A RAPID BIOLOGICAL MOLECULAR METHOD FOR IDENTIFICATION AND DIFFERENTIATION BETWEEN T. EQUINUM SPECIES ISOLATED FROM DERMATOPHYTIC HORSES.

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ABSTRACT

Dermatophytosis is considered as one of the important fungal skin diseases which infect animals and transmit from animal-to-animal, animal-to-human, and from human-to-animals. *T. equinum* is the main cause of dermatophytosis in horses, all over the world the identification and differentiation between *T. equinum* species by conventional methods has drawbacks of being very slow, often requiring weeks of culture and suffering complications of overgrowth with commensals so the molecular approaches are applied recently for diagnosis of dermatophytes. This study involved identification of *T. equinum* by conventional methods and application of PCR by using non-specific simple repeat sequence (GACA) 4. The result showed that identification of *T. equinum* var. *equinum* and *T. equinum* var. *autotrophicum* and the primer was able to amplify both species forming characteristic PCR profiles for each.

KEY WORDS: Dermatophytosis, Fungi, Mold, PCR.

1. INTRODUCTION

Dermatophytes are group of morphologically and physiologically related molds which comprise of three keratinophilic and keratinolytic genera (*Trichophyton*, *Microsporum* and *Epidermophyton*). They utilize keratin for their growth and thus the infection confined to superficial integument including outer stratum corneum of the skin, nails, claws and hair of animals and man, causing an integumentary disease called ringworm, tinea or dermatophytosis [9,12,1 3]. *T. equinum* is the main cause of dermatophytosis in horses, all over the world [13, 11] and it includes 2 varieties which identified as *T.equinum* var. *equinum* and *T.equinum* var. *autotrophicum*. The differentiation between them relaye on the conventional methods of identification that included nicotinic acid requirements for the growth of *T.equinum* var. *equinum* [7] and hair perforation test [15]. Even these methods are accurate their major drawback is that they are very slow, often requiring weeks of culture and suffering complications of overgrowth with commensals [5].

Many molecular approaches have been applied for the identification of different dermatophyte species and strains. Such approaches are considered more stable and precise than are phenotypic characteristics [3].

This study involved identification of *T.equinum* by conventional methods and
application of rapid molecular diagnostic methods on the isolates.

2. MATERIAL AND METHODS

2.1. Isolation and Identification of *T. equinum* by conventional methods [10].

15 hairs and skin scraping were collected from suspected ringworm skin lesions of infected horses. The specimens were subjected to direct microscopic examination by using KOH 20%, cultivated on sabouraud’s dextrose agar with and without enrichment by nicotinic acid and incubated at room temperature for 15 days. The grown colony was examined microscopically by lactophenol cotton blue stain. Urease test and hair perforation test were done.

2.2. DNA extraction for application of PCR [6].

Genomic DNA was prepared from fungal colony with a rapid mini-preparation procedure for fungal DNA. To a 1.5-ml Eppendorf tube containing 500 µl of lysis buffer (400 mM Tris-HCl [pH 8.0], 60 mM EDTA [pH 8.0], 150 mM NaCl, 1% SDS), a small lump of mycelia grown on Sabouraud’s dextrose broth was added by using a sterile toothpick, with which the lump of mycelia was disrupted. The tube was then left at room temperature for 10 min. After adding 150 ml of potassium acetate (pH 4.8; which was made of 60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid, and 28.5 ml of distilled water), the tube was vortexed briefly and spun at 10,000 ×g for 1 min. The supernatant was transferred to another 1.5-ml Eppendorf tube and centrifuged again as described above. After transferring the supernatant to a new 1.5-ml Eppendorf tube, an equal volume of isopropyl alcohol was added. The tube was mixed by inversion briefly. The tube was spun at 10,000 ×g for 2 min, and the supernatant was discarded. The resultant DNA pellet was washed in 300 ml of 70% ethanol. After the pellet was spun at 10,000 ×g for 1 min, the supernatant was discarded. The DNA pellet was air dried and dissolved in 50 ml of Tris-EDTA.

2.3. PCR amplification for fungal origin identification [14].

The non-specific simple repeat sequence (GACA) 4 was used as a single primer in the PCR amplification. Amplification reactions were performed in 50 µl volumes containing 25 ng template DNA, 10 µl 5 X Taq master, 160 ng of primer; and fill up to 50 µl PCR grade water. PCR was performed for 35 cycles in a DNA thermal cycler with 1 min denaturation at 93°C, 1 min annealing at 50°C, 1 min extension at 68°C, and a final elongation for 10 min at 68°C. PCR products (20 µl/sample) were separated by electrophoresis in 1% agarose gels. Amplification products were detected using ethidium bromide staining and were visualized under UV light for the classification of the infectious origin.

3. RESULTS

3.1. Isolation and Identification of *T. equinum* by conventional methods

The direct microscopic examination of the hairs and scales revealed presence of the large ectothrix spores (photo 1).

