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Original Article

Microbiology Section

Prevalence of Onychomycosis in Hail Region, Saudi Arabia

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ABSTRACT

Introduction: Dermatophytes, yeast and non-dermatophyte molds causes onychomycosis, which is the fungal infection of the nail bed and skin beneath, have four clinical types: Distal subungual, proximal subungual, superficial and total dystrophic.

Aim: Isolation and identification of causative agents by conventional and molecular methods in order to examine the relative prevalence of dermatophytes, yeast and non dermatophyte molds among patients and correlate the clinical pattern to the agents.

Materials and Methods: From dermatology private clinics, beauty salons and female students of Hail University, adults(15-70 years old) crusts and nail clipping particles of 450 nail samples were collected from toe nails and finger nails in sterile petri dishes. Direct microscopic examination was applied with KOH 20% and gentle heating. Culture, API 32 C® and PCR-ITS, PCR-RFLP were carried out.

Results: Positive cases for fungal elements were 296 (65.8%), DLSO were the major type (50.6%), TDO (31.5%), PSO (10.5%) and SWO (1.7%). As causative agents *Candida albican* was 33.1%, *Trichophyton rubrum* 18.2%, *Trichophyton mentagrophytes* complex 16.9%, mixed infection 14.5%, *Epidermophyton floccosum* 9.5% and *Aspergillus niger* was 7.8%.

Conclusion: Dermatophyteic onychomycosis were mostly found followed by yeast and non dermatophyte molds. Accurate identification of the causative agents help managing onychomycosis by giving the correct antifungal treatment, patients awareness prevent relapse and malformation of nails.

Keywords: Dermatophytes, Nail infection, Non dermatophyte molds, Yeast

INTRODUCTION

Onychomycosis is a fungal infection affecting primarily finger nails and toe nails characterised by thickening, roughening and discoloration of the nail palate [1]. If it is not treated well it can lead to discomfort and may spread to the nearby tissues [2]. It is a frequently occurring disease of nails which is caused by non dermatophyte molds, yeast and dermatophytes. The geographic area, population, and mycological features or diagnostic methods employed plays a role in spreading the infection [3]. Studies on onychomycosis showed that it has four clinical types: Distal Subungual (DLSO), Proximal Subungual (PSO), Superficial (SWO) and Total Dystrophic (TDO) [4]. Most cases of onychomychosis are difficult to be treated and treatment failure about 25-40% [5]. Onychomychosis has been reported as high as 20% in East Asia [6]. Conventional methods in Saudi Arabia are usually carried out to isolate and identify the causative agents of onychomychosis [7]. At the forefront by 40.3% onychomycosis of fungal infection then followed by tinea capitis 21.9% [8].Molecular methods such as PCR amplification of Internal Transcribed Spacer (ITS) region and PCR of Restriction Fragment Length Polymorphism (RFLP) which is based on internal transcribed spacer genes used to identify species and subspecies which is more faster and specific than conventional methods that take time and effort [9]. Prior to treatment an accurate diagnosis can help to choose the right antifungal agent, especially when the causative agent may vary in its response to the chosen anti-fungal therapy [10].The aim of this research was to isolate and identify onychomycosis causative pathogens from patients by conventional and molecular methods in order to examine the relative prevalence of dermatophytes, yeast and non dermatophyte molds among patients and correlate the clinical pattern to the agents.

MATERIALS AND METHODS

This aetiological study was performed according to International ethical guidelines for epidemiological studies prepared by Council for International Organisations of Medical Sciences (CIOMS) in collaboration with World Health Organisation [11].This survey research were carried out in Hail KSA, Department of Clinical Laboratory Sciences, College of Applied Medical Science, Hail University and the Research Protocol was approved by the Ethical Committee in the College of Medicine, Hail University, Saudi Arabia, from January 2012 till March 2016. Total of 450 nail samples

