DIVERSITY AND MOLECULAR CHARACTERIZATION OF KERATINOPHILIC FUNGI FROM DIFFERENT SOIL SAMPLES

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ABSTRACT

Keratinophilic fungi have been reported as one of the principal causes of cutaneous and sub cutaneous mycoses among individuals living in developing countries. This study was undertaken to determine the diversity, and molecularly characterized keratinophilic fungi from different soil samples in Uli community, Ihiala L.G.A, Anambra State. A total of 60 soil samples were collected randomly from different sites; poultry farm, goat farm and garden, and screened for the presence of keratinophilic fungi using hair baiting technique. The isolates obtained were characterized based on their macroscopic, microscopic and molecular characteristics. *Microsporum audouinii* strain INI48063 (MAINI), *Microsporium gypsuem* strain ATCC58595 (MGATCC), *Microsporum nanum* 5.8S rRNA gene (MNRNA), *Trichophyton mentagrophytes* strain DSM 108623 (TMDSM), *Trichophyton rubrum* strain AVMF11 (TRAVMF), *Trichophyton megninii* strain UAMH8542 (TMUAMH) were isolated from the soil samples, and the organisms were detected most in goat farms. TMDSM recorded significantly ($p<0.05$) the highest occurrences among the soil samples collected from poultry (9.26%) and goat (20.37%) farms, and TRAVMF recorded was significantly ($p<0.05$) seen most in the soil samples collected from garden (9.26%). TMUAMH and MNRNA were not detected in soil samples collected poultry farm. This study has shown the occurrences of keratinophilic fungi in the studied soil samples, and TMDSM was mostly seen in the samples.

INTRODUCTION

The term keratinophilic denotes fungi that use keratin as a susbstrate, dermatophytes, and exhibiting affinity for keratin. Keratinophilic fungi are natural colonizers of keratine substrates. They are ecologically an important group of fungi which could be found in soil. Some groups of these fungi are causative agents of cutaneous fungal infections named dermatophytosis. The prevalence of these fungi depends on different factors such as presence of creatine in soil, Ph and geographical location. Some of these fungi are well known to cause tinea infections which could be considered as a reservoir for human infection (Deshmukh and Verekar, 2006). During the past years, many researchers reported about isolation of keratinophilic fungi around the world. Also, a lot of reports were available about isolation of geophilic dermatophytes and keratinophilic fungi from soils of many parts of the world. Nowadays, most people spend their time having fun and are potentially at risk for direct contact with soil and being exposed to keratinophilic fungi (Ali-Shtaye and Jamous, 2000).

The human body is covered by skin, hair and nails which given their location in the body, are continuously exposed to the environment and consequenty, a wide variety of environmental microbes. The keratinized epithelia which comprise the outer layers of the skin constitute an effective barrier that excludes microorganisms from gaining entry into deeper tissues. However, a small number of fungi species have evolved mechanisms of overcoming defensive mechanisms and can actively colonize the surface, becoming established as members of the normal skin microbial flora. Fungi that can use the surface protein keratin are found on the skin and especially in nails and hair and they are so called keratinophilic fungi. Human to human transmission of keratinophilic fungi is the usual means of acquiring skin, hair and nail infections. Occasionally, animal to human transmission occurs called zoophilic infection. Recurrent infection after treatment is likely for zoophilic infection, if contact with the animal continues and that animal is not treated. In farmers and others working outdoors, infection may be acquired from soil. The most important such fungi are *Micosporum gypseum*, *M. fulvum* and *Sporothrix schenckii*, all known as geophilic fungi (Deshmukh and Verekar, 2006).

