MICROBIOLOGY

Distribution and In Vitro Pathogenic Profile of Melanized Fungi Isolated from Rotten Wood and Soil

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Abstract

Melanized fungi have captivated most researchers due to emerging subcutaneous infections globally. This study was conducted to investigate the distribution (prevalence), identification, and in vitro pathogenic potential of melanized fungi isolated from loamy soil, clay soil, and rotten wood at Amorka, Eziama, and Ndikeokwu communities, Anambra State, Nigeria. Forty five (45) samples comprising loamy soil (15), clay soil (15), and rotten wood (15) were collected and cultured on Sabouraud dextrose agar supplemented with chloramphenicol (0.05%). The fungal isolates were characterized based on their cultural and microscopic features. An in vitro pathogenic potential of the fungal isolates was evaluated using haemolysis on blood agar, thermotolerance potential, and Congo red test. Three different isolates of melanized fungi with diverse occurrences were isolated and identified as Exophiala jeanselmei 18(23.1%), Cladosporium species 32(41.0%), and Alternaria alternata 28(35.9%). Rotten wood harboured the highest proportion of the isolates (48.7%), followed by loamy soil (32.1%) and clay soil (19.2%). Thermotolerance test revealed strong growth of E. jeanselmei at 37 °C, moderate growth of A. alternata at 37 °C, and poor growth of Cladosporium. Congo red test at 100 µg/ml revealed resistance in E. jeanselmei (78-85%), moderate resistance in A. alternata, and sensitivity in Cladosporium (25-35%). Complete haemolysis on 5% sheep red blood cells was observed in E. jeanselmei, partial haemolysis was observed in A. alternata while gamma (zero) haemolysis was observed in Cladosporium species. The study has established that rotten wood is a reservoir of potentially pathogenic melanized fungi, with Exophiala jeanselmei exhibiting the highest virulence potential. However, further study is highly essential to evaluate an in vivo pathogenic potential of the fungal isolates, especially E. jeanselmei and molecular characterization.

Keywords: melanized fungi; in vitro; pathogenic; distribution; prevalence; Exophiala jeanselmei

1 Introduction

Melanized fungi are globally known as group of fungi that have melanin in their cell walls, which enables them to produce dark colouration, especially in reverse side of culture plates (Yew et al., 2014). Melanin is a heterogenous polymer containing phenols, quinone, carboxyl, aromatic rings among others (de Hoog et al., 2011). The pigmented structure has been described as vital component

to the survival of fungi in their environment, especially when there is an adverse environmental condition such as dryness, ultra violet light, changes in temperature, and poor availability of nutrients (Revankar and Sutton, 2010).

Melanized or black fungi are highly ubiquitous in the environment but are mostly found in the soil, dead plants and animals remain and in other substrates that have abundant organic matter (Pakshir et al., 2013). It is worthy to note that loamy, clay soil, and rotten wood contain large polymers that serve as substrate to these group of fungi due to their ability to produce extracellular enzymes that breakdown the large polymers to release carbon needed for several biological processes (Yew et al., 2014). Also, the availability of moisture in clay soil enables the fungi to proliferate and carry out biodegradation. These ecological roles have been described as beneficial to the ecosystem as it encourages natural purification of environment (de Hoog et al., 2011).

Apart from the ecological functions of melanized fungi, it is imperative to note that some have been incriminated in fungal infections, especially subcutaneous type that involves destruction of subcutaneous tissues in humans, especially those that have weak immunity (Seyedmousavi et al., 2014). Their ability to cause infection in man has been ascribed to the presence of genetic materials that code for melanin, hydrolytic enzymes, survival at body temperature (37 °C), and haemolysis (Seyedmousavi et al., 2014). Some of these potential pathogens have been identified as *Bipolaris, Exophiala, Cladosporium*, among others (Vicente et al., 2014).

In developing countries such as Nigeria, rural dwellers that engage in farming activities, fetching of firewood, and hunting are highly exposed to these infectious agents through traumatic inoculation (Revankar and Sutton, 2010). Due to traditional belief of some villagers, an infected person is regarded to have incurred the wrath of the gods. Most infected persons fail to take treatment and this has increased the burden of the infections. Though several researchers have documented on fungal infections caused by filamentous fungi such as Madhu et al. (2019) yet there is inadequate documentation on the pathogenic potential of melanized fungi, which mostly target the lower limbs of individuals that are frequently exposed to soil and rotten wood. The aim of this study is to evaluate the distribution (prevalence) of melanized fungi in the selected communities and to ascertain their *in vitro* pathogenic potentials.

