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Incidence of Fungalysins Virulence Genes (*MEP*1-5) in Dermatophytes Isolated Form Infected Cases in Egypt

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Abstract: Dermatophytes are filamentous fungi that belong to genera *Trichophyton, Epidermophyton and Microsporium*. Dermatophytes not only can cause a variety of dermatophytosis in animals but also can transmit to humans. During the course of infection, the severity of infection requires the release of specific enzymes that facilitate the penetration into the host tissue. Several proteases including fungalysins (*MEP1*-5) have been considered as a potential virulence factors. In the present study dermatophyte was isolated and identified from infected samples and then samples were examined for the presence of these genes (fungalysins) by Polymerase Chain Reaction (PCR). The data collected from this study showed that, the incidence of *MEP*1-4 was only 10% of the screened samples, while it was 20% for *MEP*5. Although, to our knowledge these findings considered as the first data carried out to screen the incidence of these genes in dermatophytes (*Trichophyton verrucosum)* isolated from local strains, however, further investigations are still required to verify their precise role in dermatophytes infection in Egypt. Moreover, sequence analysis is required as well to verify the genetic difference between the isolated strains. Collectively, this will be the basal block in order to understand the dermatophytes infection virulence attribute and helps in developing of the appropriate therapeutic intervention.

Key words: Dermatophytes · Fungalysins · Pcr · *Mep*1-5

INTRODUCTION

Dermatophytes are specialised filamentous fungi that considered as the most common cause of superficial mycoses in humans and animals [1]. Although Dermatophyte infection is mainly superficial, however, immunocompormised patients can experience severe, disseminated disease [2]. Despite the fact that dermatophyte infection is treatable, however, there is a high rate of reinfection either because of relapse or new infection [3]. Dermatophytes induced superficial mycoses resulting in treatment costs of close to half a billion dollars annually in USA [4]. The scarcity of information concerning the dermatophytes virulence characteristics might be because the fact that these fungi have not to date been intensively studied at the molecular level comparing with other fungal pathogens such as *Candida albicans* and *Aspergillus Fumigatus.* Recently, the dermatophyte full genome sequence has only become available and introduction of new molecular tools like PCR and microarray have been used as an accurate reliable method. The recent advances in the molecular diagnosis of dermatophytes have been reviewed in details in Achterman and white [5]. The better understanding of this mechanism of virulence is the rational basis for the development of effective treatment and prophylactic strategies.

The host-fungus relationship in dermatophytes infection is complex and still required further investigation. There are many studied have carried out to characterise the secreted dermatophytic proteases at the molecular level. The newly introduced genetic tools such as PCR and microarray that allow rapid, effective and accurate functional investigation of dermatophytes genes and identification of the most important virulence factors involved in dermatophytes pathogenesis. The keratin, collage, elastin and other skin proteins may be substrate for the endo-and exoprotease produced by dermatophytes

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[6, 7] and the invasion of dermatophytes into the tissue requires the elongation of germ tube and secretion of various proteolytic and lipolytic exoenzymes [8]. Dermatophytes are true pathogenic fungi infecting healthy animals and the production of metalloproteases during *in vivo* infection is seen to have a role in dermatophytes virulence [9].

Several proteases have already been isolated from different species of dermatophytes and showed keratinolytic, elastinolytic and/or collagenolytic activities [10, 11]. Brouta and co-workers described a gene family [*MEP1*, *MEP2* and *MEP*3] coding for three endometalloproteases in *Microsporum canis* and reported the production of MEP2 and MEP3 proteases during *in vivo* experiences in guinea pigs. MEP3 was characterized and shows collagenolytic, elastinolytic and keratinolytic activities [12]. Other authors recognized two new genes, *MEP4* and *MEP5*, in *M. canis* as a part of a five member gene family (named *MEP*1-5) encoding for secreted endometalloproteases (fungalysins), also identified in *Trichophyton rubrum* and *T. mentagrophytes* [13].

So far, there is no data about the incidence of these fungalysins genes (MEP1-5) is *Trichophyton verrucosum* isolated samples form Egypt. Consequently, the main aim of this was to screen the incidence of five fungalysins virulence genes (*MEP*1-5) associated dermatophytes infection by using PCR. The data obtained from this study showed that, the incidence of *MEP1-4* was only 10% and 20% for *MEP5*.

