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Identification of Dermatophyte Species by PCR Restriction Fragment Length Polymorphism in Ramallah region, Palestine

RESEARCH

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A B S T R A C T

Dermatophytes are a group of fungi that cause an infection called dermatophytosis. They are mainly classified into three anamorphic genera: *Trichphyton*, *Microsporum* and *Epidermophyton*. Sixty-five samples were collected during the period from the middle of January to the end of September of the year 2019. The clinical specimens (hair, nail and skin) were collected from 58 patients who were clinically diagnosed with dermatophytosis. Molecular identification was done by using the fungus-specific universal primers (ITS1 and ITS4) for the amplification of the ITS region in the rRNA gene. For species identification, *BstN1* restriction enzyme was used for the digestion of the amplified ITS products to produce distinct band patterns. The distribution of dermatophytosis among the patients was 52.3.%, 41.5%, 3.1%, 1.5% and 1.5% for *Tinea nail*, *Tinea capitis*, *Tinea corporis*, *Tinea pedis* and *Tinea imbricate*, respectively. Nighnteen clinical isolates of dermatophytes showed positive culture. *Trichophyton* was the most common genus with 15 sample (78.9%), followed by *Microsporum* with 4 samples (21.1%) and no *Epidermophyton* were found. The most common species was *T. rubrum* (n=5, 26.3%), followed by *T. verrucosum* (n=3, 15.8%), then *T. mentagrophytes*, *M. Canis*, *M. audouinii* and *T. schoenleinii* with tow isolates for each (10.5%). The amplification of ITS regions was successful in certain samples by using the fungus-specific universal primers (ITS1 and ITS4) and PCR-RFLP technique.

Keywords: Dermatophytes, dermatophytosis, Palestine, RFLP

Introduction

Dermatophytes are a group of anamorphic, filamentous, keratinophilic and keratinolytic fungi, which means that they have the ability to utilize keratin and destroy keratinous tissue; such as hair, nail and skin (Elavarashi et al., 2013); as a source of nutrition causing an infection which called dermatophytosis, ringworm or tinea (Habeb et al., 2016). Dermatophytes are mainly classified into three anamorphic genera: Trichophyton, *Microsporum* and *Epidermophyton* (Weitzman and Summerbell, 1995). Dermatophytosis are not usually life-threatening infections (Robert and Pihet, 2008). They are cutaneous infections, where this group of fungi grows on the infected skin in an annular shape (Aneja et al., 2013), and can't go deeper in the body in the case of immunocompetent hosts. Infections by these fungi are named according to the site of infection; infection of the feet is called tinea pedis, scalp infection called tinea capitis and nail infection called tinea (onychomycosis). Several body sites could be infected by the same dermatophyte species, and several species of dermatophyte can cause the same infection (Mukherjee et al., 2011). Approximately, 20- 25% of the world population are infected with superficial mycotic infections of which dermatophytes are the commonest etiologic agent (Grumbt et al., 2011) which makes it a public health concern, where 5-6 dermatophyte species are majorly causing these infections (Sahoo and Mahajan,

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2016), where *T. rubrum*, *T. mentagrophytes*, *T. tonsurans*, *M. canis*, *M. gypseum* and *E. floccosum* are the most common etiologic agents of dermatophytosis in humans (Habeb et al., 2016). This study conducted to address part of deficient information in molecular diagnosis of dermatophytes in Palestine. Therefore, this study aimed to isolate dermatophytes from clinically diagnosed patients in cultures and to identify dermatophytes at species level by PCR-RFLP technique.

Materials and Methods

Sample collection

Sixty-five samples were collected from the National Center for Skin Diseases, Ramallah Governmental Hospital during the period from the middle of January to the end of September of the year 2019. Clinical specimens were collected from 58 patients (26 males and 32 females) who are clinically diagnosed with dermatophytosis, where five patients (four females and one male) were sampled twice and one patient (male) was sampled three times. Sample type includes hair, nail and skin. Demographic data including: gender, age, geographic area, presence of animals in the patients' environment and dealing with them, education, career, medications and patients' history were collected (Indira, 2014).

