacl), scrapping the sporulating mycelium with sterile spatula and drawing up the resultant suspension with a sterile Pasteur pipette. The suspensions were filtered through sterile gauze to remove the hyphae. The number of conidia in the suspension was counted with haemocytometer, adjusted to 10 conidia per millimeter and verified by plating dilutions of the suspension on PDA plates (Nweze and Okafor, 2010).

Inoculation into the Mice through Subcutaneous Route

The mice were grouped into three; A, B, and C. Each group consists of 7 adult albino mice. Group A were infected subcutaneously with 0.5 ml of the normal saline, the immune system of group B were suppressed with antibiotic (Hydrocortisone) and after 24 hours were infected subcutaneously with 0.5 ml of the normal saline. The group C is the control group.

Cross Examination of the Morphologies of the Internal Organs of the Mice

The adult albino mice of mixed sex from each group were randomly selected and sacrificed at the end of the experiment. The livers, lungs, and hearts were harvested. The morphological changes and pathological signs associated with these organs were observed and recorded (Aziz and Seema, 2015).

Re-isolation of the test Organism from the Infected Organs

Portions of the harvested organs were homogenized using sterile mortar and pestle, 1g portion was suspended into 10 ml normal saline and cultured in Sabouraud Dextrose Agar (SDA) supplemented with chloramphenicol (0.05 mg/ml) and incubated at room temperature for 3-5 days. The number of colonies of the test organisms recovered from the sample were counted and recorded (Aziz and Seema, 2015).

Statistical Analysis

The data generated from this study were represented as mean ± standard deviation and then charts. The statistical analysis of data generated from protective study was carried out using chi-square at 95% confidence limit (Adeniyi et al., 2000). The statistical analysis of other valuable data generated from this study was examined using SPSS package program version 20.0, while the data were analyzed by one-way Analysis of Variance (ANOVA) to determine the significant difference of the mean values at 95% confidence limit (Adeniyi et al., 2000).

RESULTS AND DISCUSSION

The study revealed that 7(60%) samples out of 12 samples collected from poultry and goat droppings were positive to Aspergillus fumigatus and Aspergillus niger (Table 1). Thus, shows that the animal waste samples collected from poultry and goat farms, showed significant occurrences of Aspergillus fumigatus and Aspergillus niger.

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Table 1: Animal waste samples that were positive to A. fumigatus and A. niger specie

Sample source	No of sample	Positive sample (%)	Negative sample (%)	Total (%)
Poultry	6	4(66.67%)	2(33.33%)	6(50.00)
Goat	6	3(50.00%)	3(50.00%)	6(50.00)
Total	12	7(58.00%)	5(42.00%)	12(100.00)

The result shows that poultry droppings is positive to A. fumigatus and A. niger specie with 4(66.67%) and negative to both fungi with a percentage of 2(33.33%) and a total of 6(50.00%). Also, the goat droppings showed a lower percentage to positivity with 3(50.00%), negative with 3(50.00%) and a total of 6(50.00%). Although both waste samples have the same 50.00% for both their presence and absence.

Table 2: Prevalence of A. fumigatus and A. niger species in the animal waste samples

Isolates	Poultry	Goat	Total
Aspergillus fumigatus	2	3	5
Aspergillus niger	4	2	6
Total	6	5	11

The result showed the prevalence of A. fumigatus and A. niger in the animal waste samples. Thus A. fumigatus has an occurrence of 2 and 3 for poultry and goat droppings respectively. While for A. niger, there was a slight higher occurrence of 4 and a sharp drop of 2 for both poultry and goat droppings respectively. The total occurrence of A. fumigatus and A. niger was 11, in which 6 occurred in poultry droppings and 5 was seen in goat droppings.

Table 3: Macroscopic and microscopic characteristics of Aspergillus fumigatus and Aspergillus niger isolated from animal waste samples

Isolate	Macroscopic Characteristics	Microscopic Characteristics
Aspergillus fumigatus	The surface growth is velvety, downy or powdery, showing various shades of green, most commonly a blue-green to a grey green with a narrow white border. The colour typically darkens with age. The reverse is white to tan to pale yellowish. Colouration or shade can be dependent on the media on which the fungus is cultured upon.	Hyphae are septate with smooth walled conidiophore (usually less than 300 µm in length and 5-10 µm wide). Vesicles are subclavate in Shape, roughly 20-30 µm in width. Conidiogenous cells or phialides are flask shaped, uniseriate, compact (closely spaced), usually forming on the upper two-thirds of the vesicle and mature parallel to the axis of the conidiophores (columnar formation).

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Aspergillus niger	Rapidly growing on sabouraud dextrose agar starting with a white to yellow felt-like mat of mycelia, quickly turning black as conidia develops.	This shows large, globose, dark brown conidial heads, which become radiate, tending to split into several loose columns with age. Conidiophores are smooth walled, hyaline or turning dark towards the vesicle. Conidial are globose to subglobose, dark brown to black and rough-walled.

Table 4: Obvious pathological signs of the test isolates on the infected immunocompetent mice.

Parameter	Aspergillus fumigatus	Aspergillus niger	Control
Alopecia	2	1	0
Redness of skin	2	1	0
Skin lesions	1	1	0
Anorexia	1	1	0
Respiratory distress	0	0	0
Diarrhoea	1	0	0
Bloody diarrhoea	0	0	0
death	0	0	0

Table 5: Pathological features of the internal organs of the infected immunocompetent mice.

