Evaluation of Pan-Dermatophyte Nested PCR in Diagnosis of Onychomycosis in Comparison with Direct Microscopy and Culture

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ABSTRACT

This work aimed to compare nested PCR using novel primers targeting the pan-dermatophyte-specific sequence of the chitin synthase 1 gene (CHS1) with KOH microscopy and culture isolation for diagnosis of clinically suspected onychomycosis. This study was conducted during the period from December, 2012 to October 2013. Forty patients attending Outpatient Dermatology and Andrology Clinic in Benha University Hospital. This study was done on forty patients 15 cases were female and the other 25 cases were males with abnormal nails .Their ages ranged from 22 to 77 years. As many as 19 patients were living in rural areas, while 21 patients came from urban areas. Nail scrapings were collected and examined using direct KOH microscopic examination, culture and PCR using double sets of primers. As regard direct microscopy by KOH examination; 33 (82.50%) cases were positive, while 7 (17.5%) were negative. Culture was positive only in 19(47.5%) of nail samples revealing different fungi. Dermatophytes were isolated from 15(37.5%) cases; most of them were T. mentagrophytes. And in 4 cases the only isolated non dermatophytic organism was Aspergillus Niger spp. (10.00%). Nested PCR was positive in 26 (65.00%) nail samples. ***Conclusion:*** *It is concluded that nested PCR targeting the CHS1 gene may be considered the gold standard for detection of dermatophytes in patients with onychomycosis and can aid the clinician in initiating prompt and appropriate antifungal therapy.PCR is a very powerful tool for microbiology and clinical mycology. It can detect very small amounts of nucleic acids. This technique may also play an important role in large-scale studies and in the management of problematic cases of onychopathies.*

INTRODUCTION

Onychomycosis refers to a fungal infection that affects the toenails or the fingernails [(1)]. It is the most common nail disorder and is present in 2% to 13% of general population increasing up to 48% by 70 years of age [(2)].

The incidence of onychomycosis has been rising sharply over the last few years. This is due to an increase in the number of immunocompromised patients, the extensive use of immunosuppressive chemotherapy and broad-spectrum antibiotics, the population aging, avid sport participation is increasing the use of health clubs, commercial swimming pools and occlusive foots wears for exercise [(3)].

Treatment of onychomycosis is expensive. It requires long-term therapy with an oral antifungal medication with potential side effects. Therefore, a proper diagnosis of infection is needed [(4)].

Direct microscopic examination of nail material is often sufficient for the diagnosis of a fungal infection but does not provide genus or species identification and hence does not differentiate unquestionably between dermatophytes and other molds. Furthermore, although rapid and economical, this technique gives false-negative results in 5 to 15% of the cases [(5)].

Conventionally, a definitive diagnosis depends on culture isolation. The culture is, however, negative in up to 40% of the microscopy-positive cases and is time-consuming due to the slow growth and sporulation of the causative organisms and the need for additional physiological tests [(6)].

Culture requires up to 3 to 4 weeks to obtain typical macroscopic and microscopic features for specific dermatophyte identification[(7)]. This method is often associated with poor sensitivity and delayed results [(8)].

Application of extraction of DNA directly from nail specimens and a nested PCR-based diagnosis of any dermatophyte and/or T. rubrum with increased sensitivity compared to conventional diagnostic procedures allow for the first time integration of a molecular biology-based method into the routine examination of nail dermatophytosis also for diagnostic laboratories receiving specimens on a larger scale[(9)].

Aim of the work:

This work aimed to compare nested PCR using novel primers targeting the pan-dermatophyte-specific sequence of the chitin

synthase 1 gene (CHS1) with KOH microscopy and culture isolation for diagnosis of clinically suspected onychomycosis

SUBJECTS AND METHODS

Subjects:

This study was conducted during the period from December, 2012 to October 2013. Forty patients attending Outpatient Dermatology and Andrology Clinic in Benha University Hospital were enrolled in this study with clinically suggestive symptoms and signs of onychomycosis (discoloration, thickening, foul-smelling debris, subungual keratosis, onycholysis, longitudinal and transverse grooves, sulcated nail, pitting, brittle nail, cracks, crumble or friable nail).

Methods:

A-Samples:

The suspected nails were cleaned with 70% alcohol to remove contaminants, Scrapings were taken with a sterile scalpel blade and collected in a sterile clean paper, the collected specimens were sent to the Mycology Unit, Medical Microbiology and Immunology Department at Faculty of Medicine, Benha University.

