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Application of Molecular Techniques for Rapid Diagnosis of Dermatophytes Infection in Horses

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Abstract: Dermatophytes are a group of keratinophilic fungi that cause Dermatophytosis which is a highly contagious fungal infection of the skin that affects horses of all ages and breeds. In the present study, A total of 30 horses out of 216 (13.8%) were clinically examined; they were subjected to mycological examination. Direct microscopic examination of samples resulted in 20 (66.6%) positive samples out of 30, while culture examination revealed that 13 (43.3%) were culture positive within 2-4 weeks. Culture identification resulted in 4 species namely *T. verrucosum* (9, 69%), *T. mentagrophytes var. erinacei* (2, 15.3%), *T. equinum var. autotrophicum* (1, 7.6%) and *M. canis* (1, 7.6%). After performing a rapid DNA extraction method directly from the hair samples the total 30 DNA extracts were subjected to PCR using specific primers for dermatophytes group. This PCR resulted in 22 (73.3%) positive samples within 8 hours. The present work assured that dermatophytes can be molecular biologically diagnosed by an accurate, sensitive, specific and very rapid method within hours instead of weeks.

Key words: Ringworm · Dermatophytosis · Polymerase Chain Reaction · Chitin synthase 1 gene

INTRODUCTION

Dermatophytes are a group of morphologically and physiologically related moulds some of which cause well-defined infections, dermatophytosis (Tinea or ringworm). They are both keratinophilic and keratinolytic. This means that, they have the ability to digest keratin *in vitro* in their saprophytic state and utilize it as a substrate and some may invade tissues *in vivo* and provoke tinea. However, their morphology in the parasitic growth phase, *in vivo*, is different from the tmorphology exhibited *in vitro* [1] Dermatophytes comprise three genera, *Trichophyton, Microsporum* and *Epidermophyton,* they are responsible for the majority of superficial fungal infections of the skin, hair and nails [2]. Dermatophytosis is generally cutaneous and restricted to non-living cornified layers as they are keratinophilic and can't penetrate deeper tissues or organs of immunocompetent host. The reaction to dermatophytes infection ranges from mild to severe according to reaction of the host to metabolic products of the fungus, virulence of the species, anatomic location of infection and local environmental factors [3]. Dermatophytosis is a highly contagious fungal infection of the skin that affects horses of all ages. In horses it is caused by 2 main genera of fungi *Trichophyton* and *Microsporum*. [4]. Typical dermatophytosis in horse includes focal lesions, scaling, crusting, alopecia and pruritis (may or may not present), the lesions are usually multiple and most commonly occur in saddle and girth region [5]. The lesions of dermatophytes infection in horses may be superficial or deep [6]. Dermatophytosis in equines commonly caused by *Tricophyton equinum* (Trichophytosis) which characterized by typical numerous small and rounded patches covered by small bran like asbestos colored scales and *Microsporum equinum* (Microsporosis) which adequately characterized by irregular limited patches, often overlapping by pronounced desquamation and accumulation of large, lime white scales firmly adheres to the base. These differences made clinical diagnosis so difficult and so the mycological analysis is required,

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which includes both direct microscopic examination and cultural examination [7]. Ringworm lesions had many adverse effects on horses; as a horse with ringworm may be uncomfortable; ringworm is really more of an unsightly nuisance. It also can prevent the horses from working and interfere with their use in riding and racing purposes as horse with ringworm will not be welcome at shows or other events, so the cost value of horses with ringworm decreased. Dermatophytosis has considerable zoonotic importance as animals serve as reservoirs for the zoophilic dermatophytes and their infections. Zoophilic dermatophytes such as *M. canis, T. mentagrophytes* and *T. verrucosum* are significant causal agents of human ringworm in many areas of the world. The incidence of dermatophytosis varies according to climate and natural reservoirs. However, the pattern of the species of dermatophytes involved in dermatophytosis may be different in similar geographical conditions, both in humans and animals. It has been related, among other factors, to the decline in the incidence of animal ringworm in these areas or the degree and closeness of animal to human contact [8]. It is important to have rapid and accurate etiological identification of dermatophytosis because many antimycotic agents have different activity spectra. Therefore, treatment strategies must target specific species [9]. A simple, rapid and specific method for the diagnosis of dermatophyte infections would obviously be a major improvement. Introduction of a PCR-based methodology would increase specificity, simplicity, speed and potentially even reduced cost [10]. The aim of the present work was comparing the traditional methods of isolation and identification of dermatophytes in equine with those of nucleic acid based techniques such as PCR.

