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Genotypic identification and antifungal susceptibility pattern of dermatophytes isolated from clinical specimens of dermatophytosis in Egyptian patients

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Abstract:

Background: Dermatophytosis accounts for the majority of fungal infection all over the world. The conventional laboratory methods for identification of dermatophytes are slow and lack specificity. Genetic amplification has made rapid and precise identification of dermatophytes possible. With the increasing variety of drugs available for the treatment of dermatophytosis and with the lack of effective and safe antifungal, the need for a reference method for testing the antifungal susceptibilities of dermatophytes has become apparent.

Aim of the study: The current study was conducted to compare the rapid diagnostic molecular technique arbitrarily primed polymerase chain reaction (AP-PCR) with the conventional culture method for identification of the dermatophyte fungal infections of hair, nail and skin. Also to determine the antifungal susceptibility pattern of different dermatophyte isolates to Terbinafine, Griseofulvin, Itraconazole and Ketoconazole as the routinely used antifungal agents.

Patients and Methods: The present study included 115 patients with dermatomycosis of the hair, skin and nail. Their age ranged from 3 to 50 years (mean 19.8±12.5 SD). Specimens from the infected sites were collected and subjected to conventional examination by direct (potassium hydroxide) KOH microscopic examination, culture on primary and selective media. Dermatophyte isolates were identified by their characteristic morphology, physiological tests and AP-PCR. Antifungal susceptibility was tested for all isolates according to the National Committee for Clinical Laboratory Standards NCCLS microdilution

method M38-A for filamentous fungi with modifications in temperature and incubation period.

Results: Out of the 115 cases with ringworm infection, dermatophytes were isolated in culture from 46.1% of specimens and nondermatophytes from 18%. Trichophyton (T) rubrum (32.1%) was the most commonly isolated dermatophyte from all types of skin fungal infection except tinea capitis (P<0.001). T. mentagrophytes and T. violaceum were the main causes of tinea capitis. By genotypic identification (AP-PCR) of dermatophytes, all isolates formed distinct DNA band patterns on gel electrophoresis which was in agreement with the conventional methods in 86.8% of isolates. Out of the eleven phenotypically identified T. mentagrophytes; two only were diagnosed to the strain level, two strains were genotypically identified as T. rubrum and one as T. tonsurans. Also two isolates of T. violaceum were diagnosed by PCR as T. schoenleinii, one T. rubrum was diagnosed as T. ajelloi and one T. soudanense as T. violaceum. The direct KOH examination had sensitivity of 88% and specificity of 74%. The antifungal susceptibility pattern of the isolated dermatophytes were for terbinafine 0.06-0.5 (0.121) µg/ml, itraconazole 0.06-4 (0.62) µg/ml, ketoconazole ranged from 0.06-4 (0.857) µg/ml and griseofulvin from 0.5-8 (2.151) µg/ml. Terbinafine was the most powerful antimycotic and T. rubrum had the highest (minimal inhibitory concentration) MIC values for the four antifungal agents.

Conclusion: The genotypic differentiation by AP-PCR provides a rapid and practical tool for identification of dermatophyte isolates to the species and strain level within one day that is independent of the culture variations. The standard NCCLS M38-A broth microdilution method with the modifications in temperature and incubation period is convenient for antifungal susceptibility testing of dermatophytes.

Introduction

Dermatophytes infections of the skin affects a large proportion of population and have emerged as important causes of morbidity especially in aging population and in immunocompromised patients (Marie et al., 2001 [46] and Osborne at al., 2003[59]). These dermatophytes are of the genera Microsporum, Trichophyton and Epidermophyton. The genus Epidermophyton is represented by a single pathogenic species (E. floccosum), the genera Microsporum and Trichophyton are complex and made of multiple species (Koichi et al., 1999)[38].

For many years they were accustomed to diagnose dermatophytes on the basis of morphological and biochemical characteristics by using direct microscopic examination and in vitro culture. Although they are economic, these procedures suffer from the drawbacks of being either slow or non specific showing false negative results (Howell et al., 1999)[33]. Also the application of chemotherapy has contributed to the occasional modification and alteration of the morphological characteristics of dermatophytes cultures and complicating laboratory identification procedures based on phenotypic features (Faggi et al., 2001)[22].

Using molecular methods for differentiation between the genotypic characteristics of the species of dermatophytes are more specific, precise, rapid and

are less likely to be affected by external influences such as temperature variations and chemotherapy. These molecular methods, such as restriction fragment length polymorphism analysis of mitochondrial DNA (Kano et al., 2000)[36], sequencing of the internal transcribed spacer (ITS) region of the ribosomal DNA, sequencing of protein- encoding genes, and polymerase chain reaction (PCR); random amplification of polymorphic DNA [RAPD], arbitrarily primed PCR [AP-PCR] (Elisabetta et al., 2001)[18], and PCR fingerprinting, have brought important progress in distinguishing between species and strains. However, most of these techniques require additional manipulation such restriction endonuclease digestion, hybridization or sequencing after amplification, so they are complex, laborious, relatively time-consuming, and not easily employable for routine identification of dermatophytes (Graser et al., 1999)[29]. In contrast, AP-PCR technology is simple, rapid and in the absence of specific nucleotide sequence information for many dermatophyte species, AP-PCR is able to generate species- specific or strain specific DNA polymorphism on the basis of characteristic band patterns detected by agarose gel electrophoresis (Faggi et al., 2001[22]& Baeza and Giannini, 2004[7]).

Dermatophytes are eukaryotic and have machinery for protein and nucleic acid synthesis similar to that of higher animals. It is, therefore, very difficult to find out compounds that selectively inhibit fungal metabolism without exhibiting any toxicity to humans. Also there is evidence that dermatophytes have acquired resistance to certain antimycotic drugs. So, with the increasing variety of drugs available for the treatment of dermatophytoses and with the lack of effective and safe antifungal , the need for a reference method for testing of antifungal susceptibilities of dermatophytes has become apparent. Such standard method is not yet available. Establishment of a reference susceptibility testing method may allow the clinician to select the appropriate therapy for the treatment of infections caused by dermatophytes (Jessup et al., 2000 [35] and Augustine et al., 2005[5]).