B-Mycological investigation:

The collected specimens were divided into three portions.[1] The first portion of the specimens was examined microscopically using 20% KOH. [2] The second portion was cultured on into two sets of media: SDA containing chloramphenicol(0.5%) with/without cycloheximide (0.5%).[3] DNA extraction was performed on the third portion of specimen.

C- Molecular detection of fungal DNA by PCR:

1- Genomic DNA extraction from nail samples by using animal and fungi DNA preparation kit (Thermo Scientific, Germany).
2- For each of the samples, two sets of single PCR were performed.

First-round PCR was performed using dermatophyte-specific chitin synthase 1 gene (CHS1). The sequences of the primer CHS1 1S primer were (5′-CAT CGA GTA CAT GTG CTC GC-3′; nucleotides [nt] 70 to 89).

The sequences of the CHS1 1R (5′-CTC GAG GTC AAA AGC ACG CC-3′; nt 485 to 504).

Nested PCR was done by designing a novel set of primers, JF2 and JR2. This was done using the following primers:

JF2 (5′-GCA AAG AAG CCT GGA AGA AG-3′; nt 111 to 130)

JR2 (5′-GGA GAC CAT CTG TGA GAG TTG-3′; nt 378 to 398).

PCR amplification:

Procedure:

For a total 50 µl reaction volume, the following materials were added in a thin walled PCR tube:

-25 µl of Taq PCR Master Mix(Taq DNA polymerase, PCR buffer, dNTPs, and magnesium chloride (MgCl2) (Fermentas). After being briefly vortexed to avoid localized differences in salt concentration.
-The primer solutions (Bioneer) were thawed and mixed well before use. 2.5 µl of each primer (sense and antisense) was added to the PCR tube.
-10 µl of template DNA was added to each tube.
-10 µl of nuclease-free water were added.(Fermentas)
-The samples were gently vortexed and briefly centrifuged to collect all drops to the bottom of the tube.
-The samples were placed in the thermal cycler.(Biometra, Goettingen, Germany).
-Parameters for amplification cycles were:

One cycle at 95°C for 5 min (initial denaturation)
Followed by 40 PCR cycles, each consisting of three steps: Denaturing step (30 seconds at 95˚C), Annealing step (30 seconds at 56˚C), Primer extension step (1 min at 72˚C).
Followed by one cycle at 72°C for 15 min as final extension step.

- A reagent blank, which contained all components of the reaction mixture with the exception of template DNA (which was substituted with sterile distilled water), was included in every PCR procedure.
- The samples were then stored at -20˚C.

The running conditions of nested PCR were similar to the first-round PCR except that an annealing temperature of 52°C was used.

3- Agarose gel electrophoresis: DNA fragments were loaded into 2% agarose gel and visualized by ethidium bromide staining.

RESULTS

This study was conducted during the period from December, 2012 to October 2013. Forty patients attending Outpatient Dermatology and Andrology Clinic in Benha University Hospital**.**

A total number of 40 patients who were clinically suspected of having onychomycosis were included in the study. The age of the study population ranged between 22 and 77 year. The study population comprised 25 males and 15 females. Urban participants represented 21 cases

of the study population while rural participants represented 19 cases. (table 1)

Patients with anaemia represented 35% of study population while those with DM, PVD and trauma accounted for 27.5%, 20% and 12.5% respectively. Patient with associated nail disease accounted for 47.5% of study population. Patients who reported that they received treatment represented 37.5% of the study population. (table 2)

Of the 40 patients with clinically suspected cases of onychomycosis, 82.5% (33/40) were positive for fungal elements by KOH microscopy. Dermatophytes were detected in 65% (26/40) of the cases by nested PCR and isolated in 37.5% (15/40) of the cases by culture. Nondermatophytic molds were isolated in 10% (4/40). (table 3)

The proportion of patient with positive fungual culure was lower than the proportion of patient with positive fungal element on direct KOH microscopy (47.5% vs. 82.5% respectively). This difference was of significance (P = 0.001). (table 4)

The proportion of patient with positive nested PCR for dermatophyte was higher than the proportion of patient with positive fungal culture for dermatophyte (65% vs. 37.5% respectively). This difference was statistically significante (P=0.01) .(table 5)

Table (1): Socio-demographic characteristics of the study population

Variable	Definition	N=40
Age	Mean ± SD; (range)	43.53 ± 15.14; (22-77)
Sex	Male/female; (% male)	25/15; (62.50)
Residence	Urban/rural; (% urban)	21/19; (52.50)

Table (2): The distribution of the study population by their disease.

