

1 Isolation and Molecular Characterization of Dermatophytes In Donkeys

2 **Abstract:**

3 Aim: the study was conducted to investigate into the cause of skin infection among
4 donkeys in Eldamazine State, Central Sudan.

5 Study design: this a case history studies over one year duration.

6 Dermatophytosis is a fungal infection of skin, hair and nail caused by dermatophytes. The
7 zoonotic nature of the disease requires early detection for implication of treatment.

8 Materials &Methods: A total of 31 samples of skin scrapings from donkeys with an age
9 ranged 2-20years, from Eldamazine state, Sudan, were collected. Samples were cultured
10 onto Sabouraud'S (SDA) media. The developed colonies were identified by convention
11 method and characterized by molecular techniques using polymerase chain reaction.

12 Results: *Trichophyton mentagrophytes* and *Trichophyton verrucosum* were the
13 predominant isolated dermatophytes revealed on investigation. Amplification of the β -
14 tubulin gene, by polymerase chain reaction, and analysis of the amplicon sequence,
15 further confirmed their identification. The study revealed first report on characterization
16 of these isolates by molecular methods using β tubulin primers.

17 Conclusion: the present investigation showed that the integration of different methods
18 and techniques led to identification of useful molecular marker for standardization of
19 taxonomical studies of *dermatophyte* species.

20 **Keyways:** Dermatophytes, β 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].

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

humidity but the geographic distribution varies according to the species [13, 14]. Reports on ring worm 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 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. mentagrophytes* var. *mentagrophytes* causing Ringworm in Horses in Al Ahsa Province, Kingdom of Saudi Arabia has been isolated [21].

1. Materials and methods:

1.1. Clinical Examination:

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

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

1.2. 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 0.05mg/ml chloramphenicol and 0.5mg/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].

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].

2.2.2. Molecular study:

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

89 **2.3.3.1. Molecular identification of isolates:**

90 **2.3.3.1.1. DNA extraction and purification:**

91 The genomic DNA was extracted and purified from skin scrapings 10 days- old
92 culture media following Möller *et al.*, method [24]. Briefly, mycelia were harvested from
93 the surface of Sabouraud's dextrose agar culture, transferred to a mortar, supplemented
94 with liquid nitrogen and ground to powder with a pestle. About 45 mg of the mycelia
95 powder, was transferred to 2.0 ml microtube, supplemented with 500 μ l TES (100
96 mM Tris, pH 8.0, 10 mM EDTA, 2% SDS) followed by addition of 3.75 μ l Proteinase K
97 (stock 20mg/ml; w/v; Promega), before incubation for 1 hour at 60°C with occasional
98 gentle shaking of the tube. The reaction mix was then supplemented with 140 μ l 5M
99 NaCl, 65 μ l 10% incubated for 10 min at 65°C, supplemented with 700 μ l
100 chloroform:isoamylalcohol (24:1, v/v;). Tubes were gently shaken, incubated for 30 min
101 at 0°C and then centrifuged for 10 min in 4°C centrifuge adjusted at 12,000 rpm. The
102 supernatant was then transferred to 1.5 ml micro-tube, supplemented with 225 μ l 5 M
103 NH₄Ac (Ammonium Acetate), mixed by gently finger mixing, placed on ice for one hour,
104 before centrifugation at the same former conditions. The supernatant was transferred to a
105 fresh 1.5 ml micro-tube, supplemented with 500 μ l isopropanol, placed on ice for 30
106 minutes, centrifuged for 5 min at the same former conditions. Finally the supernatant was
107 decanted, the pellet washed twice with cold 70% ethanol, dried at room temperature,

108 dissolved with 50 µl TE (10 mM Tris-HCl, 1 mM disodium EDTA, pH 8.0) and stored at
109 -20°C for later experiments.

110 **2.3.3.1.3. Amplification of the β -tubulin gene PCR:**

111 **Primers:**

112 A pair of primers, β t2a and β t2b synthesized by Invitrogen, were used [25]. Their
113 sequences are

114 5'-GGTAACCAAATCGGTGCTGCTTTC-3' and

115 5'-ACCCTCAGTGTAGTGACCCTTGGC-3' respectively.

116 **Reaction components:**

117 DreamTaq Green PCR Master Mix (2X) (Fermentas, Cat. # K1072) that contains all
118 components in a volume of 25 µl, except template DNA and primers was used. A number
119 of four master mix tubes were used to make the reaction mix in a volume of 50 µl; each
120 tube received 1.5 µl of each primer, 5 µl of the respective template DNA and 17 µl
121 nuclease free H₂O.

122 **Thermocycling conditions:**

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

127 **Agarose gel:**

128 1.5% agarose (Vivantis) in 0.5x TBE (Tris Borate EDTA) supplemented with ethidium
129 bromide (10 mg/ml, Vivantis) to a final concentration of 0.5 µg/ml was used.

130 **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).

2.3.3.1.4. Sequencing and sequence analysis:

The PCR amplicons were sent to JBMI (JEONJU BIOMATERIALS INSTITUTE) for automated bidirectional sequencing using the βt2a and βt2b 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 withers and saddle area, but the infection was spread to the neck, chest and head with one or more legs being involved (Fig. 1&2).