2 Materials and methods

2.1 Study area

The study was conducted at Amorka, Eziama, Ndikeokwu, Uli, Ihiala Local Government Area, Anambra State. Uli is a village located between latitudes 5.47°N and 5.783°N and longitude 6.52°E and 6.87°E on the South eastern part of Nigeria. Uli extends westward to the confluence of the rivers of Atammiri and Eyinja, and across Usham lake down to the lower Niger region. Uli has rainforest vegetation with two seasonal climatic conditions: rainy season and dry season, which is characterized by the harmattan between December and February. Uli is characterized by double maxima of rainfall with a light drop in either July or August known as dry spell or August break. The annual total rainfall is about 1,600 mm with a relative humidity of 80 % at dawn.

2.2 Sample collection

Two different soil samples (Loamy and clay soils) and rotten wood were collected from three communities at Ihiala Local Government Area, Anambra State namely: Eziama, Ndikeokwu and

Amorka using soil auger, which was disinfected using 70 % ethanol. The soil samples were collected at the depth of 15 cm and were transferred into a sterile polyethene bag while rotten wood samples were collected using a disinfected sharp cutlass and were put in polythene bag. The samples were transported to Microbiology Laboratory, Chukwuemeka Odumegwu Ojukwu University (COOU), for immediate analysis. The samples were processed within 2 h of collection (Umedum and Iheukwumere, 2013).

2.3 Preparation of media for isolation

The media used for this study were potato dextrose agar (PDA) which were accurately weighed according to the manufacturer's instruction, and sterilized by autoclaving at 121 °C, 15 psi for 15 minutes (Yew et al., 2014).

2.4 Isolation and purification of the isolates

A stock solution of the soil and rotten wood samples was prepared by weighing one gram using a weighing balance **(**DC-300), a little normal saline was added to the soil samples in a test tube, the mixture was shook and normal saline was added to make up 10 mL. The stock sample was serially diluted tenfold in which 9.0 mL of normal saline was dispensed into test tubes (pyrex) and 1.0 mL of the stock solution was dispensed into the first test tube, and was stirred after which 1.0 mL was taken from the tube using another pipette, and was transferred into the second test tube. The process continued until the last tube after which 1.0 mL was discarded. Inoculation was carried out by aseptically inoculating 0.1 mL of the diluted sample (10^{-5}) on Potato Dextrose Agar (PDA) using spread plate method. The plates were incubated in inverted position at 30±2 °C for 7 days. After incubation, the grown colonies were sub-cultured by streaking a single colony aseptically on sterile poured plate containing SDA (60 mm OD × 55 mm ID × 13mm high). All the plates in triplicates were incubated inverted at 30±2 °C for 7 days (Yew et al., 2014).

2.5 Identification of the isolates

The sub- cultured isolates were identified using their colonial and morphological descriptions. The colonial description was carried out to determine the appearances of the isolates on agar media plates, edges, texture, surface, and reverse pigmentation (Yew et al., 2014).

2.5.1 Slide culture technique

The slide culture technique was used to visualize the structural endowment of the dematiaceous fungi as elucidated by Umedum and Iheukwumere (2013). Sabouraud dextrose agar (SDA) was prepared, followed by an agar block which was cut at $4m^2$ area using a sterile glass slide. A Petri dish containing filter paper, glass slides (75 mm× 25 mm× 1 mm) and coverslip was put in an oven at 200 °C for 30 mins. After heating, the filter papers in the Petri dishes were soaked with sterile water. The agar block was placed at the center of two slides that formed angle 90°. The colony of the fungi was inoculated at the margins of the agar, and a sterile coverslip was placed on the inoculated agar. The preparation was incubated at 30±2 °C for 4 days as illustrated by Umedum and Iheukwumere (2013).

2.5.2 Lactophenol cotton blue staining

After incubation, the coverslip was removed from the agar block using a sterile forceps and was placed on a clean slide containing a drop of lactophenol cotton blue. The margin of the coverslip was sealed using candle wax to conserve the stain. The slide was then viewed using X10 and X40 objective lenses of digital microscope (Umedum and Iheukwumere, 2013).

