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Molecular identification of dermatophytes isolated from sheep and goats in Zaria, Nigeria

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Abstract

Dermatophytosis is a contagious superficial mycosis caused by a group related fungi in the Genera *Trichophyton*, *Microsporum* and *Epidermophyton*. Early and accurate identification of the causative agent of dermatophytosis is essential for effective treatment and prevention of the disease. Genotypic techniques are more rapid, sensitive and specific than the conventional methods. However, there is a paucity of information on molecular characterization of dermatophytes from domesticated ruminants, particularly in northern Nigeria. The aim of this work was to carry out molecular identification of dermatophytes isolated from sheep and goats in Zaria. Ninety-four goats and 63 sheep skin scrapings were aseptically collected from suspected cases of deramtophytosis. Each specimen was processed for direct microscopic examination and, cultural isolation and phenotypic identification of etiologic agents. The identified dermatophyte isolates were subjected to DNA extraction followed by amplification of the internal transcribed spacer (ITS) regions of their ribosomal DNA. The dermatophytes were identified by comparing the sizes of the amplified ITS regions of each isolate with ITS regions sizes of dermatophytes reported in the literature. Out of the 34 dermatophyte isolates from both sheep and goats, 23 were phenotypically identified as *Trichophyton mentagrophytes* while 11 were *T. verrucosum.* The PCR based technique identified 23 of the isolates as *T. mentagrophytes* while 11 were identified as *T. verrucosum* in complete agreement with the culture method. The sizes of the ITS regions of all the *T. mentagrophytes* and *T. verrucosum* were approximately 600bp except one isolate of *T. mentagrophytes* which had ITS region size of approximately 550bp. *Trichophyton verrucosum* and *T. mentagrophytes* isolated from sheep and goats were successfully identified by PCR method targeting the ITS regions of their ribosomal DNA. As far as we know, this is the first report of molecular identification of dermatophytes isolates from small ruminants in northern Nigeria.

Keywords: Dermatophytes, Goats, Identification, Molecular, Sheep, Zaria

Introduction

The dermatophytes are a group of closely related keratinophilic fungi that have similar appearance as well as infectivity and pathogenicity (Weitzman & Summerbell, 1995). Infection with any of the pathogenic dermatophyte species result in dermatophytosis, a highly contagious superficial skin disease that affects both man and animal with grave economic impact (Hubka *et al*., 2018; Fawzi *et al*., 2023). Dermatophytosis is zoonotic and affects about 25% of the world population, with an annual USD500 million spent worldwide for diagnosis, treatment and prevention of the disease (Hameed *et al*., 2017; Dalis *et al*., 2018).

The dermatophytes are classified based on their microscopic appearance into three anamorphic genera as *Microsporum*, *Trichophyton* and *Epidermophyton* (Weitzman & Summerbell, 1995; Hubka *et al*., 2018). However, a mutilocus phylogenetic study of the family *Arthrodermataceae* revealed that the genus *Trichophyton* is polyphyletic and therefore, cannot be placed in the same taxonomic group (De-Hoog *et al*., 2017). The multilocus phylogenetic studies recognized seven genera of dermatophytes and dermatophyte relatives, including: *Trichophyton*, *Microsporum*, *Epidermophyton*, N*annizzia*, *Lophophyton*, *Paraphyton* and *Arthroderma*. Nevertheless, all anthropophilic dermatophyte species together with several zoophilic species usually associated with human infections are retained in the first three genera, whereas most geophilic species as well as zoophilic organisms which rarely cause disease in humans are divided among *Nannizzia*, *Lophophyton*, *Paraphyton* and *Arthroderma* (De-Hoog *et al*., 2017).

Accurate identification of the dermatophyte isolated from clinical specimen is essential for appropriate antifungal therapy because of the length of treatment, potential side effects of the drug and their high cost. Moreover, having information on zoophilic or anthropophilic sources of the causative organism may allow prophylactic measures such as treatment of both human and animal reservoirs (Frias-De-Leon *et al*., 2020).

The conventional methods for the diagnosis of dermatophytosis are usually based on clinical signs, macroscopic and microscopic morphology of isolated dermatophyte supplemented with physiological and biochemical tests (Robert & Pihet, 2008; Hameed *et al*., 2017). However, these phenotypic characteristics are easily affected by external factors such as variation of incubation temperature, isolation media and antifungal treatment which make identification difficult. Molecular methods which are based on genotypic differences are considered more stable and precise and are therefore, better for the characterization of dermatophyte species and strains (Frias-De-Leon *et al*., 2020).