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

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163 **Fig.2:** Generalized skin lesions of dermatophytes

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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 creamy colony on obverse and a pale yellowish on reverse was observed (fig. 4).



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



Fig. 4: Yellowish reverse 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 for isolate 2 showed creamy, glabrous, flat, convoluted, lightly downy, heaped up with grayish reverse side, suggestive of *T. verrucosum*.

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 microconidia 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 500 bp as shown in figure (6)

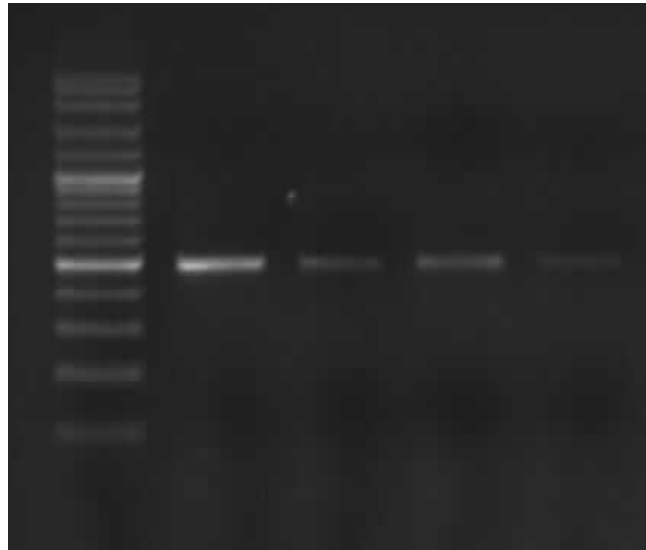


Fig: 5. Gel image of dermatophyte DNA amplified with β tubulin primers with a product size of approximate 500 bp.

3.3.1. Sequence analysis:

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*.

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      10      20      30      40      50      60
      |-----|
GGTAACCAAA TCGGTGCTGC TTTCGCCTTC CCAGGCTTTG CTAATTGTGT CCACGTACAG 60
GCAAAACATC GCTGGTGAAC ATGGTCTCGA TGGTTCCGGC ATGTAAGTGT GCTGGTTGAT 120
GGACATCTCC AACGATTCAC CTTTGACAAC CTTGAAACAG GTATGCTGGG TCTTCTGAAC 180
TCCAGCTTGA GCGTATGAAC GTTTATTTCA ACGAGGTGAA TTTGAATTGG CAATATCCTA 240
CACTCAGTCC TTGCTTACCA CTCTCCAGGC CTCTGGCGGA AAATATGTCC CCCGTGCCGT 300

      310      320      330      340      350      360
      |-----|
CCTCGTCGAC CTTGAACCCG GTACCATGGA AGCCGTCAAA GCTGGACCAT TCGGTGAACT 360
ATTCCGTCCC GATAACTTCG TCTTCGGACA ATCCGGTGCT GGAAACAAC GGGCCAAGGG 420
TCACTACACT GAGGGT 436

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

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.

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      10      20      30      40      50      60
      |-----|
TTGCAGCATA ATTGTATATC TCGTGTCAAT TGTTACTGAC TTGATTTGCA GGCAAACCAT 60
TGCCGGTGAG CACGGTCTCG ATGGATCCGG CCAGTGAGTG ATTCTGCAGG GGAGACAAAG 120
TCCCCGAGTC TCGAGGGACT TGAATGTTGA CGATTGGGAT TTCTTTAGCT ACACCGGATC 180
TTCTGACCTC CAATTGGAGC GCATGAATGT CTACTTCAAC GAGGTGTGCA CGACCAAGAC 240
CCTTCCCTTC AGCAG 255

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

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 enzymes that secreted by dermatophytes which favor digestion of skin tissue

and hair resulting in hair loss, scaly and crusty lesions [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 culture. The conventional procedure 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

242 to confirm dermatophytes infection recovered from culture [31, 32]. Thus, PCR- based
243 technique is recommended to be applied to dermatophytes recovered from
244 environmental sources to increase sensitivity and specificity and the cost of diagnosis
245 [33, 34].

246 In the current study, the use of β - tubulin primers a&b to amplify the obtained DNA
247 was similarly used [26] to determine sequences for 180 strains representing all accepted
248 species of *Penicillium*. Previous studies were carried with different primers to
249 characterize Dermatophytes targeting various genes. (panDerm1 5'-
250 GAAGAAGATTGTCGTTTGCATCGTCTC- 3' and panDerm2 5'-
251 CTCGAGGTCAAAAGCACGCCAGAG- 3') targets the chitin synthase-encoding gene
252 (chitin synthase 1 – chs1) and served for detection of dermatophytes in general, whereas
253 (Trubrum-for 5'-TCTTTGAACGCACATTGCGCC- 3' and Trubrum-rev 5'-
254 CGGTCCTGAGGGCGCTGAA- 3) targets internal transcribed spacer gene 2 (its2) for
255 the specific detection of *T. rubrum* were used [35,36].

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

262 **Conclusion:** The present investigation showed that the integration of different methods
263 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 dermatophyte species more valuable.

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