MOLECULAR IDENTIFICATION OF DERMATOPHYTES AMONG CLINICAL ISOLATES

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ABSTRACT

Aim: *This study aimed to identification of dermatophyte species in clinical isolates by both classical and molecular methods, using universal primers for amplification of ITS gene. In addition, identification at a species level was carried out by using PCR-restriction fragment length polymorphism (RFLP) technique.*

Methodology and Results:*In the present study, out of 220 suspected patients (157 females and 63 males) with dermatophytosis were identified by the supervision of specialized dermatologist in the derma unit of the General hospital in Kalar district/Sulaimania province\Iraq during the period from middle of November 2014 to the end of June 2015. Samples were collected from patients included hair fragments, skin scraping and nail clipping which transferred to the research laboratory of Biology Department at Faculty of Education \ University of Garmian, where they immediately examined. Based on the conventional laboratory methods, 80 clinical isolates of dermatophytes showed positive culture which belonging to three genera (Trichophyton, Microsporum and Epidermophyton) and 13 species, , Trichophyton rubrum (downy and granular types) was the most common species 35% followed by T.mentagrophytes 17.5%, M.canis 10% and T.tonsurans 7.5%. Other species T. soudanese; T.interdigitale; T. terrestre represented 5% of dermatophytes while T.concentricum; T.schoenleinii; T.verucosum; Microsporum audouinii; M.gypsium and Epidermophyton flocosum conistitute 2.5%. Molecular identification of dermatophytes was carried by using the primers ITS1 and ITS4 to amplify the internal transcript spacer (ITS) region of ITS1 5.8S ITS2 in rDNA gene, later the species identification was made by digestion of amplified ITS regions by BstN1 restriction enzyme in restriction fragment length polymorphism (PCR-RFLP) to produce distinct band patterns.*

Conclusions: *Survey of dermatophytes showed that PCR-RFLP is a rapid and easy method for dermatophytes identification; therefore we suggest using the PCR-RFLP corresponding to the conventional laboratory identification including macroscopic and microscopic examination.*

Keywords: PCR, RFLP, Molecular

INTRODUCTION

Dermatophytes referred as a unique group of superficial keratinophilic and filamentous fungi which have the ability to invade keratinised tissue of the skin, hair and nail in human and animals leading to dermatophytosis or tinea (ring worm) (Alkahafajii, 2014). They are based on the keratin as a source of nutrient and cause its hydrolysis by releasing number of enzymes such keratinase as a proteolytic enzyme of keratin protein which is very hard and strong (Sharma and Swati, 2012). Dermatophytes mainly classified into three anamorphic genera *Trichophyton*, *Microsporum* and *Epidermophyton*in as well as teleomorphic genus

Arthroderma, and they include more than 30 species. Among the most common etiological agent of dermatophytosis in human include *T.rubrum, Tmentagrophytes, T.tonsurans* as well as *M.canis, M.gypseum*and *E.flocosum* (Weitzman and summerbell, 1995).The conventional laboratory methods for identification still based on the results of macroscopic and microscopic examination. An important characters in the microscopic examination based on the characteristic features of macro and microconidia, hyphal properties and colony properties which seen on SDA (Kane*et al.*, 1997).Based on Davison *et al*., (1980), numerous molecular approaches have been developed for identification of dermatophytes at the level of species and strains. Consequently, some biotypes were identified preciously as species, at the present cannot be distinguished molecularly (Nenoff*et al.*, 2013). At the same time, these methods cause confusion of taxonomical classification of dermatophytes and lead to novel classification (Gräser and Summerbell, 2008). Development of molecular technique provides better understanding in classification, epidemiology and ecology. At present, methodologist depends on DNA amplification and sequencing analysis for identification of dermatophytes (Cafarchia *et al*., 2013) and the most abundant approach for identification include gene-specific PCR, PCR-RFLP, PCR fingerprinting, DNA hybridization and sequencing of ITS region in rDNA gene which provide useful method for identification and phylogenetic analysis of dermatophytes (Li *et al.*, 2008).