Aim of the Study

The current study was conducted to compare the rapid diagnostic molecular technique AP-PCR with the conventional culture method for identification of the dermatophyte fungal infections of hair, nail and skin. In addition, to determine the antifungal susceptibility pattern of different dermatophyte isolates to Terbinafine, Griseofulvin, Itraconazole and Ketoconazole as the routinely used antifungal agents.

Patients and Methods

The present study was conducted on 115 patients attending the Dermatology Outpatient Clinic of Ain Shams University Hospitals. Their age ranged from 3 to 50 years (mean 19.8 ± 12.5 SD). They were 59 females and 56 males. The patients were clinically diagnosed as having tinea capitis (47), tinea corporis (29), tinea pedis (23), onychomycosis (9), tinea cruris (5) and tinea manuum (2). All patients were subjected to full history taking including age, sex and antifungal treatment.

Specimen collection and processing:

Clinical examination was done to differentiate between different types of ring worm infection. Specimens were collected from all patients after disinfection with 70% alcohol and were kept in dry sterile containers. The collected specimens were:

- Hair: suspiciously infected hairs were plucked with forceps.
- Cutaneous skin scales: scrapings were taken from the definite edge of the lesions with sterile scalpel.
- Nail: nail eclipses, scrapings or subangular curette.

The specimens were then subjected to the following (Milne, 2001)[50]:

1. Direct microscopic examination using 10-20% KOH solution with methylene blue.
2. Culture on:

    a) Sabouraud's dextrose agar medium with chloramphenicol (Oxoid, U.K.).
    b) Dermasel agar medium with chloramphenicol and cycloheximide (Oxoid, U.K.).

3. Subculture on:

c) Potato dextrose agar (PDA)with chloramphenicol (Oxoid, U.K.).
d) Oatmeal cereal agar for antifungal susceptibility (Sigma Chemicals Co., St., Louis, Mo., U.S.A.).
e) Potato agar slants for storage of dermatophyte isolates at -80oC until time of use.

Identification of the fungal isolates was done through

1. Macroscopic examination of colonies on different media
2. Microscopic examination of cultures.
3. Physiological tests including urease and pigment production tests.
4. Arbitrarily primed polymerase chain reaction (AP-PCR).

Antifungal susceptibility testing of dermatophyte isolates:

It was done according to the standard microdilution method for filamentous fungi M38-A of the National Committee for Clinical Laboratory Standards (NCCLS, 2002) against four antifungal agents; Terbinafine, Griseofulvin, Itraconazole and Ketoconazole with some modifications according to Favre and colleagues (2003)[23].

I. Arbitrarily Primed Polymerase Chain Reaction (AP-PCR):

Principle:

Arbitrarily primed PCR technique relies on arbitrarily designed sequences of short primers (usually 10 nucleotides long) that anneal specifically to DNA templates in a target organism. If these primers anneal to target DNA sequences, the intervening segments that are proximal enough to these annealing sites will be amplified and will generate products of variable molecular weights. Such products

can be resolved by agarose gel electrophoresis, which will display a band pattern for each strain. The primer sequences are determined empirically, since the target DNA are usually not known and the entire genome of the organism serves as the target for the strain comparison and differentiation (Liu et al., 2000a [43]& Rilley, 2004[60]).

Procedure:

The procedure was done according to Liu et al. (2000b)[44].

a-DNA extraction and precipitation:

Fungal isolates were subcultured in 100 ml of Sabouraud's broth (Oxoid, UK) and incubated with shaking for up to 7 days at 25 °C. Hyphal growth was harvested by filtration and washed twice with 100ml of sterile saline. Strains, which could not be processed immediately, were frozen at -80°C prior to extraction. Liquid nitrogen was added to 2-3g of frozen hyphae and the cells were ground finely. Approximately 200mg of frozen ground mycelium was placed in a 1.5 ml microcentrifuge tube. Fungal DNA was extracted from fungal cell suspension by using the Puregene DNA purification kit (supplied by Gentra system, U.S.A.).

b-cDNA amplification:

The primer pair used for Dermatophyte DNA amplification were OPAA 17 (5'-GAGCCCGACT-3') were prepared by ABI Applied Biosystem 394 DNA, RNA synthesizer, USA.

Six microliters of cDNA was added with 1.0µl of the primers sense and antisense (50pM/µl) to 50 l reaction mixture. The reaction mix included 5µl of 1 PCR buffer (50mM KCl, 10mM Tris-HCl, pH 8.3), 2.5mM MgCl2, 0.25µM dATP, 0.25 µM dCTP, 0.25 µM dGTP, 0.75 µM dUTP, 0.125 U of UNG, and 2U of Taq polymerase (0.4µl) (Promega, USA) and Sterile nuclease free water (38.6µl).

After initial denaturation at 95 C for 2 min, the GenAmp 9700 thermocycler was programmed for 30 cycles of amplification and a final extension period of 5 min at 72oC. Each cycle consists of:
* denaturation at 94°C for 30s,
* annealing at <40°C for 30s, and
* extension at 72°C for 30s.

The amplified product were then stored at -20°C .

Negative control of sterile nuclease-free water and positive control of Trichophyton (T). mentagrophytes var erinacei identified by conventional methods, were included in the procedure.

c-Detection and interpretation of the amplified products:

A100-bp DNA ladder (Pharmacia Biotech, U.S.A.) was used as a molecular size marker. Ten microliters of the PCR amplicon was mixed with 2µl of gel

loading dye (Promega, U.S.A.) and the mixture was electrophoresed on 1.5% agarose gel in Tris-acetate EDTA buffer and stained with ethidium bromide (Amersco, U.S.A.). The gel was viewed under ultraviolet light and photographs were taken. The high intensity bands produced by AP-PCR for each isolate and the positive control were compared with the bands of the DNA ladder **(figure 1)**.