For isolation of keratinophilic fungi from soil, keratin or hair baiting technique is widely used. As for this procedure, different keratinous substrates are used as a bait to lure keratinophilic species (Sandeep and Geeta, 2016). Some species are potential pathogens, and can cause infections in the skin and scalp of mammals (the dermatophytes). These dermatophytes include the genera Microsporum and Trichophyton. It is thought that dermatophytes were initially saprophytic and lived in the soil, but due to increasing interactions with animals, they gradually evolved a parasitic lifestyle. The dermatophytes have been classified into three ecological groups based on their habitat preference they are geeophilic (soil loving), zoophilic (animal loving) and anthropophilic (human loving). Molecular studies based on the DNA sequence analysis of the ribosomal ITS region have shown that these three groups are also phylogenetically distinct (Kushwaha *et al*., 2007). It is in almost in any place in nature where there is possibility of having keratin, it can be found in cattle sheds, garbage, animal burrows, sewage, bird's nest,

barber's hair dumping area, public places like parks, schools, marketplace, etc., poultry sheds, herbivore or carnivore dung.

Several incidence of fungi diseases caused by keratinophilic fungi have been reported which were traced back to poor personal hygiene, direct contact with soil contaminated pig, goat, cow, waste and poultry droppings. Previous studies on keratinophilic fungi focused on the isolation and characterization of the organisms from different waste soil samples and the application of ethanol extract to them (Ali-Shtaye and Jamous, 2000). Also the resistance implications of some commercially synthetic antifungal agents pose the populace in serious threat. Some of the antifungal agents are rarely seen, some are not cost effective, and there are also cases of counterfeits among the available drugs. Researches had shown that most of these drugs are not environmental friendly. Therefore the need to search for non-resistance, readily available, cost effective and environmental friendly compounds from botanical origin could serve as alternative source.This study was designed to evaluate the susceptibility patterns of some selected medicinal plants against keratinophilic fungi isolated from different waste soil samples and to access the efficacy of water and ethanol extracts of *Eupatorium odorantum, Azadirachta indica* and *Garcinia kola* on the growth isolated fungi.

MATERIALS AND METHODS

Study Area: Uli town is located between latitudes 5.7833^0N and 6.8667^0E on the South eastern part of Nigeria. Uli is predominantly a low lying region on the Western plain of the Manu River with all parts at 333 metres above sea level. Uli has rainforest vegetation with two seasonal climatic conditions. They are the rainy and the dry season which is characterized by the harmattan between December and February. Uli is characterized by the annual double maxima of rainfall with a slight drop in either July or August known as dry spell or August break. The annual total rainfall is about1600 mm with a relative humidity of 80% at dawn. Uli has mean daily temperature of 18ºC, annual minimum and maximum temperature ranges are about 22ºC, and 34ºC respectively.

Collection of Samples: A total of 30 samples comprising of 10 samples each from the poultry farms, cattle farms, public gardens and sheep farms were collected randomly from Uli and its environs. The samples were collected from the superficial layer of soil at a depth not exceeding 3-5cm with sterile stainless spoon in sterile polythene bags, brought to the laboratory and processed within four hours of collection to ensure maximum recovery of the organism.

Hair Baiting Technique: Here, the soil samples from different wastes were used. The petri dishes were half filled with the soil and sterile defatted human or animal hair (not exceeding 20 years) was spread over the surface of the soil. Sterile distilled water was then added to the soil and then incubated at room temperature for 4 weeks in the dark. Sterlization of 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 autoclaved at 121ºC, 15 Psi for 15

minutes. The colonies generated from the plates were subcultured on SDA (Biotech) supplemented with chloramphenicol (50mg/L) and cyclohexamide (50mg/L) and incubated at room temperature for 7 days (Aziz and Seema, 2015).

Identification of the Isolates

Macroscopy: The colonies were carefully examined. The rate of growth, consistency, colour and texture of the surface growth, nature of the reverse side and other peculiar features of the colonies were noted (Aziz and Seema, 2015

Slide culture technique: Riddel's method as described by St-Germain and Summerbell (2000) was used. A filter paper was cut and placed on the bottom of the 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 121^0C 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 cover slip and incubated at room temperature for 7-10 days. After 10 days of growth, the cover slip 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 cover slip. The edges of the cover slip were sealed with nail polish to prevent evapouration of the stain. The slides were examined under the microscope. The isolates were identified using standard descriptions given by St-Germain and Summerbell, (2000) and Aziz and Seema, (2015).