were collected randomly from toe nails and finger nails from both adult male and female patients age between 15 and 70 years old, visiting dermatology private clinics, beauty salons and female students of Hail University/female section main campus who suffer from any abnormality in their nails. The research concentrated more on female samples because females were affected more frequently with onychomycosis than males (1.68/1.0) [12]. Nail clipping, nail cuts, debris and skin surrounding the nail after swabbing the affected area with 70% alcohol were collected in sterile petri dish. Crusts and nail clipping particles were treated by KOH 20% with gentle heat then direct microscopic examination after half an hour to detect fungal elements [13]. Sabouraud's Dextrose Agar (SDA) with chloramphenicol and cycloheximide supplement (Oxoid® supplement), Potato Dextrose Agar (PDA) to enhance sporulation and pigmentation and Derm-Duet™ II J175 rapid sporulation media dermatophyte test media (HARDY DIAGNOSTICS)™ were used for cultivation of positive samples [14]. Cultures were incubated at 25-27°C for 24 hour-3 weeks. Macro morphology and micro morphological examination were carried out by using Lactophenol Cotton Blue stain LPCB for identification of isolates [15,16]. Biochemical identification of isolated yeast API ID 32C® BioMérieux were applied. For dermatophytes and non dermatophyte molds strains, DNA extraction were done [17]. For yeast Nexttec™ kit Biotechnology GmbH for Genomic DNA were used. PCR Mixture contained reaction buffer (10 mM Tris-HCL, 50mM KCl;1.5 mM $MgCl_2$ pH 8.0) of each desoxynucleotide triphosphates, 50 pmol of each primer, the primers used for dermatophytes and non dermatophyte mold were universal primers Mass 266 (5`GCATTCCCAAACAACTCGACTC3`) and V9D (5`TTACGTCCCTGCCCTTTGTA3`) [18] and the primers used for yeast samples were ITS3 as a forward primer (5`GCATCGATGAAGAACGCAGC3`) and ITS4 reverse (5`TCCTCCGCTTATTGATATGC3`) for ITS2 region DNA [19]. 2U of Taq-polymerase (AmpliTaq DNA-polymerase with GeneAmp 10×PCR-Buffer, Roche®), 10 % dimethylsulfoxide and 50 ng of template DNA. The mixture was overlaid with mineral oil and amplified in thermo cycler (Biometra).

Thirty cycles of the following protocol were performed: initial denaturation at 95°C for 5 min, annealing for 1 min at 55°C and extension for 1 min at 72°C. All strains amplifying fragment size approximately 1000 bp including parts of 18S and 28S rDNA as well as the whole ITS1, ITS3, 5.8 SrDNA and ITS2, ITS4. For positive control used CBS reference strain 118892 *T.rubrum* for dermatophytes and non dermatophyte molds and for *C.albicans* CBS 2696 ref., strain. PCR master mix as shown in [Table/Fig-1]. For negative controls only using master mix without adding DNA.

PCR products were separated on 1.4% Agarose gel for 40-50 min at 140V, with Gene ruler DNA Ladder Mix Fermentas® stained with Ethidium Bromide and photographed under UV light by BIO-Rad ChemiDoc™ Imaging system [20]. RFLP were done for *E.floccosum* by taking 20 µL of PCR product and precipitated with 1/10 volume of sodium acetate (3M, pH 5.2) and 2.5 volume of 90% ethanol for 16 hours at 4°C. The templates were centrifuged for 15 min at 13,000 rpm, the pellets were washed with 70% ethanol centrifuge again and remaining ethanol were evaporated. The DNA were redissolve in 18 µL of sterile water. 2 µL of R™ buffer(10mM Tris-HCL pH 8.5,10 mM $MgCl_2$, 100 mM KCl;0.1mgXmL^{-1}) Thermo Fisher Scientific® and 1µL of restriction enzyme Mva1 Fermentas® were added. The templates were incubated for 4 hours at 37°C. the digested DNA was separated on 2% Metaphor Agarose Cambrex Bio Science® for 2 hours and half at 5.8VXcm^{-1} with 1kb Ladder Invitrogen® 1µg/µL then stained with ethidium bromide and photographed under UV [21]. Standard strain *E.floccosum* were from Central Bureau voor Schimmel cultures (CBS) ref.,14 CBS number 130793 and ref.,15 CBS number 130802.

Master Mix and DNA Mixture	Concentration		Final Concentration
PCRH2O	-	25.5µL	-
10×PCR-Buffer	10×	5µL	1.11×
dNTPs	2.5 mM	4 µL	220 µM
Primer T1for	10 Pmol/µL	3 µL	0.67 Pmol/µL
PrimerT1 Rev DIG	10 Pmol/µL	3 µL	0.67 Pmol/µL
Taq polymerase	5 U/µL	0.5 µL	0.056 U/µL
DNA	-	4 µL	-
Total		45 µL	

[Table/Fig-1]: PCR mixture performed in 45µL.