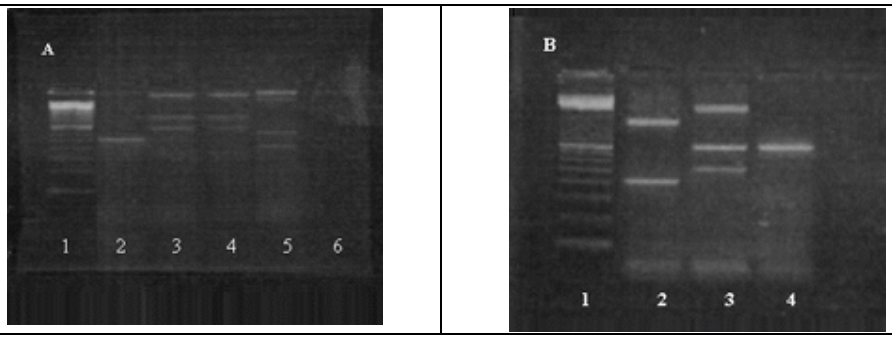

Fig 1: Agarose gel electrophoresis of PCR products of the amplified Dermatophytes DNA

Lane 1A&B: 100-bp DNA ladder
Lane 2A: T. verrucosum; 500bp
Lane 3A: M. canis; 700, 1200, 3000bp
Lane 4A: M. canis; 700, 1200, 3000bp
Lane 5A: T. rubrum; 400, 600, 2500, 3400bp
Lane 6A: negative control
Lane 2B: T. mentagrophytes var, erinacei; 400, 1500bp
Lane 3B: T. ajelloi; 500, 800, 1900bp
Lane3B: E. floccosum; 800bp

The identification and differentiation of the 53 isolates were done by comparing the molecular size of the DNA bands (bp) of the amplified arbitrarily primed PCR products with that performed by Liu et al. (2000b)[44] and with the culture results. Table (1) shows the examination of the DNA products from dermatophyte fungi by AP-PCR.

Dermatophyte species and varieties	Number tested	DNA products (bp) obtained with random primer OPAA17
T. rubrum	18	400, 600, 2500, 3400
T. violaceum	9	400, 1100, 2600, 3500
T. mentagrophytes var. mentagrophytes	6	2800, 3500
T. schoenleinii	4	900, 3400
M. audouinii	3	1300, 2000, 3000
M. canis	3	700, 1200, 3000
M. ferrugineum	2	1300, 3000
T. tonsurans	2	900, 2800, 3400
E. floccosum	2	800
T. mentagrophytes var. interdigitale	1	1300, 2800, 3500
T. mentagrophytes var. erinacei	1	400, 1500
T. ajelloi	1	500, 800, 1900
T. verrucosum	1	500

Table (1): Examination of DNA products from dermatophyte fungi by AP-PCR obtained by OPAA17 primer (Liu et al., 2000b)[44]

II. Antifungal Susceptibility Testing of Dermatophyte isolates: (NCCLS, 2002):

A standard method for antifungal susceptibility testing of Dermatophytes is not yet available, mainly the NCCLS (M38-A) standard method for conidium forming filamentous fungi was followed with some modifications recommended by Favre et al., (2003)[23] such as changing temperature and incubation time.

a) Antifungal agents:

Terbinafine (Novartis Pharma, Basel, Switzerland), Griseofulvin (Pharco Pharmaceutical- Alexandria), Itraconazole and Ketoconazole (Janssen- Cilag Beerse, Belgium) were used in this study.

Stock solutions of Terbinafine, Itraconazole and Ketoconazole were prepared by dissolving 16µg of each antifungal in 10ml of 100% dimethyl sulfoxide (DMSO) (Sigma chemicals Co., St. Louis, Mo., U.S.A.) in separate tubes to get a concentration of 1600µg/ml. For terbinafine 5% Tween 80 was added to the 100% DSMO. While a stock solution of Griseofulvin was prepared by dissolving 32µg in 10ml of 100% DMSO to get a concentration of 3200µg/ml. Stock solutions were kept frozen in 1ml aliquots at -70oC.

A working solution of each antibiotic was prepared by diluting 100 µl of the stock solution in 900 µl of RPMI-1640 medium containing L-glutamine and

0.165M morpholine propane sulfonic acid (MOPS) without bicarbonate (GIBCO BRL, Life Technologies, Paisley, Scotland) to get a concentration of 32µg/ml for Terbinafine, Itraconazole and Ketoconazole and 64 µg/ml for Griseofulvin.

b) Quality Control Strains:

Candida parapsilosis ATCC 22019 (The American Type Culture Collection (ATCC) (Rockville, Md) was used as quality control strain to test for the used antifungal drugs. According to NCCLS M27-A standard method (2000) for antifungal susceptibility of yeast, the reference MIC range for C. parapsilosis is 0.06-0.5 µg/ml for both itraconazole and ketoconazole after 48h incubation (Jessup et al., 2000)[35]. Reference strain was grown in 10ml brain heart infusion broth (Difco) at 35oC overnight. The suspension was diluted two folds with brain heart infusion broth containing 20% glycerol (Sigma), dispensed in screw-capped tubes, sealed and stored at -70oC. The reference strain was tested with every batch of antifungal susceptibility of the isolated species (Gupta and Kohli, 2003)[30].

c) Preparation of the microdilution plates:

o Serial two fold dilution of the antifungal agents were prepared with RPMI 1640 medium. The final concentrations of the antifungal agents ranged from 64 to 0.125 for Griseofulvin and from 32 to 0.06µg/ml for Itraconazole, Terbinafine and Ketoconazole. Sterility control (negative control) and growth control (positive control) were included in each plate. With each batch of antifungal susceptibility, antifungal control using C. parapsilosis ATCC 22019 reference strain was inoculated to test for the validity of the four antifungal agents according to the NCCLS M27-A standard method (NCCLS, 1997).

o Uninoculated microtitration plates containing antifungal dilutions were kept covered for approximately 6 months at -70°C.

d) Preparation of the Dermatophyte inoculum:

Dermatophyte isolates were grown on oatmeal cereal agar slants for 7 days at 28°C; the best medium to support conidial growth (Jessup et al., 2000)[35]. Sterile normal saline (0.85%) was added to the slant culture and was gently swabbed with a cotton tip applicator to dislodge the conidia from the hyphal mat. The suspension was adjusted to 5 mL with sterile normal saline. The cell density was adjusted to give final inoculum concentration of 104CFU/ml. The suspension was counted on a hemocytometer and was diluted in RPMI 1640 to the desired concentration. 100µ1 of the organism suspension was transferred into all wells of the microdilution plates except for the negative control wells. Plates were incubated aerobically at 30°C, except for E. floccosum and M. canis at 35°C. All were incubated for 4-10 days according to the growth in the control wells.

e) Reading and interpretation of the panel:

The minimal inhibitory concentration (MIC) endpoints were determined according to NCCLS M38-A standards as the point at which no visual turbidity where the organism was inhibited 80% when compared to the growth control. For

the quality control Candida parapsilosis the MIC endpoint was determined as ≥80% inhibition of the positive growth control for Itraconazole and Ketoconazole (NCCLS M27-A).

