

# **A Rapid molecular method for identification and differentiation between *T.equinum* species isolated from dermatophytic horses.**

**Ali M. El-Ged, Adel M. Khalid, Ashraf A. Abd El-Tawab and Enas Abd El-Baset.**

Bacteriology, Immunology and Mycology Department, Faculty of Veterinary Medicine, Banha University, Qalyobia, Egypt.

## **Abstract**

*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)<sup>4</sup>. 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.

## **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 [1,2,3].

*T. equinum* is the main cause of dermatophytosis in horses, all over the world [3,4] 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* [5] and hair perforation test [6]. 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 [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 are phenotypic characteristics [8].

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

## **Materials and methods:**

### **1- Isolation and Identification of *T. equinum* by conventional methods [9].**

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- DNA extraction for application of PCR [10].**

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 10min. 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 rpm for 1 min, the supernatant was discarded. The DNA pellet was air dried and dissolved in 50 ml of Tris-EDTA.

### **3- PCR amplification for fungal origin identification. (Shehata et al., 2008)**

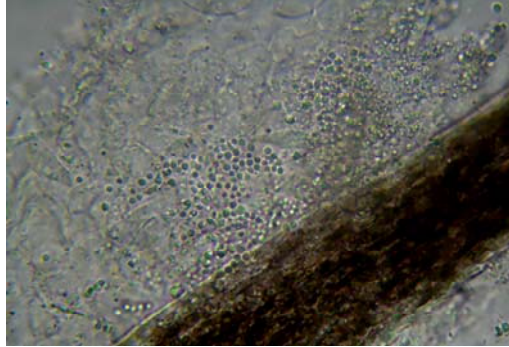
The non-specific simple repeat sequence (GACA)<sub>4</sub> was used as a single primer in the PCR amplification.

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.

## **Results**

### **1- Isolation and Identification of *T.equinum* by conventional methods**

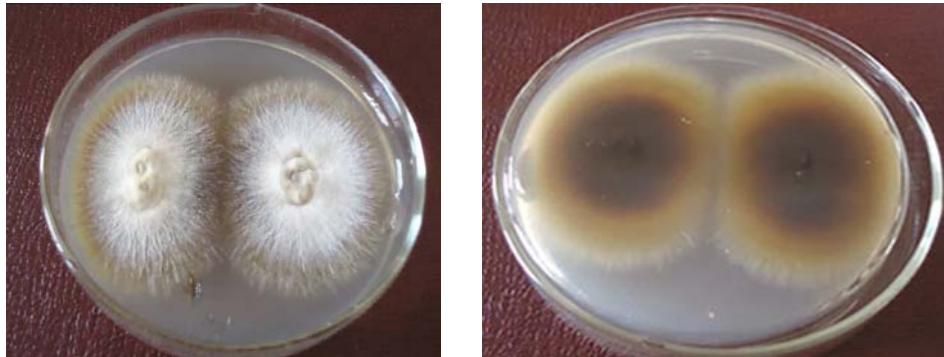
The direct microscopic examination of the hairs and scales revealed presence of the large ectothrix spores (**photo 1**).



