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ORIGINAL ARTICLE

Molecular Identification and Phylogenetic Analysis of Dermatophytes Isolated from Small and Large Ruminants in Egypt

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Abstract
Dermatophytosis is an infectious disease of worldwide importance caused by pathogenic keratinolytic fungi named dermatophytes in both animals and humans. In Egypt, the mixed grazing of small and large ruminants increases the threat of various dermatophytes interspecies transmission among animals and consequently to humans. The current study motivates on the importance of rapid and accurate diagnosis of dermatophytosis among animals and the limitations associated with conventional methods of diagnosis. The current study was performed to investigate the prevalence of dermatophytosis among calves, cows, and sheep reared together in a private farm from September 2021 through April 2022 and identify the causative dermatophytes by both conventional cultural and molecular approaches. The overall prevalence of dermatophytosis in the investigated farm was 15.2% (40% in calves, 14.3% in sheep and 10% in adult cows). On mycological examination, colonies appeared rapidly and become powdery to granular with white to cream color on the surface. The recovery of *T. mentagrophytes* from clinical samples was confirmed by PCR targeting ITS region followed by sequencing analyses. Phylogenetic analysis revealed that *T. mentagrophytes* sequences obtained in the current study are clustered along with KY765897.1 and KY765898.1 isolated from cattle. Results of blood serum urea and creatinine didn't show statistical difference between control non infected and infected animals. The current investigation concluded that *T. mentagrophytes* is the most prevalent species of dermatophytes in the farm which poses a high predicted risk to the humans either through direct or indirect contact. Sequencing analysis of the ITS region is a faster, more accurate, and more reliable diagnostic tool that can replace the conventional methods for rapid recognition of dermatophytes species.

Keywords
Cattle, Dermatophytes, kidney functions, Sheep, Sequencing

1. Introduction
Dermatophytosis is a highly contagious disease caused by dermatophytes fungi that are the most ubiquitous fungal pathogens worldwide **(Ahmed and Saber, 2008).** The infection is superficial in its nature and confined to the keratinized epithelium of skin, hair and nails. The breakdown of the keratin protein complex by keratinases and other proteolytic enzymes produced by dermatophytes enables them to penetrate more deeply into the host's stratum corneum and thereby trigger an inflammatory response **(Copetti et al., 2006).** The severity of the clinical symptoms depends on the degree of inflammation and the host-fungus interaction **(Dahdah and Scher, 2008).**

Based on their principal habitat relationships, dermatophytes are categorized into anthropophilic, geophilic and zoophilic species. Currently, seven genera of dermatophytes have been identified; *Epidermophyton*, *Trichophyton*, *Paraphyton*, *Lophophyton*, *Nannizzia*, *Arthroderma* and *Microsporum* **(De Hoog et al., 2017).** *Microsporum* (*M*), *Trichophyton* (*T*) and *Epidermophyton* (*E*) representing the most prevalent genera of public health concern **(Coelho et al., 2008; Dahdah and Scher, 2008).** Although the infection is superficial in healthy man, disseminated dermatophy-tosis and invasive dermatitis may occur particularly in immunocompromised patients **(Rouzaud et al., 2015;**

Benedict et al., 2019). The diseases can occasionally spread to humans directly by contact with affected animals or fomites **(Aghamirian and Ghiasian, 2011),** so, high prevalence of infections is frequently found in overcrowded farms.

Dermatophytosis (Ringworm) in animals is a substantial issue for veterinary medicine. Ringworm is a prevalent illness of enzootic scenarios that commonly affects cattle herds **(Chermette et al., 2008).** Animals, like people, get infected by direct close contact with the skin or hair of infected animals. Diseased animals have a high capacity for distributing infectious fungal spores in the environs, which stick to hair or skin scabs. Additionally, the strong resilience of the dermatophyte conidia to the environment for months or maybe even years that allows for the potential for bouts of infections indirectly via contaminated fomites (**Chermette et al., 2008; Bond, 2010)** raising the risk of infection among farmworkers and those who manage the diseased animals **(Papini et al., 2009).** Even though dermatophytosis is benign and a self-limiting disease, it still causes economic losses **(Moretti et al., 2013).** Extensive fungal infection of the animals could cause renal or hepatic damage because of the oxidative damage in such vital organs **(Elgazzar et al., 2011).** In sheep flocks, dermatophytosis is an emerging problem associated with several outbreaks of exudative dermatitis that accompanied with severe inflammation. Lesions might last for up to 24 weeks with control difficulty **(Sargison et al., 2002).**

