A molecular Study of the *Microsporum Canis* and *Trichophyton Mentagrophytes* Associated Fungal Infection: Athlete's Foot among Farmers

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Abstract

Athlete's foot (*Tinea pedis*) and toe nails infection (onychomycosis) are disease conditions caused by dermatophytes; both diseases are prevalent in adults, especially in farmers who often wear robber shoes during farming. Proper treatment of dermatophytes related skin diseases needs a proper understanding of susceptibility of the causative agents to the intended treatment option. This knowledge can only be derived from proper identification and characterization of the related fungi. In the present study, both traditional and molecular identification approaches were applied on cultured samples for detection and identification of *Tinea pedis*. From the PCR analysis, *Microsporum canis* and *Trichophyton mentagrophytes* were identified as the two commonest species implicated in Tinea pedis basing on their DNA banding patterns. From the fifty two identified dermatophyte isolates via the conventional identification methods, only 45 isolates were confirmed via molecular approach, with 25 isolated being confirmed as *M. canis* while 20 isolates were *T. mentagrophytes*. The same was confirmed with real time PCR quantification.

Keywords: *Tinea pedis, Athlete's foot, M. canis, T. mentagrophytes, Ringworm.*

Introduction

Dermatophytes are the commonest cause of superficial mycoses in human; the lesions of dermatophyte infection are characterized by desquamation, alopecia, circular disposition, and erythma of the edges (1) Among the infections caused by dermatophytes are athlete's foot, ringworm, and jockey itch; these infections result from direct contact with either the hyphae or spores of any Microsporum, Epidermophyton, or Trichophyton. Typically, the Trichophyton species (such as *T. mentagrophytes & T. rubrum*) and *M. canis* are commonly implicated in hair, nails and skin infections. Candidiasis and tinea infection remain the commonest forms of skin fungal infections; they are common in all regions of the globe and their incidence has continued to increase. (2, 3). Dermatophytes-related superficial infections can occur in both healthy and immune-compromised persons (4); they also affect a wide range of mammals but not commonly found in birds. Dogs and cats are often infected with fungal dermatophytosis, including the cattle (5). The very reason for the high incidence of dermatophytosis, is it is more contagiousness and difficult to identify along with costly treatment. This worms are easily transmitted to humans, especially farmers owing to their close contact with such infected

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animals. [6] The main lesions of dermatophytosis are alopecia, crusting, and erythema. These dermatophytes usually feed on keratin as such called as keratinophilic pathogenic fungi. These pathogens invade the epidermal stratum corneum and the related keratinized tissues (e.g., skin, hair, nails, fur) [6, 7]

Farmers were considered the best subjects for this study because the working conditions in the farm encourage the emergence of antropophilic fungal infections. Being that farmers spend long working days in the humid farming conditions, they are required to wear rubber footwear during such long periods as they continuously contact with organic matter. When compared to other professions, farmers are more exposed to pathogenic fungi; they can be infected by pathogenic fungi present in the soil or zoonotically [8, 9]; hence, dermatophyte-related infection, such as tinea pedis is commonly prevalent among farmers. Other conditions that can be caused by dermatophytosis complex include interspace fissuring, leukokeratosis, and hyperkeratosis. [10]

Taxonomically, these fungi belong to *Trichophyton* and *Microsporum*, where individual species which propagate by sexual methods are placed into *Arthroderma* of the *Ascomycota*. These are zoophilic, soil-adapted and mostly anthropophilic. [11]

