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Isolation and Molecular Characterization of Dermatophytes in Donkeys

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Authors' contributions

This work was carried out in collaboration between all authors. Author EAS designed the study, wrote the protocol and the first draft of the manuscript. Author WGA and EAS managed the analyses of the study. Author AHA managed the literature searches. All authors shared the laboratory work read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Background: Dermatophytosis is a fungal infection of skin, hair and nail caused by dermatophytes. The zoonotic nature of the disease requires early detection for implication of treatment.
Aim: The study was conducted to investigate into the cause of skin infection among donkeys in Eldamazine State, Central Sudan.
Study Design: This a case history studies over one year duration.
Methodology: A total of 31 samples of skin scrapings from donkeys with an age ranged 2-20years, from Eldamazie state, Sudan,were collected. Samples were cultured onto Sabouraud'S (SDA) media. The developed colonies were identified by convention method and characterized by molecular techniques using polymerase chain reaction.
Results: *Trichophyton mentagrophytes* and *Trichophyton verrucosum* were the predominant isolated dermatophytes revealed on investigation. Amplification of the β-tubulin gene, by polymerase chain reaction, and analysis of the amplicon sequence, further confirmed their

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identification. The study revealed first report on characterization of these isolates by molecular methods using β- tubulin primers.
Conclusion: The present investigation showed that the integration of different methods and techniques led to identification of useful molecular marker for standardization of taxonomical studies of *dermat*ophyte species.

Keywords: Dermatophytes; βeta tubulin primers; conventional methods; PCR.

1. INTRODUCTION

Dermatophytosis (ringworm or tinea), is a superficial skin infection caused by closely related keratinophilic fungi [1]. They have the ability to degrade keratin and invade the skin and its appendages [2]. Ringworm is a major Public and Animal Health problem in various regions of the world resulting in great economic loss [3]. *T. mentagrophytes* var, erinacei accommodates the anthropophilic and zoophilic strains [4]. In man, *T. mentagrophytes* and *Microsporum canis* were reported to be the most common dermatophytes responsible for tinea infections in man [5,6]. Animals can get infection from soil while digging, rolling, and lying down. Infection depends on the fungal species, age, and health, condition of exposed skin surfaces, grooming behavior, and nutrition of the animal. However, broken hairs with associated spores are important sources for spread of the disease [7]. The identification of dermatophytes is based on methods that focus on morphological, physiological, ecological, and genetic characteristics [8,9,10].

1.1 Prevalence of Dermatophytosis in Donkeys

Donkeys (*Equus asinus*) are of great economic value being used for income generating activities. They can be used for riding, pack transport, pulling carts and for farm work [11]. Pack donkeys are an actual promising choice for transport in third world countries [12]. Despite their great value, they are unfortunately not given enough veterinary attention although they suffer many health problems and diseases such dermatophytosis affecting their viability and ability to work. Dermatophytes are known to grow best in warm and humid environments and are therefore more common in tropical and subtropical regions and may present in epidemic proportions in areas with high rates of humiditybut the geographic distribution varies according to the species [13,14]. Reports on ringworm in donkeys are scanty and refer to zoonotic agents such as *T. mentagrophytes* and *T. verrucosum*. Recently *Microsporum racemosum* has been isolated from naturally infected donkeys [15]. In the Sudan, *T. verrucosum* has been isolated from donkeys ringworm [16]. Additionally, a severe outbreak of ringworm among 69 adult domestic donkeys in Sudan was described due to *T. mentagrophytes* [17]. However, in horses infections are common. *Trichophyton equinum* and *T. mentagrophytes* were identified as the primary cause of ringworm in horses [18,10]. *M. gypseum*, *M canis*, and *T.verrucosum* have also been isolated [19,6]. Moreover, *Trichophyton* and *Microsporum* species associated with apparently normal horses' hair from Riyadh, KSA were isolated [20]. Recently, *T. mentagrophyes var. mentagrophyes* causing Ringworm in Horses in Al Ahsa Province, Kingdom of Saudi Arabia has been isolated [21].

2. MATERIALS AND METHODS

2.1 Clinical Examination

Thirty-one donkeys from Eldamazine city, central of the Sudan, in the age of 2-20 years, clinically suspected as having dermatophytosis were randomly selected for this study.