Molecular Characterization of the Isolates

Extraction and purification of DNA: All strains were plated on Nutrient Agar (Biotech) and incubated at room temperature (30±2°C) for 5 days. Fungal genomic DNA was then extracted and purified using Zymo Research (ZR) DNA miniprepTM kit (Category No. D6005; Irvine, California, USA) (Lee *et al.,* 2009)

Determination of the quality of extracted DNA: This was carried out using mass spectrophotometer (Nanodrop). One micro litre (1µL) was aseptically dropped into a clean aperture in the chamber and the chamber was gently closed. The system was then connected to a computer system which displayed the window that revealed the quality of the sample at 260/280nm (Mohammed *et al.,* 2011)

Amplification of DNA and gel electrophoresis of PCR product: This was carried out using Master cycler Nexus Gradient (Eppendorf). A mixture of primer (20 µl), template DNA (20µl), water (72 µl) and master mix (108 µl), which comprises taq polymerase, DimethylSulfoxide (DMSO), magnesium chloride ($MgCl_2$) and nucleotides triphosphates (NdTPs), was prepared in 1.5 ml tube and homogenized using vortex mixer (Eppendorf). This was then placed in the block chamber of the master cycler and then programmed for the initial incubation, denaturation,

annealing, extension and final extension. The amplified products were electrophoresed in 1.0% agarose gel and a1kb DNA ladder was used as a size reference. After staining with 3µl of nucleic acid stain (GR green), the gel was documented with gel documentation apparatus (Mohammed *et al.,* 2011).

DNA sequencing of the amplicons: The amplicons generated were used for the sequencing using ABI DNA sequencer (Applied Biosystem Inc) at International Institute of Tropical Agriculture (IITA), Ibadan using the method of Mohammed *et al.* (2011).

Computational analysis: This was carried out using the modified method of Mohammed *et al.* (2011). The chromatograms generated from the sequences were cleaned to obtain regions with normal sequences. The cleaned nucleotides were aligned using pair wise alignment tool. The consensus sequences formed by the alignment of the forward and reverse sequences were used to perform the Basic Local Alignment Search Tool (BLAST) using National Centre for Biotechnology Information BLAST over the internet. The sequences of the isolates with 95% and above similarities were accepted. Also the maximum scores, total scores and accession numbers of the isolates were assessed. The relatedness of the isolates was determined by tracing their phylogenetic tree using DNA distance neighbor phylogenetic tree tool.

Prevalence of the Identified Isolate

The occurrences of the identified isolates was determined by tracing the macroscopic, microscopic and molecular identity of each isolates from individual sample, determining the number and percentage occurrences in each sample and total samples (Iheukwumere *et al*., 2018)

Statistical Analysis

The data obtained from this study was presented in percentage, and assessed for the significance using one way Analysis of variance (ANOVA) at 95% confidence level. The pairewise comparison was carried out using unpaired “T” test (Iheukwumere *et al*., 2018).

RESULTS

The present study revealed significant occurrences of keratinophilic fungi in the soil samples collected from poultry farms, goat farms and garden, of which the occurrence of the keratinophilic fungi was significantly ($P < 0.05$) seen most in the samples collected from goat farms whereas the samples collected from poultry farms showed least occurrences of keratinophilic fungi. Also, it was observed that 46.67% of the studied soil samples were positive for keratinophilic fungi (Table 1). Isolates 3MA, 3MB and 3MC showed similar microscopic characteristics. Macroscopically, isolate 3MA had slow growth rate cottony, grayish-white colour on the surface but rose-brown in reverse side. Isolate 3MB had rapid growth rate, powdery, whit- to buff colour on the surface but red in reverse side. Isolate 3MC had moderate-to-rapid growth rate, powdery, white to yellow colour on the surface but red-brown in reverse side. Isolates 3TA, 3TB and 3TC shared similar microscopic characteristics. They have pyriform and numerous micro conidia. Isolate 3TC had spiral hyphae. Macroscopically, isolates 3TA and