The most commonly seen dermatophyte species within animals are *T. verrucosum, M. canis, T. mentagrophytes, M. gypseum, and M. nanum.* [12] Normally, routine laboratory diagnosis of dermatophytosis requires a direct examination of the clinical samples under a microscope, followed by in-vitro culturing of the sample. [13] A quick microscopic method of identifying fungal elements is direct emulsification of the clinical samples in 10% KOH solution; however, the sensitivity and specificity of this approach remains an issue as both depends on the skills and experience of the analyst. As per S Jarraud,et al. (2013) [14], the KOH smear approach can only approach a sensitivity level of 73.3% (CI: 66.3 to 79.5%) while the culture approach can achieve a sensitivity level of 41.7% (34.6 to 49.1%). Although the culture method is somewhat specific, it is a time-consuming approach [15], taking up to 4 weeks to achieve the final diagnosis. Furthermore, the polymorphism of dermatophytes couls make morphological identification misleading [16] Hence, PCR-based techniques are devised to reduce the diagnostic accruable process and achieve good results with a reasonable level of specificity and sensitivity in comparison to the conventional methods. [15, 17]

There are numerous PCR-based techniques, such as Random Amplification of Polymorphic genomic DNA (RAPD) [18], PCR fingerprinting [19] , Restriction Fragment Length Polymorphism (RFLP) [20] and real-time RTPCR [21] which are widely used to screen the dermatophytes *in vitro*. Moreover, TRFLP (PCR-terminal restriction fragment length polymorphism) [22] , nested PCR, and PCR-ELISA [23] are some of the other advanced techniques that have been found useful in identifying dermatophytes. But still there is a need to design a sensitive molecular-based approach to easily handle large amounts of clinical isolate samples within a short time. But the major hindrance to the cause is lack of a consistent method to extract fungal DNA from the clinical specimens. The aim of the current study is to use PCR to detect Tinea pedis using a method as described by [24] BL Wickes, (2018) for onychomycosis identification among suspected farmers. The process involves extracting fungal DNA and electrophoresing the PCR products.

M. canis is among the common causes of the simplex interdigital types of infections among the general populace, while *T. mentagrophytes* is implicated in inflammatory/vesicular Tinea pedis which is typically characterized by tense, hard vesicles on the mid-anterior plantar surface. [10] The vesicular lesions present diameters of about 1 to 5 mm and settle deep within the epidermis, while chronic moccasin or hyperkeratotic tinea pedis due to *T. mentagrophytes* infection exhibits chronic plantar erythema that ranges from slight scaling to severe hyperkeratosis.

Dry hyperkeratotic scaling normally affects the entire plantar surface and could extend to the lateral foot, with the foot's dorsal surface normally unaffected. Meanwhile, there could be manifestation of thick hyperkeratotic scales with fissures. [13, 25, 26] Mild to severe pruritus may be observed and painful fissures may be experienced while walking. This is normally encountered in patients with low immune status, such as those living with diabetics. [27] Both anthropophilic Microsporum and Trichophyton species are implicated in ulcerative tinea pedis; this condition is characterized by vesiculopustular lesions, erosions and ulcers that spreads rapidly; it is often associated with a secondary bacterial infection. The characteristic lesions usually have maceration and scaling border, normally starting in the 3rd to 4th interdigital spaces and progressing to the lateral dorsum, plantar surface, and sometimes to the entire sole. [10, 28]

Materials and Methods

Clinical samples were procured from microbiology diagnostic laboratory at Baghdad Training Hospital. Samples from fifty two patients suspected of ringworm infection were initially examined by the macro- and micromorphology.

Microscopic analysis: Samples for fungal microscopic identification, piece of acetate tape was gently touched on the wound surface and carefully applied onto a glass slide with a drop of methylene blue stain and two to three drops of the KOH+DMSO. [29] The mount used for microscopy was equal volumes of 10% KOH and 40% Dimethyl sulphoxide (DMSO). A clean cover slip was placed on the sample and squashed to prevent any air bubbles from forming. 'The slides were then examined under low power (10X) and high power (40X) for the presence of hyphae and arthroconidia.

Molecular analysis: Clinical samples are procured from the microbiology diagnostic laboratory at Baghdad Training Hospital. Samples from 52 patients suspected of ringworm infection were initially examined via direct microscopy, followed by culturing on Sabouraud dextrose agar (SDA was supplemented with 0.05g/L chloramphenicol and 0.4g/L cycloheximide) at 30°C for 14days. Pure cultures were derived based on macro- and micro-morphology analysis. The pure cultures were used for the DNA extraction process from the isolation regions as described by [30].