The main genetic markers that are targets for the identification of dermatophytes are: the internal transcribed spacer (ITS) regions of ribosomal RNA (rRNA) gene, (Jackson *et al*., 1999; Ebihara *et al*., 2009), chitin synthase 1 (*CHS1*) (Hirai *et al*., 2003; Dhib *et al*., 2012), DNA topoisomerase II (*TOP-II*) (Kamiya *et al*., 2004), beta tubulin (*BT2*) (Rezaei-Matehkolaei *et al*., 2014) and translation elongation factor 1-*α* (*Tef-1α*) genes (Rezaei-Matehkolaei *et al*., 2012).

Phylogenetic analysis and identification of dermatophytes based on sequencing of the internal transcribed spacer (ITS) regions of rDNA has proven to be useful as a gold standard method for characterization of dermatophyte (Graser *et al*., 1999; Dalis *et al*., 2018; De-Hoog *et al*., 2018). This is because these regions contain sufficient sequence heterogeneity to provide differences at the species level. In general, strains of the same species have less than 1% nucleotide substitutions in the region of interest, whereas strains that are separate species have more than this number of substitutions (Kurtzman, 1994; Valente *et al*., 1999).

In spite of the obvious need to adopt the nucleic acid technology for diagnosis of dermatophytosis in our society, reports on molecular identification of dermatophytes from animals particularly in northern Nigeria, are scanty (Emenuga & Oyeka, 2013; Dalis *et al*., 2018;). This paper describes the identification of dermatophytes isolated from sheep and goats using PCR, targeting the ITS regions of the ribosomal RNA gene.

Materials and Methods

Study area

This study was performed on sheep and goats in Zaria Local Government Area, Kaduna State, Nigeria. Its geographical coordinates are 11o 4’ 0” North and 7^{o} 4’0” East

Selection of sampling units and determination of sample size

The purposive sampling method of Martin *et al*. (1994) whereby only animals showing observable skin lesions such as circumscribed alopecia, erythema, scaling or thickly- crusted, greyish-white skin lesions suggestive of dermatophytosis were included in the sample. All other animals which did not meet the inclusion criteria were excluded from the experiment.

Assuming an expected prevalence of 8.9% for goats and 7.0% for sheep (Nweze, 2011), the number of specimens to be collected for each animal species was determined using the formula: $n= Z^2pq/L^2$, where n= required sample size, Z = 1.96, p = expected prevalence, q = (1-p) and L = allowable error of 5% (Thrusfield, 1997).

One hundred and fifty seven skin scrapings including 94 from goats and 63 from sheep suspected of clinical dermatophytosis were aseptically by first, cleaning the lesion with cotton wool soaked in 70% alcohol and the samples were obtained by scarping skin scales at the edges of the lesions into clean envelops, labeled and transported at room temperature to the Veterinary Microbiology Laboratory, Faculty of Veterinary Medicine, Ahnadu Bello University, Zaria, for processing, and ensuring that the samples were not exposed to moisture to prevent the growth of bacteria and saprophytic fungal contaminants.

Laboratory processing of samples

Each of the specimens was divided into two parts. One part was used for direct microscopic examination while the other part was used for isolation of the causative dermatophytes in culture.

The portion for direct microscopic examination was placed on a clean glass slide containing two drops of 10% potassium hydroxide. A coverslip was applied to the preparation and allowed to stand on the bench for 5 to 10 minutes for proper digestion and clearing of the tissue cells to allow visualization of fungal elements and then examined microscopically for the presence of hyaline septate hyphae in skin scales or arthrospores occurring inside or outside infected hair as described by Tandon *et al*. (2023).

Isolation of etiologic dermatophytes in culture

Isolation of the dermatophytes was carried out by placing each of the samples in a Petri dish containing Sabouraud's dextrose agar (SDA), (OXOID CM0041; Oxoid Ltd, Basingstoke Hampshire-England) incorporated with chloramphenicol at the rate of 16µg/mL and cycloheximide at the rate of 0.5mg/mL using a pair of sterile forceps. The chloramphenicol was included to inhibit the growth of bacteria while cycloheximide prevented the growth of unwanted saprophytic fungal contaminants (Fawzi *et al.,* 2023). The specimen was pressed down gently to ensure adequate contact between the sample and medium. The plates were sealed with masking tape, incubated at room temperature and observed for fungal growth every three days for a period of 14 to 30 days. Macroscopic features of the isolates such as the colony pigmentation, topography, texture and rate of growth were noted.