Parameter	Aspergillus fumigatus	Aspergillus niger	Control
Air sacculitis	0	0	0
Pericarditis	0	0	0
Perihepatitis	1	0	0
Liver congestion	1	1	0
Liver haemorrhage	0	0	0
Heart congestion	0	0	0
Heart haemorrhage	0	0	0
Lungs congestion	0	0	0
Lungs haemorrhage	0	0	0
Liver hypertrophy	0	0	0
Heart hypertrophy	0	0	0
Lung hypertrophy	0	0	0

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Table 6: Total mean viable plate counts of the isolates in the internal organs of the infected immunocompetent mice.

Isolate	Liver (CFU/g)	Lungs (CFU/g)	Heart (CFU/g)
Aspergillus fumigatus	4.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Aspergillus niger	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

DISCUSSION

Keratinophilic fungi are very closely related to dermatophytes, having the ability to cause infection. They parasitize hard keratin tissues and can also invade and degrade them. Thus, they are keratinophilic and keratinolytic in nature. Animal wastes enriched with keratin substrate like hairs, feathers, horn and hoofs, skin are good reservoir of dermatophytes as well as keratinophilic fungi, thus agreed with Marchisio, 2000; Ajello, 2004. Dermatophytes are classified into three genera namely Epidermophyton, Microsporum and Trichophyton according to Summerbell, 2000.

In many parts of the world, isolation of geophilic dermatophytes and keratinophilic fungi from the animal waste have been reported. However, anthropophilic infection, zoophilic infection and recurrent infection have been persistent in immuno-compromised individuals The most reported includes: Microsporum gypseum, Aspergillus fumigatus and Aspergillus niger. This is in line with this study as seen in table 3. These fungi are present in the environment with variable distribution patterns that depends on different factors such as humans or animal presence (Ali-Shtayeh and Jamous, 2000).

The study revealed that 50% samples out of 12 waste samples collected randomly from the farms of poultry and goat from Uli community in Ihiala Local Government Area of Anambra State were positive to keratinophilic fungi. The presence of keratinophilic in the animal waste samples could be traced back to poor personal hygiene, direct contact with the surfaces contaminated with goat, cow, and poultry dropping. Similar findings were reported by many researchers (Ellis et al., 2007; Van-Oorschot, 2000). Hence, researchers had shown that poor personal hygiene and direct contact with soil can also harbor keratinophilic fungi and this contributes to the contamination of waste soil (Deshmukh and Verekar, 2006).

The significant pathological signs such as alopecia, anorexia, redness of skin, skin lesions, respiratory distress and bloody diarrhea as reported by Ali-Shtayeh and Jamous, (2000). This report was also seen on the internal organs of albino mice under used in this study. The occurrence of air sacculitis, pericarditis, and perihepatitis among those mice infected by Microsporm gypseum could be due to the organism's capability of invading the lungs, liver and hearts of the infected mice. Microsporum gypsem is a frequent geophilic dermatophyte commonly distributed in soil, and finally gets to the intestine of these animals; which are in turn ejected as feces. Although non dermatophyte fungi isolates were more common than dermatophytes according to a report by Simpanya, 2000; Zaki et al., 2009, though did not agree with the present study in which Aspergillus fumigatus and Aspergillus niger was identified and was supported by .the work done by Abanmi A, et al., 2008

The presence of Aspergillus fumigatus (AM) and Aspergillus niger (AN), in the organs of the infected mice supported the findings of many researchers (Gentles and Holmes, 2007; Auger et al., 2003; Djeridane et al., 2007). The significant total mean viable plate counts of Aspergillus fumigatus (AM), Aspergillus niger (AN), recorded from the internal organs of the infected mice supported the reports of (Degreef, 2008). The presence of these keratinophilic fungi in the lungs, liver and heart suggests that the organs contain sufficient nutrients and favourable environment for the growth of the keratinophilic fungi. The activities of the invaded organisms on the organs might cause degradation of the nutrients, obstruction of the lumen of the organs, deterioration and deformation of the organs thereby producing pathological lesions that can clinically manifest on the infected albino mice. This is in respect to the report of Ghosh, (2000)

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The total mean viable plate counts of Aspergillus fumigatus (AM), P) ,and Aspergils niger (AN), in the internal organs of the infected immuno-competent mice were detected mostly in the lungs of the immuno-competent mice, This is in consonance with the report by Alischul et al., 2000. Mugge et al., 2006, also supported the significant decrease in the total mean viable plate counts of keratinophilic fungi as the age of the mice increased in this study, thus was due to the increase in maturation of the immune cells and organs as the mice continually responds to natural gut colonization.

CONCLUSION

This study has revealed the presence of Aspergillus fumigatus (AM) and Aspergillus niger (AN),) in the animal excreta randomly collected from 6 different farms in Umu-aku village Uli in Ihiala Local Government Area, Anambra State, The isolates Aspergillus fumigatus was most pathogenic in the infected immuno-competent compared to Aspergillus niger. The prevalence of these fungi depends on different factors, such as the presence of creatinine in soil surface, pH, and geographical location. Some of these fungi such as dermatophytes are well known to cause tinea infections which could be transmitted from animal excreta to humans. In general, animal excreta could be considered as a reservoir for human infection, hence should handle with altmost hygiene

Competing Interest

The authors hereby declare that no competing interests exist regarding the publication of this research.

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