MATERIALS AND METHODS

Samples: A total of 30 hair and skin scraping samples were collected from different horses clinically showing ringworm lesions from different hospitals and clubs in Cairo.

Media: Sabouraud's Dextrose agar (SDA); SDA enriched with thiamine and inositol (SDAE) and that enriched with nicotinic acid (SDAN) media were used in this study according to Kwon-Chung and Bennett [11].

KOH (20%) solution was used for direct microscopic examination of the specimens [12] and Lactophenol cotton blue stain was used for staining of moulds for microscopic examination [13].

Preparation of the Samples: The area of the skin to be sampled was disinfected with 70 % ethanol and superficial material (dirt) was removed before sampling. Mycological samples were taken by scraping the hairless skin and plucking the hair (if present) from the margin of lesions in affected skin with sterile forceps. Each sample was divided into two portions, one for conventional method of diagnosis and the other for molecular diagnosis. For samples used in molecular diagnosis, the distal hair tips were removed aseptically to prevent contamination; only the hair roots were used for fungal DNA extraction. Each sample was collected in a sterile labeled envelop, the samples were stored at room temperature till mycological examination. Hair and some skin scraping samples of the collected specimens were placed on a clean glass slide then 1-2 drops of 20 % KOH solution were added, covered with cover slide, heated gently and left in a humid chamber for 2 hours. This preparation was then examined under the low and high dry objective lenses of light microscope for detection of fungal elements.

Conventional Methods Used for Identification of Dermatophyte Species: Each sample was inoculated into nine slopes; three slopes of Sabouraud's dextrose agar with cyclohexamide and chloramphenicol, three slopes of Sabouraud's dextrose agar enriched with nicotinic acid and three slopes of Sabouraud's dextrose agar enriched with thiamine and inositol. The inoculated slopes were incubated at 30°C except the slopes enriched with thiamine and inositol were incubated at 37°C for growing and subsequently isolating of *T. verrucosum* if present. The inoculated slopes were observed weekly up to 4 weeks for the presence of fungal growth. The isolated dermatophyte species were identified according to their macroscopic and microscopic characters [14, 15].

DNA Extraction: DNA was extracted directly from prepared samples using GeneJet Genomic DNA Purification Kit (Fermentas, Germany) according to the manufacturer's instructions.

PCR Analysis of Dermatophytes Strains: The PCR assays were performed using two selected oligonucleotide primers as indicated in Table 1. Primers were purchased from (Biolab, Swedan). Each reaction mixture contained 9 µl of DNA template (20 ng/µl), 12.5µl of ready to use Master Mix (Biolab, UK) and 0.4µM from each primer. The final volume reached by adding 0.5µl of nucleic acid free distilled water to 25 µl final volume.

Table 1: Primers used for PCR analysis of the isolated strains according to Brillowska *et al.* [10]

Primer	Sequence
-Pan derm1	(5′-GAAGAAGATTGTCGTTTGCATCGTCTC-3′)
-Pan derm 2	(5′-CTCGAGGTCAAAAGCACGCCAGAG-3′).

PCR assays were carried out in DNA Thermal cycler 9600 (Applied Biosystems, USA) for *T. verrucosum, T. mentagrophytes, T. equinum* and *M. canis* strains at an initial 5 min denaturation step at 94°C followed by 40 cycles each consists of denaturation step at 94°C for 40 second, annealing step at 60°C for 1 min and extension step at 72°C for 2 min. After the 40 cycles, there was an additional extension step at 72°C for 7 min. A negative control in which DNA was replaced by sterile distilled water was also included. Aliquots of 10 µl of amplified products were analyzed by electrophoresis on 1.5 % agarose containing 0.5 µg/ml Ethidium Bromide at 80 V for 90 min and PCR products were detected by UV transilluminator.