DNA extraction: The clinical isolates were allowed to grow in 2ml of Sabouraud liquid medium with cycloheximide and chloramphenicol (HiMedia, India) and incubated in a shaker incubator for 8 days at 27°C. Following incubation, the cells were centrifuged at maximum speed, and the pellet obtained was re-suspended in 500μl of lysis buffer (400mM Tris-HCl [pH 8.0], 60mM EDTA [pH 8.0], 150 mM NaCl, 1% sodium dodecyl sulfate) for 10min RT. To the suspension, 150μl of potassium acetate (pH 4.8) was centrifuged at 12,000 × *g* for 1min following vortexing. The supernatant obtained was transferred to a fresh tube and the DNA pelleted with equal volumes of isopropyl alcohol was later stored in 50μl of TE (10mM Tris, 1mM EDTA) buffer.

Multiplex PCR amplification: PCR primers were designed using the primer 3 software, and used for amplifying Chitin synthase1 of dermatophytes. panDerm_F (5'GAAGAAGATTGTCGTTTGCATCGTCTC3') and panDerm_R (5'CTCGAGGTCAAAAGCACGCCAGAG3') were used in this study. Multiplex PCR mixtures consisted of (2x PCR Mix [HiMedia, India], Taq DNA polymerase 0.1U/µL, MgCl2 4.5mM, dNTPs (dATP, dCTP, dGTP, dTTP) 0.5mM of each dNTP). The total volume of the reaction mixture was 15µL with primer of 0.1µL (both FW and RV) and 2µL of DNA was used for the reaction. The amplification was carried out thermocycler (Eppendorf make) with time-temperature profile for the PCR as follows: Initial denaturation at 95 °C for 3min, followed by 45s at 94 °C, 45s at 55 °C, and 45s at 72 °C for about 35cycles. Following amplification, the PCR products were run a 3% agarose gel. The PCR products would be

approximately 361 and 643bp for *T. mentagrophytes and M. canis* respectively.

RNA extraction: Total RNA was extracted according to the protocol as described by [31] TRIzol-mediated method was adopted so as to attain good quality RNA for qRT-PCR analyses. Each sample (*M.canis* and *T. mentagrophytes)* from the culture plates were placed into 2ml microfuge tubes with single 3mm tungsten bead. Following which the tubes were freeze into liquid nitrogen for 1min. following incubation, the microfuge tubes were placed in homogenizer and ground for 3min at 25-30Hz/s. To the extraction, 1ml of TRIzol was added and vortexed briefly for 5sec. The contents were then incubated for 5 min at RT, and centrifuged for 10min at 12,000 × *g* at 2–8°C. About 0.2ml of chloroform was added and tubes were vigorously vortexed for 15sec in a shaker. Following vortexing, the contents were centrifuge for 15min at 12,000 × *g* at 2–8 °C. The upper aqueous phase was transferred to a new 2ml vial, and added with 0.25ml of isopropanol. The contents were mixed thoroughly and incubated for 10min at RT, followed by centrifuge 10min at 12,000 × *g* at 2–8°C. The pellet obtained was washed with 1ml of 70% ethanol and centrifuged for 5min at 7500 × *g* at 2–8°C. The pellet obtained was air-dried and re-suspended in 20–50µl of RNase-free water.

cDNA synthesis: The cDNA synthesis was done with RT PCR kit using Superscript TMII Reverse Transcriptase, 200U/µl (HiMedia). About 2µg of the total RNA obtained was used along with random primers and 1µl of RT enzyme. The contents were incubated at 25°C for 10 min and then by incubating the contents at 70°C for 45min. This cDNA was used in real time PCR.

Real-time PCR: Primers used for the traditional PCR were used for the real time PCR also. The real-time PCR assay was then performed according to Salam Abbas *et al* (2019) [32] using SYBR Green (HiMedia). The primers of 600nM and 1µl of the cDNA was used with a total volume of 12.5µl. The assay was done in duplicates alongside with negative control. Housekeeping gene β-actin (β-act) [Accesion no: XM_002845542] was used in the study.