MATERIALS AND METHODS

Animals and Samples Collection: A total of 100 hair and skin scrapings were collected from suspected ringworm lesions of infected horses and cattle from different localities in Minufyia and Shargia Governorates. The skin lesions were cleaned and disinfected with 70 % ethyl alcohol to remove surface contaminants. The scales were scrapped from the edges of the lesions by using blunt scalpel blade until blood was drawn. The basal root portion of stubby or damaged looking hair which contains the most useful diagnostic materials was collected by plucking the hair with sterile forceps. Samples were then collected in a sterile labelled closed envelop and transferred as soon as possible to laboratory for mycological examination [14].

Laboratory Isolation and Identification of Dermatophytes: Samples were cultivated on Sabouraud's dextrose agar medium with cyclohexamide and antibiotics [15] Sabouraud's dextrose agar medium enriched with thiamine and inositol [16]. The antibiotics, thiamine and inositol were added to the sterile media under full aseptic conditions.

Direct Microscopical Examination: The skin scrapings and the broken hairs were place on a clean glass slide, then a drop of 20% KOH was added and covered with a cover slide and gently heated and left for 1 hour. The slides were than examined for fugal elements (hyphea and spores around (ectothrix) or within the hairs (endothrix)) by using low and high power.

Isolation of Dermatophytes: The collected specimens from different animals were cultivated Sabouraud's dextrose agar supplemented with antibiotics, actidion, thiamine and inositol, the inoculated media were then incubated at 25°C and 37°C for 3-4 weeks for rapid isolation of *Trichophyton verrucosum*.

Identification of the Isolated Dermatophytes [17]: The isolated fungi were identified according to macroscopical and microscopical morphology of the isolates.

Macroscopical Examination of the Cultures: The examination involves, rate of growth, colour, texture of the colony or consistency (Cottony, fluffy, suede-like and wiry), its surface topography (flat, folded, plicate, rugose) and reverse side of colony (pigmentation of the medium), margins, elevation and detachability from the agar surface.

Microscopical Examination of the Cultures

B1-wet Mount Preparation [18]: A small part of the colony was gently teased out on the slide with a drop stain [lactophenol cotton blue] using a flamed bent inoculating needle or straight wire. A cover slip was applied with gentle pressure then examined by low and high power.

B2-slide Culture Technique [15]: The test was carried out to examine the presence of macro and micro conidia. A sterile glass slide was placed over a bent glass rod in bottom of sterile Petri dish, block of SDA. 1cm × 1cm was inoculated with the examined fungus. Few millilitres of sterile distilled water were added in the bottom of the Petri dish to keep its humidity. The plates were then incubated

at 25°C for 2-3 weeks and examined daily for identification of micro and macro conidia of *T. verrucosum*.

Selection of *T. Verrucosum* Used for DNA Isolation Preparation: The selection of *T. verrucosum* samples was based on the conventional isolation and identification methods (macroscopically and microscopically, urease production…etc) The selected stains should be have the following criteria, strain should be a local strains, highly virulent strains isolated from badly infected animals, rapid grower *in vitro* with intensive spore forming (microcondia) [19]

DNA Isolation and PCR Technique: The PCR technique was carried on isolated samples to verify *Trichophyton verrucosum*. DNA was extracted from the positive samples (10 positive samples were verified macroscopically and microscopically)

Identification of *Trichophyton Verrucosum:* The Identification of *Trichophyton verrucosum* was primarily based on the detection of Simple repetitive Oligonucleotide (GACA)4 as a single primer for identification of species of dermatophytes. DNA was extracted from the culture samples according to method previously described by Liu *et al*. [20] and from the direct hair and skin scraping using Genejet Plant Genomic DNA extraction kits [Sigma Scientific services Co, Egypt] [21]

Amplification Reaction Using Simple Repetitive Oligonucleotide [GACA]4 [22]: The dermatophyte genomic DNA samples (2 µl) were amplified by PCR in a reaction mixture (20 µl) containing 10 µl of Maximo Taq Pol. 2 X pre-mix, 1 µl of each primer and fill up to 20 µl PCR grade water. Samples were amplified as follows: initial denaturation at 95°C for 5 min, followed by 39 cycles of I min at 93°C, annealing for 1 min at 50°C and extension for 1 min at 72°C. This was followed by a final extension step of 7 min at 72°C The PCR products (10 µl /sample) were electrophoresed through 1% agarose gel and then stained with ethidium bromide and were visualized under UV light.