Skin lesions of suspected animalswereexamined and a complete clinical examination of all affected animals, was performed. The shape, size, position and distribution of lesions were observed.

2.2 Mycological Investigations

2.2.1 Macro and microscopic study

These included collection of samples, direct examination and culture. Affected areas were cleaned and disinfected with 70% ethyl alcohol. Then, skin scrapings and hair plucks were taken from the active margins of the lesions using sterile disposable scalpel blades as described previously [22]. The samples were transported to Central Veterinary Research Laboratory (CVRL)

located at Soba using clean, dry sterile Petri dish. Wet mounts were prepared from specimens with 20% potassium hydroxide for direct microscopic examination. Cultures were made onto duplicate sets of Sabouraud's dextrose agar (SDA) plates supplemented with 0.05 mg/ml chloramphenicol and 0.5 mg/ml cycloheximide and incubated aerobically at 27and 37°C. Cultures were observed daily for growth of dermatophytes for up to four weeks. For more studies on cultural features, potato dextrose agar (PDA),) cornmeal agar (Oxoid) and 5% horse blood agar were used. The plates showing no growth were discarded after 28 days of incubation while those showed growth were indentified on the basis of their colonial and morphological characteristics including the growth rate, colony morphology; colour, shape, size and observe and reverse sides [8].

2.2.2 Biochemical study

Urease hydrolysis was done by inoculated parts of colonies from SDA on Christensen urea agar (Difco) to further confirm the identity of the isolated dermatophyte species [23].

2.2.3 Molecular study

Further, the isolated fungi were characterized by molecular markers using (ßeta tubulin gene).

2.3.3.1 Molecular identification of isolates

2.3.3.1.1 DNA extraction and purification

The genomic DNA was extracted and purified from skin scrapings10 days- old culture media following Möller *et al.,* method [24]. Briefly, mycelia were harvested from the surface of Sabouraud's dextrose agar culture, transferred to a mortar, supplemented with liquid nitrogen and ground to powder with a pestle. About 45 mg of the mycelia powder, was transferred to 2.0 ml micro tube, supplemented with 500 µl TES (100 mMTris, pH 8.0, 10 mM EDTA, 2% SDS) followed by addition of 3.75 µl Proteinase K (stock 20mg/ml; w/v; Promega), before incubation for 1 hour at 60°C with occasional gentle shaking of the tube. The reaction mix was then supplemented with 140 µl 5M NaCl, 700 µl chloroform: isoamyl alcohol (24:1, v/v;) and incubated for 10 min at 65ºC. Tubes were gently shaken, incubated for 30 min at 0ºC and then centrifugated for 10 min in 4ºC centrifuge adjusted at 12,000 rpm. The supernatant was then transferred to 1.5 ml micro-tube, supplemented with 225 µl 5 M NH_4Ac (Ammonium Acetate), mixed by gently finger mixing, placed on ice for one hour, before centrifugation at the same former conditions. The supernatant was transferred to a fresh 1.5 ml micro-tube, supplemented with 500 µl isopropanol, placed on ice for 30 minutes, centrifuged for 5 min at the same former conditions. Finally the supernatant was decanted, the pellet washed twice with cold 70% ethanol, dried at room temperature, dissolved with 50 µl TE (10 mMTris-HCl, 1 mM disodium EDTA, pH 8.0) and stored at -20ºC for later experiments.

2.3.3.1.2 Amplification of the β-tubulin gene PCR

Primers

A pair of primers, ßt2a and ßt2b synthesized by Invitrogen, were used [25].Their sequences are

5'-GGTAACCAAATCGGTGCTGCTTTC-3' and
5'-ACCCTCAGTGTAGTGACCCTTGGC-3'respectively.

Reaction components

DreamTaq Green PCR Master Mix (2X) (Fermentas, Cat. # K1072) that contains all components in a volume of 25 µl, except template DNA and primers was used. A number of four master mix tubes were used to make the reaction mix in a volume of 50 µl; each tube received 1.5µl of each primer, 5 µl of the respective template DNA and 17µl nuclease free H_2O.