Expression of pander F members: Real-time PCR was carried on the samples in the Corbett Research cycler (Bio-Rad). The panDerm_F primers (both FW and RV) of 600nM concentration was used in the program. 1.1µl of RNA was used for about 40 cycles at 94°C for 45s, 62°C for 50s, and with an elongation at 71°C for 55s. Beta actin primers (FW: CTCCTGAGGCTCTCTTCC; RV: GTAGTACCGCCGGACATG; Product length 142bp] was used (Ciesielska, A, 2018) [33] was also amplified for a comparative analysis of the expression. The comparative analysis was done by ΔΔCt method. The Ct values thus obtained for the desired gene was normalized to its housekeeping gene.

Results:

All samples were first processed via direct microscopy and culturing prior to multiplex PCR analysis. The direct microscopic evaluation using KOH was performed on all the 52 subjects with physical manifestation of Athlete's foot. However, 19 clinical samples turned out negative upon culturing while the rest of the samples were positive. *M. canis* was identified as the main causative agent, accounting for 25 (80.5%) of the positive cases, while *T. mentagrophytes* was implicated in 20 positive cases. The data in figure 1 showed that the PCR process improved the detection rate of dermatophytes by about 21.1 %.

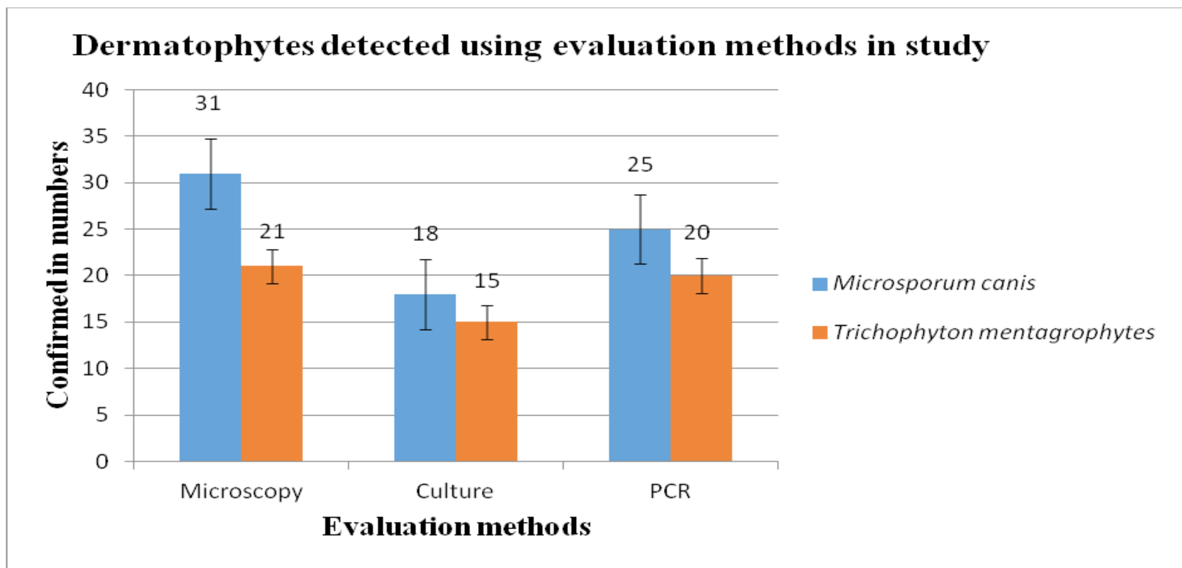

Figure 1: Graph showing the confirmed Dermatophytes in numbers using the three methods of evaluation Microscopy observation, culturing and PCR.

