Species Identification and Strain Differentiation of Dermatophyte by $(GACA)_4$-Primer based PCR

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Abstract

Dermatophytes are a group of morphologically and physiologically related molds some of which cause well defined infections: dermatophytoses (tineas or ringworm). The present study aims at identification of dermatophytes species and varieties from patients in Wasit province-Iraq using molecular approach based PCR fingerprint.
The short oligonucleotide $(GACA)_4$ is a microsatellite primer was used in this study for identification of dermatophyte isolates. The results identified different species and varieties among dermatophytes. The numbers of resulting PCR bands ranged from 1 to 4 (size range, 600bp to 1600bp) for each species. The resulting patterns were distinct for *Trichophyton* and *Microsporum* species and varieties.

Trichophyton soudanense and *Trichophyton verrucosum* revealed distinguishable profiles with four largest bands (800bp to 1900bp) among *Trichophyton* species. (GACA)4 is able to amplify all species of *Microsporum* producing intraspecies variation which is observed with *Microsporum canis* (rough strain) and *Microsporum canis* (smooth strain), all of which had different band pattern. We conclude that $(GACA)_4$-based PCR has utility as a rapid method for identification of dermatophytes species as well as its utility for differentiation of *Trichophyton* and *Microspmoru* species and varieties.

Keywords: *E.* (*Epidermophyton*), *M.* (*Microsporum*), Polymerase Chain Reaction (PCR), *T.*(*Trichophyton*).

تشخيص انواع الفطريات الجلدية وتمايز سلالاتها باستخدام التفاعل التسلسلي $(GACA)_4$للبوليمراز المعتمد على البادئ

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الخلاصة:

تعد الفطريات الجلدية اعفان مرتبطة من الناحيتين الشكلية والوظيفية ويسبب بعضها أصابات واضحة منها القوباء الحلقية . تهدف هذه الدراسة الى تشخيص انواع وضروب الفطريات الجلدية المعزولة من مرضى محافظة واسط في العراق باستخدام طريقة البصمة الوراثية المعتمدة على التفاعل التسلسلي للبوليمراز . اظهر التشخيص الوراثي على المستوى الجزيئي باستخدام البصمة الوراثية انماط للحامض النووي منقوص الاوكسجين DNA لكل نوع من الفطريات الجلدية: أستخدم البادئ القصير قليل النيوكليوتيدات $(GACA)_4$

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لإيجاد البصمة الوراثية بوصفه بادئا لتشخيص عزلات الفطريات الجلدية . شخصت بهذه الطريقة انواع وضروب مختلفة بين الفطريات الجلدية وتراوح عدد حزم التفاعل التسلسلي للبوليمراز الناتجة من 1-4 حزمة لكل نوع ومدى حجم تراوح ما بين 600 زوج قاعدي الى 1600 زوج قاعدي. كانت نماذج الحزم الناتجة متميزة لأنواع وضروب الـ*Trichophyton* و *Microsporum* التي شخصت بهذه الطريقة.

أظهر النوعين *Trichophyton soudanense* و*Trichophyton verrucosum* طرز متميزة بأربع حزم كبيرة (800 زوج قاعدي الى 1900 زوج قاعدي)بين أنواع الـ *Trichophyton*. وتمكن البادئ $(GACA)_4$ من تضخيم جميع انواع الـ *Microsporum* وادى ذلك الى ظهور اختلافات ضمن النوع الواحد والذي لوحظ في النوع *M. canis* بين سلالته الخشنة والملساء والتي اظهر كل منها حزم مختلفة . نستنتج ان طريقة البصمة الوراثية باستخدام البادئ $(GACA)_4$ طريقة سريعة لتشخيص الفطريات الجلدية كما لها الفائدة في التمايز بين انواع وضروب الجنسين *Trichophyton, Microsporum*.