RESULTS

Incidence of Ringworm among Clinically Examined Horses: A total of 30 out of 216 (13.8%) clinically examined horses showed cutaneous lesions characteristic for ringworm. The diagnosis was confirmed by direct microscopy, culture examination and PCR diagnosis.

Direct Microscopic Examination of Hair and Skin Scraping: Specimens were subjected to direct microscopic examination using 20 % KOH, which resulted in 20 (66.6%) positive samples out of 30. The positive samples characterized by the presence of ectothrix hair invasion with fungal spores. Also, some hairs showed parallel chains of fungal spores along the hair shaft.

Results of Culture Examination: Culture examination of horse samples showed that 13 (43.3%) out of 30 (total number of clinically infected horses) were positive for culture isolation (Table 2).

Culture identification resulted in 4 species namely *T. verrucosum var ochraceum* (9, 69%), *T. mentagrophytes var. erinacei* (2, 15.3%), *T. equinum var autotrophicum* (1, 7.6%), *M. canis* (1, 7.6%).

Morphological Characteristics of the Isolated Dermatophyte Species

Microsporum Canis Isolate: Macroscopically, first colonies grown on SDA slants were scattered then become associated with each other; at last they covered the whole surface of the slants. In subcultures on plates of the same medium, fungal growth was rapid and colonies were about 3 cm in diameter within 10 days. The surface of the colonies was white and silky at first and then it became dense, tan and cottony with bright yellow coloration, which was visible from the surface through the thin radial mycelial growth in the peripheral parts. Closely spaced radial grooves were observed (Fig. 1.A). The reverse of the colonies was brown at the center and bright yellow in the peripheral parts (Fig. 1.B). Microscopically, numerous large multiseptated, spindle-shaped, rough-walled macroaleuriospores with curved or hooked ends showing septation that varied from 6 to 10 cells were seen. Few small clavate one-cell microaleuriospores were also present (Fig. 1.C).

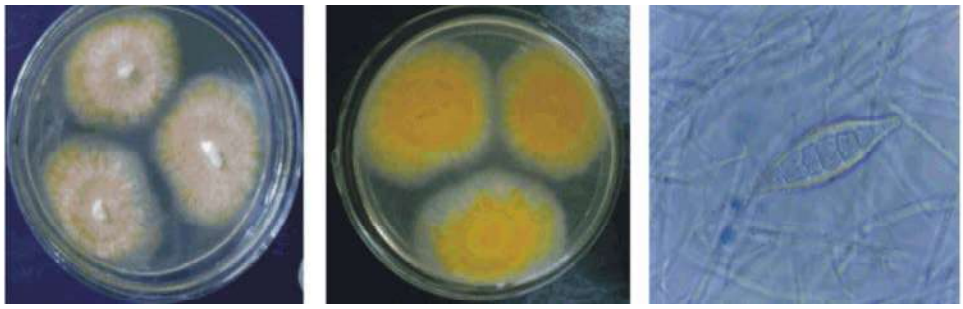
Table 2: Correlation between positive microscopy and positive culture of examined horse samples

	Positive microscopy		Positive culture	
No. of examined horse Samples	No.	%*	No.	%*
30	20	66.6	13	43.3

* = Percentage to the total no. of examined horse samples

Trichophyton Equinum Var. Autotrophicum Isolate: The fungus grew rapidly on SDA slants, after 3 weeks it was subcultured in plates of the same medium and incubated at room temperature. Macroscopically, it grew as white downy to fluffy colony. At the beginning surface of the colonies was flat and by the end of the second week, it showed irregular heaping and folding with raised center. The fungus showed a pale yellow to pink creamy colour (Fig. 2.A). The reverse of the colonies was lemon yellow (Fig. 2.B). Microscopic examination of the isolated fungus revealed the presence of spherical single-cell microaleuriospores, which were born on short stalks. Macroaleuriospores were rarely found (Fig. 2.C).

