

Microbes and Infectious Diseases

Journal homepage: <https://mid.journals.ekb.eg/>

Original article

Molecular identification of *Microsporum canis* isolates and detection of subtilisin genes from dermatophytosis

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ARTICLE INFO

Article history:

Received 28 June 2022

Received in revised form 20 July 2022

Accepted 26 July 2022

Keywords:

PCR

Real-time PCR

velB gene

ABSTRACT

Background: *Microsporum canis* (*M.canis*) is the most common dermatophyte causing tinea capitis and corporis in humans. Proteases encoded by subtilisin (SUB) virulence genes are from the keratinases leading to dermatophytosis. Subtilisin 1 and SUB3 were expressed during the adherence of arthroconidia to corneocytes by *M. canis*. Few *M. canis* molecular identification assays were used. There is a new target gene for identification of *M.canis* such as the velB gene. The aim of our study is to assess the presence of SUB (1-3) virulence genes in *M.canis* isolates and to assess the ability of the velB gene in identification of *M.canis* isolates compared with the traditional Mc gene. **Methods:** Thirty *M.canis* isolates were collected from 120 suspected cases of dermatophytosis attended the Dermatology outpatient clinic in Suez Canal University Hospitals. Polymerase chain reaction (PCR) for detection of MC gene was done. Real time PCR was done for detection of the velB and SUB (1-3) genes. **Results:** The number of positive isolates detected of MC gene were 28(93.3%) isolates. Thirty isolates (100%) were positive for velB gene. Subtilisin 1 gene was detected in 21 isolates (70%), while SUB 2 gene was positive in 3 isolates only (10%) and SUB 3 gene showed the highest percentage as it was positive in 29 isolates (96.7%). **Conclusion:** We concluded that the new primer Mc-VelB showed high specific results in detection of *M.canis* isolates. Subtilisin genes 1- 3 were detected with high percentage in our study that proves having a major role in the infection by *M.canis*.

Introduction

Microsporum canis (*M.canis*) is one of the pathogenic and most common dermatophytes that may cause *tinea capitis* and *tinea corporis* in humans [1]. Dermatophytes have great potential to attack the keratinized tissues (skin, hair, and nails) in humans and animals, leading to dermatophyte infections (dermatophytosis) [2].

Conventional methods that are based on microscopy and culture are mostly used to diagnose *M. canis* dermatophyte infections. However, these

methods have a lot of limitations, such as the slow growth (2-4 weeks) [3] and the low sensitivity (20%) [4]. Nowadays new molecular diagnostic methods are preferable to overcome these limitations, such as increasing the specificity and sensitivity of detection, as well as reduction of the time and cost needed for the diagnosis [5]. There were only a few *M. canis* molecular identification assays described in the literature, in which the ITS1 sequence of the ribosomal DNA or β -tubulin gene was used as molecular markers (example: *Mc gene*)

[6]. Other researchers started to assess a new target gene for identification of *M.canis* such as the velvet-like B (*velB*) gene that uses a set of two primers—Mc-VelB-F and Mc-VelB-R. The amplified product is specific due to the presence of (CAGCAC)₈ microsatellite sequence only in the *velB* gene of *M. Canis*. The VelB protein is a light-dependent regulator of the velvet family proteins, which plays a key role in regulating secondary metabolism and fungal development [7].

Infection stages of dermatophytosis are adherence, invasion, and inflammation in keratinized tissues [8]. Serine proteases encoded by subtilisin (*SUB*) virulence genes are from the keratinases which lead to dermatophytosis [9]. The serine proteases which are secreted by pathogenic fungi belong to S8A subfamily (subtilisin-like proteases), and their role in infection and inflammation should be further studied and explored [10]. Three subtilisin genes (1, 2 and 3) were detected from *M.canis* species isolated from humans. Subtilisin 1 and *SUB3* were expressed during the adherence of arthroconidia to corneocytes by *M.canis* [8].

The aim of our study is to assess the ability of the new *velB* gene in identification of *M.canis* isolates compared with the traditional *Mc* gene and to assess the presence of *SUB* (1-3) virulence genes in *M.canis* isolates that caused dermatophytosis.

Patients and methods

Study design and target population

This is a cross-sectional descriptive study conducted in Suez Canal University Hospitals in Ismailia. It included 30 *M.canis* isolates collected from 120 clinically suspected cases of dermatophytosis (*Tinea capitis*- *Tinea corporis* and *Tinea cruris*) attended the Dermatology outpatient clinic. Consent was taken from each patient before history taking, examination and collection of the specimens.