2.6 In vitro pathogenic screening of the fungal isolates

The *in vitro* pathogenic potentials of the isolates were ascertained by testing for the ability of the isolates to grow at body temperature (37 °C) haemolyze red blood cells on blood agar as described in the study published by Pakshir et al. (2013).

2.6.1 Haemolysis on blood agar

Blood agar was prepared following the manufacturer's instruction using SDA and whole blood from sheep. The media were allowed to solidify and aseptically streaked using the test isolates. This was incubated in an inverted position at 30±2 °C for 72 h. The presence of clear zones around the colonies was an indication of beta haemolysis, partial zones of inhibition were detected by greenish to gray zones which indicated alpha haemolysis while absence of clear zones as described by Pakshir et al. (2013) indicated no haemolysis.

2.6.2 Thermotolerance

This test was carried out to ascertain the ability of the isolates to grow at high temperature including its ability to survive at body temperature. This was performed by subculturing the fungal isolates by streaking method on a solidified PDA. Incubation was carried out at 37 °C for 7 days. Physical examination of the plates was employed in growth measurement at different incubation temperature (Revankar and Sutton, 2010).

2.6.3 Congo red test

Congo red binds to β-1,3-glucan and chitin in fungal cell wall. Fungi that have large composition of melanin in their cell walls resist the dye and grow while fungi that have little or no melanin are inhibited. Congo red was prepared at 100 µg/ml concentration. To prepare 100 µg/ml, 100 mg (0.10 g) of Congo red was weighed using an electronic weighing balance and dissolved in 5 ml of distilled water, then poured into 1000 ml of already sterilized and cooled PDA (45 °C), thoroughly mixed, and poured on sterile plates, and was allowed to solidify before spread plate technique was used to inoculate the fungal isolates followed by incubation at 30 °C for 7 days. Control plate was also prepared without the Congo red. Growth % was calculated thus (Vicente et al., 2014; Saleh et al. 2018);

$$\text{Growth}\left(\%\right) = \frac{\text{diameter of growth on Congo red}}{\text{diameter of growth on control}} \times 100 \quad (1)$$

2.7 Statistical analysis

Data obtained in the study were analyzed using Statistical Package for Social Sciences (SPSS version 22). Percentage prevalence was calculated and chi-square was used to test association between substrate and isolate distribution at $P<0.05$.

3 Results

The distribution and prevalence of the fungal isolates in the sampled communities are presented in Table 1. A total number of 45 samples comprising of 15 loamy soil, 15 clay soil, and 15 rotten wood produced 78 isolates of melanized fungi. Rotten wood produced the highest prevalence of 48.7% followed by loamy soil (32.1%) while the least was clay soil (19.2%). The difference in overall distribution across different samples was statistically significant ($\chi^2 = 10.23$, $P = 0.006$).

The cultural and microscopic features of the fungal isolates are presented in Table 2. Isolate A produced mucoid, black yeast-like colonies with the presence of annelidic conidia, and septate hyphae while isolate B showed velvety, olivaceous-brown colonies with the presence of branched chains of conidia, septate hyphae. Isolate C exhibited grey-black colonies with large, muriform conidia bearing transverse and longitudinal septa and septate hyphae.

An *In vitro* pathogenic potential of the fungal isolates is presented in Table 3. The result revealed that *Exophiala jeanselmei* exhibited the highest pathogenic potential, showed strong growth at 37 °C, high resistance to Congo red (78-85%), and complete haemolysis of blood cells. Also, *Alternaria alternata* exhibited moderate virulence portraying incomplete thermotolerance and alpha haemolysis while *Cladosporium* species exhibited the lowest virulence, as revealed in its sensitive nature to Congo red, and gamma haemolysis.

Table 1. Distribution and prevalence of melanized fungi in different samples.

Sample	NS	NPS (%)	Isolate A	Isolate B	Isolate C	Total/Pre %	Overall χ^2	P value
Rotten wood	15	15 (100)	12(31.6)	14(36.80)	12(31.60)	38 (48.70)	10.23	0.006
Loamy soil	15	13(86.70)	4(16.00)	11(44.00)	10(40.00)	25(32.10)		
Clay soil	15	10(66.70)	2(13.30)	7(46.70)	6(40.00)	15(19.20)		
Total	45	38(84.40)	18(23.1)	32(41.00)	28 (35.90)	78(100)		

Note: NS, number of samples collected; NPS, number of positive samples; Pre, prevalence; isolate A, Exophiala jeanselmei; B, Cladosporium species; C, Alternaria alternata.