Thermocycling conditions

The PCR was performed with a TC-512 (Techne) thermocycler following Samson, *et al.,* method [26]. The heat lid temperature was adjusted to 104°C. The thermocycling conditions were one cycle of 94°C for 1 min; 32 cycles of 94°C for 1 min, 68°C for 1 min and 72°C for 1 min; and one cycle of 72°C for 5 min.

Agarose gel

1.5% agarose (Vivantis) in 0.5x TBE (Tris Borate EDTA) supplemented with ethidium bromide (10 mg/ml, Vivantis) to a final concentration of 0.5µg/ml was used.

Electrophoresis

A Horizontal mini-gel electrophoresis system was used. One well was loaded with 5µl of 100 bp DNA size marker (Vivantis), and the other wells loaded with 8 µl of each PCR amplicon mixed with 2 µl of loading dye 6x (Vivantis). The power supply was adjusted to 80 V and the DNA was left to be separated for 45 min.

Visualization of resolved DNA

The separated DNA bands were visualized and photographed by a gel documentation system (Ingenius, Syngene Bio Imaging).

2.3.3.1.3 Sequencing and sequence analysis

The PCR amplicons were sent to JBMI (JEONJU BIOMATERIALS INSTITUTE) for automated bidirectional sequencing using the ßt2a and ßt2b primers. The sequences were analyzed by the BioEdit software package and the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov).

3. RESULTS

3.1 Case History

Infected animals showed skin lesions started as alopecia, severe incrustation, scaling, on the flanks backs, face, ears legs and gluteal areas similar to dermatophyte lesion. Other lesions of ringworm were observed on the withersand saddle area, but the infection wasspread to the neck, chest and head with one or more legs being involved (Figs. 1 and 2).

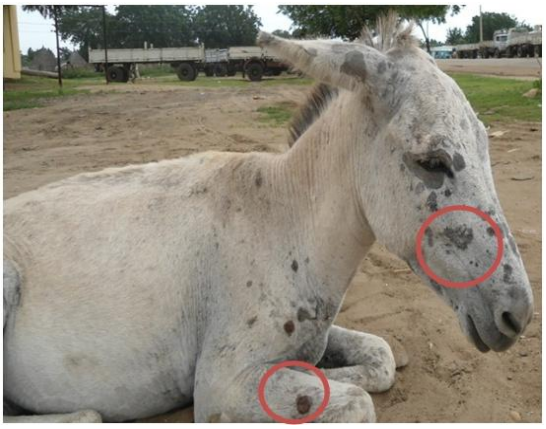

Fig. 1. Localized skin lesions of dermatophytes on the face, neck and fore limbs

3.2 Morphological Characterization

3.2.1 Macroscopic characters

Two different isolated colonies were obtained on SDA, macroscopic feature of the isolated fungi for isolate 1 showed moderate rapid growth of a white to creamy flat powdery colony (Fig. 3). At 7 days on incubation, nodular granular white to creamycolony on obverse and a pale yellowish on reverse was observed (Fig. 4).

Fig. 2. Generalized skin lesions of dermatophytes

Fig. 3. White to creamy powdery colony of *T. mentagrophytes* on SDA

On potato dextrose agar and corn meal agar creamy colonies with granular surface on obverse and pale grey on reverse were obtained. On blood agar, as from days 5 a clear zone of β-haemolysis was observed around the colony.

Macroscopic feature forisolate 2 showedcreamy, glabrous, flat, convoluted, lightly downy, heaped up with grayish reverse side, suggestive of *T.verrucosum*.