MATERIALS AND METHODS:

Sample Collection

Two hundred twenty clinical specimens (63 Male and 157 Female) including hair, skin, and nail were collected from patients between the periods from middle of November 2014 to the end of June 2015, where suspected by dermatologist at Kalar General Hospital, Kalar, Kurdistan region, Iraq. Each sample was divided into two parts one for direct microscopic examination by KOH and the other for culturing on PDA and SDA with cyclohexamide and chloramephinicol(SDACC), then incubated at 25-30°C for 7-21 days and examined at regular intervals. Macroscopical, microscopical and physiological features studied on the developing colonies and the positive diagnosed dermatophytes preserved for molecular diagnosis.

Extraction of Fungal DNA

Fungal genomic DNA was extracted by using OMEGA bio-tek E.Z.N.A. ® SP (USA) fungal DNA Mini Kit according to the instructions recommended by the manufacturer. About 100 mg of fungal tissue was collected in a 1.5 or 2 mL micro centrifuge tube and freeze by dipping in liquid nitrogen then grinded to powder, 4µLRNase A and 600µL SFG1 Buffer was added, vortexed vigorously to mix thoroughly. The mixture was incubated at 65°C for 10 min. The sample was mixed twice during incubation by inverting tube then 140 µL SFG2 Buffer was added and vortexes to mix thoroughly and Centrifuged at 10,000 x g for 10 minutes. The Homogenizer Mini Column was inserted into 2ml collection tube then the supernatant was transferred to a new microcentrifuge tube and centrifuged at 10,000 x g for 2 min. and the supernatant was removed. The lysate was transferred to a new 1.5 ml microcentrifuge tube then 1.5 volume of SFG3 was added and vortexes to obtain a homogenous mixture. The HiBind DNA Mini Column was inserted into 2ml collection tube then 650 µL of the sample was transferred to HiBind DNA Mini Column and centrifuged at 10,000 x g for1 min., the filtrate was discarded and the collection tube reused This step was repeated until the remaining sample has been transferred to the HiBind DNA Mini Column. The column was transferred into a new 2ml Collection tube then 650 µL of SPW Wash Buffer was added and centrifuged at 10,000 x g for1 min. The filtrate was discarded and the Collection tube was reused. This step was repeated. The empty HiBind DNA Mini Column

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was centrifuged at maximum speed for 2min. to dry the membrane then the HiBind DNA Mini Column was transferred into a nuclease-free 1.5 or 2ml microcentrifuge tube. 100 µL of Elution Buffer (heated into 65 °C) was added then left for 3-5 min. at room temperature and centrifuged at10,000 x g for 1 min.(This step was repeated) and the DNA sample was stored at -20.

Primers and PCR Analysis

The ITS region in rDNA gene of different dermatophyte species was amplified by using one pair of primers, symbolized as ITS1 forward primer (5' TCC GTA GGT GAA CCT GCGC-3'(TCC GTA GGT GAA CCT GCGC) and ITS4 revers primer ITS4 5'(TCC TCC GCT TAT TGA TAT GC -3'). PCR was performed in 25 µL of PCR reaction mixture containing was prepared by mixing12.5 µL of Prime Taq DNA Polymerase (2X master mix) containing: Prime Taq DNA polymerase, 10 X reaction buffer containing MgCl2, Tris HCL, (NH4) 2SO4 and PCR enhancer. dNTPmixture, protein, stabilizer and sediment and 2 X loading dye (GeNet Bio\Korea) which mixed with1.5 µL of each primer, 3.5µL of DNA template, 1.5 µL of each primer and 6 µL of deionized water was added to made up the total volume of 25 µL PCR reaction. The PCR reaction were spun down shortly for 5 sec. and placed in thermal cycle (TCY, Crealcon, and NL). Amplification conditions of each isolate were: initial denaturation (1 cycle 95°C for 5 minutes) followed by 35 cycle: denaturation (95°C for 30 seconds), annealing (58-61°C for 1 minute), extension (72°C for 2 minute) followed by final extension (72°C for 5 minutes). Then the PCR products were loaded visualized on 1.5% agarose gel and stained with ethidium bromide and visualized by UV transilluminator. A 100bp DNA marker was used as a reference to determine the size of fragments.