Introduction

Dermatophytes comprise a group of related fungi that belongs to three genera, *Epidermophyton*, *Microsporum*, and *Trichophyton*, each of which includes several recognized species. These fungi are keratinophylic, as they infect the superficial keratinized tissues (skin, hair, and nails) of humans and animals [1] and can cause cutaneous mycoses which are called dermatophytoses, tinea, or ringworm infections. Health care costs associated with management of these mycoses are high [2,3]. Moreover, dermatophytosis are widespread and increasing in prevalence on a global scale and the recent increase in their incidence has been attributed to the increase of immunocompromised states, such as those associated with AIDS, diabetes mellitus, organ transplantation, and the use of corticosteroids and antineoplastic agents [4-7].

Many molecular approaches have been applied for the identification of different dermatophyte species and strains. Such approaches are considered more stable and precise than those using phenotypic characteristics [8]. One such approach employs PCR technology, which is simple, rapid, and able to generate species-specific DNA polymorphisms with many dermatophyte species on the basis of the characteristic band patterns detected by agarose gel electrophoresis [9, 10]. PCR-based approach employing the simple repetitive oligonucleotide $(GACA)_4$ as a single primer for identification of species of dermatophytes.

The present study aimed at identification of dermatophytes species and varieties from patients in Wasit province-Iraq by using molecular approaches based- PCR: PCR**.**

Materials and Methods

Two hundred forty five samples including hair and skin were collected from attending Out Patient Dermatology Department of Al-Zahraa teaching hospital, Al-Karama teaching hospital, Al-Nu'maniya General hospital in Wasit governorate. Furthermore, samples were collected from schools located in Wasit governorate which includ (City center, Al-Hay, Al-Bataar, Sheikh Saad, Al-Azizia and Al-Suwaira districts). We started to collect samples since October 2012 till the end of April 2013. The collected specimens induced: Tinea capitis, Tinea corporis, Tinea cruris, Tinea manuum and Tinea faciei. All patients were subjected to full history taking including age, gender and antifungal treatment. Clinical examination was done to differentiate between different types of ringworm 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.

-Skin scales: scrapings were taken from the definite edge of the lesions with sterile scalpel [11].

The samples were diagnosed by direct examination using 10% KOH solution and laboratory culture on Sabouraud's Dextrose Agar with Chloramphenicol and Cycloheximide (SDACC) (BDH, England).

Identification of fungal isolates were done through

1- Macroscopic examination of colonies on different media.
2- Microscopic examination of cultures.
3- Physiological test including urease and pigment production test.
4- Fingerprinting primed polymerase chain reaction.

DNA Extraction

DNA was extracted from mycelium using (BIO BASIC INC. Protocol extraction Kit) as described by the manufacturer of BIO BASIC INC., Canada.

DNA Finger printing

PCR finger printing used a one-step PCR-based approach employing the simple repetitive oligonucleotide $(GACA)_4$ prepared by BIONEER, AccuOligo®, Korea as a single primer for identification of species of dermatophytes according to the [10].

Primer Preparation

The $(GACA)_4$ primer were provided in lyophilized form, were suspended in sterile distilled water to give a final concentration of 100 pmol/ µl as recommended by Alpha- DNA company protocol and stored in deep freezer until used in PCR amplification.

PCR Working Solution

Optimization of polymerase chain reaction was accomplished after several trials, thus the following mixture were adopted Table (1).

Table 1- The Mixture of PCR Working Solution.

Working Solution	
Go *Taq*® master mix	25.0 µl
Template DNA	4.0 µl
Primers	2.5 µl
D.W	18.5 µL
Final Volume 50 µL	

Programmable Thermal Controller

A thermal cycler was used for DNA amplification as follows in Table-2.

Table 2- Temperature Cycling Program for AP-PCR.