Trichophyton Mentagrophytes Var. Erinacei Isolate: Macroscopically, the fungus colony appeared flat with white colored powdery surface in subculture on SDA plates it showed a raised center (Fig. 3.A). The reverse colour was brilliant yellow (Fig. 3.B). Microscopic examination of the isolated fungus revealed the presence of small spherical, thin, elongated, variable sized microaleuriospores. Macroaleuriospores were absent.

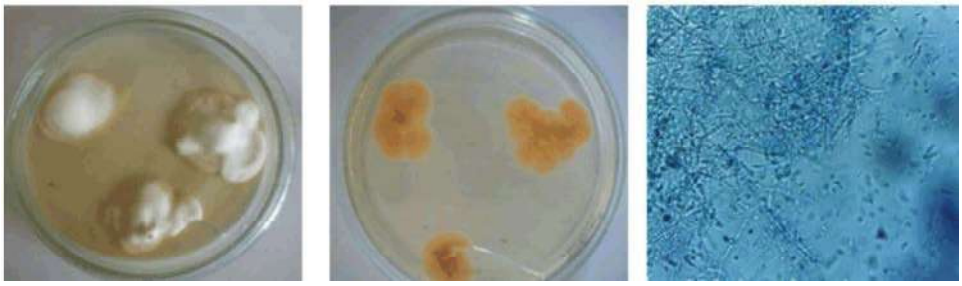


(A) (B) (C)

Fig. 1: Macroscopic and microcscpic morpholgy of three weeks old culture of *Microsporum canis* o SDA

(A) (B) (C)

Fig. 2: Macroscopic and microcscpic var. erinacei isolate



(A) (B) (C)

Fig. 3: Macroscopic and microcscpic morpholgy of morthology of T. Mentogrophytes var. erinacei

An intermediate forms between macro-and microaleuriospores were numerous (Fig. 3.C). When compared with *T. mentagrophytes*, *T. mentagrophytes var. erinacei* were characterized by the brilliant yellow pigment shown on the reverse of the colony and by the intermediate forms between macro- and microaleuriospores.

Trichophyton Verrucosum Var Ochraceum Isolate: The fungus grew very slowly on SDAE slants after incubation at 37°C. Macroscopically, the colony appeared as yellowish white, waxy, raised, rounded growth with convoluted surface and the size ranged from small (Fig. 4.A) to medium sized colonies (Fig. 4 B,D) which attached firmly to the surface of the medium. Reverse was white to colorless (Fig. 4.E). Microscopic examination of the isolated fungus revealed the presence of numerous thick-walled large chlamydospores (Fig. 4.C) and an antler like structure on the hyphal tips (Fig. 4.F). Few rounded microaleuriospores were observed. Macroaleuriospores were absent.

Molecular Diagnosis of Examined Horse Samples: As shown in Fig. 5. 22 (73.3%) samples out of 30 were positive for polymerase chain reaction (PCR) performed for the diagnosis of dermatophytes using set Panderm primers derived from the chitin synthase1 (Chs1) gene, which is common for all dermatophytes. PCR amplification produced a product of 366 bp molecular weight as expected.