STATISTICAL ANALYSIS

Biodata and examination results were inserted in SPSS software (version 16.0, Chicago, IL, USA) for evaluation.

RESULTS

Out of 450 collected nail samples 296 (65.8%) were positive for fungal infection by direct microscopic examination using KOH 20% with gentle heating. The fungal isolates from nails showed in [Table/Fig-2]. Nails clinical examination indicate that distal and lateral subungual were the most common type 167 cases (56.4%), followed by total dystrophic by 93 cases (31.4%). Proximal subungual 31 cases (10.5%) caused mainly by *Candida albicans* as chronic paronychia. Superficial white onychomycosis were 5 cases (1.7%). [Table/Fig-3] shows dermatophytes were the major 132 cases (44.6%) then yeast 98 cases (33.1%), mixed infection were 43 cases (14.5%) and non dermatophyte molds were 23 cases (7.8%), of non dermatophyte molds infection associated with preungual inflammation and dark brown to black pigmentation of the nail. Fungal structures were observed under microscope with LPCB and macroscopic examination were done for the cultures. API ID 32 C® bioMérieux results positive or negative depend on the turbidity were transformed into numerical biocodes and identified through the use of ID 32C analytical profile index which showed that all isolated yeast samples were *C.albicans*.

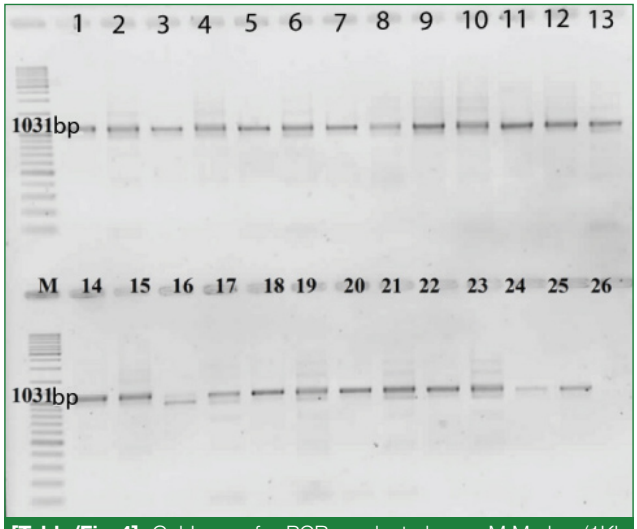
Gel images of PCR products from DNA of dermatophytes and non dermatophyte molds shows that bands of DNA at 1031 bp [Table/Fig-4-6] while bands of yeast at 300 bp [Table/Fig-7], RFLP patterns shows identical bands with the standard strains of *E.floccosum* as in [Table/Fig-8].

Causative Agents	DLSO	PSO	SWO	TDO	Total (n=296)
Dermatophytes					
T.rubrum	35	-	-	19	54
T.mentagrophytes	25	-	5	20	50
E.floccosum	-	-	-	28	28
Yeast					
C.albicans	67	31	-	-	98
Non Dermatophyte Molds					
A.niger	23	-	-	-	23
Mixed infection	17	-	-	26	43

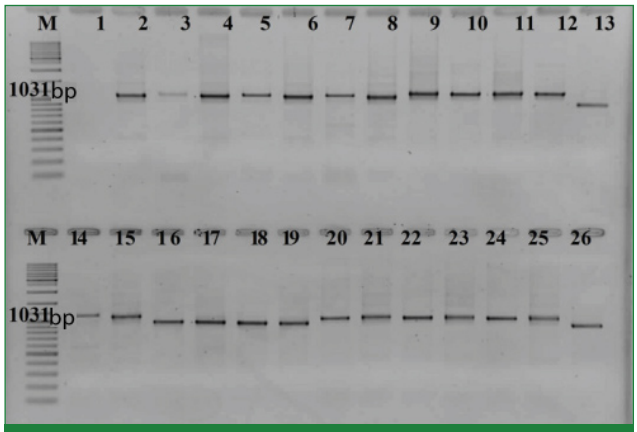
[Table/Fig-2]: Number of isolated fungi and clinical infections.