PCR-RFLP

In order to identification at a species level by specific PCR, all PCR products were subjected to digestion with restriction enzyme *BstN1* (BioLabs, England). The mixture contained 10 µL of PCR product, 1.5 µL of 10 X NEB buffer, 0.5 µL of *BstN1* enzyme,which recognizes the sequence 5' CC (T/A) GG 3' to a final volume 12 µL. Subsequentlyreactions were incubated at 60° C in dry oven for one hour. All PCR digested products were electrophoresed in a 2 % agarose gel, after placing 5 µL of the ladder in the first well and 10 µL of the PCR products in the other wells of the gel which supplied to the power at 90 volt for 90 min. The gel was observed under a UV trans-illuminator and identification of the isolates was carried out through comparing the electrophoretic RFLP patterns with those profiles (Jackson *et al.*, 1999; Matehkolaei, 2012; Elavarashi *et al*, 2013).

RESULTS

A total of two hundred twenty clinical specimens (63 Male and 157 Female) including hair fragments, skin scraping and nail clipping, 200 (90.9%) of specimens showed positive result by 10% KOH examination and 80 (36.4%) of clinical specimens revealed positive cultures of dermatophytes(Table 1).

Table 1. Positive and negative results of the direct examination and laboratory culture of dermatophytes

Total Number	*Direct Examination by KOH*				*Laboratory Culture*			
220	*Positive result*	*%*	*Negative result*	*%*	*Positive result*	*%*	*Negative result*	*%*
	200	90.9	20	9.09	80	36.4	140	63.6

From 200 cases were showed positive KOH examination, only 80 (36.4%) revealed positive culture for dermatophytes which belonging to three genera *Trichophyton, Microsporum and Epidermophyton* and 13 species as seen (Figure 1 & 2). Conventional identification of dermatophytes based on macroscopic features (colony surface, colony reverse and texture of colony), microscopic examination (macroconidia, microconidia, vegetative hyphae and arthroconidia) and some physiological characters. *T.rubrum* (downy and granular strains) was the most common isolate (35%) followed by *T.mentagrophytes* (17.5%), *M.canis* (10%) and *T.tonsurans* (7.5%) (Table 2).

Table 2. Frequency and ratio of fungal species were positive culture

N.	*Isolates*	*Number of isolates*	*%*
1.	*Trichophyton rubrum*	28	35%
2.	*Trichophyton mentagrophytes*	14	17.5%
3.	*Trichophyton tonsurans*	6	7.5%
4.	*Trichophyton soudanese*	4	5%
5.	*Trichophyton interdigitalae*	4	5%
6.	*Trichophyton concentricum*	2	2.5%
7.	*Trichophyton schoenleinii*	2	2.5%
8.	*Trichophyton terrestre*	4	2.5%
9.	*Trichophyton verucosum*	2	5%
10	*Microsporum canis*	8	10%
11	*Microsporum audouinii*	2	2.5%
12	*Microsporum gypsium*	2	2.5%
13	*Epidermophyton floccosum*	2	2.5%
14	Total	80	