3TC were powdery, moderate to rapid growth rate whereas isolate 3TB was slow to moderate in its growth rate (Table 2). The present study revealed the concentrations and the qualities of DNA from the test fungal organisms (Table 3). The extracted DNA from 3MA, 3MB, 3MC, 3TA, 3TB and 3TC were within the stipulated range (1.80-1.90) as presented in Table 3. The agarose gel of electrophoresed amplicons is shown in figure 1. The regions coding for internal transcribed spacer rDNA (ITS1-1.58 ITS2), B-tublin (Ben A) and Calmodulin (Cam) were amplified and electrophoresed using 1.5% agarose. The photograph of the gel revealed clear amplification of the selected regions for sequencing of the isolates. The sequencing of the amplified regions of isolates 3MA, 3MB, 3MC, 3TA, 3TB and 3TC showed 100% identities of each of the isolates (Table 4). The study of revealed the presence of *Microsporum audouinii* strain INI48063 (Isolates 3MA), *Microsporum gypseum* strain ATCC58595 (Isolate 3MB), *Microsporum nanum* 5.8S rRNA gene, ITS1 and ITS2 (Isolate 3MC), *Trichophyton megninii* strain UAMH8542 (Isolate 3TA), *Trichophyton rubrum* strains AVMF11 (Isolate 3TB) and *Trichophyton mentagrophytes* strain DSM108623 (Isolate 3TC).

The present study revealed the distribution of the keratinophilic fungi in soil samples collected from poultry farms, goat farms and garden (Table 5.) The study revealed that *Tichophyton mentagriophytes* skin DSM 10863 (TMDSM) significantly ($P < 0.05$) recorded the highest occurrences among the six isolates encountered in the studied soil samples. TMDSM significantly ($P < 0.05$) occurred most in the samples collected from poultry and goat farms. *Microsporum andouinil* strain INI 148063 (MAINI), *Microspirum gypseum* strain ATCC 58595 (MGATCC), *Trichopyton megninil* strain UAMH 8542 (TMUAMA) and *Microspurum nanum* (MNITS) significantly ($P < 0.05$) recorded the highest occurrences from the soil samples collected from goat farms whereas soil samples from poultry farms whereas soil samples from poultry farms recorded their least occurrences. TVAMH was not seen in the samples collected from poultry farms and garden. Similarly MNITS was seen in the samples from poultry farms. *Trichophyton rubrum* strain AVMF 11 (TRAVMF) was seen most in the soil samples collected from garden whereas the least occurrence was seen in the samples collected from poultry.

Table 1: Soil samples that was positive for keratinophilic fungi

Soil source	**N = 20 Positive sample (%)**	**Negative sample (%)**	**Total (%)**
Poultry farm	40 (20.00)	16 (80.00)	20 (100.00)
Goat farm	16 (80.00)	4 (20.00)	20 (100.00)
Garden	8 (40.00)	12 (60.00)	20 (10.00)
Total	28 (46.67)	32 (53.33)	60(10.00)

Table 2: Macroscopic and macroscopic characteristics of the isolates

Isolate	Growth rate	Texture	Surface colour	Reverse colour	Macro conidia	Micro conidia
3TA	Moderate rapid	Powdery	Pale pink	Red	Pencil shape	Pyriform to round shape. Numerous
3TC	Moderate rapid	Powdery velvety	White to creamy-tan	Reddish-brown	Club-shape and few	Pyriform to round shape. Numerous, and with spiral hyphae
3TB	Slow to moderate rapid	Fluffy	White to pale pink	Wine red	Few. Smooth walled pencil-shaped	Pyriform and numerous
3MA	Slow	Cottony	Grayish-white	Rose-brown	Thick wall and spindle shape	Absent. Presence of terminal chlamydospore
3MB	Rapid	Powdery	White to buff	Red	Rough, thin walled and elliptical. Numerous	Club-shape and few
3MC	Moderate rapid	Powdery	White to yellow	Red-brown	Ovoid shape and numerous	Club-shape and few