Initial denaturation	Temp:94ºC	Time:5min
No. of Cycles =39 cycles		
Denaturation	Temp: 93 ºC	Time: 1 min
Annealing	Temp: 50 ºC	Time: 1 min
Extension	Temp: 72 ºC	Time: 1 min
Final Extension	Temp: 72 ºC	Time: 7 min

Results

The PCR-based method used in this study utilized the short oligonucleotide $(GACA)_4$ as a primer for identification of dermatophyte isolates. The studied strains were amplified with this simple, repetitive primer, and the numbers of the resulting PCR bands ranged from 1to 4 (size range, 600bp to 1600bp). The resulting pattern were distinct for *Trichophyton* and *Microsporum* species and varieties. In general , our results revealed that the (GACA)4-based PCR band profiles were more complex with *Microsporum* species and varieties than with *Trichophyton* species. *Trichophyton mentagrophytes* var. *granular* and *Trichophyton mentagrophytes* var. *quinckeanum* gave two bands for each species, they showed the same (600bp) band. *Trichophyton rubrum* (downy type) and *Trichophyton rubrum* (granular type) revealed nearly the same pattern which consisted of three faint bands, there was a (450bp) for each species. *Trichophyton soudanenes* could be distinguished from other *Trichophyton species* by the size of the four largest band (1900bp, 1600bp, 1200bp and 800bp) and the last two band were bright. *Trichophyton tonsurans* displayed only one faint band (1600bp). *Trichophyton verrucosum* revealed a complex profile with four large bands ranging from (550bp to 1100bp). No band was detected with *Trichophyton equinum*, *Trichophyton mentagrophytes* var. *mentagrophytes* and *Epidermophyton floccosum* figure-1 and table-3.

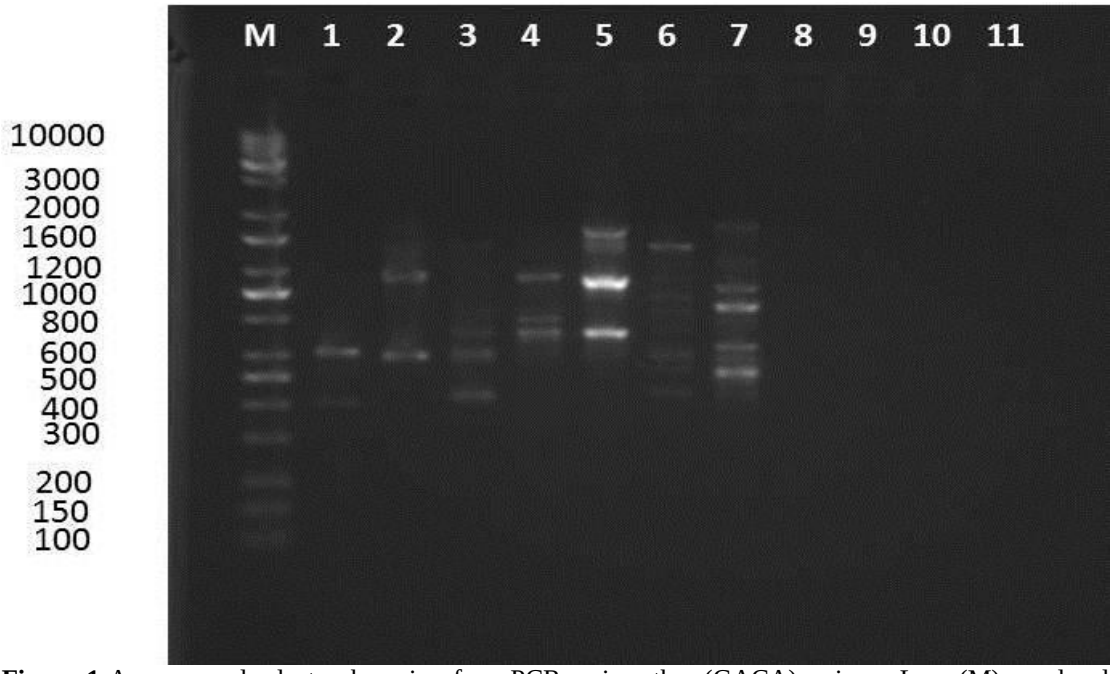

Figure 1-Agarose gel electrophoresis for PCR using the $(GACA)_4$ primer. Lan (M) , molecular weight marker (Fisher Scientific International , Inc.) ; 1-*T. mentagrophytes var. granular*; 2-*T. mentagrophytes var. quinckeanum; 3-T. rubrum* (downy type)*; 4-T. rubrum* (granular type)*; 5-T. soudanense; 6-T. tonsurans; 7-T. verrucosum; 8-T. equinum; 9-T. mentagrophytes* var.*mentagrophytes;* 10-Negative *controle (no template DNA)*; 11-*E. floccosum.*