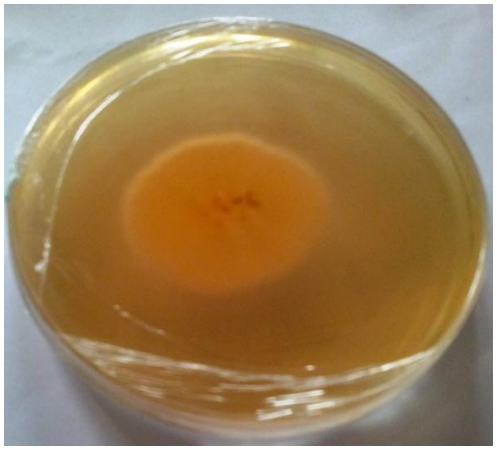

Fig. 4. Yellowish reverse colony of *T. mentagrophytes* on SDA

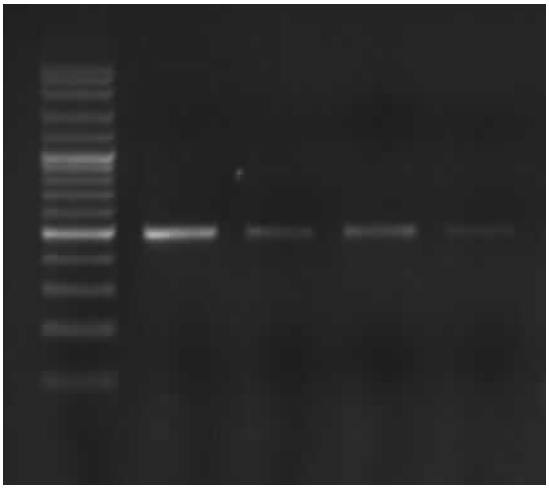

Fig. 5. Gel image of dermatophyte DNA amplified with β tubulin primers with a product size of approximately 500 bp

3.2.2 Microscopical investigation

On direct microscopic examination of hair and skin scrapings 19 samples showed fungal elements in the form of ectothrix arrangement of spores for some samples (isolate 1)and large numbers of endothrix spores and hyaline hyphae inside the hair for others (isolate 2).The microscopic feature (for isolate 1) showed numerous round micro conidia clustered on branched conidiophores and coiled hyphae.For isolate 2: Chains of chlamydospores with antler hyphae were observed. Moreover, a positive urease reaction was developed on Christensen agar urea within 9 days for isolate 1(*T.mentagrophytes*) and a negative one for isolate 2(*T. verrucosum*).

3.3 Molecular Characterization

Analysis of the PCR amplicons by agarose gel electrophoresis revealed amplicon fragments of about 500bp as shown in Fig. 5.

3.3.1 Sequence analysis

3.3.1.1 Sequence analysis for *T. mentagrophytes* (isolate 1)

Analysis of the sequences of the isolate1 using BioEdit software package showed that the clean sequence is 436 bp (Fig. 6). The sequence is unique and has similarity to the partial sequence of the beta-tubulin gene of many fungi including *T. mentagrophytes*. But the isolate was thoroughly investigated using the conventional mycological and biochemical assays that shown its similarity to *T. mentagrophytes.*

3.3.1.2 Sequence analysis for *T. verrucosum* (isolate 2)

Only 255 bp is clean sequence (Fig. 7). The clean sequence is similar to the partial sequence of the beta-tubulin gene of the *T. verrucosum* accession number KJ606180.1.

4. DISCUSSION

Donkeys (*Equus asinus*) are considered as important animals. They play an essential role in the economy of underdeveloped countries including Sudan [11]. In the present study, the clinical signs of alopecia, scaling and crusting, which appeared to varying degrees, suggested dermatophyte infection [8,27]. This finding was due to proteolytic and lipolytic enzymesthat secreted bydermatophytes which favor digestion of skin tissue and hair resulting in hair loss, scaly and crusty lesions [5,8]. Furthermore, infection among equines showed that equine ringworm is highly contagious which transmitted by direct contact or indirect route through contaminated fomites.This indicates, possibility of existence of dermatophyte spores in the environment.

Mycological investigation revealed isolation of *T. mentagrophytes* and *T.verrucosum*. This finding is similar to that obtained [16,17,18] when investigated equines for skin infection.