A

B

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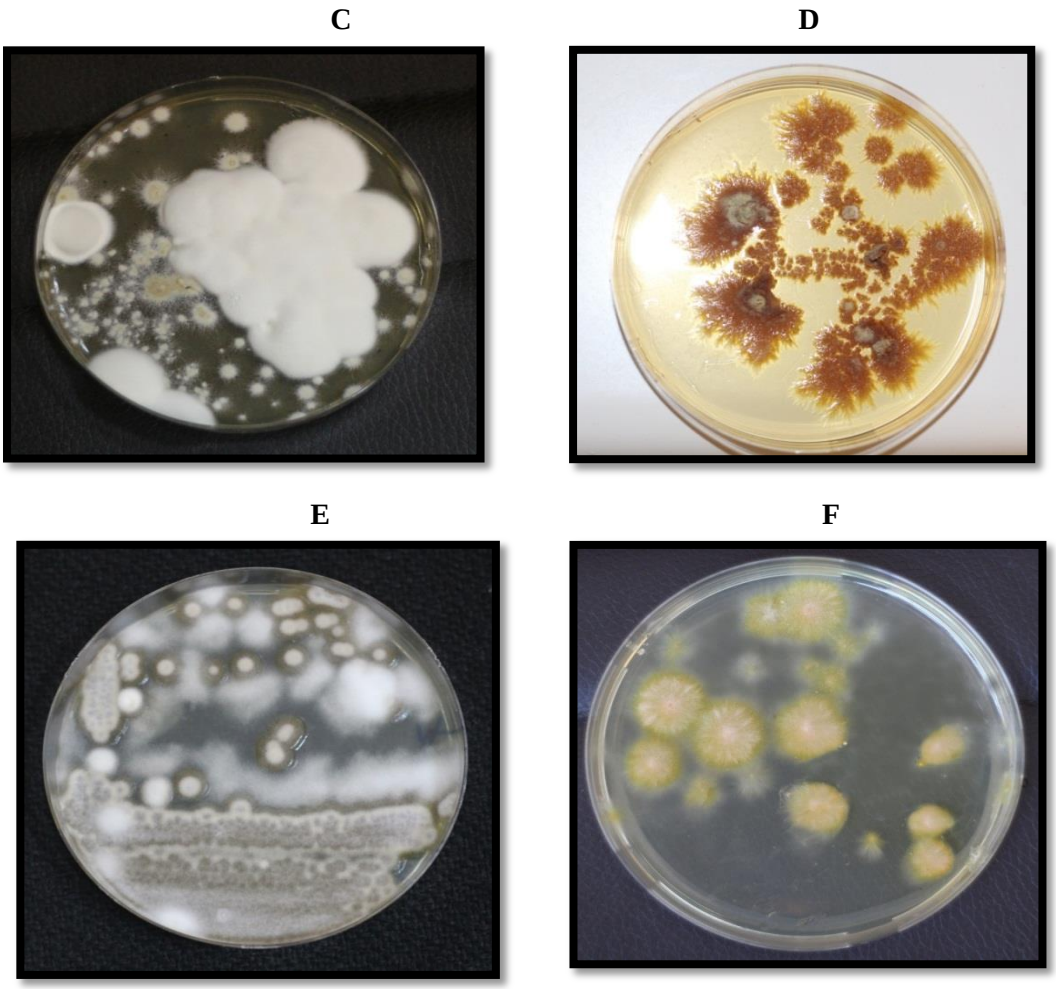
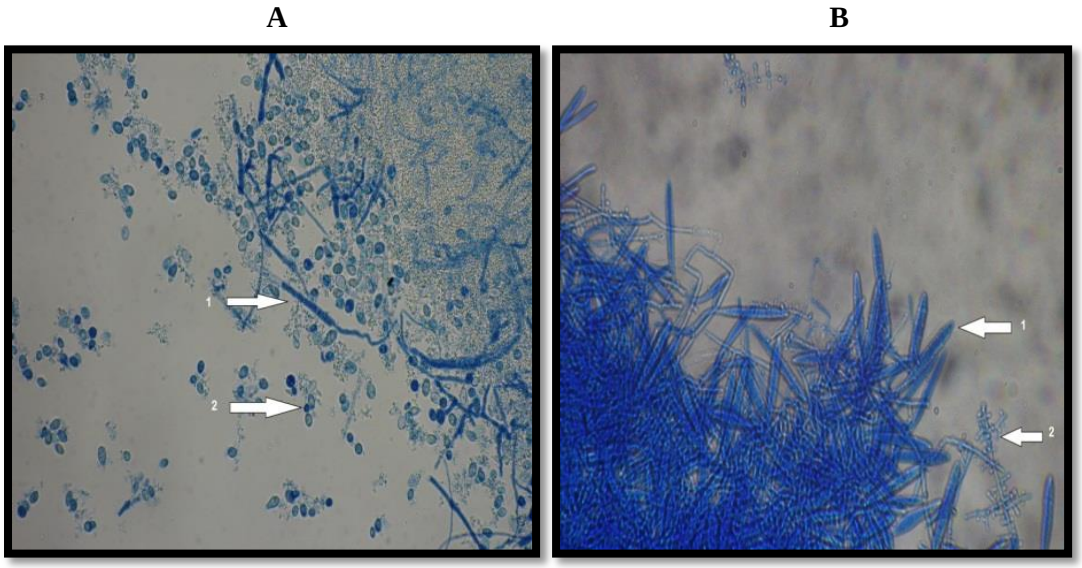