Amplification Reaction Using MEP1-5 Primers [23]: The dermatophyte genomic DNA samples (2 µl) were amplified by PCR in a reaction mixture (20 µl) containing 10 µl of Maximo Taq Pol. 2 X pre-mix, 1 µl of each primer; and fill up to 20 µl PCR grade water. PCR cycling conditions consisted of: 94°C for 3 min followed by 35 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C and a final extension step of 10 min at 72°C. The PCR products (10 µl) were resolved by agarose gel electrophoresis (1%) and then stained with ethidium bromide and were visualized under UV light. The following table shows the primers sequences [23]. These primers were designed mainly for T*richophyton rubrum* based on the gene sequence of MEP1-5 available in genebank database.

Gene		Primer sequence F and R (5´- 3´)	Reference
MEP1	For	GCCACTGAGCTGGTTAAG	[23]
	Rev	CTTTGGATCGAACTTAGC	
MEP2	For	AGAGTTCCTGACTCGGAC	
	Rev	ACTCGTGGATGACAATACC	
MEP3	For	GCCATGTCCTTCTCCAAG	
	Rev	AGACCACGCTTAGCAAAG	
MEP4	For	ATCGTGATTCCTTTAGCACC	
	Rev	TCGCCCATGGTATAGTCAG	
MEP5	For	CCAGCTACATGAGTTCAGATG	
	Rev	ACAGGATGTGTAGACCAATGG	

RESULTS

Isolation and Identification of *T. Verrucosum* Strains: In this study we identified *T. verrucosum* depend on macroscopic appearance. Where it was very slow growing with heaped up, button like with folded white colony and non pigmented reverse side as shown in photo (1) and on microscopic appearance, the fungus gave characteristic chlamydospores arranged in chains as shown in photo (2A) and using slide culture technique for demonstration of macro and micro conidia, where it give Clavate to pyriform microconidia as shown in photo (2B).

Molecular Identification of *T. Verrucosum* by Using Simple Repetitive Oligonucleotide (GACA)4 Primer: Hair and skin scraping samples from affected horses and cattle that give positive result by conventional methods were tested by using Simple repetitive Oligonucleotide (GACA)4 primer. The primer was able to amplify two DNA fragments of about 200 and 600 bp that specific to T. *verrucosum* from all tested samples as shown in photo (3).

Amplification of MEP1-5 Genes Using Specific Primers: In the present study, the incidence of metalloproteases enzymes encoding genes was screened using PCR. The test was carried out on 10 positive samples that were verified using simple repetitive Oligonucleotide $(GACA)_4$. The amplification of *MEP*1-4 was successful in 10% while *MEP*5 was 20% of the screened samples (photo. 4A-C).

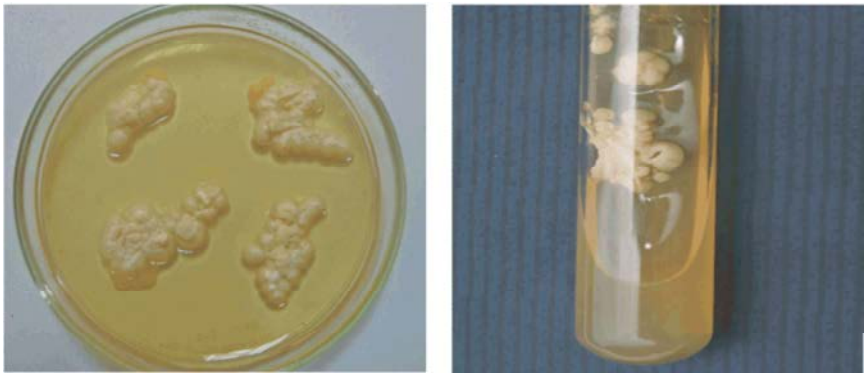

Photo 1: Showing the growth character of T.verrucosum on saboround dextroes arge, colonies are very showing growth, with heaped up, button like appreance folded white colored colony.