Table 3: Quality of nucleic acid (DNA) used for study

Sample	Concentration of Nucleic acid (mg/ml)	A_{280}	A_{260}	260/280
3MA	68.40	0.4413	0.7980	1.81
3MB	78.50	0.5368	0.9966	1.86
3MC	76.60	0.5319	0.9980	1.88
3TA	71.70	0.5168	0.9420	1.82
3TB	64.30	0.4351	0.7880	1.81
3TC	84.10	0.5289	0.9760	1.85

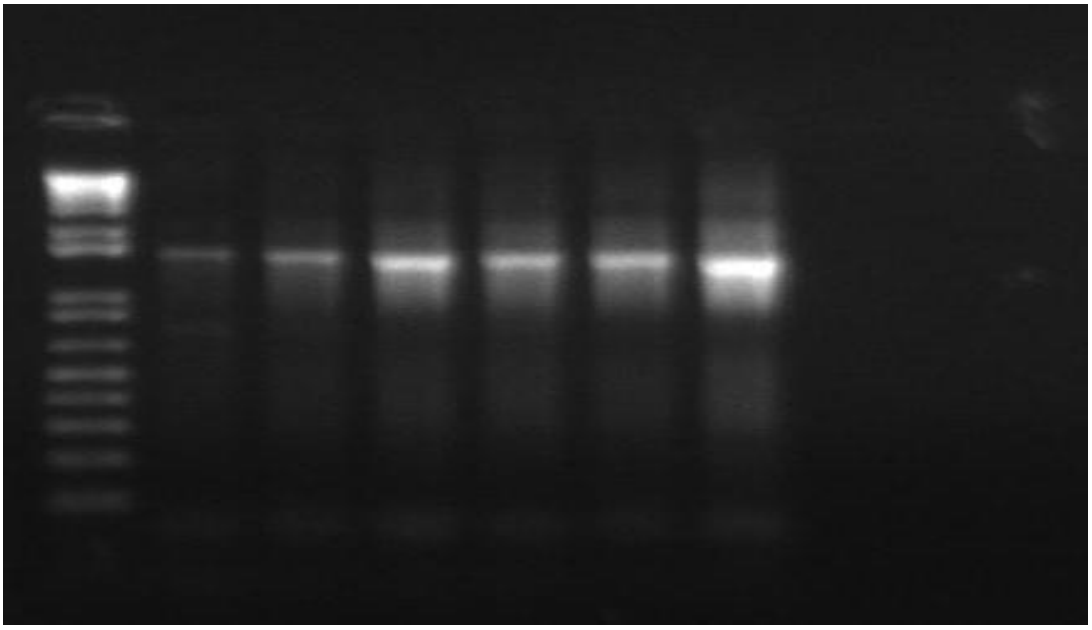

Figure 1: Agarose gel representation of cleaned amplicons for sequencing

Table 4: Molecular characteristics of the isolates

Isolate	Max Score	Total Score	Query Cover	E-Value	Identity	Description
3TA	1567	1567	100%	0.0	100%	*Trichophyton megninii* strain UAMH8542 (AF170463.1)
3TC	1269	1269	100%	0.0	100%	*Trichophyton mentagrophytes* strain DSM 108623 (MK 460545.1)
3TB	1114	1114	100%	0.0	100%	*Trichophyton rubrum* strain AVMF 11 (MK 461916.1)
3MA	1376	1376	100%	0.0	100%	*Microsporum audouinii* strain INI 48063 (KX 681682.1)
3MB	23176	23176	100%	0.0	100%	*Microsporum gypseum* strain atcc 58595 (FJ798800.1)
3MC	1208	1208	100%	0.0	100%	*Microsporum nanum* 5.85 S rRNA gene, ITS1 and ITS2