Results

This study was carried out on 115 patients with ringworm infection. Their age ranged from 3-50 years. Forty seven patients were below age of ten years (41%), eleven were in the second decade (9.4%), thirty one patients were in the third (27%), twenty three patients were in the fourth (20%) and three patients were in the fifth decade (2.6%). The patients under study were 59 (51.3%) females and 56 (48.7%) males. Tinea capitis infection 47 (41%) was only prevalent below the age of ten years and double in females 31 (27%) than males 16 (13.9%). Also tinea pedis was higher in males 13 (11.3%) than females 10 (8.7%).

Table (2) shows the distribution of the Dermatophyte positive cultures among patients with ringworm infection. Tinea capitis represented 47 (41%) of cases followed by tinea corporis 29 (25.2%) and tinea pedis 23 (20%). Twenty three (43%) of dermatophyte isolates were separated from tinea capitis patients, followed by 14 (26.4%) and 12 (22.6%) from tinea corporis and tinea pedis respectively.

	Clinical Cases		Positive Cases by culture	
Diagnosis	**No.**	**%**	**No.**	**%**
T. capitis	47	41.00%	23	43.40%
T. corporis	29	25.20%	14	26.40%
T. pedis	23	20.00%	12	22.60%
Onychomycosis	9	7.80%	2	3.80%
T. cruris	5	4.30%	1	1.90%
T.mannum	2	1.70%	1	1.90%
Total	115	100.00%	53	100.00%

Table (2): Distribution of Dermatophyte Positive Cultures Among Patients with Skin fungal Infection

In spite that there was no statistically significant association between the isolation of dermatophytes from the infected sites and the sex of the patients (P>0.5), dermatophytes isolated from tinea capitis patients were higher in females 15 (28.3%) than males 8 (15.1%). Males show higher prevalence of tinea pedis than females.

Out of the 115 specimens of the patients under study, 53 (46.1%) yielded dermatophyte growth on culture and 21 (18%) specimens grew nondermatophytes; nine Candida, eight Aspergillus spp. (five A. fumigatus, three A. niger) and four cases showed Acremonium. Three (2.61%) of the patients showed both Dermatophytes and non dermatophytes growth. The later group grew mainly on Sabouraud's dextrose agar (SDA).

In the present study there was a highly significant association (P<0.001) between the results of direct microscopic examination by KOH and dermatophyte culture. Out of the 53 positive cases by culture, there were 38 positive cases (33%) by direct KOH examination and the remaining 15 cases (13%) were negative and out of 62 negative cases by culture, there were 16 (14%) cases positive by direct KOH examination. Out of those 16 dermatophyte culture negative specimens and KOH positive; five cases were on antimycotic therapy (two tinea cruris and three tinea capitis), five cases grew Aspergillus (three obtained from tinea capitis and two from onychomycosis), five cases Candida (two were onychomycosis, one tinea pedis, one tinea capitis and one tinea cruris) and one case of tinea capitis showed mixed growth of Aspergillus and Candida. The KOH method had a sensitivity of 88% and specificity of 74%. By direct KOH microscopic examination no definite identification was reached. However, hyphae ,arthrospores and chlamydospores were seen in some preparations. Acremonium species gave the fronded appearance and Aspergillus branched dichotomously at acute angles.

Out of the three cases that yielded growth of both dermatophytes and rapidly growing non Dermatophyte fungi, one was onychomycosis which showed the fruiting bodies of Aspergillus together with the hyphae of dermatophytes on KOH examination. The second two cases were tinea corporis which showed budding yeast cells of Candida species on direct microscopy together with the hyphae of dermatophytes.

Table (3) shows the dermatophyte species isolated from each type of fungal infection. The most common type was T. rubrum 17 (32.1%) which showed a highly significant association with almost all types of dermatophytosis except tinea capitis (P<0.001). There were a highly significant association between T. mentagrophytes 11 (20.8%), T. violaceum 10 (18.9%) with tinea capitis, corporis and pedis patients (P<0.001).

Species of Dermatophytes	T. capitis No (%)	T. corporis No (%)	T. pedis No (%)	T. unguium No (%)	T. cruris No (%)	T. mannum No (%)	Total No (%)
T. rubrum	0	5 (35.7%)	8 (66.7%)	2 (100.0%)	1(100.0%)	1 (100.0%)	17 (32.1%)
T. mentagrophytes	7 (30.4%)	2 (14.3%)	2 (16.7%)	0	0	0	11 (20.8%)
T. violaceum	6 (26.1%)	3 (21.4%)	1 (8.3%)	0	0	0	10 (18.9%)
T. verrucosum	1 (4.3%)	0	0	0	0	0	1 (1.9%)
T. schoenleinii	2 (8.8%)	0	0	0	0	0	2 (3.8%)
T. soudanense	1 (4.3%)	0	0	0	0	0	1 (1.9%)
T. tonsurans	0	1 (7.1%)	0	0	0	0	1 (1.9%)
M. canis	2 (8.8%)	1 (7.1%)	0	0	0	0	3 (5.6%)
M. ferrugineum	1 (4.3%)	1 (7.1%)	0	0	0	0	2 (3.8%)
M. audouinii	3 (13.0%)	0	0	0	0	0	3 (5.6%)
E. floccosum	0	1 (7.1%)	1 (8.3%)	0	0	0	2 (3.8%)
Total No.	23 (100%)	14 (100%)	12 (100%)	2 (100%)	1 (100%)	1 (100%)	53 (100%)