Type of Infection	n (%)
Distal and Lateral Subungual	167 (56.4%)
Total Dystrophic	93 (31.4%)
Proximal Subungual (Chronic Paronychia)	31 (10.5%)
Superficial White	5 (1.7%)
Total	296 (100%)

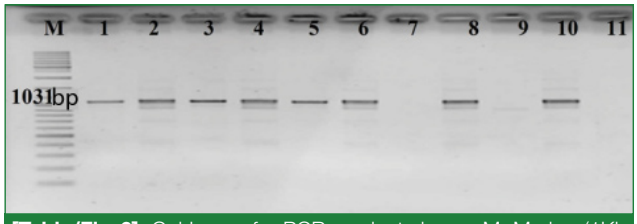
[Table/Fig-3]: Types of clinical infections.



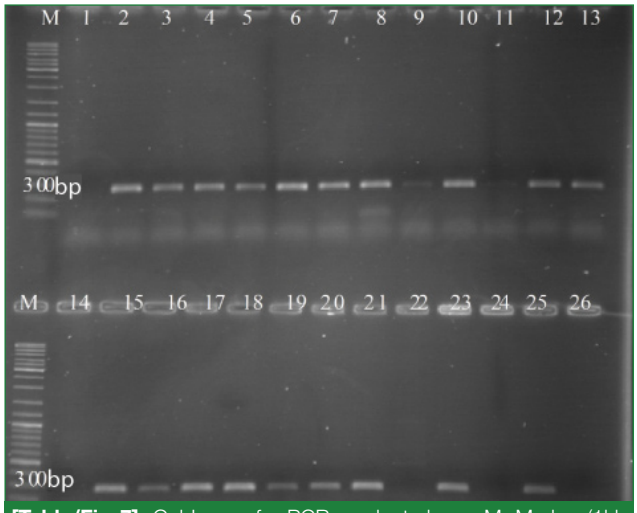
[Table/Fig-4]: Gel Image for PCR product shows: M:Marker (1Kb Gene Ruler DNA Ladder Mix, Fermentas®)
1:*T. rubrum* 33, 2:*T. rubrum* 29,3: *T. rubrum* 49, 4: *T. rubrum* 13, 5: *T. rubrum* 54, 6: *T. rubrum* 28, 7: *T. rubrum* 3, 8: *T. rubrum* 15, 9: *T. rubrum* 11, 10:*T. rubrum* 7,11: *T.rubrum* 21, 12: *T.rubrum* 14, 13: *T. rubrum* 17,14: *T. rubrum* 35, 15: *T. rubrum* 10, 16: *T. rubrum* 8,17: *T. rubrum* 2,18: *T. rubrum* 41, 19:*T. rubrum* 30, 20:*T.rubrum* 9, 21:*T.rubrum* 16 , 22: *T.rubrum* 19,, 23: *T.rubrum* 20, 24:*T.rubrum* 22, 25: (+) control, 26: (-) control



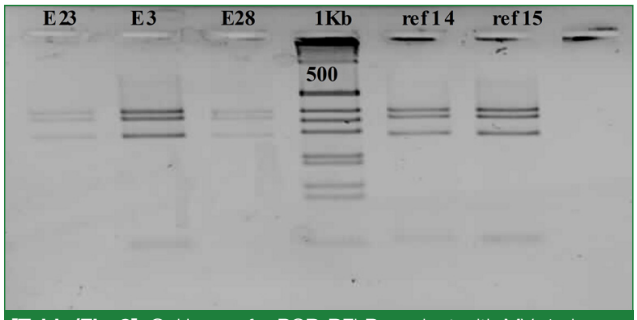
[Table/Fig-5]: Gel Image for PCR product shows :M: Marker (1Kb Gene Ruler™ DNA Ladder Mix, Fermentas®)
1: (-) control,2: (+) control, 3: *T. rubrum* 18, 4: *T. rubrum* 50, 5: *T. rubrum* 23, 6: *T. rubrum* 55, 7: *T. rubrum* 12, 8: *T. Rubrum* 4, 9:*T.mentagrophytes* 5, 10: *T.mentagrophytes* 7, 11: *T.mentagrophytes* 9,12: *T.mentagrophytes* 14, 13: *A.niger* 3, 14: *T. mentagrophytes* 13,15 :*T.mentagrophytes*1, 16: *T.rubrum* 1,17:*T.rubrum* 5,18:*T.rubrum* 6, 19:*T.rubrum* 44, 20: *E.floccosum* 11, 21: *E.floccosum* 4, 22: *E.floccosum* 3, 23: *E.floccosum* 2, 24: *E.floccosum* 13, 25: *E.floccosum* 5, 26: *A.niger* 11