Table 5: Occurrences of the keratinophilic fungi in the studied soil samples

Isolate	**Poultry farm (%)**	**Goat farm (%)**	**Garden (%)**	**Total (%)**
Microsporum audouninii strain INI48063	2 (1.85)	8 (7.41)	4 (3.70)	14 (12.96)
Microsporum gypseum strain ATCC 58595	4 (3.70)	10 (0.26)	8(7.41)	22 (20.37)
Trichophyton rubrum strain AVMFII	6 (5.56)	8 (7.41)	10 (9.26)	24 (22.22)
Trichophyton mentagrophytes strain DMS 108623	10 (9.26)	22 (20.37)	2 (1.85)	34 (31.48)
Trichophyton megninii strain UAMH 8542	0 (0.00)	8 (7.41)	(0.00)	8 (741)
Microsporum nanum 5.8S rRNA gene, ITS1 and ITS2	0 (0.00)	4 (3.70)	2 (1.85)	6 (5.56)
Total	22 (20.37)	60 (55.56)	26 (24.07)	108 (100.00)

DISCUSSION

The significant occurrence of keratinophilic fungi in the soil samples from goat farms, poultry farm and garden in this study supported the findings of many researchers (Tanbekar *et al.,* 2007; Jain *et al.,* 2012; Pontes *et al.,* 2013; Sharma *et al.,* 2015; Jangid and Begum, 2018). The significant occurrence of the keratinophilic fungi in the soil samples collected from the goat farms agrees with the work of El-Said *et al.* (2009) and Awad (2007) but disagrees with the work of the Sharma and Choudhery, (2015). The occurrence of these keratinophilic fungi could be attributed to the abundant of keratinous materials in the soil samples, the warmth, humidity and the pH of the soil samples (Shukla, 2012), and these conditions favour the growth of these organisms most in the goat farms as seen in the present study.

The presence of *Trichophyton mentagrophytes* strain DSM 10863 (TMDSM), *Microsporum audouinii* strain INI 148063 (MAINI), *Microsporum gypseum* strain ATCC 58505 (MG ATCC), *Trichophyton megninii* strain UAMH 8542 (TMUAMH), *Microsporum nanum* (MN ITS) AND *Trichphyton rubrum* strain AVMF 11 (TRAVMF) supported the findings of many researchers

(Ganaie *et al.,* 2010; Kumar *et al.,* 2013; Pakshir *et al.,* 2013; Jangid *et al.,* 2018). Traditionally, the use of the selective or general purpose media in isolation of keratinophilic fungi does not provide hundred percent assurance of the identity of the isolate but the introduction of molecular technique provides more sensitive and rapid way of identifying the isolates (Iheukwumere *et al.,* 2017).

The highest occurrence of TMDSM in the soil samples collected from poultry and goat farms could be attributed to the soil conditions such as pH, moisture, warmth nature and the abundant of keratinous materials which favour the growth TMDSM in thr soil samples more than other keratinophilic fungi isolated from the samples. Several researchers (Hossein *et al.,* 2006; Ganaie *et al.,* 2010; Jangid *et al.,* 2018) reported the most abundant of *Trichophyton mentagrophytes* in the soil samples. Also *Trichophyton mentagrophytes* is both zoophilic and anthropophilic dermatophytes and this could make this organism more diverse than other isolates.

The predominant of TRAVMF in the soil samples collected from garden could be attributed to human activities in the garden. *Trichophyton mentagrophytes* is an anthropophilic dermatophyte that causes about 70% infection in humans. Several researchers (Ganaie *et al.,* 2010; Sharma and Choudhary, 2015; Jangid *et al.,* 2018) reported predominant occurrence of *Trichophyton mentagrophytes* in the soil samples.

CONCLUSION

This study has shown the presence of *Microsporum audouinii* strain INI48063 (MAINI), *Microsporium gypsuem* strain ATCC58595 (MGATCC), *Microsporum nanum* 5.8S rRNA gene (MNRNA), *Trichophyton mentagrophytes* strain DSM 108623 (TMDSM), *Trichophyton rubrum* strain AVMF11 (TRAVMF), *Trichophyton megninii* strain UAMH8542 (TMUAMH) in studied soil samples, of which TMDSM was the most predominant isolate mostly in goat and poultry farms. The abundant of this organism warrants a renewed look into the antibiogram of the isolates in order to combat their infections.

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