Accurate identification of dermatophytes to the species level can be useful not only in controlling disease outbreaks through tracking the infection source but also reducing transmission to humans or other animals. Direct microscopic detection of fungi from clinical samples is considered a quick diagnostic method however; it yields false negative results as much as 15% of cases **(Chung et al., 2010).** Although isolation is considered the gold standard method of diagnosis of dermatophytosis, it may be hampered by the lengthy cultivation period (up to 8 weeks). Additionally, the identification based on cultural results is difficult and time-consuming because dermatophytes exhibit morphological variability, similarity, and polymorphism **(Kanbe, 2008).** Molecular technology like PCR, is a highly sensitive and specific technique to detect a variety of pathogens, including dermatophytes **(Monod et al., 2006).** Measuring of serum biochemical markers could be used as a beneficial tool for assessment of the animal health as well as to help in better understanding of the pathophysiology of the disease **(Olaogun and Onwuzuruike, 2018).**

In Egypt, the mixed grazing of small and large ruminants increases the threat of various dermatophyte interspecies transmission among animals and consequently to humans. Therefore, the current study aimed to investigate the incidence of dermatophytosis among calves, cows and sheep reared together in a private farm from September 2021 through April 2022 and identify the isolated dermatophytes by both conventional and sequencing approaches.

2. Materials and Methods

2.1. Studying Area

The present study was done on a mixed private farm located in Beni Suef province, Egypt (coordinates: 28° 54' 22.9" N, 30° 56' 18.7" E) from September 2021 to April 2022. The farm housed 140 sheep and 90 dairy cattle (20 calves and 70 adult cows). Animals of all ages were housed together and reared in an open system with overcrowded condition. The farm permits the mixed grazing system where cattle and sheep were grazing together and assisted with the same workers. All animals in the farm were thoroughly and clinically examined for evidence of dermatophytes infection according to the methods described by **Radostits et al., (2000).**

2.2. Animals

A total of 35 animals (8 calves, 7 cows and 20 sheep) exhibited the typical clinical signs of dermatophytosis. All affected animals were subjected to sample collection for detection and identification of the causative dermatophytes.

2.3. Ethical Approval

The present study was approved by "Institutional Animal Care and Use Committee" (IACUC), Ref. No: IORG 022-337), Beni-Suef University, Egypt.

2.4. Sample Collection

2.4.1. Hair and Skin Scrapings

Hair was plucked from its root using forceps while skin scrapings were scraped by a sterile scalpel from center to boundary, crossing the margins of lesions after gently decontaminated with 70% alcohol **(Papini et al., 2009).** All samples were individually placed in sterile plastic cups and were transported to Lab of Veterinary Medicine department, Faculty of Veterinary Medicine, Beni-Suef University for microscopic, cultural and molecular analyses.

2.4.2. Blood Samples

Blood samples were drained from the jugular vein of the diseased calves (n=8) as well as from eight clinically healthy calves to be used as a control group. Blood was left without anticoagulant to obtain serum that was carefully collected and stored at -20ºC until analyses. Blood serum urea and creatinine were determined spectrophotometrically using available commercial chemical test kits **(Bio-diagnostics Co., Cairo, Egypt),** following the manufacturer instructions.

2.5. Mycological Examination

2.5.1. Direct Microscopical Examination of Collected Samples

Upon a clean dry glass slide, one portion of the collected hair and skin scrapings samples were mixed with a drop of KOH (10% conc) then, covered using cover slide, heated gently, let for at least 30 to 60 min and examined for detection of fungal structures (i.e. hyphae, ectothrix or endothrix spores) using

both low and high power of microscopic examinations **(Ellis et al., 2007).**

2.5.2. Dermatophytes Isolation and Identification

The collected specimens were cultured individually on the surface of Sabouraud's dextrose agar (SDA) containing thiamine (10mg/L), cycloheximide (400mg/L), chloramphenicol (500mg/L) and inositol (50mg/L). The inoculated media were incubated at 30ºC for up to 4 weeks and continuously examined every 72hrs for evidence of growth. The isolated dermatophytes were identified on the basis of macroscopic examination of grown colonies including growth rate, consistency and the surface and reverse color of the colony **(Cheesbrough, 2003).** Microscopic morphology of the isolates was performed by using Lactophenol cotton blue (LPCB) preparation to determine whether chlamydospores, macroconidia, hyphae and other fungal structures are present **(Collee et al., 1996).**

2.5.3. Molecular Identification and Sequence Analysis

2.5.3.1. DNA Extraction

The suspected fungus colonies were taken out and crushed in a mortar with liquid nitrogen. The obtained mycelial powder was transferred to 1.5ml capacity Eppendorf tube and subjected to extraction of DNA according to the product's instructions of the QIAamp DNeasy Plant Mini kit (supplied by Qiagen, Germany, GmbH).