**Photo 1:** KOH preparation showing horse hair surrounded with chain of large ectothrix spores X400

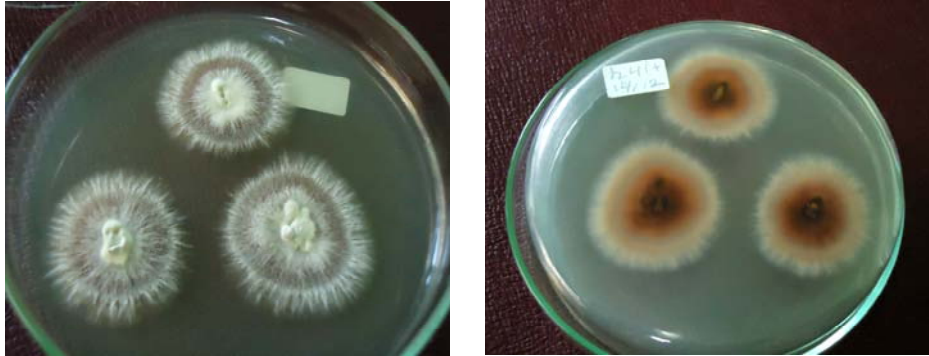
The culture identification revealed isolation of *T.equinum* var. *equinum* (1 isolate) and *T.equinum* var. *autotrophicum* (14 isolates). The morphological characters of the 2 colony were nearly similar but varied in requirement of *T. equinum* var *equinum* for nicotinic acid in the culture for optimum 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.



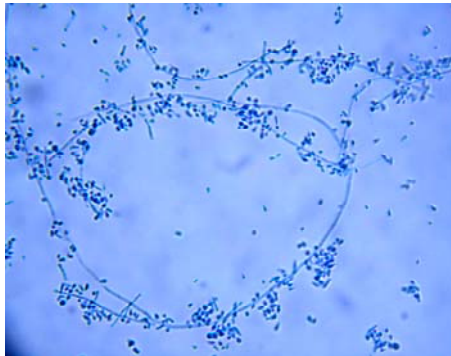
**Photo 2:** Colony surface of *T.equinum* var *autotrophicum* on Sabouraud's dextrose agar after 15 days.

*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.

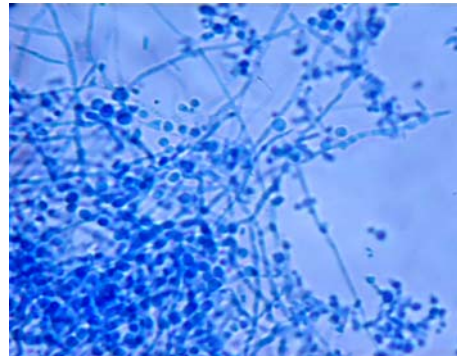


**Photo 3:** Colony surface of *T. equinum* var *equinum* on Sabouraud's dextrose agar after 12 days.

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.



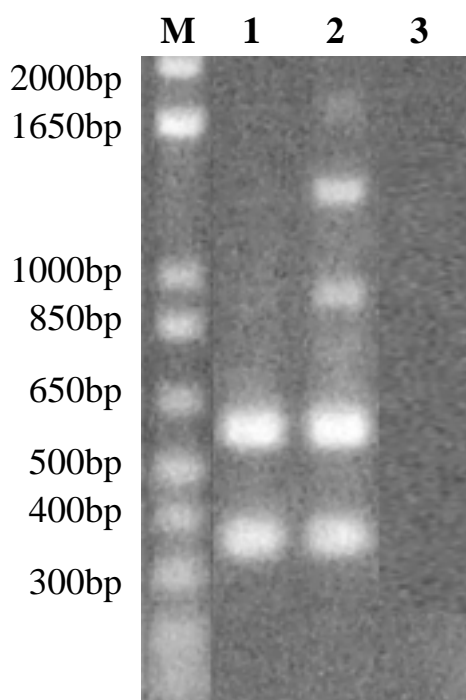
**Photo 5:** Abundant Chlamydospores and macroconidia of *T. equinum* var *equinum* stained with LCB, 400X.

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

## 2- PCR amplification

The non-specific simple repetitive oligonucleotide (GACA)<sub>4</sub> 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)<sub>4</sub>. Lanes: M, molecular weight marker; 1, *T.equinum* var autotrophicum; 2, *T.equinum* var equinum; 6, and 3, control negative.

## Discussion:

The results of the morphological, microscopical and physiological characters were coincided with those reported by [9, 1, 11]. 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 [10, 2].