Figure 1: colonial morphology of dermatophytes on SDACC at 25°C after one week of incubation. A & B) Colony surface and reverse of *T.rubrum.* C) Colony surface of *T.mentagrophytes* . D) Colony surface of *E.flocosum.* E & F) Colony surface of *M.canis*



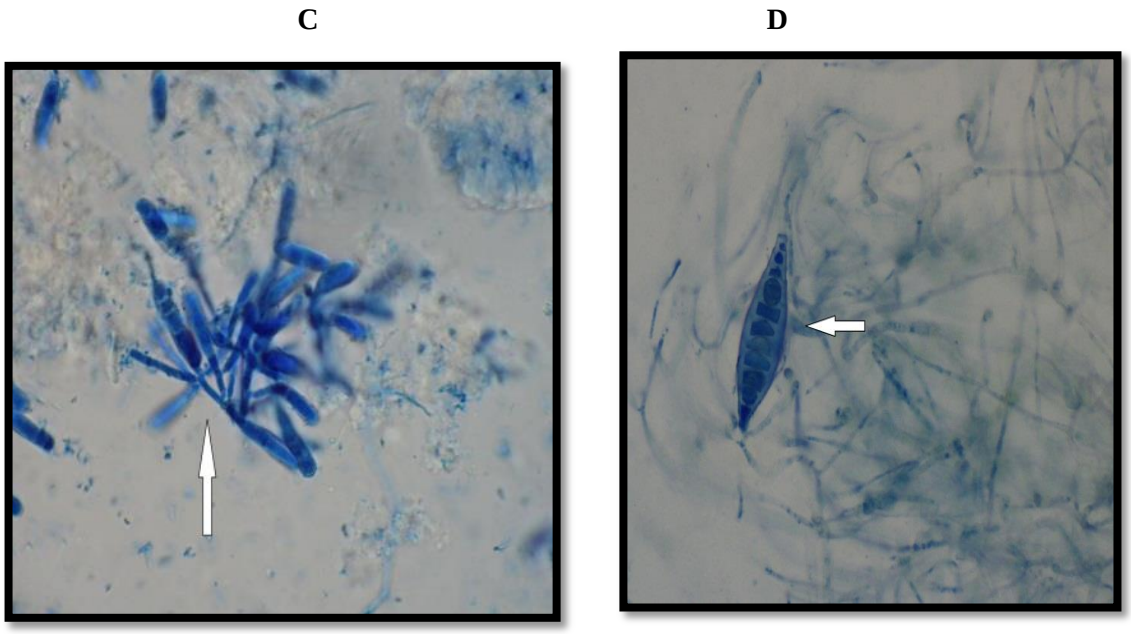
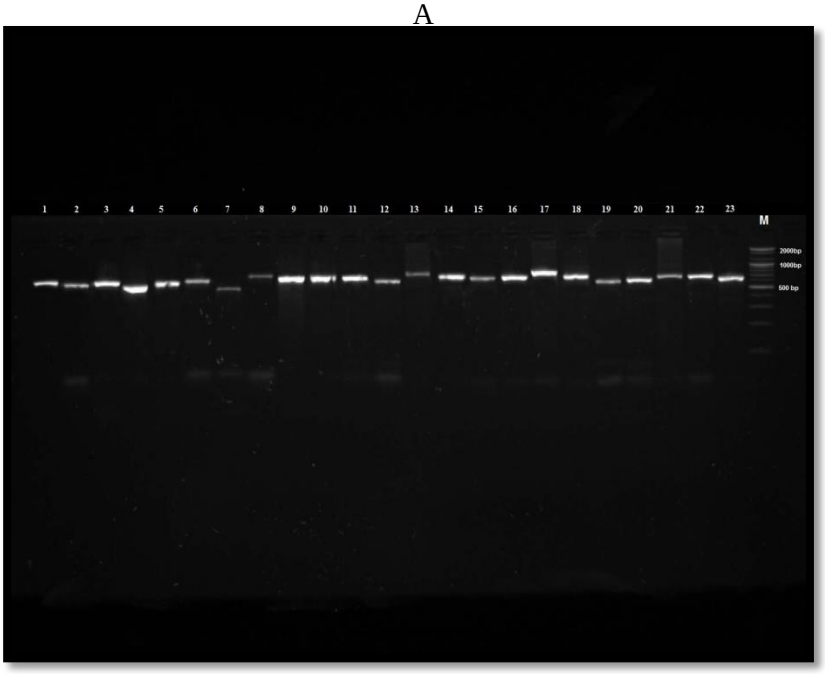