The clinical isolates were differentiated based on their gross colonial and microscopic presentations. *T. mentagrophytes* produces powdery to downy colonies while *M. canis* produces powdery to granular colonies. Owing to the inconsistency in the cultural characteristics of the fungi, further testing was performed to establish their identity. Both *T. mentagrophytes* and *M. canis* can hydrolyse urea but only *T. mentagrophytes* can produce yellow diffusible pigments on Littman Oxgall agar and this differentiates it from *M. canis*.

Table 1: Classification of lesion changing patterns resulting in the detection of dermatophytes scraping skin samples

Fungal causative agents	Dermatophytes lesion patterns.	No.	Percentage
Microsporum canis	Inflammatory or vesicular (vesiculobullous)	10	32.2%
	Chronic hyperkeratotic (moccasin)	18	58%
	Ulcerative Tinea pedis	3	9.7%
Trichophyton mentagrophytes	Inflammatory or vesicular (vesiculobullous)	3	14.2%
	Chronic hyperkeratotic (moccasin)	17	80.9 %
	Ulcerative Tinea pedis	1	4.7
Total		52	100%

Dermatophytosis simplex lesions typically manifests between the 4th and 5th digits and commonly presents with itching, malodour, and burning as observed in the 52 patients included in this study. The most common clinical type was chronic hyperkeratotic (moccasin) is seen in 18 (58%) and 17 (80.9%) of patients infected with *M. canis* and *T. mentagrophytes,* respectively. This syndrome is common in immunocompromised persons, such as diabetic patients and normally involves *Trichophyton* species [34](Leung et al., 2020). (See Table 1).

Microscopic analysis: Samples obtained from the infection sites were viewed under low (10X) and high power (40X) for screening the hyphae and arthroconidia with methylene blue. From the staining, it was found that 31 of them are *M.canis* and 21 of them are *T. mentagrophytes* out of the 52 samples isolated [Figure 2].

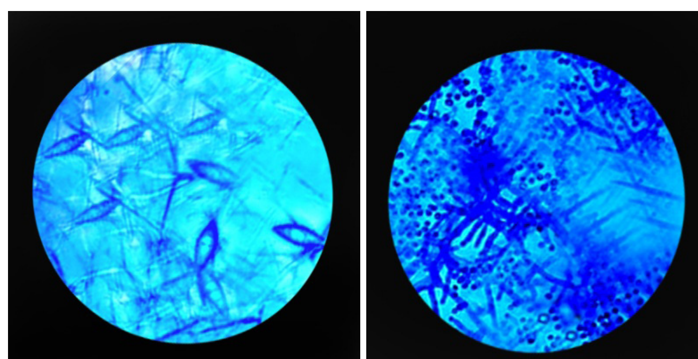

Figure 2: Images as viewed under 40X power, Left: *M. canis*; **Right:** *T. mentagrophytes.*

PCR amplification: The results indicate that the PCR could be the most accurate one to detect dermatophyte-specific DNA from the microbes (*M. canis and T. mentagrophytes*) in the study. All samples were first processed via direct microscopy and culturing prior to multiplex PCR analysis. On running the products on 3% agarose gel, it was found that specified bands were obtained at 361 and 643bp for *T. mentagrophytes and M. canis* respectively. This confirms the presence of the genes. However, some bands seems to be varying in intensity for both the genes used in the study [Figure 3, 4].