Disease	Definition	N=40
Trauma	Yes/no; (% yes)	5/35; (12.50)
DM	Yes/no; (% yes)	11/29; (27.50)
Anaemia	Yes/no; (% yes)	14/26; (35.00)
PVD	Yes/no; (% yes)	8/32; (20.00)
Associated nail disease	No disease; (%) Psoriasis; (%) Contact dermatitis; (%)	21; (52.50) 12; (30.00) 7; (17.50)

Table (3): Status of direct KOH microscopy, fungal culture and nested PCR results for the study group

Variable	Definition	N=40
Direct KOH microscopy	Yes/no; (% yes)	33/7;(82.50)
Fungal culture	Negative; (%) Non-dermatophyte;(%) Dermatophyte (%)	21; (52.50) 4; (10.00) 15; (37.50)
Nested PCR	Yes/no; (% yes)	26/14; (65.00)

Table (4): Comparing the fungal culture results and direct KOH microscopy results

Fungal culture Dermatophytes & non Dermatophytes Yes/no (% no)	Direct KOH microscopy Yes/no; (% yes)	Z	P
19/21; (47.50%)	33/7; (82 .50%)	3.28	0.001

Table (5): Comparing the fungal culture results with the nested PCR results

Fungal culture Dermatophyte/non-dermatophyte; (% yes)	Nested PCR Yes/no; (% yes)	Z	P
15/25; (37.50%)	26/14; (65.00%)	2.46	0.01

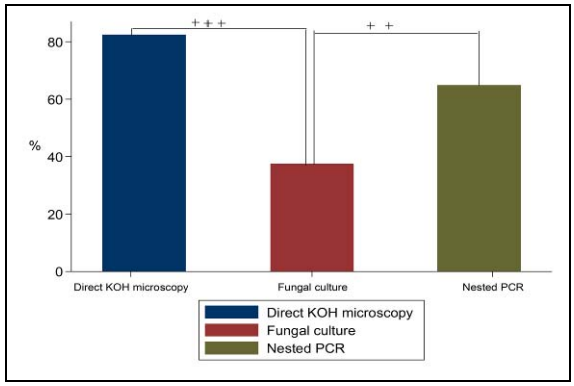

Fig. (1): Comparison between the likelihood of disease using the direct KOH microscopy and the nested PCR vs. the reference test (fungal culture)

Fig. (2): Culure of Trichophyton rubrum on Sabouraud's dextrose agar

DISCUSSION

Because onychomycosis can be caused by several pathogens giving appearance that may be indistinguishable clinically, it is essential that the causative organism is positively identified so that the appropriate course of treatment will be initiated[10] .

KOH test is easy-performed and economic, but the sensitivities are inconsistent. Factors that influence the sensitivity of KOH test include inadequate sampling, different sampling methods, and experience of the doctors [11].

KOH procedure is only a screening test for the presence or absence of fungi and can't identify the type or the species [11].

Fungal culture can identify specific pathogen but it takes a long incubation period. The false negative rate of fungal culture is approximately 30% and the sensitivity was about 60% [12].

In the present study , the number of positive samples for fungi were 33 (82.50%) samples by 15% KOH microscopic examination which is in accordance with **Pontes et al.**[13] (68.4%) . On the other hand, **Kam et al.**[14] (14.3%), **Ellis**[15] (40%), **Brilhante et al.**[16] (48%) and **El- Batawi et al.**[17] (21.8%) found low percentage that may be due to preparation of the specimen and selection of cases in their studies.

The number of positive samples for fungi by culture on SDA were 19 (47.50%) samples which is near to that detected by **Ellis**[15] (30%) and **Brilhante et al.**[16] (36%). However, the percentage of positive samples for fungi by culture found by **Lopes et al.**[18] (56.6%), **Pontes et al.**[13] (66.5%), and **El- Batawi et al.**[17] (68.7%) were higher; this may be due to selection and large number of cases.

Dermatophytes were detected in 15 (37.50%) samples of positive cultures and they were of T.Mentagrophytes and T.rubrum species.

NDMs represent the second common isolated organisms constitute 10.00% (4 samples). Aspergillus species detected in all these 4 samples of positive culures representing the only isolated NDMs.

Nested PCR was done for the 40 nail samples using douple sets of primers. Nested PCR was positive in 26 (65.00%) nail samples.

In agreement with results of our study**, Arca et al.**[19] found that 40(77%) out of 52 specimens were positive by KOH microscopic examination, 12(23%) by culture and 20 (38%) by PCR.