Direct examination

Each sample was divided into two parts, one part was used for the direct examination by microscopy. A part of the sample was placed on a sterile microscope slide with a few drops of 20% (KOH). Hair and skin samples were examined after 15-20 minutes and nail samples were examined after 30 minutes. Each sample was examined under high dry (400x) magnification power for the presence of mycelium and /or spores.

Cultivation and isolation of Dermatophytes

The other part of each sample was used for culture on Sabouraud's dextrose agar (SDA) without being supplemented with any antibiotics. Then they were incubated at room temperature for 7- 28 days. Macroscopic and microscopic features of the colonies were determined. Culture was considered as negative after four weeks (Emam and Abd El-salam, 2016). Dermatophytes-positive samples were preserved at -20˚C for further molecular studies.

Extraction of fungal DNA

The fungal genomic DNA was extracted from fungal tissues with NucleoSpin Plant II Kit (MACHEREY-NAGLE, Germany) according to the manufacturer's instructions. For the lyses of fungal cells, fungal tissues were collected in a 1.5 ml Eppendorf tube. Glass beads and 200 µl of PL1 buffer were added, the sample was homogenized by vortex. PL1 buffer (100 µl) and *RNase A* (10 µl) were added with continuous homogenization by vortexing. The homogenate was incubated over night at 65˚C with shaker. Then, 100 µl of chloroform was added, vortexed for 10 sec, then the tube was centrifuged for 10 min at 10,000 RPM. The aqueous supernatant layer was transferred into a NucleoSpin Filter (violet ring) constructed in new collection tube, centrifuged for 2 min at 10,000 RPM, then the filtrate was collected and the NucleoSpin Filter discarded. For DNA binding, 450 µl of PC buffer was added and mixed thoroughly by gentle vortexing, then transferred into a NucleoSpin Plant II column (green ring) that was inserted into a new collection tube, centrifuged for 1 min at 10,000 RPM and the filtrate was discarded. For wash and dry the membrane of the filter; 400 µl of PW1 buffer was added to the NucleoSpin Plant II column and centrifuged for 1 min at 10,000 RPM then the flow-through was discarded. This step was repeated two more times by adding 700 µl of PW2 buffer at the first time and 200 µl of the same buffer at the second time and centrifuged for 1 min. and 2 min. at 10000 RPM, respectively. In each time the filtrates were discarded. For DNA elution, the NucleoSpin Plant II column was then inserted into a new Eppendorf tube, 50 µl of the elution buffer that was kept at 65˚C was added, and kept again at 65˚C for 5 min. Then, the micro centrifuge tube was centrifuged for 1 min at 10,000 RPM according to the manufacturer instructions. DNA concentration was less than 50 ng/µl except in two samples where they were 57 and 67 ng/ µl. The extracted DNA was then stored at -20˚C until being used.

PCR amplification

The ITS region in rRNA gene of different dermatophyte species was amplified using one pair of primers ITS1 F 5'- TCC GTA GGT GAA CCT GC-GC-3'and ITS4 R 5'-TCC TCC GCT TAT TGA TAT GC -3' as described by (Habeb et al., 2016). The PCR reaction mix with a final volume of 25 µL, was performed with 12.5 µL of 2X Prime *Taq* Premix (GeNet Bio\Korea). This master mix contains Prime *Taq* DNA polymerase, 10X reaction buffer containing $MgCl_2$, Tris HCl, (NH_4) $2SO_4$ and PCR enhancer. dNTP mixture, protein, stabilizer and sediment and 2X loading dye, and then 1µL of each primer (conc. 10µl), 5.5 µL of deionized water and 5µL of DNA template were added. The amplification was carried out using the thermal cycler (Tprofessional Basic Cradient) according to the following thermal conditions: initial denatur-

ation for 5 min at 95ºC was followed by 40 cycles of denaturation at 95ºC for 30 sec, annealing at 58ºC for 1 min and extension at 72ºC for 30 sec, followed by a final extension step at 72ºC for 3 min (Habeb et al., 2016). The PCR products were resolved by electrophoresis through 2% agarose gel to determine the size of amplified fragment after ethidium bromide staining (0.5 µg/ml) and visualized by UV transilluminator. A 100-bp DNA ladder was used to determine the size of fragments.