Figure 2: microscopic morphology of dermatophytes showing micro and macroconidia (40X). A) 1) Macroconidia 2) Microconidia in *T.rubrum*. B) 1)Macroconidia . 2) Microconidia in *T.mentagrophytes* . C) Macroconidia in *E.flocosum*. D) Macroconidia in *M.canis*

Fungal genomic DNA was extracted from clinical isolates of dermatophytes using liquid nitrogen and Omega genomic DNA extraction kit and the genomic DNA was analyzed by electrophoresis on 1% agarose gel**.** In Species –Specific PCR the ITS region on rDNA was amplified by using primers ITS1 and ITS4 which resulted in PCR product size in the range between 550 in*T.tonsurans* and 740bp in *M. canis* and *E.flocosum*as shown in table (3). The PCR products were obtained previously by amplification of the ITS region subjected to the digestion by *BstN1* restriction enzyme. This analysis showed the polymorphism of ITS region for different species of dermatophytes. Digestion of ITS PCR products revealed a number of recognition patterns with the exception of *M.audouinii* which was none. The size of ITS PCR products before and after digestion demonstrated in table (3) and figure (3).

A



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B

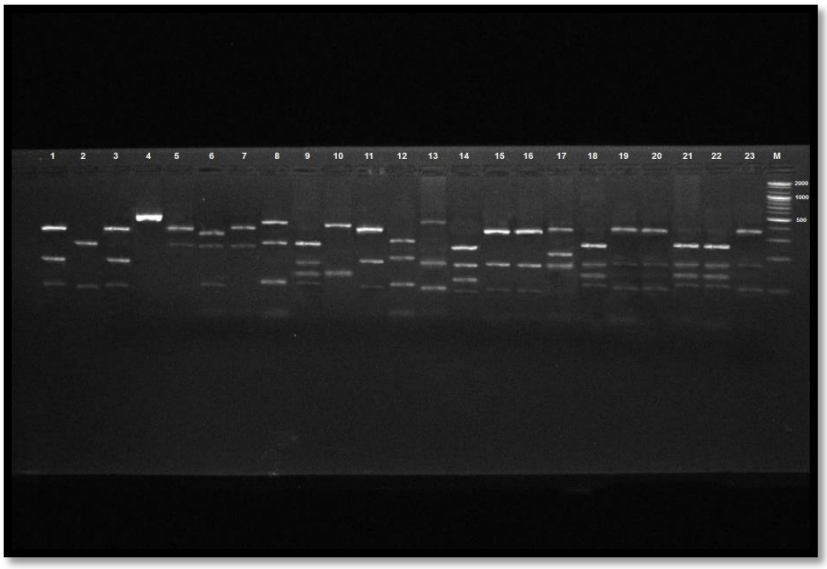

Figure 3. (A) PCR amplified of ITS regions. (B) Digestion of amplified ITS product by *BstN1* restriction enzyme (PCR-RFLP) for 23 clinical isolates of dermatophytes. All products were electrophoresd in 2% agarose gel with ethidiumbromide.Lanes: *1,T.rubrum; 2, T.tonsurans; 3,T.rubrum; 4, M.audouinii; 5, T.concentricum; 6, T.rubrum7, M.gypseum; 8, M.canis; 9,T.mentagrophytes; 10, T.schoenleinii; 11,T.rubrum; 12,T.terrestre; 13,M.canis; 14, T.interdigitalae; 15,T.rubrum; 16, T.rubrumvar.rubitiskii; 17,E.flocosum; 18,T.mentagrohytes;19, T.soudanense; 20,T.soudanence; 21,T.mentagrophytes; 22, T.mentagrophytes; 23, T.verucosum*

Table 3. PCR product of ITS region in dermatophyte species before and after digestion

N.	*Dermatophyte species*	*Size of amplified ITS region befor digestion*	*Size of digested amplified ITSproduct*
1	*T.rubrum*	690bp	380, 180 , 100 and 30bp.
2	*T.mentagrophytes*	690bp	250,180,160 and 120bp.
3	*T.tonsurans*	550bp	280,100,100 and 70bp
4	*T. soudanense*	680bp	350,180,100 and 50bp
5	*T.concentricum*	650bp	360 and 290bp
6	*T.verucosum*	650bp	380,180,100 and 10bp.
7	*T.schoenlenii*	650bp	400 and 250bp
8	*T.interdigitalae*	700bp	250,180,150,120
9	*T.terrestre*	630bp	250,190,120 and 80bp
10	*M. canis*	740bp	440,160 and 100 and 40bp.
11	*M.audouinii*	600bp	600bp
12	*M.gypseum*	650bp	400,250bp
13	*E.flocosum*	740bp	400,250 and 180bp.

DISCUSSIONS

The results we have obtained revealed that among 220 suspected patients with clinical symptoms of dermatophyte infection, 200 cases (90.9%) were found to be positive by KOH examination while the positive cultures were 80 cases (36.4%) and the remaining cultures were suspected to be contaminated by *Rhizopus* , *Aspergillus* , *Penicillium Alternaria* and *Fusarium*. These data revealed that the KOH examination was false positive for 120 cases and this result is unacceptable. This variation between the results of KOH test and laboratory culture may be due to that the microscopic examination by KOH is a simple technique which giving fast presumptive diagnosis (Sarika *et al*., 2014) and therefore the laboratory culture was more sensitive than the direct examination by KOH. This study showed similarity with the results of Singh