Table 3- Trichophyton species and varieties examined in PCR-Fingerprint with the primer $(GACA)_4$.

Lane 1 ***T. mentagrophytes*** **Var.*granular***	**Lane 2** ***T. mentagrophytes var. quinckeanum***	**Lane3** ***T. rubrum*** **(downy type)**	**Lane 4** ***T. rubrum*** **(granular type)**	**Lane 5** ***T. soudanense***	**Lane 6** ***T. tonsurans***	**Lane 7** ***T. verrucosum***
600bp	**1200bp**	**750bp**	**1200bp**	**1900bp**	**1600bp**	**1100bp**
450bp	**600bp**	**600bp**	**800bp**	**1600bp**		**900bP**
		450bp	**450bp**	**1200bp**		**700bp**
				800bp		**550bp**

Figure-2 and table-4, showed the pattern profiles of *Microsporum* species amplified by $(GACA)_4$ primer. *Microsporum canis* (rough strain) and *Microsporum canis* (smooth strain) gave intraspecies variation, all of which had different band pattern. *Microsporum canis* (rough strain) gave three faint bands (350bp to 900bp) while *Microsporum canis* (smooth strain) gave two large bright bands (700bp and 1200bp).

There was no intraspecies variation among *Microsporum persicolor* and *Microsporum audouinii* var. *revalieri*, they had the same band pattern. *Microsporum gypsum* revealed three largest bands ranging from (800bp to 1300bp) whereas *Microsporum nanum* had three bands (600bp to 900bp). There was no band observed with *Microsporum audouinii* var. *langeronii* using this primer.