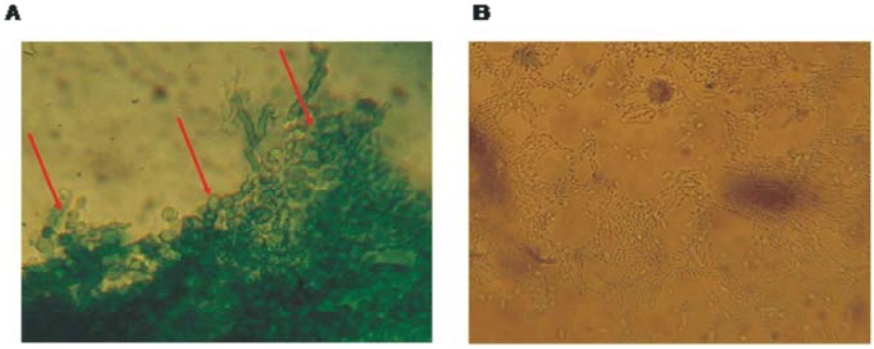

Photo 2: A) The typical chaimydocoridia of T. Verrucosum, B) Glavate to pyiforms microconidia of T. Verrucosum.

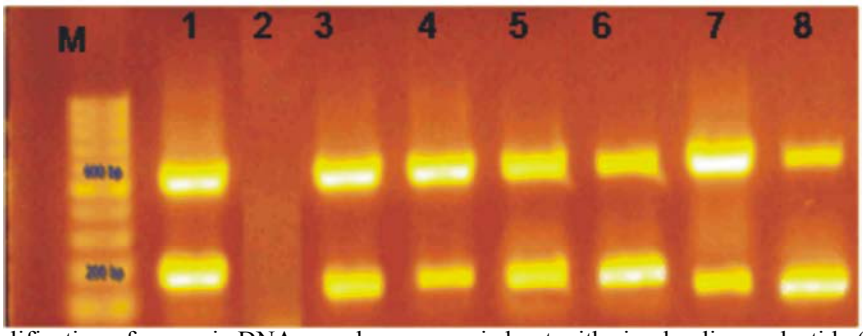

Photo 3: PCR amplification of genomic DNA samples was carried out with simple oligonuclectide (GACA) 4 primer, lanes. M. Molecular weght market. C+ control positive, C-, control negative and 1,2,3,4,5 and 6 tested samples.

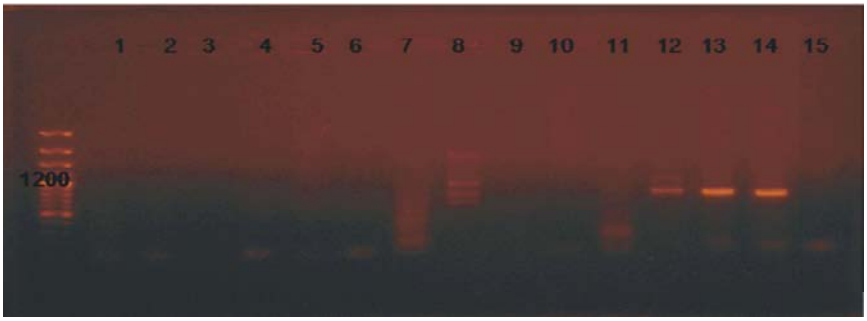

Photo 4: A) MEP1 (Sample 1-2), MEP2 (Sample 3-4), MEP3 (Sample 5-7), MEP4 (Sample 8-10), MEP5 (Sample 11-13), + ve color (Sample 14). and -ve control (Sample 15).