![Photo 1 KOH preparation showing horse hair surrounded with chain of large ectothrix spores X400](image-url)
Diagnosis of *T. equinum* isolated from dermatophytic horses

Characters of the 2 colony were nearly similar but varied in requirement of *T. equinum* var equinum for nicotinic acid in the culture for optimum growth. *T. equinum* var autotrophicum (photo 2) grew moderately rapid (after 5 days). The colony was initially fluffy; white with raised center, later it became velvety, with folded center. The reverse side was yellow brown in color.

*Photo 2: Surface colony of T. equinum* var autotrophicum on Sabouraud’s dextrose agar after 15 days.

*T. equinum* var equinum (photo 3) grew moderately rapid as disk shape thallus which forms white knobs and folds in the center, with pink periphery. The reverse side had yellow brown color and the periphery of the thallus showed fine radiating extensions.

*Photo 3: Surface colony of T. equinum* var equinum on Sabouraud’s dextrose agar after 12 days.

Microscopically, there were abundant pyriform to nearly spherical microconidia, which either sessile or born on short stalk along the hyphae (photo 4). Macroconidia were rarely developed and observed as long, smooth, multicellular and thin walled, but *T. equinum* var equinum had tendency to form many chlamydospores (photo 5) in old culture more than *T. equinum* var. autotrophicum.

*Photo 4: Microconidia of T. equinum* stained with LCB, 400X.

*Photo 5: Abundant Chlamydospores and macroconidia of T. equinum* var equinum stained with LCB, 400X.

Physiologically all strains showed positive urease activity. *T. equinum* var equinum was negative in hair perforation test, while *T. equinum* var. autotrophicum was positive.

3.2. PCR amplification

The non-specific simple repetitive oligonucleotide (GACA)4 amplified both dermatophyte species with production of specific PCR profiles for every one (photo 6). With *T. equinum* var equinum, the PCR profile was consisted of 5 bands ranging from 350bp to 1800 bp and appeared as 2 bright bands at 350 bp and 580 bp, and 3 faint bands at 930, 1400 and 1800bp. While, those of *T. equinum* var
autotrophicum was consisted of 2 bright bands at 350 bp and 580 bp.

Photo (6): PCR amplification of genomic DNA samples was carried out with Simple repetitive Oligonucleotide (GACA)₄. Lanes: M, molecular weight marker; 1, T. equinum var autotrophicum; 2, T. equinum var equinum; 6, and 3, control negative.

4. DISCUSSION

The results of the morphological, microscopical and physiological characters were coincided with those reported by [4, 9, 10]. The result of direct microscopic examination revealed presence of dermatophyte infection but it was unable to identify the species. This result was in agreement with [6, 12]. In concern with the culture characters and physiological test, they gave definitive identification of the T. equinum and were able to differentiate between its verities. But, it was time consuming which conceded with [5, 6, 8], who stated that in-vitro culture of clinical samples was capable of providing a species-specific determination of dermatophytes on the basis of morphological and biochemical criteria in 10-15 days in >95% of cases examined, but it was cost and time-consuming.

The results obtained by usage of non-specific simple repetitive oligonucleotide (GACA)₄ showed formation of specific PCR profiles for each isolate. This application on this species was first time to be done.

The results showed that (GACA)₄-based PCR was a simple, easy, rapid, and reproducible molecular technique that had utility for identification of dermatophyte fungi to the species level as well as intra-species varieties, which conceded with finding of [1, 2, 14].

4. REFERENCES

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الطريقة البيولوجية الجزيئية السريعة للتعرف والتفرقة بين عترات الترياكوفيتون ايكوينم المعزولة من الخيول المصابة

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الملخص العربي

مرض القوباء الحلقي أحد أهم الأمراض الفطرية الجلدية التي تصيب الحيوانات وتنتشر بينهم بسهولة، وذها من الحيوان إلى الإنسان والعكس. تعتبر الترياكوفيتون ايكوينم من أهم السبب لفطر القوباء الحلقي في الخيول في جميع أنحاء العالم. ولكن تشخيص الترياكوفيتون ايكوينم بالطرق الميكولوجية المختلفة يستغرق وقتا طويلاً بجانب احتمالية ظهور نمو الفطريات الأخرى، ولذلك كان الاتجاه نحو التشخيص السريع باستخدام الطرق البيولوجية الجزيئية السريعة. تتضمن هذه الدراسة التعرف والتفرقة بين انواع عترات الترياكوفيتون ايكوينم بالطرق الميكولوجية المعتادة وتفاعل البممرة المستمر باستخدام بريمر غير متخصص (GACA)⁴. وقد أوضحت النتائج التعرف على الترياكوفيتون ايكوينم فار ايكوينم والبريمرات المتسمسلة في اختبار البممرة المستمر بالطرق الميكولوجية واستطاع اختيار البمرة المستمر أن يفرق بين المعزولات.