Table (3): Dermatophytes Species Isolated From different types of fungal infections by culture

Table (4) shows the results of the phenotypic and genotypic identification of dermatophyte isolates. Out of the 115 specimens the highest rate of dermatophyte isolation on primary culture media was on Dermasel agar (46%) after incubation at 28oC, followed by incubation at 37oC (12%) then SDA (7%) at 28oC. T. mentagrophytes and M. canis were the fastest to grow on Dermasel media (primary culture media) (6 days) at 28°C while T. violaceum was the slowest to grow (23days). All dermatophytes grew better on 28°C with exception of T. violaceum and T. verrucosum which grew faster at 37°C . Subculture of dermatophyte isolates on PDA showed that the fastest growth was for T. mentagrophytes and M. canis (3 days) and the slowest were for T. violaceum, T. schoenleinii and T. soudanense (10 days).

Comparison between the identification of dermatophyte isolates from culture by the ordinary phenotypic methods (morphology and physiological tests) and the genotypic method by the AP-PCR was done in this study. The genotypic identification was in agreement with the conventional phenotypic methods in 46 (86.8%) out of 53 Dermatophyte isolates while the disagreement was in 7 cases (13.2%). Out of the eleven isolates of T. mentagrophytes only two were diagnosed phenotypically to the strain level and confirmed genotypically, var. mentagrophytes and var. erinacei. By the genotypic AP-PCR five were found to be var. mentagrophytes and one var. interdigitale. The last three isolates, two of them were diagnosed by PCR as T. rubrum, and one as T. tonsurans. In addition two isolates of T. violaceum were diagnosed by PCR as T. schoenleinii. One T. rubrum isolate was genotypically diagnosed as T. ajelloi. The last T. soudanense isolate was genetically diagnosed as T. violaceum.

No.	Primary Culture media				PDA Sub-culture	Culture Results	PCR Results
	SDA	SDA	DS	DS			
	28°C	37°C	28°C	37°C			
2	-ve	-ve	7d	-ve	3d	T. mentagrophytes	T.ment v. mentagrophytes
3	-ve	-ve	9d	-ve	6d	T. mentagrophytes*	T. rubrum
6	-ve	-ve	16d	-ve	9d	T. rubrum	T. rubrum
7	8d	-ve	7d	-ve	3d	T.ment v. mentagrophytes	T.ment v. mentagrophytes
11	-ve	-ve	20d	17d	7d	T. violaceum*	T. schoenleinii
12	-ve	-ve	7d	-ve	5d	T. mentagrophytes	T.ment v. mentagrophytes
15	15d	16d	14d	16d	7d	T. rubrum	T. rubrum
21	Candida	Candida	15d	-ve	8d	T. violaceum	T. violaceum
22	Candida	-ve	11d	-ve	7d	T. rubrum	T. rubrum
28	-ve	-ve	7d	8d	5d	M. audouinii	M. audouinii
31	-ve	-ve	6d	-ve	6d	T. mentagrophytes	T.ment v. mentagrophytes
32	-ve	-ve	9d	13d	6d	E. floccosum	E. floccosum
34	-ve	-ve	6d	-ve	3d	M. canis	M. canis
39	-ve	-ve	23d	-ve	10d	T. violaceum	T. violaceum

No.	Primary Culture media	PDA	Culture Results	PCR Results

	SDA 28°C	SDA 37°C	DS 28°C	DS 37°C	Sub-culture		
40	11d	-ve	19d	-ve	9d	T. violaceum	T. violaceum
41	-ve	-ve	15d	-ve	10d	T. violaceum	T. violaceum
42	-ve	-ve	10 d	-ve	8d	T. rubrum	T. rubrum
45	-ve	-ve	12d	16d	8d	T. mentagrophytes*	T. rubrum
46	-ve	-ve	12d	15d	9d	T. rubrum	T. rubrum
47	-ve	-ve	10d	-ve	7d	T. rubrum	T. rubrum
52	-ve	-ve	17d	-ve	10d	T. violaceum	T. violaceum
53	12d	-ve	10d	-ve	7d	T. tonsurans	T. tonsurans
55	-ve	-ve	15d	-ve	8d	T. violaceum*	T. schoenleinii
58	14d	-ve	10d	-ve	5d	E. floccosum	E. floccosum
61	-ve	-ve	7d	-ve	4d	T. mentagrophytes	T.ment. v. interdigitale
65	-ve	-ve	15d	-ve	8d	T. violaceum	T. violaceoum
68	-ve	-ve	10d	10d	6d	T. rubrum	T. rubrum
71	-ve	-ve	6d	7d	4d	T. mentagrophytes*	T. tonsurans
72	-ve	-ve	6d	-ve	4d	M. canis	M. canis
73	-ve	-ve	17d	-ve	10d	T. schoenleinii	T. schoenleinii
75	-ve	-ve	10d	-ve	7d	T. rubrum	T. rubrum
77	10d	-ve	8d	-ve	5d	M. ferrugineum	M. ferrugineum
79	-ve	-ve	11d	-ve	7d	M. audouinii	M. audouinii
82	-ve	-ve	20d	-ve	10d	T. schoenleinii	T. schoenleinii
84	-ve	-ve	14d	13d	9d	T. rubrum	T. rubrum
86	15d	-ve	10d	12d	7d	T. rubrum	T. rubrum
87	-ve	-ve	17d	-ve	9d	M. ferrugineum	M. ferrugineum
88	-ve	-ve	9d	-ve	5d	T. mentagrophytes	T.ment v. mentagrophytes
89	-ve	-ve	10d	-ve	5d	T. rubrum	T. rubrum
92	10d	-ve	7d	-ve	4d	M. canis	M. canis
93	-ve	-ve	22d	-ve	10d	T. soudanense*	T. violaceum
95	-ve	9d	7d	-ve	5d	T.ment. v. erinacei	T.ment var. erinacei
98	-ve	-ve	20d	22d	10d	T. violaceum	T. violaceum
101	-ve	-ve	10d	12d	8d	T. rubrum	T. rubrum
103	-ve	-ve	10d	-ve	7d	T. rubrum	T. rubrum
105	-ve	-ve	19d	17d	8d	T. verrucosum	T. verrucosum
106	-ve	-ve	19d	-ve	9d	T. violaceum	T. violaceum
107	Aspergillus	Aspergillus	11d	-ve	6d	T. rubrum	T. rubrum
108	-ve	-ve	10d	-ve	6d	M. audouinii	M. audouinii
109	-ve	-ve	6d	8d	4d	T. mentagrophytes	T.ment v. mentagrophytes
111	-ve	-ve	12d	-ve	7d	T. rubrum*	T. ajelloi
114	-ve	-ve	10d	-ve	9d	T. rubrum	T. rubrum
115	-ve	-ve	10d	-ve	7d	T. rubrum	T. rubrum