2.5.3.2. PCR Amplification of ITS Region

PCR amplifications using primer sequence targeting internal transcribed spacers (ITS) region **(Table, 1)** were carried out in reactions with a total volume of 25µl/reaction that contain 12.5µl of PCR master mix (supplied by Thermo, USA), one µl of each primer (ITS-1 and ITS-4) of concentration 20pmol, 5µl of DNA template and 5.5µl of water. The reaction was done in biosystem 2720 thermal cycler. Electrophoresis on agarose gel 1.5% (supplied by Applichem, Germany, GmbH) in 1x TBE was carried out to separate the PCR products. For gel analysis, in each gel slot, 20µl of the products were loaded. A gene ruler 100bp DNA Ladder (supplied by Thermofisher, Germany) was used for determination of the fragment sizes. Using gel documentation system (supplied by Alpha Innotech, Biometra's), the gel was photographed and then data were analyzed through DigiDoc-It Imaging System's software.

2.5.3.3. Sequence and Sequence Analysis

A Gene Aid Gel extraction kit (supplied by New Taipei City, 22180 Taiwan, and China) was used to purify the PCR products of the selected isolates according to the instruction of manufacturer. Purified PCR amplicons were commercially sequenced. Using the BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST/), the sequence data of nucleic acid were compared with previously published data in GenBank. Sequence and phylogenetic analyses were performed using MEGA X software. Maximum likelihood method was used to design the tree with 1,000 boot-strapped data sets. Using Kimura 2-parameter as a model and Neighbor-Join and BioNJ algorithm, the tree was initially obtained **(Kimura, 1980).**

2.6. Statistical Analysis

Using SPSS (version 22.0), the obtained data were recorded, and the prevalence was calculated using Chi-Square Test. Regarding serum parameters, the student's T-test was utilized to detect the difference between means using boxplots (mean ± SD).

3. Results

Clinical examination of the animals revealed that, 40% of the calves, 10% of the adult cows and 14.3% of sheep were affected with dermatophytosis **(Table 2, Fig. 1).** The lesions were distributed on the head region, especially around their eyes, and neck **(Fig. 2a, b).** Lesions were gray, whitish, and crusty ranged in size from 1-5cm and in some areas coalesce to form a diffuse area of alopecia **(Fig. 2c, d).** In some calves the lesions were observed all over the body, involving the head, neck, dewlap and legs **(Fig. 2).** In adult cows, Ringworm lesions were few in the form of circular alopecic areas in neck and dewlap **(Fig. 3).** Regarding sheep, the overall infection rate was 14.3% and the lesions were limited to the head and ears in form of defined, circumscribed parts of scaly alopecia with greyish crusty scales measuring 2-4cm in diameter **(Fig. 4).** No lesions were observed in the fleece and other regions of the body.

Table 1. Primers sequences, amplicon size, target gene and cycling conditions for conventional PCR.

Target gene	Primers sequences	Amplified segment (bp)	Amplification (35 cycles)				Final extension	Reference
			Primary denaturation	Secondary denaturation	Annealing	Extension		
ITS	ITS1 TCCGTAGGTGAACCTGCGG ITS4 TCC TCC GCT TAT TGA TAT GC	Variable	94˚C 5 min	94˚C 30 sec	56˚C 30 sec	72˚C 30 sec	72˚C 7 min	Luo and Mitchel (2002)

Table 2. Distribution of dermatophytosis among the examined animals.

Animal species		No. examined	Positive	Negative
Cattle	Calves	20	8 (40%)	12 (60%)
	Adult	70	7 (10%)	63 (90%)
Sheep	Adult	140	20 (14.3%)	120 (85.7%)
P- value = 0.004*				

* p-value significant at (p<0.05)

Table 3. Kidney functions in control and ringworm infected calves.