and Beena (2003) who's found that KOH test was less sensitive than the laboratory culturing of dermatophytes. On the other hand the negative results were obtained from the laboratory culture of dermatophytes may be due to error in the sampling process or inadequate specimen which subjected to splitting to perform microscopic examination and laboratory culturing. In addition, the presence of saprophytic fungi which accompanied with the dermatophytes at the infected site, possibly compete with it and prevent the dermatophytes from growing in the agar medium plate (Milne,1996 ; Robson, 2012). The most common cause of negative culture was belonged to inappropriate topical use of corticosteroid drug which had been taken from the patient (Colle*et al.*,1996;Hayette and Sacheli, 2015). In the present study *Trichophyton* species represented 82.5% (66 cases) followed by *Microsporum s*pecies 15% (12 cases) and *Epidermophytonflocosum* which conistitute 2.5% (2 cases) from the positive culture. Among *Trichophyton* species, *T.rubrum* was the predominant dermatophytes 35% and this is similar to the result of Hay, (1999); Enemuor and Amedu, (2009); Salman, *et al.,* (2013) and Bhatia and Sharma, (2014) followed by *T.mentagrophyte* 17.5%, *M.canis* 10% and *T.tonsurans* 7.5% and the same results were reported by Hanumanthappa *et al.,(*2012). From 28 clinical isolates of *T.rubrum,* 6 isolates were identified as downy types which characterized by absence of macroconidia, 22 isolates were granular types which revealed oval shaped microconidia and cylindrical shaped macroconidia with appendix or tail. Of the 14 clinical isolates of *T.mentagrophytes*, 5 isolates were identifies as downy type and 9 isolates were granular type. Morphologically, the granular type revealed powdery to granular colonies with white color at the surface and brown color at the reverse, while the downy variant exhibited white colonies with velvety to cottony texture and no pigmentation at the reverse and microscopically showed typical coiled and areal hyphae which absent in the granular type. The alterations which have been seen in the physiological and morphological properties of dermatophytes complicate or hamper the diagnosis process. This variation may be due to the culture technique, incubation temperature and the use of pharmaceutical (Brilhante *et al.*, 2005). In the present research there were many difficulties in the phenotype identification of dermatophytes. So, molecular techniques were employed to confirm the identification. Fungal genomic DNA of was extracted by using Omega DNA kit in addition to the liquid nitrogen which utilized to lysis the fungal cell wall. Later, the genomic DNA was analyzed by electrophoresis on 1% agarose gel. Molecular identification of the positive cultures of dermatophytes was achieved by gene-specific PCR followed by PCR-RFLP for digestion the PCR products which resulted from amplification of ITS region in rDNA gene. In the species or gene specific PCR, the ITS region was amplified by using the primers ITS1 and ITS4 which resulted in PCR products ranged between 550-740bp. The variation which have been seen in the length of ITS region among dermatophytes confirmed the results of laboratory conventional identification and these results showed good similarity with the results of Jackson *et al.*, (1999); Li *et al*., (2008), Al-Khafajii (2014), Ahmadi *et al.*, (2015), Ghojogh *et al*., (2015). Several studies