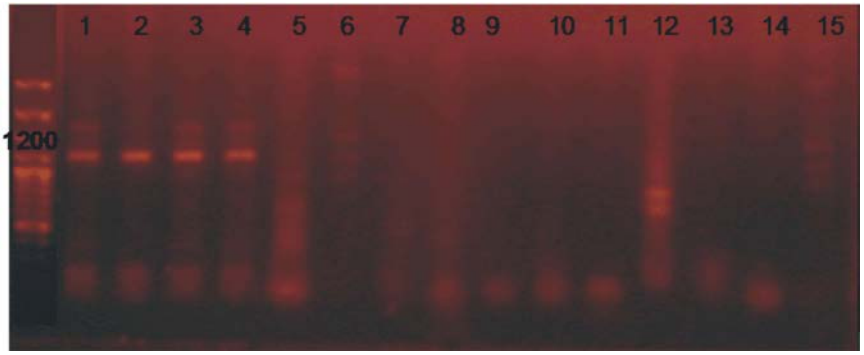

Photo 4: B) +ve control (Sample 1), MEP5 (Sample 2,3), MEP4 (Sample 4,5), MEP3 (Sample 6-8), MEP2 (Sample 9,10) MEP 1 (Sample 11-13), -ve control (Sample 14), MEP4 (Sample 15).

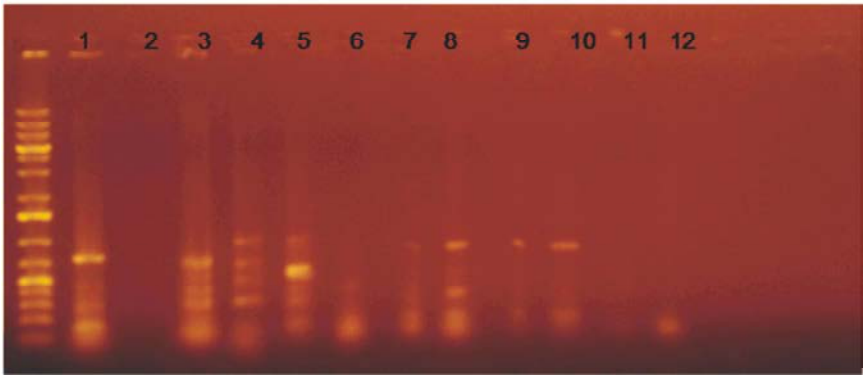

Photo 4: C) +ve control (Sample 1), -ve control (Sample 2), MEP 5 (Sample 3-5), MEP 1 (Sample 9), MEP 3 (Sample 10), MEP 4 (Sample 11,12)

DISCUSSION

Dermatophytosis is one of the most frequent skin diseases of pets and other livestock including cattle and horses. Dermatophytosis spread among animal by direct and indirect contact and also its zoonotic importance has been revealed. Although, the contagiousness among animal communities, high cost of treatment, difficulties of control measures and public health consequences of animal ringworm explain their great importance. However, the molecular basis of the Pathogenicity of theses fungi is largely unclear. Thus, the better understanding of this basis is considered the rational element in the developing of the appropriate therapeutic intervention.

In the present study we identified T. verrucosum depending up on macroscopic and microscopic appearance based in the identification key of the veterinary mycology laboratory manual [24] and the laboratory handbook of dermatophytes [25]. T. *verrucosum* characterized by slow growing with heaped up, button like with folded white colony and non pigmented reverse side as shown in photo (1): and microscopically, it gave characteristic chlamydospores arranged in chains as shown in photo [2] this agree with koop *et al*. [26] and Clavate to pyriform microcondia, as shown in photo (2): and this agree with Hassan [27]and with Calina *et al*. [28] who depend on culturing on selective medium (Sabouraud,s dextrose agar medium and dermatophyte test medium) morphological and culture characterization for isolation and identification of Trichophyton strains. In addition, the positive samples [conventional isolation and identification methods] were verified as T. verrucosum using simple repetitive Oligonucleotide $[GACA]_4$ [photo, 3]. The usage of simple repetitive Oligonucleotide $[GACA]_4$ as fingerprint for identification of Trichophyton was reviewed in Faggie *et al*. [22].