Parameters	Control group	infected animals	P-value
Urea (mg/dl)	34.57± 2.32	35.24±2.14	0.651 (NS)
Creatinine (mg/dl)	0.71±0.02	0.77±0.04	0.134 (NS)

NS= p-value non-significant at (p<0.05)

On macroscopic examination of the cultures, Colonies appeared rapidly and become powdery to granular with white to cream color on the surface. Fungal growth microscopic examination based on the characteristic shape of fungal hyphae, macroconidia and microconidia was carried out to identify the type of dermatophytes. Microscopic examination revealed numerous unicellular, round to pyriform microconidia clustered in grape like clusters highly suggestive to *T. mentagrophytes.*

By Conventional PCR targeting ITS region, a fragment of 680bp has been amplified from all DNA extracts **(Fig. 5).**

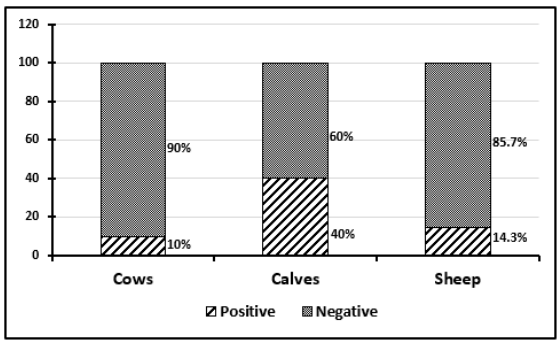

Fig. 1. Distribution of dermatophytosis in examined animals.

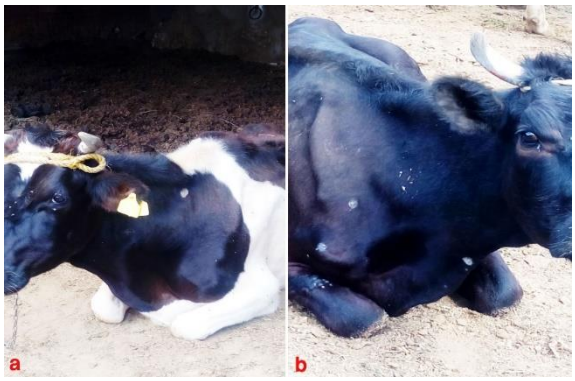

Fig. 3. Localized dermatophytosis lesions in adult cows.

Sequencing analyses of the ITS gene disclosed that sequences obtained in this study are belonging to *T. mentagrophytes*. Phylogenetic analysis revealed that *T. mentagrophytes* sequences obtained in the current study are clustered along with KY765897.1 and KY765898.1 isolated from cattle **(Fig. 6).**

Concerning the operated kidney function parameters, serum urea and creatinine, the clinically diseased animals (calves with diffused lesions) and those apparently healthy ones did not show statistical difference at ($p < 0.05$) as clarified in **Table (3)** and **Fig. (7).**

Fig. 2. Dermatophytosis lesions in the face, neck and body of calves with different ages.

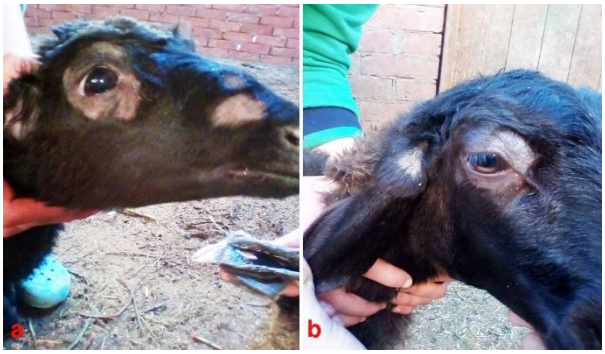

Fig. 4. Typical dermatophytosis lesions on ear and around the eyes of sheep

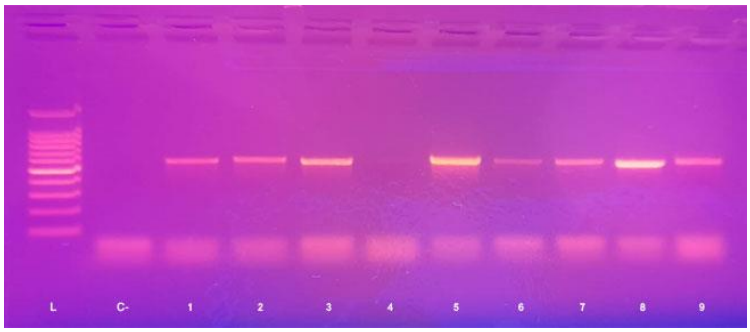

Fig. 5. Gel electrophoresis of internal transcribed spacers (ITS) gene-based PCR assay for detection of dermatophytes; Lane L: 100bp DNA ladder, Lane C-: control negative, Lane 1:9 clinical isolates of dermatophytes (680bp)