In concern with the culture characters and physiological test, they gave definitive identification of the *T.equinum* and were able to differentiate between its varieties. But, it was time consuming which concurred with [7, 10, 12], who stated that 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)<sub>4</sub> 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)<sub>4</sub>-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 concurred with finding of **[13. 14.15]**.

### Reference:

- 1- **Quinn, P. J.; Carter, M. E.; Markey, B. and Carter, G.R. (1994):** Section 3: Mycology in Clinical veterinary microbiology. Copyright Elsevier limited. P. 381-390.
- 2- **Simpanya M.F., (2000):** Dermatophytes: Their Taxonomy, Ecology and Pathogenicity. Revista Iberoamericana de Micología Apdo. P. 1-12.
- 3- **Saad, A.M.A.A. (2010):** Some studies on skin affections in equine. M.V.Sc. thesis (Infectious Diseases), Fac. Of Vet. Med., Mansoura University.
- 4- **Robinson N.E and Sprayberry K.A., (2009):** Chapter 150: fungal skin diseases in Current therapy in equine medicine, Sixth edition. Saunders Elsevier, USA. P. 687.
- 5- **Pereira, D.I.B.; de Oliveira, L.S.S.; Bueno, A.; Cavalheiro, A.S.; Schwendler, S.E.; de Azevedo, M.I.; Júnior, J.C.E.; Aguiar, L.C.; Santurio, D.F.; Santurio, J.M. and Alves, S.H.(2006):** Outbreak of Trichophyton Equinum Var. Equinum in Horses in the South of Brazil. Ciência Rural. **36**:1849-1853.
- 6- **WWW.Provlab.ab.ca.**
- 7- **Liu, D.; Coloe, S.; Baird, R. and Pedersen, J. (1997):** PCR identification of Trichophyton mentagrophytes var. interdigitale and T. mentagrophytes var. mentagrophytes dermatophytes with a random primer. J. Med. Microbiol. **46**: 1043-1046
- 8- **Gräser, Y.; Kuijpers, A.F.A.; Presber, W. and de Hoog, G.S. (2000) <sup>a</sup>:** Molecular taxonomy of the Trichophyton rubrum complex. J Clin Microbiol. **38**: 3329–36.
- 9- **Rebell, G. and Taplin, D. (1979):** Dermatophytes. Their recognition and identification. 4<sup>th</sup> printing, page 110. Dermatology foundation of Miami Press. U.S.A.
- 10- **Liu, D.; Coloe, S.; Baird, R. and Pedersen, J. (2000):** Application of PCR to the identification of dermatophytes fungi. J. Med. Microbiol. **49**: 493-497.
- 11- **Hungerford, L.L.; Campbell, C.L. and Smith, A. R. (1998):** veterinary mycology laboratory manual. Copyright Iowa State University Press. P. 34.
- 12- **Petrini, B. and von Rosen, M. L. (2002):** Optimal dermatophyte diagnosis requires both microscopy and culture. Lakartidningen **99**:4084.

- 13- **Faggi, E.; Pini, G.; Campisi, E.; and Bertellini, C.; Difonzo, E. and Mancianti, F. (2001):** Application of PCR to Distinguish Common Species of Dermatophytes. *J of Clin. Microbiol.* **39:** 3382–3385.
- 14- **Shehata, A.S.; Mukherjee, P.K.; Aboulatta, H.N.; El Akhras, A.I.; Abbadi, S.H. and Ghannoum, M.A. (2008):** Single-Step PCR Using (GACA)<sub>4</sub> Primer: Utility for Rapid Identification of Dermatophyte Species and Strains. *Journal of Clinical Microbiology.* **46:** 2641–2645.
- 15- **Chung, T.H.; Park, G.B.; Lim, C.Y.; Park, H.M.; Choi, G.C.; Youn, H.Y.; Chae, J.S. and Hwang, C.Y. (2010):** A rapid molecular method for diagnosing epidemic dermatophytosis in a racehorse facility. *Equine vet. J.* **42:** 73-78.