PCR-RFLP analysis

In order to identify dermatophytes to the species level by specific PCR, all PCR products were subjected to digestion with restriction enzyme *BstN1*. The reaction mixture contained 10 µL of PCR product, 1 µL of 10 X *NEB* buffer and 0.5 µL of *BstN1* restriction enzyme, which recognizes the sequence 5'-CC (T/A) GG-3' (Habeb et al., 2016). Subsequently reactions were incubated at 60°C in dry oven for 80 min. All PCR digested products were analyzed by 2% agarose gel electrophoresis.

The DNA fragments were visualized under a UV trans-illuminator and identification of the isolates was carried out through comparing the electrophoretic RFLP patterns with the previously published Habeb et al. (2016) profiles. Size of amplified ITS region before and after digestion are presented in Table 1.

Table 1: The profile regarding to PCR product of ITS region in dermatophyte species before and after digestion (Habeb et al. 2016).

Dermatophyte species	PCR product size before digestion	Size of digested amplified ITS product
T. rubrum	690	380,180,100 and 30bp
T. mentagrophytes	690	250, 180, 160 and 120bp
T. tonsurans	550	280, 100, 100 and 70 bp
T. soudanense	680	350, 180, 100 and 50 bp
T. concentricum	650	360 and 290 bp
T. verrucosum	650	380, 180, 100 and 10 bp
T. schoenleinii	650	400 and 250 bp
T. interdigitalae	700	250, 180, 150 and 120 bp
T. terrestre	630	250, 190, 120 and 80 bp
M. canis	740	440, 160, 100 and 40 bp
M. audouinii	600	600 bp
M. gypseum	650	400 and 250 bp
E. flocosum	740	400, 250 and 180 bp

Results

Identification of Dermatophytes by conventional methods

This study was done on a total number of 65 samples that were taken from 58 (26 males (44.8%), 32 females (55.2%)) patients who suffered from dermatophytosis. The distribution of dermatophytosis among the patients was 52.3.%, 41.5%, 3.1%, 1.5% and 1.5% for Tinea nail, Tinea capitis, Tinea corporis, Tinea pedis and Tinea imbricate, respectively (Table 2).

Table 2: Distribution of dermatophytosis based on gender among 65 patients.

Clinical manifestation (Tinea types)	Gender		Total (n\%)
	Male n(%)	Female n(%)	
Tinea nail	11 (32.4%)	23 (67.6%)	34 (52.3%)
Tinea capitis	16 (59.3%)	11 (40.7%)	27 (41.5%)
Tinea corporis	1 (50%)	1(50%)	2 (3.1%)
Tinea pedis	1 (100%)	0	1 (1.5%)
Tinea imbricate	0	1 (100%)	1 (1.5%)
Total	29 (44.6%)	36 (55.4%)	65 (100%)

Results of this study showed that 62 (95.4%) samples were positive based on microscopy (KOH) method. However, 19 (29.2%) samples were positive based on the culture method. Initially, fungal hyphae were seen by direct examination with (KOH) so the samples were recorded as positive. From the 19 samples that were positive in culture, *Trichophyton* was the most common with 15 (78.9%) samples, followed by *Microsporum* with 4 (21.1%) samples and no *Epidermophyton* were found.