Table (4): Results of Phenotypic and Genotypic Identification of Dermatophyte isolates

Table (5) shows the pattern of antifungal susceptibility of the dermatophyte isolates and the range of their MIC endpoints by broth microdilution method (NCCLS M38-A with modifications) towards the four antifungal drugs. The MIC

ranges (mean) were as follows: for terbinafine from 0.06 to 0.5 (0.121) µg/ml, itraconazole from 0.06 to 4 (0.62) µg/ml, ketoconazole ranged from 0.06 to 4 (0.857) µg/ml and griseofulvin from 0.5 to 8 (2.151) µg/ml. Terbinafine was the most powerful antimycotic. T. highest MIC values for the four antifungal agents.

Rubrum had the Dermatophytes Species	MIC endpoints			
	Terbinafine	**Itraconazole**	**Griseofulvin**	**Ketoconazole**
T. mentagrophytes	0.06 - 0.25	0.125 -2	1 - 4	0.25 -2
T.ment,var erinacei	0.06 - 0.125	0.25 – 0.5	0.5 - 2	0.06 - 0.25
T. rubrum	0.06 - 0.5	0.125 - 4	1 - 8	0.06 – 4
T. violaceum	0.06 - 0.125	0.06 – 0.5	0.5 - 2	0.06 - 0.5
T. verrucosum	0.06	0.125	2	1
T. schoenleinii	0.06	0.125 - 0.5	0.5 - 1	0.125
T. soudanense	0.06	0.5	2	1
T. tonsurans	0.5	2	1	4
M. canis	0.06 -0.5	0.125 – 2.	2 - 4	0.25 – 1
M. ferrugineum	0.06 - 0.125	0.06 - 0.125	0.5	0.125
M. audouinii	0.06 - 0.125	0.06 - 0.125	1 - 2	0.125 - 2
E. floccosum	0.06	0.125	4	2
Range	0.06 -0.5	0.06 – 4	0.5 - 8	0.06 – 4
Mean	0.121	0.620	2.151	0.857
SD	0.122	0.973	1.637	0.991

Table (5): Antifungal Susceptibility Pattern of Dermatophyte isolates by NCCLS M38-A method

Discussion

With the increasing incidence and mortality of fungal infection, the requirement for strict diagnostic approaches became a very urgent issue. In addition, the traditional detective techniques, such as culture, give poor diagnostic approaches, accordingly, the molecular tools of classification and identification of pathogenic fungi such as dermatophytes considered of great help (Li et al., 2004)[41].

As fungal infection of the skin is the most common infectious dermatologic condition throughout the world (Gupta et al., 2003)[31] and because of the difference in distribution of these types of infections from country to country (Chan and Friedlander, 2004)[12], our study was done to detect Dermatophytes distribution in Egyptian patients. In this study, tinea capitis accounted for 41% of all cases, followed by tinea corporis (25.2%), tinea pedis (20%), onychomycosis (7.8%), tinea cruris (4.3%) and tinea manuum (1.7%). This copes with results done by Omar, (2000)[58] who emphasized that tinea capitis is considered as the major

type of fungal infection in Egypt. In Libya, tinea corporis accounted for 45.9% of cases followed by tinea pedis (8.1%) and tinea manuum (2.6%) as discussed by Ellabib et al., (2002)[19]. In Yemen, tinea corporis accounts for the majority of cases followed by tinea capitis as mentioned by Mahmoud, (2002)[45]. The endemic nature of scalp infection in the developing countries is perpetuated by lack of knowledge about prevalence, carrier state, and the absence of control measures (Moubasher et al., 2000)[51]. In the developed countries, tinea pedis is the major type of fungal infection. In Japan Takahashi and Nishimura, (2002)[66] revealed that tinea pedis accounts for 64.2%, followed by tinea unguium (14.6%), tinea corporis (11.9%), tinea cruris (5.4%), tinea manuum (3.6%) and tinea capitis (0.2%). This was assured by results of Seebacher, (2003)[61], who found increase in the incidence of tinea pedis in Central and North Europe. Aste et al., (2003)[4] reported that tinea pedis accounts for 23.4% in Italy. Wearing shoes for long time as in athletes and excessive use of water in gymnasiums will result in maceration of toe clefts predisposing to tinea pedis infection (Tietz, 2003[68]).

In the present study, tinea capitis was found only in children below the age of ten years (41%). Our results were in accordance with the studies done by Omar (2000)[58] in Alexandria (54%), Fuller et al., (2003)[28] in south-east London and by Mounkassa, et al., (2004)[52] in France (70%). Aste, et al., (2003)[4]; Gupta, et al., (2003)[31]; Hubert and Callen, (2003)[34]; Shibaki and Shibaki, (2003)[62] and Sigurgeirsson and Steingrimsson, (2004)[63] all agreed that above the age of ten years tinea infections are other types than tinea capitis, a fact that was explained by the started sebum production about this age, with its antifungal properties.