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showed thatPCR-RFLP analysis by using single restriction enzyme *BstN1* was able to distinguish all species of dermatophytes during the formation of different band patterns. The PCR- RFLP targeting ITS region is a rapid, inexpensive, easy to application and reliable method for dermatophyte differentiation at species level (Mochizuki et *al*., 2005; Ahmadi *et al*., 2015). In this work, the amplified products of ITS regions from all species were subjected to digest by the *BstN1* endonuclease restriction enzyme provides between two to four recognition sites with the exception of *M.audouinii* which was none and only revealed the original band 600bp. In addition, the morphological and microscopic examination of *T.interdigatale* revealed similarity with those in *T.mentagrophytes*, so much often difficulty to distinguish between them however, the patterns were obtained from ITS RFLP in *T.interdigitale* exhibited variation from those obtained by *T.mentagrophytes*. The profiles of electrophoretic analysis of patterns were obtained from ITS- RFLP in all clinical isolates showed similarity with those of Jackson *et al*., (1999); Mochizuki *et al*., (2003); Elavarashi *et al.,* (2013); Al-Khafajii (2014); Ahmadi *et al.,* (2014); Ghojoghi *et al*., (2015) who have identified dermatophytes by PCR RFLP and showed the same patterns of ITS RFLP. However, an important fact which observed in the present study during the performance of PCR- RFLP and differ from the previous studies, that the patterns bands were below 100bp not revealed on the gel during the electrophoretic analysis and this is may be due to the small molecular weight of these packages bands and cannot be shown which needs to increase the molecular weight of the agarose gel.. Furthermore, the results were obtained from ITS RFLP in *T.concentricum* showed two bands 360 and 290bp and the clinical isolate number 6 of *T.rubrum* revealed three bands and these results differ from the results of the other isolates in the same species of our study and also showed variation from the previous studies. This alteration may be due to mutation in the ITS region or mistakes in the identification process. Consequently, the analysis of ITS region by *BstN1*endonuclease enzyme was provided simple method for dermatophytes characterization and this analysis showed the polymorphism of ITS region for strains and species of dermatophytes (Jackson *et al.,* 1999).

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