In our study, mycological culture was chosen as the reference method to assess the performance of each test. The sensitivity, specificity, accuracy, PPV and NPV of the used tests were assessed. PCR, and KOH have 73.33% and 80% sensitivity respectively. The specificity was 40 % and 16 % for PCR and KOH respectively.

This is in harmony with **Winter et al.**[20] who calculated the diagnostic sensitivity of the PCR assay as 79.0%.

In the past few years several molecular methods for the detection and identification of dermatophytes from clinical samples have been developed. Major difficulties of PCR methods are that it requires training, sophisticated equipments and standardization and it is also expensive [19].

On the other hand, it is not only sensitive and specific, but also has the potential to decrease the time taken for the laboratory identification of pathogens that grow slowly or are difficult to culture **.**By the use of PCR, reliable rapid results within 24 hours in contrast to the 21 days of incubation required for the isolation of dermatophytes by culture**.**

The application of PCR technology directly to the clinical specimens would allow early and accurate identification of agents of onychomycosis. This would permit prompt and targeted initiation of antifungal therapy[21] .

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تقييم استخدام تفاعل سلسلة البوليميرات المتداخلة فى تشخيص العدوى الفطرية للأظافر بالمقارنة مع الميكروسكوب المباشر و المزرعة الفطرية بمستشفى بنها الجامعى

أمانى قاسم شحات ،احمد عمر شفيق ، محمود عبد الصبور محمود ، شيرين محمد امام
قسم الميكروبيولوجى والمناعة ، كلية طب بنها ، جامعة بنها

نقوم بهذه الدراسة فى مستشفى بنها الجامعى بقسم الامراض الجلدية والتناسلية وكلية الطب بقسم الميكروبيولوجى والمناعة على ٤٠ من المرضى المشتبه باصابتهم بالعدوى الفطرية للاظافر وذلك عن طريق:

١. أخذ التاريخ المرضى للمريض.
٢. الفحص الاكلينيكى .
٣. التحاليل اللازمة ومنها :
   - تحاليل عامة مثل نسبة الهيموجلوبين ونسبة السكر بالدم .
   - تحاليل فطرية وذلك عن طريق اخذ عينة من الظفر المصاب وتحليلها بثلاث طرق :

الطريقة الاولى: الفحص المباشر بالميكرسكوب باستخدام هيدروكسيد البوتاسيوم.
الطريقة الثانية : فحص نتيجة المزرعة الفطرية بالميكرسكوب وبالعين المجردة .
الطريقة الثالثة:استخدام سلسلة تفاعل البوليمرات المتداخلة وذلك عن طريق استخلاص الحمض النووى من العينة وتكبيرها وفحصها .

و كانت النتائج كالتالى :

- هناك 40 حالة اجريت عليهم الدراسة كان منهم 15 (34.50%) حالة من الاناث و٢٥ (65.50%) حالة من الذكور و قد تم اخذ عينة من الاظافرالمصابة لكل حالة.
- كانت الاعمار تتراوح بين ٢٢ سنه و ٧٧ سنة و كان متوسط الاعمار ٤٣.٥ عاما .
- هناك ١٩ (47.5 %.) حالة كانت تقطن المناطق الريفية بينما٢١ (52.5%) حالة كانت تقطن الحضر.
- الحالات الايجابية للفطريات : ٣٣ عينة باستخدام١٥ % بوتاسيوم هيدروكسيد , ١٩ عينة بالزرع , ٢٦ عينة باستخدام تفاعل البلمرة المتسلسل.
- كان اكثر الفطريات وجودا هو الدرماتوفيت فى ١٥ عينه يليه فطر الاسبريجلس وهو الفطر الوحيد الموجود باربع عينات فقط.
- اظهر سلسلة تفاعل البوليمرات المتداخلة حساسية عالية فى تشخيص الاصابة اكثر من الفحص المجهرى باستخدام ١٥% بوتاسيوم هيدروكسيد واكثر من المزرعة الفطرية .

بعد دراسة ٤٠ حالة وجد ان:

- الديرماتوفايت هو المسئول الرئيسى عن معظم حالات فطار الاظافر لذا لا يمكن تجاهل دوره كمسبب لفطار الاظافر.
- ان سلسلة تفاعل البوليمرات المتداخلة ذو حساسية عالية فى تشخيص فطار الاظافر لذا نوصى باستخدام هذا الاختبار فى التشخيص لسرعة اختيار مضاد الفطريات المناسب للعلاج .