Collection and processing of specimens

Clinical samples (skin and hair) were collected under aseptic conditions from patients suffering from dermatophytosis. Suspected lesions were cleaned with 70% ethyl alcohol. Skin scales and crusts were collected from the erythematous, peripheral, actively growing margins of the lesions by scraping across the inflamed margin of the lesion into the apparently healthy tissue using the blunt edge of a sterile surgical blade onto clean glass slide. Hair specimens were collected by using epilating

forceps to pluck along the base of the hair shaft, and scales were scraped from the surface using the blunt edge of a sterile surgical blade [11].

Direct microscopic and laboratory examination

For direct microscopy, the samples collected were screened for the presence of fungal elements using a 10% KOH for skin and hair specimens. Specimens were inoculated in Sabouraud's dextrose agar (SDA) supplemented with chloramphenicol (0.5 g/l) and cyclohexamide (0.4 g/l) (Oxoid, UK) poured in tubes and plates then incubated at temperature 25°C and examined every 4-6 days for growth and observation of surface and reverse of the plate was done. Cultures were held for 4 weeks before being considered negative [12]. For the microscopic observation, a smear was prepared from the grown colony and colored with Lactophenol Cotton Blue [13].

DNA extraction

The mycelium was collected from Sabouraud Dextrose agar and was grounded with a pestle in a mortar surrounded by ice pack and without using liquid nitrogen and the DNA was extracted by ABT fungal-yeast DNA extraction kit according to manufacturer (catalog no: ABT001, Applied Biotechnology Co.Ltd, Egypt).

Identification of *M.canis* by detection of *Mc* gene by PCR

Specific primers of *Mc* gene were used (Table 1). PCR amplifications were done in a thermal cycler (Peltier Thermal cycler, MJ Research, U.S.A.) with a total volume of 20 µl containing 2µl of 25 Mm MgCl₂, 2µl buffer for Taq polymerase, 0.2µl for primers, 2µl of dNTP, 1µl of Taq polymerase, 2µl of DNA extract and the volume was completed with distilled water. The cycling conditions for PCR was 94°C for 3 minutes then 30 cycles (94°C for 45 s + 65 °C of the primers for 45s + 72°C for 1.15 minute) + 72°C 10 mins. Amplicons obtained from PCR reactions were analyzed by gel electrophoresis (Major Science, Taiwan) and visualized under ultraviolet light.

Detection of *Mc*, *velB* and *SUB* (1-3) genes using Real-time PCR

The real-time PCR was performed using the specific primers of *Mc*, *velB* (*MC-VelB*) and *SUB* (1-3) genes in separate reactions with a final volume of 20 µL containing 10µL of Power Up SYBR Green Master Mix (catalog no. A25741, Applied Biosystems, USA), 1µL (20 pmol) of the target genes forward and reverse primers given in table (1), 4 µL of DNA

(100 ng), and 5 µL of nuclease free water. The PCR cycling conditions were performed using StepOne™ real time instrument (catalog no: 4376600, Applied Biosystems, USA) as follows: initial denaturation for 3 min at 96 °C, 40 cycle of denaturation 15 s at 96 °C, annealing for 1 min at (61-65) °C and elongation for 30 s at 72 °C. Following the accomplishment of the PCR amplification reaction, a melting curve analysis was accomplished (60 to 85°C) to confirm the target gene product detection. The PCR signals were judged as positive for the genes when the PCR test led to a sigmoidal rise in the PCR curve with a *Ct*-values equal or below to 35 cycles (Table 1).

Data analysis

The frequency distribution and the data were analyzed by SPSS software version 22.

Results

Results of demographic and clinical data

The study was carried out on 120 patients with clinically suspected tinea. It was held during the period from September 2021 till June 2022. The number of dermatophytes collected were 78 isolates (65%), included skin and hair lesions. Number of *M.canis* were 30 isolates (38.5%) (Figure 1). The age of the patients ranged from 3 to 54 with mean 21 and standard deviation 16.5. Male patients were 65%. Most of the patients were from rural areas (80%). Sixty percent of the patients confirmed contact with animals before infection. Seventy percent of the patients suffered from *Tinea capitis*, 20% from *Tinea corporis* and 10% from *Tinea cruris* lesions.