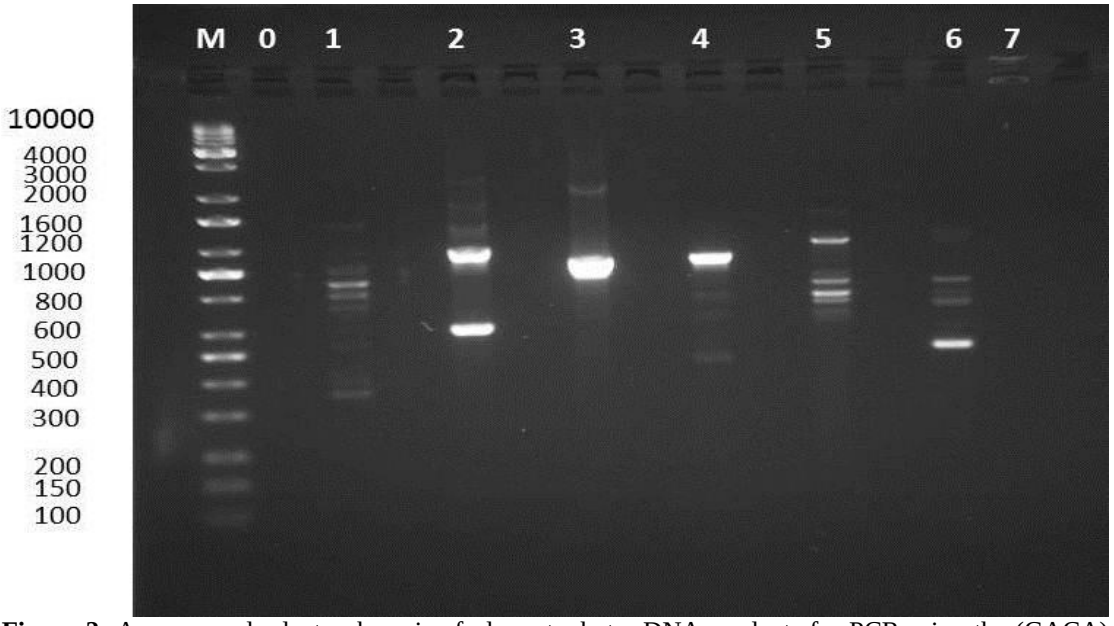

Figure 2- Agarose gel electrophoresis of dermatophyte DNA products for PCR using the $(GACA)_4$ primer. lan(M) DNA mol. wt marker (Fisher Scientific International, Inc.); 0, negative control (no template DNA); 1-*M.canis* (rough strains); 2-*M.canis* (smooth strains); 3-*M.persicolor*; 4-*M.audouinii* var. *rivalieri*; 5-*M. gypseum*; 6-*M. nanum*; 7-*M. audouinii* var. *langeronii.*

Table 4- Sum may of microsporum species examined in PCR-Fingerprint with the primer $(GACA)_4$.

Lane 1 ***M. canis*** **(rough strains)**	**Lane 2** ***M. canis*** **(smooth strains)**	**Lane 3** ***M. persicolor***	**Lane 4** ***M. audouinii*** **var. *rivalieri***	**Lane 5** ***M. gypseum***	**Lane 6** ***M. nanum***
900bp	**1200bp**	**1100bp**	**1100bp**	**1300bp**	**900bp**
800bp	**700bp**			**900bp**	**700bp**
350bp				**800bp**	**600bp**

Discussion

In this study, the method employed PCR was DNA fingerprint using the short oligonucleotide $(GACA)_4$ as a single primer. This primer has been shown to be a useful tool in molecular identification of dermatophytes [10, 12]. In the current study, we successfully identified the tested isolates to the species level. Our study also revealed that the$(GACA)_4$-based PCR band profiles were more complex with *Microsporum* species and varieties than *Trichphyton*.

The obtained profiles were characteristic of each species tested in this study, but marked similarity was observed between the profiles of the *T. mentagrophytes var. granular and T. mentagrophytes var. quinckeanum*, which can be explained by the close relatedness of these two species. These results agree with Kanbe *et al.*, 2003[13] that showed that the restriction profile of *T. mentagrophytes var. interdigitale* was identical to that of *T. mentagrophytes var. quinckeanum.*

This close relationship was also observed with *T. rubrum* (downy type) and *T. rubrum* (granular type) which revealed nearly the same pattern. [14] demonstrated similar results ,they found that $(GACA)_4$ are species- specific to the identification of dermatophytes, but cannot be used to subtype *T. rubrum* or *T. mentagrophytes*. Pereira *et al*., 2013 [15] revealed that *T. rubrum* cluster, strains were distributed into smaller highly related sub-groups with a similarity values above 85%. *Microsporum* strains (smooth and rough) in this study gave intra species variation, all of which had different band pattern. These results disagree with Faggi *et al.*, 2001[10] that found no intra species variability among 49 isolates of *M. canis* from human and animals. These results may suggest that $(GACA)_4$ primer has utility for diferentiation of *M. canis* strains isolated from patients and animals geographically isolated areas. On the other hand, there was no intra species variation among *M. persicolor* and M. aud. var. rev. This can be due to the low frequency of changes in DNA among populations of analyzed strains. This can be due to the low frequency of changes in DNA among populations of analyzed strains and may reflects the close phylogenetic relationship among them [16], accordingly, the $(GACA)_4$ -based

PCR band profiles were more complex with *Microsporum* species than *Trichophyton* It was possible to discriminate among most species with primers $(GACA)_4$. We conclude $(GACA)_4$-based PCR has utility as a rapid method for identification of dermatophytes species as well as its utility for differentiation of *Trichophyton* and *Microspmoru* species and varieties.

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