Molecular characterization of the isolates was done on the basis that encouraged the use of PCR worldwide due to insensitivity of the conventional methods to detect fungal elements by direct microscopy and a non specificity of the

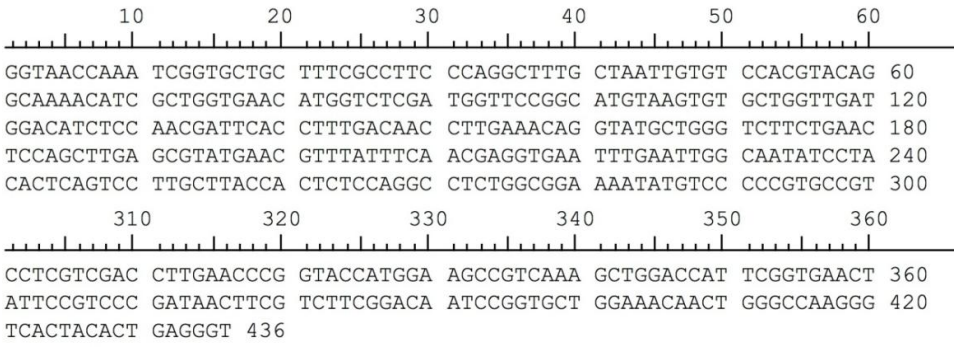

Fig. 6. Partial sequence of *T. mentagrophytes* (isolate 1)

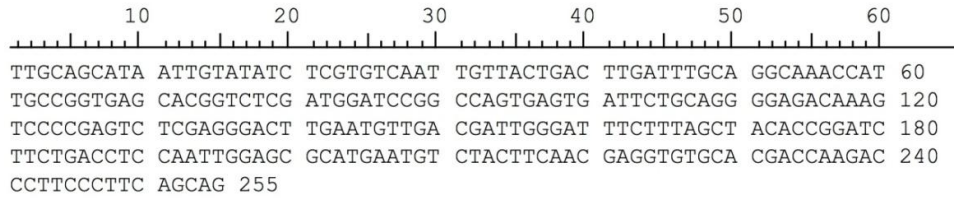

Fig. 7. Partial sequence of *T. verrucosum* (isolate 2)

culture. The conventionalprocedure is time-consuming usually requiring 10-15days or even 3-4weeks. Thus the use of molecular methods has supplemented traditional methods with DNA – based tools to examine phylogenetics and systematic of fungi [28]. This finding is similar to previous findings obtained [29] where re-classification of Penicillium species on molecular basis was performed. In the current study, the PCR technique used was found simple, inexpensive and rapid for efficiently isolated fungi. In addition, DNA was found suitable for use as a template for PCR amplification and sequences assay.Similar result was reported that shown the importance of the amount of extracted DNA as a parameter determining sensitivity of the PCR [30]. Moreover, the mycelium used in this study was directly recovered from Petri-dish cultures; the quality and quantity of DNA obtained were found suitable for molecular assays; it does not require the use of expensive and specialized equipment or hazardous reagents. Similar studies were carried to confirm dermatophytes infection recovered from culture [31,32]. Thus, PCR- based technique is recommended to be applied to dermatophytes recovered from environmental sources to increase sensitivity and specificity and the cost of diagnosis [33,34].

In the current study, the use of β- tubulin primers a&b to amplify the obtained DNA was similarly used [26] to determine sequences for 180 strains representing all accepted species of *Penicillium*. Previous studies were carried with different primers to characterize Dermatophytes targeting various genes. (panDerm1 5´-GAAGAAGATTGTCGTTTGCATCGTCTC- 3´ and panDerm2 5´-CTCGAGGTCAAAAGCACGCCAGAG- 3´) targets the chitin synthase-encoding gene (chitin synthase 1 – chs1) served for detection of dermatophytesin general, whereas (Trubrum-for 5´-TCTTTGAACGCACATTGCGCC- 3´ and Trubrum-rev 5´-CGGTCCTGAGGGCGCTGAA-3´) targets internal transcribed spacer gene 2 (its2) for the specific detection of *T. rubrum* were used [35,36].

The obtained results indicated that incorporation of PCR technique in routine laboratory processing of skin scrapings augmented detection of dermatophytes. This finding is in accordance with the findings of other workers [35,36]. Furthermore, evaluation of the importance of the use of commercial PCR, to increase the detection rate of dermatophytes compared to culture alone was reviewed [37,38].

5. CONCLUSION

The present investigation showed that the integration of different methods and techniques led to identification of useful molecular marker for characterization of dermatophyte species. Thus, the study has aided the development of molecular marker to make identification of dermatophytespecies more valuable.

CONSENT

It is not applicable.

ETHICAL APPROVAL

As per international standard or university standard written ethical approval has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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