[Table/Fig-6]: Gel Image for PCR product shows :M: Marker (1Kb Gene Ruler™ DNA Ladder Mix, Fermentas®)
1: *E.floccosum* 28, 2: *E.floccosum* 10, 3: *E.floccosum* 23, 4: *E.floccosum* 20, 5: *E.floccosum* 15, 6: (+) control, 7: (-) control, 8: (+) control, 9: (-) control, 10: (+) control, 11: (-) control.



[Table/Fig-7]: Gel Image for PCR product shows M: Marker (1kb Gene Ruler™ DNA Ladder Mix, Fermentas®)
1:(-) control, 2:(+) control, 3: *C.albicans* 24, 4: *C.albicans* 2, 5: *C.albicans* 3, 6: *C.albicans* 12, 7: *C.albicans* 7, 8: *C.albicans* 13, 9: *C.albicans* 20, 10: *C.albicans* 23, 11: *C.albicans* 15, 12: *C.albicans* 21, 13: *C.albicans* 67, 14:, (-) control, 15: (+) control, 16: *C.albicans* 31,17: *C.albicans* 33, 18: *C.albicans* 1, 19: *C.albicans* 18, 20: *C.albicans* 16, 21: (+) control, 22: (-) control, 23: (+) control, 24: (-) control, 25: (+) control, 26: (-) control.



[Table/Fig-8]: Gel Image for PCR-RFLP product with MVa1 shows: E23, E3 and E.28: *E.floccosum* samples, ref 14 CBS number 130793 and ref 15 CBS number 130802 *E.floccosum* standard strain, 1Kb Ladder Invitrogen®

DISCUSSION

Regions, environment and habits play an important role in species of dermatophytes exists and types of infections [22]. Advanced age, gender, life style and medical condition also are important factors that can increase or help relapsing infection with onychomycosis [23]. Diabetes mellitus and peripheral arterial disease may be independent predictor of onychomycosis [24]. Onychomycosis classified according to causative agent into dermatophytes, non dermatophyte molds, yeast and mixed infection [3]. Bokhari MA et al., found that 50% of positive culture were *Candida albicans* as the most common yeast causing onychomychosis specially fingernails [25] but in disagreement with this study, the results showed that *C.albicans* represent only 33.1% of the positive cases and the major pathogens belong to dermatophytes by 44.6%.The difference in this study and other previous studies was the environment and lifestyle [19]. Conventional phenotypic methods based on the reported morphological and physiological characteristic have been widely used to the genus *Candida* [26] however, such methods are usually time consuming and insufficient to separate the types. Similar to this study results dermatophytes specially *E.floccosum, T.rubrum* and *T.mentagrophyte* complex were the most important keratinophilic fungi which infect nails and cause permanent deformities to the nail palates [27]. Same results were obtained as Gupta AK et al., [28] and Mochizuki T et al., [29] that *T.rubrum* were isolated as the most common dermatophytes pathogen. In agreement with Hartman et al., [30] and Graser Y et al., [31] *T.mentagrophytes* complex were difficult to be identified with conventional methods due to the similarity between vars and to other types of dermatophytes. Molecular identification can easily separate between vars of *T.mentagrophytes* complex or other types of dermatophytes like using sequencing, which help in using correct treatment. As Nouripour-Sisakht S et al., [32] and Garcia-Martos P et al., [33] reported that non dermatophyte molds *A. niger* was the most common agent causing onychomycosis. *A.niger* and other molds are emerging cause of onychomycosis which mainly affect toe nails specially in diabetic patients [34,35].

LIMITATION

Study concentrated more on adults, samples were available from females more than males. Study population is small.

CONCLUSION

It clearly shows that dermatophytes are the major pathogens causing onychomycosis in Hail region. *C.albicans* were the most prevalent yeast and *A.niger* were the non dermatophytes mold mostly found.

In order to give the right treatment, causative agents should be diagnosed correctly and this can be achieved when the conventional methods are used along with molecular methods. Patients awareness help to prevent relapse and malformation of nails.

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