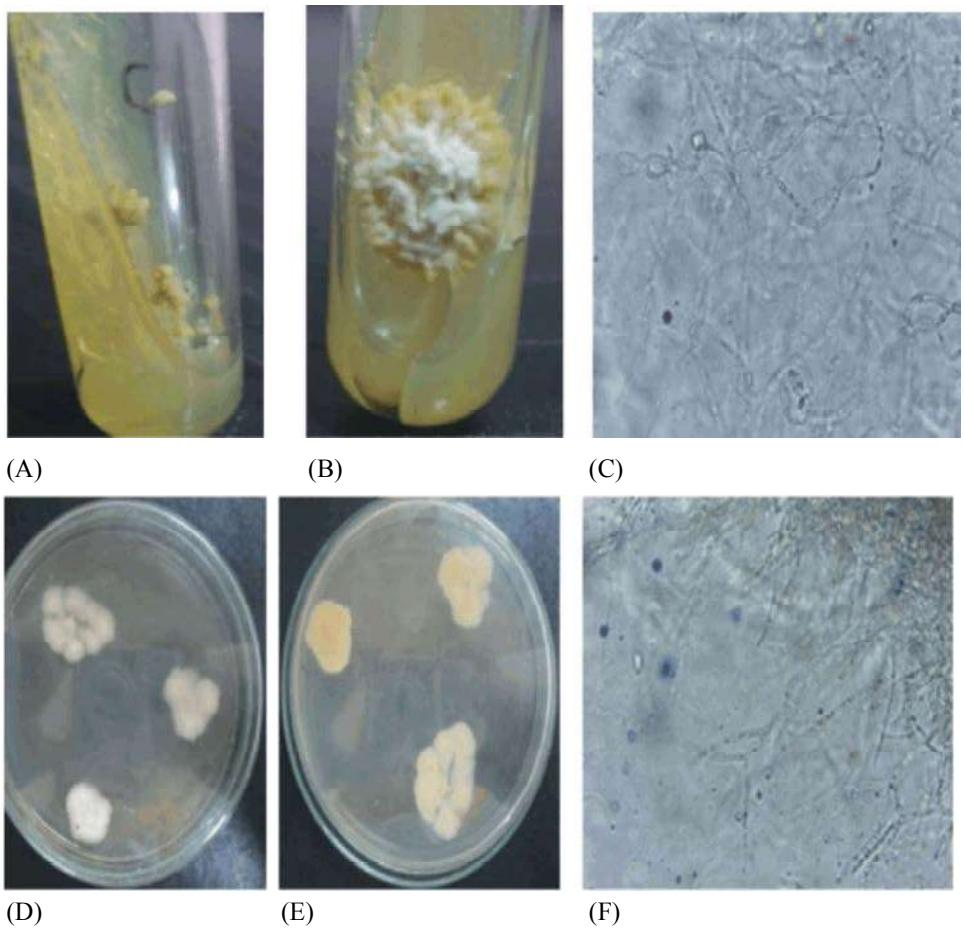


Fig. 4: Macroscopic and microcscpic morpholgy of morthology of T. Verricosum

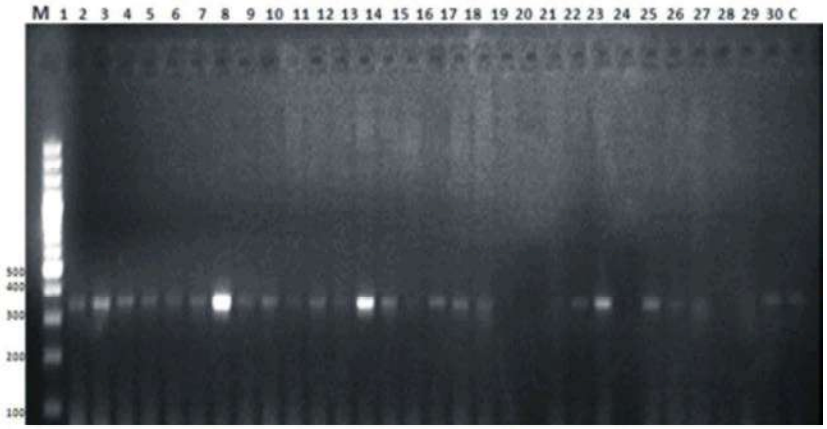


Fig. 5: Agarose gel electrophoreis showing amplification of 366 bp. Fragment of dermatophyte. M: 1 kb marker, lane 1-lane 30: NNA samples. C. Positive control