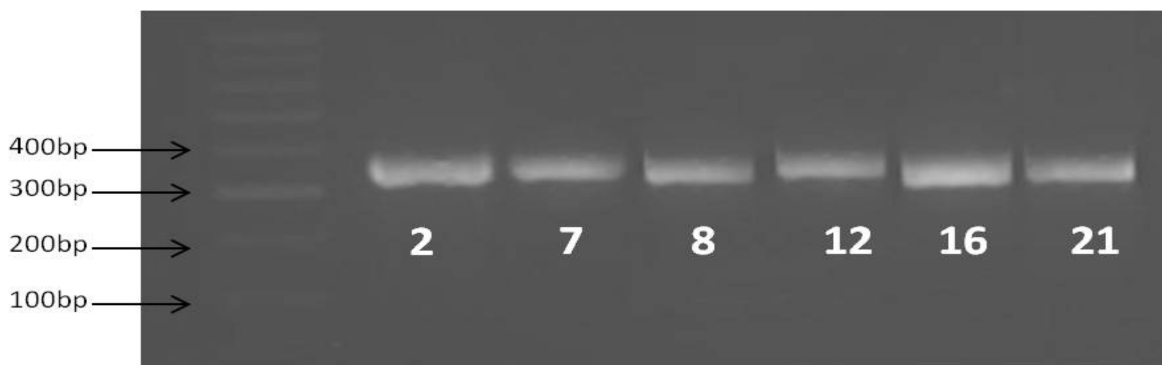

Figure 3: 3% agarose gel showing the amplified bands at approximately 361bp. Molecular marker of 100bp was used in the study. Bands of few PCR products of the mentioned sample numbers were 2, 7, 8, 12, 16 and 21.

Figure 4: 3% agarose gel showing the amplified bands at approximately 643bp. Molecular marker of 100bp was used in the study. Bands of few PCR products of the mentioned sample numbers were 1, 3, 4, 11, 17 and 20.

Real time PCR of gene members: The results obtained which are normalized to its housekeeping gene (GAPDH), reduces the differences between the samples. The level of mRNA expression of the gene for both the samples was separately studied. The sample with the lower ΔΔCt values was chosen to be the calibrator so the samples were compared with that calibrator. The Ct values of beta actin was found to be 11. The ct values for the gene of *T. mentagrophytes and M. canis* were found to be 16 and 17 respectively. Based on the Ct values, the Ct values of the samples (treatment with the drug) were normalized to the housekeeping gene (beta actin). The beta actin expression was considered to be 100% in the study. From the calculated 2−ΔΔCt values, it was found equal expression for both the samples. In *T. mentagrophytes* the gene was expressed 64 times, and in *M. canis* it was expressed 32 times.

Discussion

Till the last 2 to 3 decades, microscopy and culture methods on SDA were alone used for diagnosis of dermatophytes [35]. Sometimes, due to little sporulation, identification of dermatophytes becomes impossible or difficult in a lab setting. Such problems are addressed by isolating the DNA and sequencing with aligning to the available fungal databases.

But still such methods are time-consuming and very expensive as such we proposed a method to show good detection following the traditional methods. Even though we could end up with 100% detection of dermatophyte DNA but still the assay needs to be better optimized. And moreover, we need to use different reagents than those which are previously used [36] . And also, the gold standard methods could only permit the correct identification, if at all carried by a qualified mycologist who is having a sound knowledge on the morphological features of dermatophytes micro- and macroconidia. In addition to this, presence of chlamydospores also need to be addressed. In the present context, the detection was quite possible within 5hr of time (excluding the culturing time) and moreover, these results are objective and do not need an experienced investigator.

In addition to the above pros, this method uses a small amount of sample. And very few diagnostic laboratories round the world, use such modern molecular assays like PCR due to lack of a proper diagnostic algorithm [37]. However, the method of fungal DNA extraction previously proposed brings is simple, accurate and cost effective for diagnosing dermatophytosis from animals. This could be used as a standard method in the coming years for veterinary diagnosis.

This study explored the evolution, spread and reproduction of two major fungi (*M. canis and T. mentagrophytes*) implicated in tinea pedis. The medical practitioners will be well-guided in managing *Tinea pedis* if provided with the accurate clinical data that detailed the appropriate diagnosis and correct identification of the causative agents. These suggest that habits, such as wearing of rubber foot

wears and nylon socks, as well as practice of animal husbandry, could be the most important determinants of the frequency of superficial fungal infections and their aetiological agents among farmers and forestry workers. The outcome of this study confirmed the screening of dermatophyte-specific DNA within the clinical samples from farmers. Adaptation of this assay could pave ways to design a much more reliable method to detect the dermatophytes-specific DNA directly from the patients in less time.

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Ethical Clearance- No Need

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Conflict of Interest – the Authors have declared, **No Conflict of Interest**

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