Microscopic identification of dermatophyte isolates

A portion of mycelium was removed from the colony using a Pasteur pin and teased in a drop of lactophenol cotton blue stain on a clean glass slide. A coverslip was applied, gently pressed down to remove air bubbles which might interfere with fungal identification and then examined with the x10 and x40 objectives of a light microscope (Nikon, ECLIPSE-E100, 824592, China). Microscopic characteristics of the isolates such as size, shape and arrangement of their macroconidia and microconidia, spirals, nodular organs and pectinate branches were recorded.

The isolates were identified based on their macroscopic and microscopic morphology as described by Kane *et al*. (1997). Colonies of *Trichophyton verrucosum* on SDA are slow growing, small, button-like, white-cream colored, a raised centre and flat periphery with some submerged growth. Microscopically, *T. verrucosum* forms hyphae with many chlamydoconidia often in chains and some pectinate branches. Some strains produce small, delicate, single microconidia. Macroconidia are rarely produced, but when present they are sinuous (having many curves) and have a characteristic string bean shape (Nwiyi *et al*., 2021). However, colonies of *T. mentagrophytes* are white, flat and powdery with yellow reverse side. Microscopically, *T. mentagrophytes* produces many microconidia that tend to arrange in loose, grape-like clusters. Macroconidia are elongated, pencil-shaped, thin, and smooth walled (Tang *et al*., 2021).

The phenotypically identified dermatophyte isolates were transported in sterile containers at room temperature to the Biotechnology Division, National Veterinary Research Institute, Vom, for molecular identification.

DNA extraction

Each of the phenotypically identified dermatophyte isolate was sub-cultured into a sample bottle containing Sabouraud's dextrose broth and incubated at room temperature for 10 days. The fungal growth was harvested, washed with distilled water and grinded in a mortar. Fungal DNA was isolated using ZR Fungal/Bacterial DNA (Zymo Research Corporation), based on the manufacturer's instructions. Briefly, approximately 70mg of ground fungal mycelium was mixed in 200µL of phosphate buffered saline (PBS) in a lysis tube and 750 µL of lysis solution was added. The preparation was attached to a bead beater fitted with a 2mL tube holder assembly and mixed for 5

minutes using a vortex mixer. The lysis tube was centrifuged in a micro centrifuge at 10,000g for 1 minute. 400 µL of the supernatant was transferred to a spin filter in a collection tube and centrifuged at 7,000 g for 1 minute. 200 µL of fungal DNA binding buffer was added to the filtrate in the collection tube and centrifuged at 7,000 g for 1 minute. 800 µL of the mixture was transferred to a column in a collection tube and centrifuged at 10,000 rpm (8000 g) for 1 minute. The flow through was discarded from the collection tube and centrifuged at 10,000 rpm (8000 g) for 1 minute. 200 µL DNA pre-wash buffer was added to the column in a new collection tube and centrifuged at 10,000 rpm (8000 g) for 1 minute. 500 µL of fungal DNA wash buffer was added to the column and centrifuged at 10,000 rpm (8000 g) for 1 minute. The column was transferred to a clean 1.5mL microcentrifuge tube and 100 µL DNA elution buffer was added directly to the column matrix. The preparation was centrifuged at 10,000 g for 1 minute.The column was discarded and the eluted DNA was stored at -20^{o}C until used.

Amplification of the internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) of dermatophytes using polymerase chain reaction (PCR)

The amplification of the internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) was carried out with primers ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') forward and ITS-4 (5' TCCTCCGCTTATTGATATGC-3') reverse as described by Dalis *et al.* (2018). Amplification reactions were carried out in final volumes of 50 µL which contain 5µL of template DNA, 5x One Taq Standard Reaction Buffer (20mM Tris-HCl [pH 8.9 @ 25^{o}C],1.8mM MgCl2, 22mM NH4Cl, 22mM KCl, 0.06% IGEPAL CA-630, 0.05% Tween 20), 2.5mM (each) dTTP, dGTP, dCTP and dATP, 20µM each of ITS1 and ITS4 primers and 2.5U of One Taq DNA polymerase. PCR was performed in a 96 well Gene Amp PCR System 9700(Applied Biosciences) with initial denaturation at 95^{o}C for 2 minutes, followed by 35 cycles of denaturation at 95^{o}C for 1 minute, annealing at 55^{o}C for 1 minute, extension at 72^{o}C for 2 minutes and then a final extension at 72^{o}C for 10 minutes. PCR products were separated by electrophoresis in 1.5% agarose gels incorporated with ethidium bromide and documented using Gel Doc (BIORAD Laboratories).