Results in this study showed that *T. rubrum* is the most common species (26.3%), followed by *T. verrucosum* (15.8%). Followed by *T. mentagrophytes*, *M. canis*, *M. audouinii* and *T. schoenleinii*, which showed that the frequency for each species is (10.5%). One sample of the 19 has shown a mix fungal isolates composed from *Dermatophyte* and *Aspergillus* (Table 3). According to the colonial morphology of identified species, *T. rubrum* are characterized by fluffy, buff and downy white. The reverse side of colonies have orange-brown with yellow borders.

Table 3: Distribution of *Dermatophytes* among patients that had positive culture

Dermatophyte species	Number of isolates (%)
T. rubrum	5 (26.3%)
T. mentagrophytes	2 (10.5%)
M. canis	2 (10.5%)
M. audouinii	2 (10.5%)
T. schoenleinii	2 (10.5%)
T. verrucosum	3 (15.8%)
T. vilaceum	1 (5.3%)
T. tonsurance	1 (5.3%)
Unknown	1 (5.3%)
Total	19 (100%)

The septated macroconidia were clearly obvious, and the microconidia were existed in a large number in a teardrop shape (Figure 1).

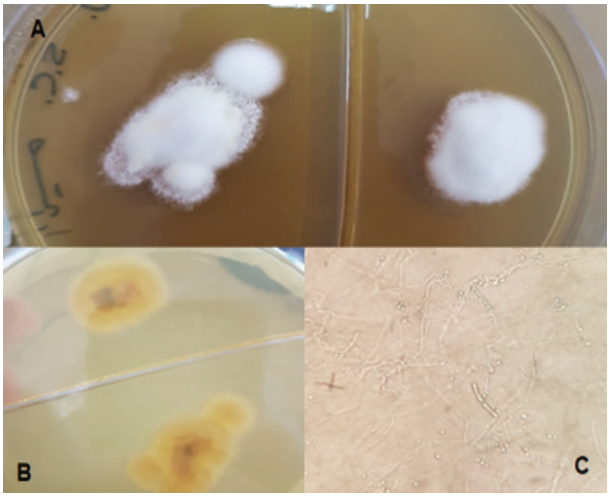

Figure 1: Colonial morphology of *T. rubrum* (A) surface, (B) reverse side and (C) macroconidia under direct microscopy (400 X)

T. verrucosum are characterized by gray to pink, wrinkled to velvety small colonies with brown reveres side, many chlamydoconidia seen in chains, macroconidia and microconidia are few to rare (Figure 2). *M. canis* are characterized by a membranous with feathery periphery colony, buff center, white to lemon-yellow in color, yellow-orange reverse side. Macroconidia are large, spindle-shaped with curved ends (Figure 3). *T. mentagrophytes* are characterized by granular, wrinkled and creamy yellow to a white surface, and yellow-brown reverse side, club-shaped and cigar-shaped macroconidia are seen. Many round microconidia can be seen (Figure 4).

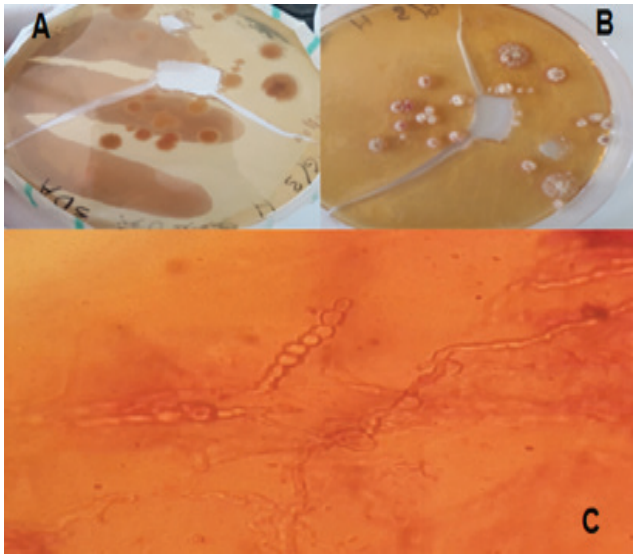

Figure 2: Colonial morphology of *T. verrucosum*; (A) reverse side, (B) surface and (C) chlamydoconidia under direct microscopy (400 X)