Isolation and identification of *M.canis* isolates

1. Microscopic and macroscopic examination: colonies grew well after incubation at 25°C for 7 days on Sabouraud dextrose agar. The texture was woolly to cottony. The colour was white to yellowish from the front and deep yellow from the reverse. By microscopy macroconidia were noticed spindle-shaped, with an asymmetrical apical knob. They were 6- to 15-celled and had thick outer cell walls.
2. Molecular Identification of *M.canis* isolates by PCR: The number of positive isolates (176bp) detected by the specific primers of *MC* gene were 28(93.3%) isolates (Figures 2 and 3).
3. Detection of the *Mc* gene by real time PCR: The number of the positive isolates were 28 isolates (93.3%).
4. Detection of the *velB* gene by real time PCR: The number of the positive isolates using the specific primers (*Mc-VelB F* and *Mc-VelB R*) were 30 isolates (100%) (Figure 3).

Detection of subtilisin gene by real time PCR

Subtilisin 1 gene was detected in 21 isolates (70%), while *SUB 2* gene was positive in 3 isolates only (10%) and *SUB 3* gene showed the highest percentage as it was positive in 29 isolates (96.7%) (Figure 3). Two isolates only were negative for the three *SUB* genes.

Considering the clinical data of the *SUB* positive isolates, most of them were isolated from males in rural areas. Results showed significant difference (p value<0.05) between the presence of *SUB 1* and *SUB3* with male gender and rural distribution. More than 60% of the lesions were severe (more than one lesion) and they were mostly *Tinea capitis*. More than 50% of the positive isolates had a history of animal contact (Table 2).

Table 1. Target genes with their primer sequences and annealing temperatures used in PCR reaction.

Gene name	Primer sequences	Annealing Temperature (°C)
SUB1	Forward (TATCTCTCTTGCGGCTGTGTCC) Reverse (GTTCCAGAATCTGCCGAAGGTC)	63
SUB2	Forward (TGAACCTCGGCCTGCTTCTC) Reverse (TTCTTGGGCAGTGTGAACTTGC)	61
SUB3	Forward (TGGGCTGCATCAAGGTTATCTC) Reverse (CCGTTGTAGAGGAGCTTGTTGG)	61
Mc	Forward (GTGTGATGGACGACCGTCCCCCT) Reverse (ATAATACATGGTTCGTTAGGCCAGCCTG)	65
MC- VelB	Forward (CTTCCCCACCCGCAACATC) Reverse (TGTGGCTGCACCTGAGAGTGG)	61

Table 2. Relation between the positive isolates for *SUB* genes and their clinical data.

	SUB1 (N=21)	SUB2 (N=3)	SUB3 (N=29)
Gender	95% male*	100% male	82.2% male*
Number of lesions	66.7% more than one	100% more than one	62% more than one
Type of infection	71.4% Tinea capitis	100% Tinea capitis	72.4% Tinea capitis
Geographic distribution	76% rural*	100% rural	79.3% rural*
History of animal contact	57%	66.7%	58.6%

*=significant difference (*p* value<0.05)

Figure 1. Percentage distribution of dermatophyte species (Total number=78).

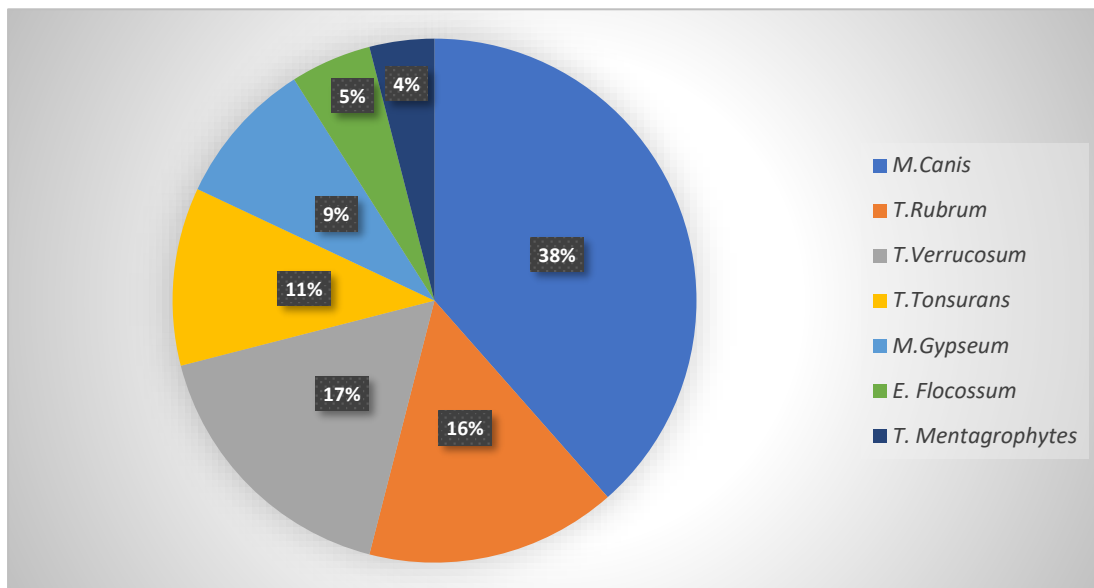


Figure 2. Positive results of 176bp of *MC* genes for identification of *M. canis*.