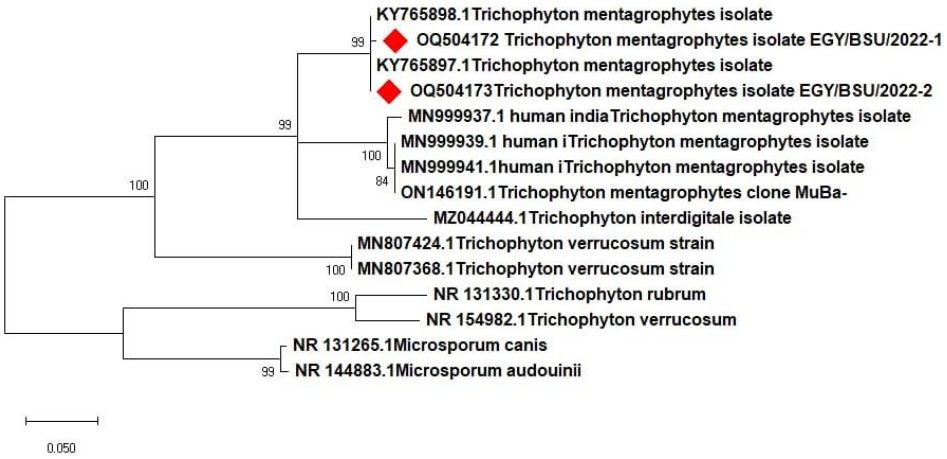

Fig. 6. Phylogenetic analysis of ITS gene sequences. Tree was created using Mega x program by the neighbor-joining analysis. The values of Bootstrap confidence were calculated on 1000 replicates in accordance with to the maximum-likelihood approach. Sequences obtained in this study are labeled with (OQ504172, OQ504173).

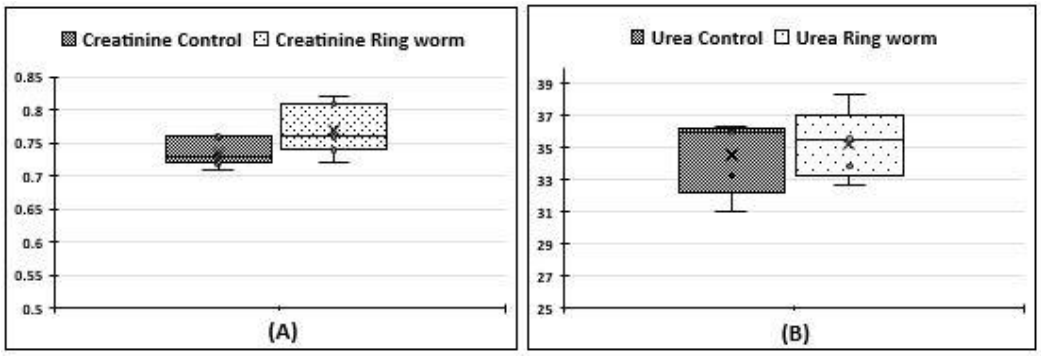

Fig. 7. Creatinine (A) and urea (B) levels in control and Ringworm infected calves.

4. Discussion

Considering animals spend most of their time in their pens and in close proximity to one another under the stress of overcrowding, the fungus can be easily spread among animals leading to exposure and consequently infection of susceptible animals **(Agnetti et al., 2014; El-Ashmawy et al., 2015).** Although dermatophytes live freely in the environment, under particular circumstances it can cause infections in animals and humans. Epidemiologically, it was confirmed that the relatively humid weather as an important component in the epidemic triad is the favorable condition for the persistence of dermatophytes infection **(Murray et al., 2005).** The current study investigates the prevalence of dermatophytosis in calves, cows and sheep grazing together in a private farm. An overall prevalence of 40% in calves, 14.3% in sheep and 10% in adult cows were obtained. High prevalence of dermatophytosis was observed among calves. According to **Havlickova et al. (2008),** since the immune system of the host hinders fungi to infect healthy epidermis, arthroconidia can't invade healthy tissues consequently; predisposing factors are essential for invasion to take place. Important risk factors for infection include young age, skin trauma, immunosuppression, nutritional deficiency, and high environmental humidity **(Chermette et al., 2008).** In addition, the sebaceous glands in adults create a slightly acidic pH which protects against dermatophytes infections while in calves the skin has a higher pH **(Radostits et al., 1997; Agnetti et al., 2014).**