In this study, tinea capitis was double in female patients than males (27% and 13.9% respectively) although there were no statistically significant correlation with sex ($P>0.05$). This was in accordance with Omar (2000), who found that tinea capitis was more common in school girl children. This may be due to sharing combs and sharing facilities in hair dresser. This result is different from that done in Kenya, by Ayaya et al. (2001)[6], who found that tinea capitis was higher in boys (60.9%) than girls (39.1%), this may be due to some bad habits as sharing caps and combs, and it cannot be correlated with specific sex. On the other hand, our results showed that the rate of tinea pedis, was higher in males than females (11.3% and 8.7% respectively). This goes with the results done by Sofia et al., (2001)[64] and Cheng and Chong, (2002)[13]. Aste, et al., (2003)[4] reported that adult males probably have about 20% chance of developing tinea pedis, while among women only 5% are likely to become clinically infected, because it occurs more common in athletes sharing washing facilities and using common swimming baths. In our country males use sport tight shoes more frequently than females. Males also wear closed shoes for long time in comparison with females.

Dermatophyte and nondermatophyte species were isolated in this study from 46.1% and 18% of the cases of fungal infection respectively. These results are nearly similar to the results reported by Al-Sogair et al. (1991)[1] in Saudi Arabia, who isolated both dermatophytes and nondermatophytes in 57.2% of cases. Candida and Aspergillus were the main nondermatophytes isolated in their study as in our study. Infection caused by yeast and moulds were involved in our study because no marked differences in their clinical picture from that of dermatophyte

infection could be observed. This reveals the role of nondermatophytes species in infection of the skin and its appendages .

In the present study, out of the positive specimens by culture, 71.7% were positive by the direct KOH microscopic examination; two of them showed mixed dermatophytes and nondermatophytes infection. The KOH direct method had a sensitivity of 88% and specificity of 74%. This was in agreement with Escobar and Carmona-Fonseca (2003)[20] and Arca et al. (2004)[3], who found that direct microscopy was positive in 92% and 77% respectively from all culture positive cases. In our study direct microscopic examination show false negative results in 13% of cases. This is in accordance with Liu et al. (2000b)[44], who stated that this method is insensitive, showing false negative results up to 15%. While Tampieri, (2004)[67], mentioned that direct microscopic examination shows false negative results up to (50%). This may be because direct microscopic examination appears to be greatly influenced by the meticulous preparation of specimens, experience of the observer and rate of contamination. On the other hand there were 16 (14%) positive specimens by direct KOH examination that showed negative results by culture This may be because 5/16 cases were on antimycotic treatment so gave no growth on culture. The remaining 11/16 cases showed either true mixed infection or contamination by the rapidly growing fungi (Aspergillus, Candida and Acremonium) than dermatophytes that covered the entire medium giving no chance for the slowly growing dermatophytes to appear. In our study, Dermatophytes were seen on microscopic examination as hyphae, arthrospores and chlamydospores. Acremonium species gave the fronded appearance and Aspergillus branched dichotomously at acute angles. However no definite identification was reached. In agreement with Liu et al., (1997)[42], Elweski (1995)[17] and Escobar and Carmona-Fonseca, (2003)[20] it seems to be difficult to rely on results of direct microscopy with KOH to establish the diagnosis of fungal infection as it could not detect the characteristic morphology of the three genera and it lacks sufficient sensitivity but, it is highly efficient as screening technique before therapy is initiated because of the expense, duration and potential adverse effects of the treatment (Tampieri, 2004 [67]& Hainer,2003[32]).

In this study Trichophyton rubrum was the most frequently isolated organism (32.1%) mainly from tinea pedis and tinea corporis. These results were in agreement with those detected in France (35.5%) (Lacroix et al., 2002)[39]. While higher isolation rates of T. rubrum were reported in Australia (69.5%) (Coloe and Baird, 1999)[14], Japan (79.4%) (Shibaki and Shibak, 2003) and Slovakia (81.6%) as reported by Buchvald and Simaljakova, (2002)[10]. On the other hand, in Libya, isolation rate of the organism was lower (13%) as mentioned by Ellabib et al., (2002)[19]. No isolates of T. rubrum was detected from tinea capitis patients in this study. This was in accordance with Fisher and Cook (1998)[26] who reported that T. rubrum is the most common cause of tinea pedis and rarely causes tinea capitis.

The most frequently isolated Dermatophytes from tinea capitis patients were Trichophyton mentagrophytes (30.4%) and Trichophyton violaceum (26.1%). In contrast Moubasher et al., (2000)[51], found that T. violaceum accounts for (62%) of all cases with tinea capitis while it accounts for (91%) in London as mentioned by Fuller et al., (2003)[28]. In Brazil, Microsporum canis accounts for (71.3%) as discussed by Dias et al., (2003)[16] and Chan and Friedlander (2004) and in

Germany it was (50%) as mentioned by Lehmann et al., (2004)[40]. In Turkey, T. verrucosum accounts for (43%) in the study done by Metin et al., (2002)[49]. This can be attributed to the fact that species of dermatophytes causing tinea capitis vary from country to country and also change with time, geography, environment, climate, occupation, ethnic group and life styles (Nweze and Okafor 2005)[56].

The highest rate of Dermatophyte isolation on primary culture media was on Dermasel agar (46%) after incubation at 28oC, with exception of T. violaceum and T. verrucosum which grew faster at 37°C . This elevates the importance of dermasel as selective medium that contains cycloheximide for the isolation of dermatophytes at 28oC which is the optimum temperature for most of dermatophytes (Aly, 1994[2] & Hainer, 2003[32]).

Trichophyton mentagrophytes and M. canis were the fastest to grow (6 days) while T. violaceum was the slowest to grow (23days). Subculture of Dermatophyte isolates on PDA showed that the fastest growth was for T. mentagrophytes and M. canis (3 days) and the slowest were for T. violaceum, T. schoenleinii and T. soudanense (10 days). In-vitro culture is capable of providing a species-specific determination of dermatophytes on the basis of morphological and biochemical criteria in 5-15days in >95% of cases. However, for some unusual and atypical isolates, identification may require a range of culture media and tests. These tests are costly, time consuming (3-4 weeks after primary isolation) and demand specialist skills. More importantly, because these conventional methods depend on measurement of the phenotypic characteristics of dermatophytes, they can be easily influenced by outside factors such as temperature variations and chemotherapy that may affect the interpretation of in-vitro culture results (Liu et al., 2000b)[44].