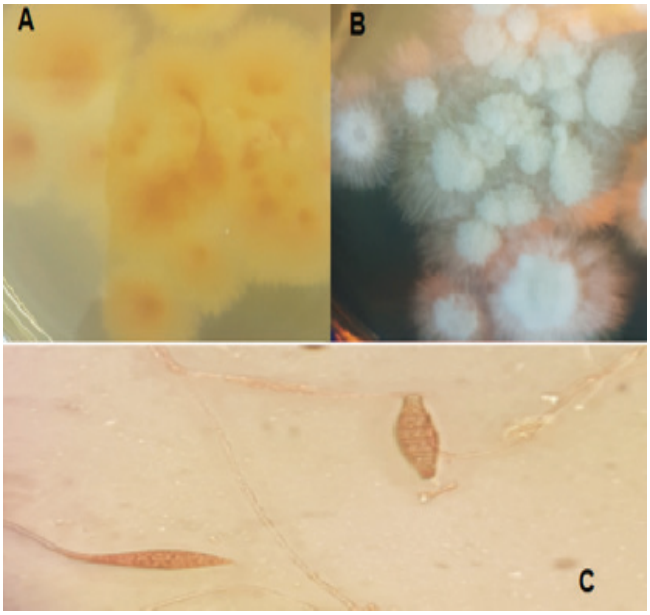

Figure 3: Colonial morphology of *M. Canis*; (A) reverse side, (B) surface and (C) macroconidia under direct microscopy (400 X)

Identification of Clinical Isolates by PCR and PCR-RFLP

In this study, only 9 (47.4%) of isolates showed PCR product (Figure 5). The size of PCR amplicon was 550-bp, 650-bp and 690-bp depending on the species. Three isolates were identified as *T. verrucosum* based on colonial morphology gave two different sizes of product, one 650-bp and two 550-bp (Table 4).

Only 3 (33.3%) amplified ITS region were restricted by *BstN1* restriction enzyme, while the rest (66.7%) were undigested. Data are presented in Figure 5 and Table 5.

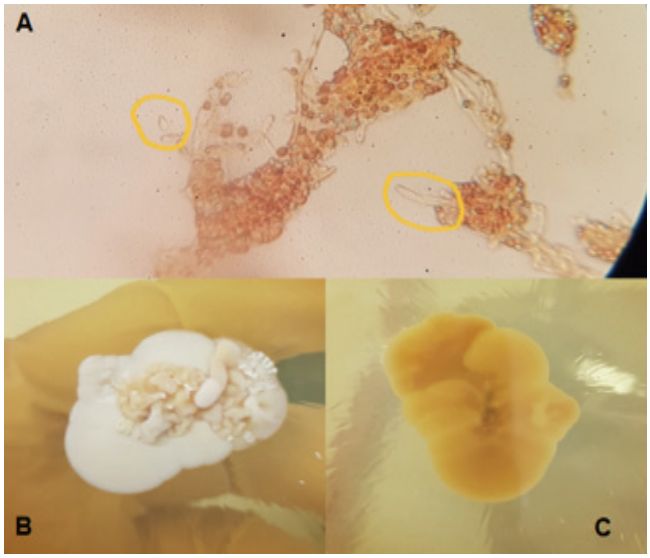

Figure 4: Colonial morphology of *T. mentagrophytes*; (A) microconidia and macroconidia under direct microscopy (400 X), (B) surface and (C) reverse side.

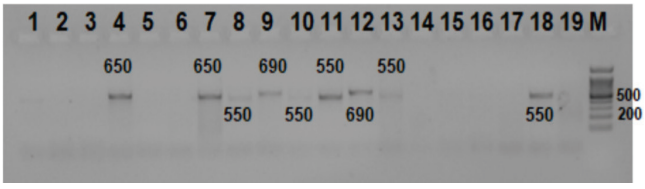

Figure 5: PCR product of amplified ITS region by primers ITS1 and ITS4 and analyzed by 2% agarose gel electrophoresis.