The dermatophytes were identified by comparing the amplicon sizes of the ITS regions of their ribosomal DNA with those reported in the literature.

Results

Out of the 94 goat samples tested, 21 (22.3%) dermatophytes were isolated including 14 (66.7%) *Trichophyton mentagrophytes* and 7(33.3%) *T. verrucosum*. Similarly, of the 63 sheep samples processed, 13 (20.6%) dermatophytes were isolated including 9 (69.2%) *T. mentagrophytes* and 4 (30.8%) *T. verrucosum*. A total of 21.7% (34/157) dermatophyte islation rate from sheep and goats was obtained in the study area (Table 1).

The PCR targeting the internal transcribed spacer regions of dermatophytes successfully identified 23 dermatophyte isolates to be *T. mentgrophytes* while 11 of the isolates were identified as *T. verrucosum* in concordance with the culture based technique. The length of the ITS regions of both *T. mentagrophytes* and *T. verrucosum* were 600bp except one isolate of *T. mentagrophytes* which had ITS regions size of approximately 550bp. This isolate may be a different genotype occurring among the *T. mentagrophytes* population in the study area, since it produces colony and microscopic features typical and inseparable from other isolates identified phenotypically as *T. mentagrophytes*. The gel picture shows bright bands in lanes 4, 5, 6, 8, 9, 10, 12, 13, 15 and 16 while faint bands were observed in lanes 1, 2, 3, 7, 11 and 14. The faint bands may be due to low DNA concentrations resulting from poor cellular content of the samples (Figure 1).

Discussion

The 20.6% dermatophyte isolation rate in sheep and 22.3% in goats, in this study is higher than the 7.0% and 8.9% dermatophyte isolation rates in sheep and goats respectively reported by Nweze (2011) who studied dermatophytoses in domestic animals in

Table 1: Isolation rates of *T. mentagrophytes* and *T. verrucosum* in sheep and goats in Zaria

Animal (No. of samples tested)	Dermatophyte species	Isolated (%)	Total (%)
	T. mentagrophytes	*T. verrucosum*	
Goats (94)	14 (66.7)	7 **(**33.3**)**	21 (22.3**)**
Sheep (63)	9 (69.2)	4 (30.8**)**	13 (20.6)
Total (157)	23	11	34 (21.7%)

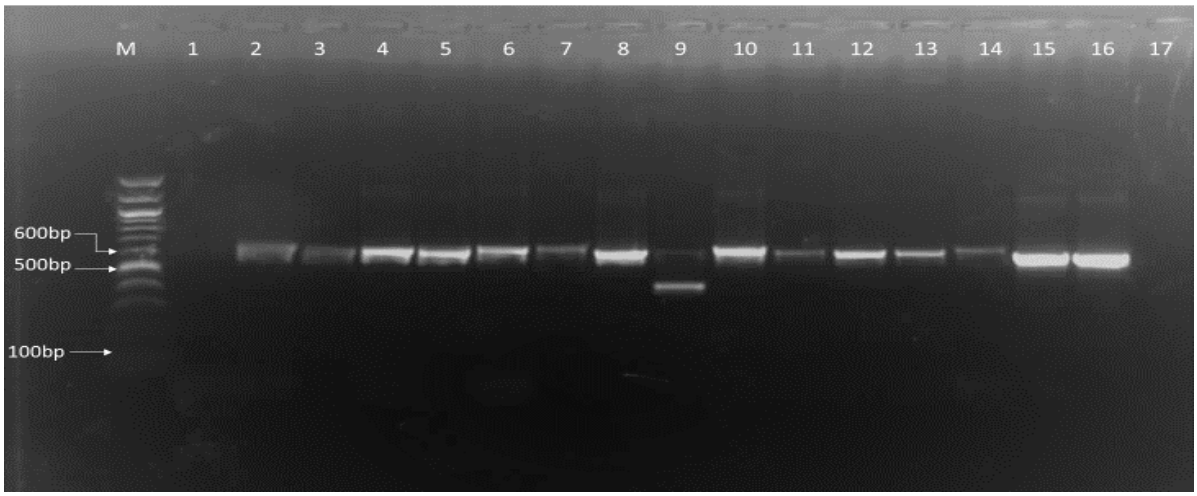

Figure 1: Agarose gel electrophoresis showing amplification of the 600bp ITS regions of *T. mentagrophytes* by PCR. Lane M (molecular marker), lanes 1-16, phenotypically identified isolates of *T. mentagrophytes*, lane 17 (negative control)