DISCUSSION

Ringworm infections are very important and cited among the most frequent causes of dermatological problems. Identification of the species and strain of dermatophyte can play an effective role in control of disease outbreaks by establishing the source of infection. Current methods of identification are based on cultural and microscopic methods, often involving weeks before a positive identification can be examined. A rapid

molecular diagnostic method would therefore be an important laboratory technique, but requires confirmation in equine clinical practice [16]. In the present study, 30 (13.8%) out of 216 clinically examined horses showed suspected ringworm lesions. Lesions appeared as multiple focal areas of alopecia and scaling could be observed as they were described by Abo El-Yazid [17] and Pilsworth and Knottenbelt [4]. Cultivation of the collected specimens revealed 13 isolates (43.3 %); these results were quite similar to other results obtained by Nweze [18] who examined 25 horses and found that 11 samples (44 %) were positive and as Hassan [19] who isolated dermatophytes from 36.5 % of total horse samples. Also similar finding was obtained by Abo El-Yazid [17] who found that the isolation rate of dermatophyte species from horses were (49 %). The culture identification of isolated dermatophytes strains resulted in 4 species; 9 isolates of *T. verrucosum* (69 %), 2 from *T. mentagrophytes var. erinacei* (15.3 %), one isolate was identified as *T. equinum var. autotrophicum* (7.6 %) and one as *M. canis* (7.6 %). These findings were in agreement with most of the published reports about the etiological agent of equine ringworm all over the world as described by Abo El-Yazid [17]; Pilsworth; Knottenbelt [4] and Nweze [18]. However, *Trichophyton verrucosum* takes the first place of the causative dermatophytes in horses (strains give result to 9 isolates out of 13, 69%), this considered as rare result; where Abo El-Yazid [17]; Kane *et al.* [20] and Nweze [18] stated that *T. equinum* and *T. equinum var autotrophicum* were the most commonly isolated species from horses and *Trichophyton verrucosum* in cattle. Indeed, such controversy is difficult to explain, but it may give indication to bad hygienic measures surrounding the horses which play an effective role in cross infection through several routes from cattle to horses as suggested by Mantovani *et al.* [21] and Abadi *et al.* [22]. *Trichophyton mentagrophytes var. erinacei* was isolated from one case only, it was interesting that Abo El-Yazid [17] isolated one *T. mentagrophytes* strain out of 33 samples with a ratio (3 %) as did White and Yu [6], who also isolated the same strain from horse samples. *Microsporum canis* was also isolated from only one case as reported by Nweze [18] while Moretti *et al.* [23] and white [24] isolated *M. audouinii* from horse samples. All isolated strains were identified on the basis of their macroscopic and microscopic morphology stated by mycological experts as Rebell and Taplin [14]. All strains showed typical morphological characters but after a long period of incubation, where identification of dermatophytes using the traditional methods difficult because of the long time of incubation as they were time consuming. Also there were difficulties in differentiation between species, these difficulties were also stated by Hall *et al.* [25], so a help from a mycological expert was a must. Many molecular based approaches have been applied for identification of different dermatophyte species and strains. Such approaches are considered more stable and precise compared to phenotypic characteristics [26]. In the present study, we used two specific primers based on the sequence of Chs1 gene [27-29] as conserved primers for all dermatophytes. In this work we tried to reach a rapid diagnosis of dermatophyte by purifying the DNA sample directly from clinical specimens to skip the period of culture that extends for weeks. The PCR amplification revealed 22 (73.3%) positive samples out of 30 (Fig. 5). This result was obtained within hours nearly similar to the results obtained by Brillowska-Da browska [30] but differ in the DNA extraction method. While Monod *et al.* [31] implemented a dermatophytes molecular diagnostic method in 2 days. However, Ohst *et al.* [32] and Kardjeva *et al.* [33] reported identification of *T. rubrum* within 14 hours. In conclusion, we have conducted a molecular diagnostic method for dermatophyte identification in 8 hours with a highly sensitive and specific result and required less efforts and time; hence time factor for diagnosis of any infectious disease is very important to start as early as possible in treatment of the infection.

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