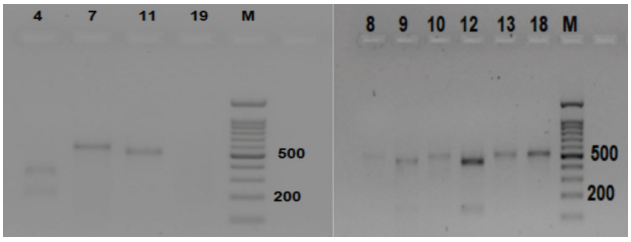

Figure 6: Bands after digestion of PCR product of ITS region by BstN1 restriction enzyme.

Table 4: The size amplicon of the amplified ITS region for 9 isolates belonged to the Dermatophyte species.

Number of DNA sample [a]	Dermatophyte species	Size of amplifies ITS region (bp)
4	*T. schoenleinii*	650
7	*T. verucosom*	650
8	*M. canis*	550
9	*T. rubrum*	690
10	*T. verrucosum*	550
11	*T. tonsurans*	550
12	*T. rubrum*	690
13	*T. mentagrophytes*	550
18	*T. verucosom*	550

Table 5: The size of PCR product and size of digested fragments for Dermatophyte species identified by culture method.

No.DNA sample [a]	Species identified based on culture	Size of PCR product	Length of digested fragments
4	*T. schoenleinii*	650-bp	400+250-bp
9	*T. rubrum*	690-bp	450+140-bp
12	*T. rubrum*	690-bp	450+140-bp

Discussion

Until today, fungal infections are considered as one of the most common skin infections all over the world, which makes it a public health concern, of which dermatophytes are the most causative agent. Dermatophytosis is a disease caused by dermatophytes, where young adults of both genders are most commonly affected (Rashidian et al., 2015). Moreover, both human and fungal cells are eukaryotes, this makes the treatment of fungal infections is difficult and not always successful (Indira, 2014) Results of this study showed that the distribution of the skin fungal infection in female is higher than males. These results are in agreement with the previously published studies carried out in different countries, such as the Kurdistan region (Habeb et al., 2016), India (Elavarashi et al., 2013) and Ethiopia (Alemayehu et al., 2016). Results of this study are in contrast to other studies which showed that the distribution of skin fungal infection in male is higher than females, such as Korea (Lee et al., 2015). In this study, the majority of clinical manifestations is Tinea nail (onychomycosis). This result is consistent with a previously published report from Sweden (Drakensjö and Chryssanthou, 2011). However, in Ethiopia (Alemayehu et al., 2016), tinea capitis was predominant among the samples that were culture confirmed as dermatophytes (18%), in India (Elavarashi et al., 2013) and Iran (Rashidian et al., 2015) tinea corporis was the predominant, 57.97% and 69.2% respectively. The simplest method for the diagnosis of dermatophytes is using KOH for the digestion of keratin tissue, therefore, fungal elements could be easily seen under the microscope, which provides only a presumptive diagnosis of fungal infection. However, it is important to know that this method can not distinguish between different genera and it can cause false negative results (Mukherjee et al., 2011). In this study, 62 samples from a total of 65 were positive in KOH test, while 19 were positive in culture, the remaining samples have shown other fungal isolates such as *Aspergillus*, yeast, others have shown bacteria growth, and some have shown no growth at all. The difference in the results between these two methods can be attributed to certain factors