seven States predominantly from southern Nigeria, suggesting that the disease may be a health problem among small ruminants in the study area. The variations in the isolation rates may be attributed to differences in location of the animals studied. It has been found that the prevalence of dermatophytosis among animals may vary from one geographical location to another (Dalis *et al*., 2018; Hubka *et al*., 2018).

The increase in superficial mycoses among human and animal population, coupled with the emergence of antifungal resistance has further emphasized the need for diagnostic methods that can rapidly and accurately identify fungal pathogens (Bishnoi *et al*., 2018).

In this study, the PCR targeting the internal transcribed spacer (ITS) regions of dermatophytes yielded amplicon sizes of approximately 600bp for both *T. mentagrophytes* and *T.verrucosum*, except one isolate of *T. mentagrophytes* which was found to have ITS size of approximately 550bp. This finding concurs with the work of Jha *et al*. (2012) who employed the use of PCR targeting the ITS regions of the ribosomal DNA of dermatophytes to identify seven *Trichophyton verrucosum* isolated from domestic animals and seven isolates of *T. verrucosum* isolated from humans. They found that, the sizes of the ITS regions of all the animal isolates of *T. verrucosum* were approximately 600bp whereas the sizes of the ITS regions of all the human isolates of *T. verrucosum* were approximately 680bp in length.

It is also in agreement with the report of Frias-De-Leon *et al*. (2020) who carried out molecular identification of *T. mentagrophytes* complex and found amplification products of their ITS regions to be approximately 600bp in size.

We do not know why one of the isolates which was found to produce colony and microscopic features, typical of *T. mentagrophytes*, should have a smaller ITS regions size of 550bp. Perhaps this may be an atypical isolate occurring among the *T. mentagrophytes* population. According to Svarcova *et al*. (2023), several *Trichophyton* species, including *T. mentagrophytes*, *T. erinacei*, *T. simii, T. africanum* and *T. benhamiae* are believed to retain their ability to reproduce sexually. Therefore, higher level of intraspecific genetic and phenotypic variability generated by the sexual reproduction is found in these species. Meanwhile, Klinger *et al*. (2021) have identified 28 ITS genotypes among *T. mentagrophytes/T. interdigitale* isolates, five of which are considered to be *T. interdigitale*.

It is pertinent to state that Mirzahoseini *et al*. (2009) had similarly reported the occurrence of an isolate of *T. verrucosum* having ITS region size of approximately 550bp when PCR-RFLP was used to identify pathogenic dermatophytes from clinical specimens. In situations where DNA is extracted directly from the clinical specimen without culturing, post PCR processing such as DNA hybridization with specific probes, restriction fragment length polymorphism or sequencing of the ITS regions may be required in order to clearly differentiate between *T. verrucosum* and *T. mentagrophytes* particularly when atypical strains are encountered. For example, Dalis *et al.* (2018) in a study of molecular characterization of dermatophytes isolated from cattle in Plateau State, Nigeria, used the restriction enzyme *Mva*1, in a PCR-

RFLP of the amplified ITS regions of dermatophytes to clearly differentiate between *T. verrucosum* and *T. mentagrophytes*.

Since treatment and prophylactic measures against dermatophytosis may be dependent on the infecting dermatophyte species (Hubka *et al*., 2018), the need for quick and accurate identification of the causative agent cannot be overemphasized. It was concluded that, the PCR base molecular technique successfully identified 23 *Trichophyton mentagrophytes* and 11 *Trichophyton verrucosum* isolated from sheep and goats in Zaria. To the best of our knowledge, this is the first report of molecular identification of dermatophytes from small ruminants in northern Nigeria. Sequencing of the amplified ITS regions and its comparison with ITS sequences available in the data base for confirmation of dermatophyte species was recommended.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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