Table 2. Cultural and microscopic features of the fungal isolates.

Isolate Code	Cultural feature	Microscopic feature	Possible fungus
A	Grew slowly, mucoid, yeast-like, black colonies, black reverse	Septate hyphae, flask-shaped annellides, small oval conidia present	*Exophiala jeanselmei*
B	Grew moderately on PDA, olivaceous brown to black colonies, powdery, black reverse	Septate hyphae, branched chains of brown blastoconidia, oval shape with moderate conidia	*Cladosporium* species
C	Grew fast, wooly surface texture, grey-black colonies, black reverse	Septate hyphae, large brown muriform conidia	*Alternaria alternata*

Table 3. In vitro pathogenic potential of the fungal isolates.

Isolate	Growth at 37 °C	Congo red (100µg/ml)	Blood agar	Virulence
Exophiala jeanselmei	+++ (25-35 mm)/ strong	Resistant (78-85% growth)	Beta (High) or complete haemolysis	High
Cladosporium species	+/- (8-12 mm)/ weak	Sensitive (25-35% growth)	Gamma (zero haemolysis)	Low
Alternaria alternata	++ (15-22 mm)/ moderate	Moderate (50-65% growth)	Alpha (moderate haemolysis)	Moderate

Note: +++, profuse growth; ++, moderate growth; +/−, weak growth.

4 Discussion

Subcutaneous mycoses have been neglected by several people due to its chronic nature but are capable of causing debilitating diseases, especially among rural dwellers and immunocompromised individuals. In this study, the distribution and in vitro pathogenic potentials of melanized fungi were examined. The cultural and microscopic features of the fungi observed in the present study corroborate to the isolation of melanized fungi reported by several researchers (Revankar and Sutton, 2010; Yew et al., 2014).

This high distribution pattern of melanized fungi in rotten wood could be ascribed to high cellulose and lignin content and moisture retention in rotten wood, which supports saprophytic proliferation of the fungi as supported by Vicente et al. (2014). Among the isolates, *Cladosporium* species was the most frequent (41.0%), followed by *Alternaria alternata* (35.9%) while the lowest was *Exophiala jeanselmei* (23.1%). The prevalence of *Cladosporium* and *Alternaria* in soil is well documented as they are ubiquitous saprophytes while *Exophiala* is more selective and associated with hydrocarbon-rich, decaying wood (de Hoog et al., 2011). The high prevalence in rotten wood confirms it as a major reservoir for potentially pathogenic melanized fungi in the sampled communities.

In the present study, *Exophiala jeanselmei* exhibited strong growth at 37 °C, showing its ability to survive body temperature and cause fungal systemic mycosis, such as phaeohyphomycosis. This observation corresponds to the findings of Vicente et al. (2014). However, in *A. alternata*, the moderate growth observed at 37 °C clearly indicates that the fungus could be moderately pathogenic while poor growth in *Cladosporium* species indicates low pathogenic potential. The fungal cell wall evaluated using Congo red stain revealed that *E. jeanselmei* was highly resistant (78-85%, growth at 100 µg/ml), showing that the cell wall of the fungus is highly saturated with melanin, which is the chief determinant of microbial resistance and evasion of immune cells as reported by Vicente et al. (2014) and Seyedmousavi et al. (2014).

Similarly, haemolytic activity which facilitates iron to be removed from the host red blood cells showed complete in *E. jeanselmei*, incomplete in A. alternata and zero haemolysis in Cladosporium. Similar observation was documented by Pakshir et al. (2013). In rating virulence, *E. jeanselmei* was the most virulent followed by *A. alternata* while *Cladosporium* species was the lowest while rotten wood was a significant source of highly pathogenic environmental isolates of public health concern.

5 Conclusion

This study has observed that loamy soil, clay soil, and rotten wood are reservoirs of melanized fungi with varying pathogenic potentials *in vitro*. The ability of *E. jeanselmei* to grow at 37 °C, resist Congo red, and cause complete haemolysis clearly showed its opportunistic pathogenic potential. However, further study is highly essential to evaluate *in vivo* pathogenic potential of the fungal isolates, especially *E. jeanselmei*.

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Conflict of interest

The author and coauthors declared that there was conflict of interest observed throughout the period of this research.

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