such as antifungal treatment before sample collection where this will inhibit the growth of the fungi on the media, and inadequate quantity of sample may inhibit or weak the growth of the fungi as well (Rudramurthy and Shaw, 2017). Moreover, co-infection of certain saprophytic fungi with dermatophyte in culture may inhibit the growth of dermatophytes due to nutrient competition (Habeb et al., 2016). Results of the current study showed that *Trichophyton* species were the most common followed by *Microsporum* while no *Epidermophyton* was recovered. these results are in agreement with the previously published study in India (Bhatia and Sharma, 2014). The identification typically based on the conventional methods such as macroscopic features and microscopic examination. Surface, reverse and texture of the colony are macroscopic features, while macroconidia, microconidia, vegetative hyphae and arthroconidia are microscopic (Habeb et al., 2016). A cultured sample has shown different fungal isolates dermatophyte and *Aspergillus* that had mixed and no separated colonies. Therefore, dermatophyte species was not been able to be correctly identified, however, it has been reckoned as *Trichophyton* because of the macroconidia proprieties that were seen, where it looked like the pencil shape which is related to the *Trichophyton* proprieties (Elmer et al., 1997). This study has shown that *T. rubrum* is most common species detected. These results are in agreement and disagreement with previously published reports. This variation in results may be due to the difference in the geographical area, socioeconomic status, time, life style and immigration. (Alemayehu et al., 2016). It has been reported that *T. rubrum* has become the most frequent species worldwide, where it was the predominant in Europe in the 1920s, and since the 19th centuray in South Asia. It has also been reported that during the period 1991-1995 *M. canis* was the predominant in Spain, and in Rome, Italy as well between 2002 and 2004. *E. floccosum* was the predominant in Tehran, Iran in 1999-2001 (Hayette et al. 2015). Identification of dermatophytes by cultural method is very difficult. This could be due to alterations in the physiological and morphological properties of dermatophytes. These alterations may be due to culture technique; incubation temperature and the absence of antimicrobial drug within the media (Habeb et al., 2016). These conditions can easily affect the accuracy and precision of dermatophytes identification by culture. From the 19 clinical isolates of dermatophytes; nine isolates have been amplified and produced bands, this means that the agreement between the two methods is 47.36%, this could be due to the fact that the molecular method is more accurate in addition to the misidentification that may happen in culture due to the great similarity between some dermatophytes (Rezaei-Matehkolaei et al., 2012). Three of the isolates that were identified as *T. verrucosum* by culture method produced two different sizes of product, one isolate produced fragment with a size 650-bp and two isolates produced a fragment with a size 550-bp. Based on Habeb et al. (2016), *T. verrucosum* has a PCR product with a fragment 650-bp, this means that the other isolates may not be *T. verrucosum*, because even between the strains of the same species the difference in the number of the nucleotide is from 1 to 2 or even from 5 to 6 at most (Kim et al., 2011). The same thing according to *M. canis* and *T. mentagrophytes*, *M. canis* in this study produced a fragment with 550-bp. In this study, *T. mentagrophytes* produced a fragment with a size 550-bp, while according to Habeeb et al. (2016), supposed to produce a fragment with a size 690-bp. These results confirm that the conventional method is not accurate compared to molecular, where misidentification happened frequently due to the high degree of similarity between certain dermatophyte species, which by turn may lead to be treated inappropriately. After the digestion of PCR products of *T. rubrum*, two fragments have been produced. According to Habeb et al. (2016) four fragments are produced, while three fragments are produced according to Maikhan et al. (2017). This alteration could be due to mutations in the ITS region, technical errors during PCR amplification or mistake in the identification process (Habeb et al., 2016; Maikhan et al.). In conclusion, from all the dermatophytes, the amplification of ITS regions was successful by using the fungus-specific universal primers (ITS1 and ITS4) (Li et al., 2008), where the variation of these regions between different species can be employed for species identification (Mukherjee et al., 2011). In general, the use of molecular methods such as PCR-RFLP is more reliable than classical methods for the identification of dermatophytes, it is a good and rapid method for the detection and identification of dermatophytes genus and species, where this is important in accelerating the therapy process regarding to the fact that some dermatophyte species are more affected by certain types of antifungal with certain doses (Sahoo & Mahajan, 2016). However, in this study, PCR amplification for some samples were failed, this may be due to poor quality and quantity of extracted DNA.

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