The most common prevalent clinical findings observed among the infected calves were patches of hair loss appearing as round gray, whitish, and crusty. Typical lesions were distributed on the head region, especially around their eyes, and neck and ranged in size from 1 to 5cm. Diffuse areas of alopecia were also observed. In some calves' lesions were distributed all over the body involving the head, neck, dewlap and legs. In contrast to calves, cows exhibited a localized lesion with 10% infection rate. Regarding sheep, the overall infection rate was 14.3% and the lesions were limited to the head and ears in form of circumscribed, defined areas of scaly alopecia with greyish crusts and scales measuring 2-4 cm in diameter. The fleece and other areas of the body were free of lesions. This obtained results are nearly similar to that have been reported by **Abd-Elmegeed et al., (2015); Al-Ani et al., (2002**) (30% in calves) and **Rahbari (1986) (12.5%).**

Laboratory diagnosis of dermatophytosis is mandatory due to the clinical presentation resemblances between dermatophytosis and other skin diseases such as dermatophilosis, mange as well as different types of dermatitis **(Outerbridge, 2006).** During isolation, colonies appeared rapidly and become powdery to granular with white to cream color on the surface suggesting the recovery of *T. mentagrophytes* from

examined clinical samples. In comparison to other fungi *T. mentagrophytes* grows rapidly. The granular colony of *T. mentagrophytes* has a powdery appearance due to the great quantity of microconidia (spores) formed **(Kim et al., 2018).** Culture followed by species identification using microscopic examination is considered the gold standard method but may take up to 4 weeks **(Kanbe, 2008).** PCR is a more sensitive and specific tool to detect dermatophytes compared with culturing process. By Conventional PCR targeting ITS region a fragment of 680bp has been amplified from all DNA extracts. Sequencing of ITS regions provides more accurate, quick, and valuable method for evolutionary identification of dermatophytes due to the presence of minor nucleotides variances in it **(Li et al., 2008; Sitterle et al., 2012).** Sequencing analyses of the ITS gene clarified that sequences obtained in this current study are belonging to *T. mentagrophytes.* Phylogenetic analysis revealed that T. *mentagrophytes* sequences obtained in the current study are clustered along with KY765897.1 and KY765898.1 isolated from cattle.

Although cattle and sheep are the main reservoir for *T. verrucosum* however, the isolation of *T. mentagrophytes* from the screened animals may be attributed to the presence of rodents in the farm under investigation. Rodents are the potential reservoir for *T. mentagrophytes* **(Cabañes, 2000; Moretti et al., 2013).** Results of the current study shed light on the ability of dermatophytes for interspecies transmission **(El-Ashmawy et al., 2015).** Our finding were in parallels to those reported by **Nweze (2011)** and **Biswas et al., (2015)** who mentioned that *T. mentagrophytes* was the most prevalent isolate detected from skin lesions of infected cattle and sheep.

Although the dermatophytes infection is superficial in nature and confined to keratinized epithelium of skin, nails and hair, **Abo-El-Foutah et al., (2012)** recorded a significant increase in serum urea and creatinine levels in camels affected with ringworm. The elevation in kidney functions may be caused by renal damage due to dermatophytosis induced oxidative stress (**Elgazzar et al., 2011).** In the current study, the results of blood serum urea and creatinine did not show statistical difference between control and affected animals. Similar findings were recorded by **Sezer et al., (2021)** who referred that normal liver and kidney function tests could be attributed to the normal organ functions in the affected animals.

5. Conclusion

Regarding the results obtained from the current investigation we concluded that *T. mentagrophytes* is the most prevalent species of dermatophytes which poses a high risk to the humans either through direct or indirect contact. Also, strict measures should be adopted to extinct the rodents to minimize their potential for dermatophytes infections between humans and animals. Additionally, sequence analysis of the ITS region is thought to be a faster, more accurate, and more reliable diagnostic tool that can replace the traditional methods for the rapid recognition of dermatophyte species and realize the source of infections in clinical and epidemiological approaches.

6. Conflict of Interest

The authors declare no conflict of interest.

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