Dermatophytes are known to infect keratinised tissue like hair, skin and nails and such ability is considered as a major virulence attribute of dermatophytes infection [5]. The secretion of proteases by dermatophytes *in vivo*, which are thought to be responsible for the colonisation and degradation of keratinised tissue during the infection. Screens have been used mainly to identify gene products

likely to play a role in virulence and dermatophytes have been shown to secrete more than 20 proteases *in vitro* when grown in media containing protein as a sole source for nitrogen [29]. Although, the molecular techniques such as PCR and microarray have elucidated many genes involved in the dermatophytosis infection like fungalysins, however, there a scarcity of data regarding the screening of these genes in *Trichophyton verrucosum*. In addition, there is no data regarding the incidence of these virulence genes in dermatophytes isolated from local strains in Egypt.

In the present study, the amplification of five genes encoding proteases protein secreted from dermatophytes and played indispensable role in its pathogenesis was screened by PCR. PCR provides a rapid and sensible tool for identification of dermatophytes isolates, that is independent of morphological and biochemical characteristics and thus enhances laboratory diagnosis of dermatophytosis [20]. The data collected form the study revealed that, the amplification of *MEP*1-4 was only successful in 10% of the screened samples and 20% for *MEP*5. The data are consistent with the findings of Burmester *et al*. [29], who found that only some of the keratin-induced proteases were strongly expressed during fungus-keratinocyte interaction. On the contrary, these findings are contradictory with that of Staib *et al*.[30], who reported none of the genes encoding the *in vitro* keratin-specific metalloproteases *MEP*1 and *MEP*3 was up-regulated during infection. Among the fungalysins family genes, *MEP*3 has already been characterised and showed elastinolytic, keratinolytic and collagenolytic activities [9]. Despite the fact that, this gene was successfully amplified only in 10% of the screened samples, knowing the proteolytic activity of *MEP*3, its effective absence has to be proven by analysis of more isolates. In case of confirmation, it can be emphasized its replacement by another gene coding for a protein with similar activities. These finding is consistent with that of Lemsaddek *et al*. [23], who reported the presence of *MEP1-3* in the screened samples of dermatophytes. Of note, among the screened samples by Lemsaddek *et al* [23]. none of them was *Trichophyton verrucosum.*

The presence of *MEP*4 was only detected in 10% of screened samples. The successful amplification of *MEP*4 in the *Trichophyton verrucosum* isolated from infected cases in Egypt is considered as a new finding. The successful amplification of *MEP*4 was detected in many studies. The presence of *MEP4* was detected in more than half of the analyzed isolates. The protein encoded by *MEP4*, was the most secreted by *M. canis, T. mentagrophytes* and *T. rubrum* on soy medium [31]. Using the same medium, Giddey *et al*.[32] detected these proteases on the supernatant of *A. vanbreuseghemii, T. equinum, Ttonsurans, T. rubrum, T. soudanense* and *T. violaceum.* mRNAs corresponding to *MEP4* was also detected on keratin medium inoculated with *T. rubrum* [33].

Regarding the amplification of *MEP*5, *MEP*5 was successfully amplified in 20% of screened dermatophytes samples. The presence of *MEP*5 in our samples is consistent with the findings of Lemsaddek *et al*.[23], who reported the successful amplification of *MEP*5 only in eight species. Although, the high incidence of *MEP5* gene among our collection of isolates seems to indicate an important role of its coding protein in the infection process. However, a future study including an additional number of isolates, representing all species, will help to unravel the putative role of *MEP*5 protein during the infectious process. Of note, the high incidence of MEP1-5 was observed in the clinical samples isolated from infected horses and mainly that obtained directly from hair samples. This observation indicates that, the pattern of virulence gene expression is varies from *in vivo* and *in vitro* conditions. In addition, this emphasises the paramount role of these genes in dermatophytes infection. Thus, further investigations are still urgently required to elucidate the crucial role of these genes and verify the most important conditions that alter the putative expression of these genes either *in vivo* or *in vitro*.

CONCLUSION

The data presented in this study for our knowledge is considered the first screening data regarding the incidence of fungalysins (*MEP*1-5) in culture samples and hair of infected cases in Egypt. The high incidence was observed in horse samples (DNA obtained directly from hair samples) emphasises the important role of these genes in dermatophytes infection in horses. Moreover, more investigations are urgently required to clarify the variation in virulence between cattle and horses.

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