In the present study, genotypic identification of Dermatophyte isolates by the AP-PCR using the OPAA-17 primer was in agreement with the phenotypic methods in 86.8% of the isolates and the disagreement was in 13.2% of them. Phenotypic identification of T. mentagrophytes to the strain level could only be done for two isolates form eleven. The disagreement between the two methods of identification was reported by Kawai, (2003)[37]. These may be explained as the conventional methods of identification require special skills, or because of atypical culture characteristics due to treatment. Two isolates of T. mentagrophytes were genotypically diagnosed as T. rubrum and one as T. tonsurans. This may be explained as the three strains were similar to T. mentagrophytes in having similar morphology, moderately slow-grower, and flat, granular, creamy, with reddish brown reverse. On microscopic examination the genetically diagnosed T. rubrum strains showed many drop shaped to round microconidia and abundant club shaped macroconidia. This morphology and genetic characters are matching with the urease positive Asiatic variant of T. rubrum which is different from the typical T. rubrum but is minimally genetically distinguished from it. It is usually separated from tinea corporis. Trichophyton tonsurans develops several types of colonies with a variety of colors and surface textures and is urease positive that resembles T. mentagrophytes. They have branched septate hyphae with terminal swellings, numerous microconidia forming loose clusters and few short blunt cylindrical macroconidia (Bassiem and El-Borhamy, 2002[9]& Summerbell, 2003[65]).

Two T. violaceum isolates were diagnosed by PCR as T. schoenleinii and one

T. soudanense was genetically diagnosed as T. violaceum. Fisher and Cook (1998) recommended that those three species must be differentially diagnosed from each others by further tests other than the usual KOH and culture methods. They were all slow growers, developed at first glabrous cream-colored colonies and as they aged they developed the deep red or purple color. They all had few macro and microconidia with short segmented and distorted twisted branching hyphae.

Finally one T. rubrum isolate was genotypically diagnosed as T. ajelloi. T. ajelloi gave purple black pigments, with smooth thick walled cylindrical macroconidia as T. rubrum. Its presence may be due to mixed infection with a slow grower organism or due to contamination of culture as Summerbell (2003)[65] reported that there is no data to say it is pathogenic.

Although most dermatophytes can be identified after primary isolation (10-15days), a few may require secondary culture on specialized media (10-15days). With some atypical, unusual or slow-growing isolates, the identification process may take even longer time. Therefore, DNA analysis using AP-PCR technique has clear advantage over conventional techniques for identification of dermatophytes. PCR made a genetic-based differentiation of dermatophytes species possible at the species and strain level, more rapid (within one day), sensitive and more precise and stable. AP-PCR can identify young culture before development of any characteristic feature of the organism, dermatophytes with atypical morphology and dead strains (Faggi et al., 2001)[22]. This is necessary to survey the current epidemiologic situation and to trace back the pathogenesis of infection in order to avoid reinfection and thus optimize the therapy.

In the present study, the NCCLS M38-A broth microdilution method was used to determine the antifungal susceptibility pattern of dermatophytes isolated from the clinical specimens. The MIC (mean) (µg/ml) endpoints obtained ranged from 0.06 to 0.5 (0.121) for terbinafine, 0.06 to 4 (0.62) for itraconazole, 0.5 to 8 (2.151) for griseofulvin and 0.06 to 4 (0.857) for ketoconazole. Our results were significantly higher than those obtained by Barros and Hamdan (2005)[8] where MICs were <0.007-0.015 for terbinafine, 0.062-1.0 for itraconazole, 0.25-2.0 for griseofulvin and. 0.125-2.0 for ketoconazole. Our results were also higher than the MIC ranges and means obtained by Pujol et al. 2005[57], Brilhante et al. (2005)[11] and Esteban et al. (2005)[21]. These higher MICs than those of other studies, possibly because of differences in culture medium used in the other studies or may be due increased use of local and oral antifungal agents or repeated intake due to patient incompliance or chronic infection. In this study T. rubrum had the highest MIC values for the four antifungal agents. This is in accordance with Mukherjee et al. (2003)[53] who detected T. rubrum strains with primary resistance to terbinafine.

Our study demonstrated that the four antifungal drugs used were active against dermatophytes, although these results were species dependent which cope with that obtained by Fernindez et al., (2002)[25]. In the present study terbinafine possessed the highest antifungal activity against all dermatophytes tested in vitro. This was in accordance with the results obtained by Fernindez et al. (2001 and 2002)[24,25], Favre et al. (2003)[23] and Gupta and Kohli (2003)[30] even though a reference method for testing dermatophytes still has not been developed. In vivo

terbinafine is the extremely potent systemic drug against dermatophytes and it provides super long term mycological and clinical efficacy and lower rates of clinical relapse as mentioned by Darkes et al., (2003)[15].

In conclusion, it seems to be difficult to rely on results of direct microscopic examination with KOH to establish the diagnosis of different fungal infections of the skin, as it lacks sufficient sensitivity; however, it is highly efficient as a screening technique. Culture on the specific media of dermatophytes will ensure the diagnosis and reach to the species level and sometimes to the strain level. However it may be time consuming, costly, as it needs different culture media for proper identification, in addition it needs special skills and experiences as the morphological character of some species are atypical.

The genotypic differentiation by AP-PCR provides a rapid and practical tool for identification of dermatophyte isolates that is independent of morphological and biochemical characteristics thus, enhances laboratory diagnosis of dermatophytes. AP-PCR represents a technological advance in the laboratory diagnosis of dermatophytosis. Further investigation of a larger number of isolates could shed light upon more details of these kinds of infections. The future development of procedure for the isolation of DNA from clinical materials such as skin, hair and nails would further enhance the potential of AP-PCR for identification of dermatophytes.

This study shows that the standard NCCLS M38-A broth microdilution method with the modifications made in temperature and incubation period are convenient for antifungal susceptibility testing of dermatophytes. Among the antifungal tested terbinafine was the most potent antifungal drug. Further studies are needed to standardize these optimal growth conditions, ensure reproducibility and allow the comparison between different antimycotics. MICs need to be correlated with clinical outcome to develop interpretive breakpoints, which in turn may clarify the reasons for lack of clinical response and detection of resistance. This is especially important for immunocompromised patients and young children.

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