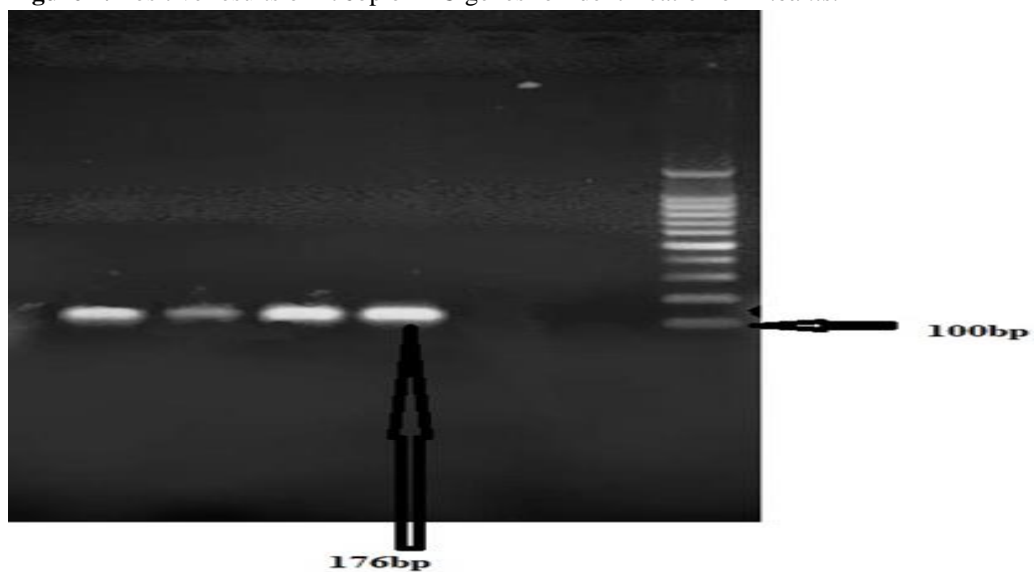


Figure 3. Comparison of *M.canis* identification using the traditional Mc primers(93.3%) and the new Mc-VelB primers (100%).

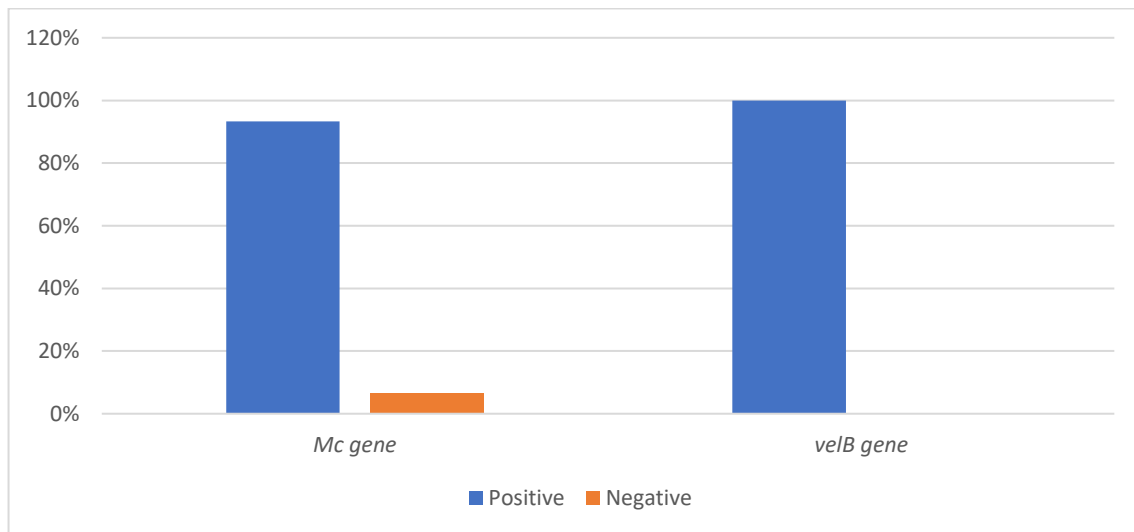
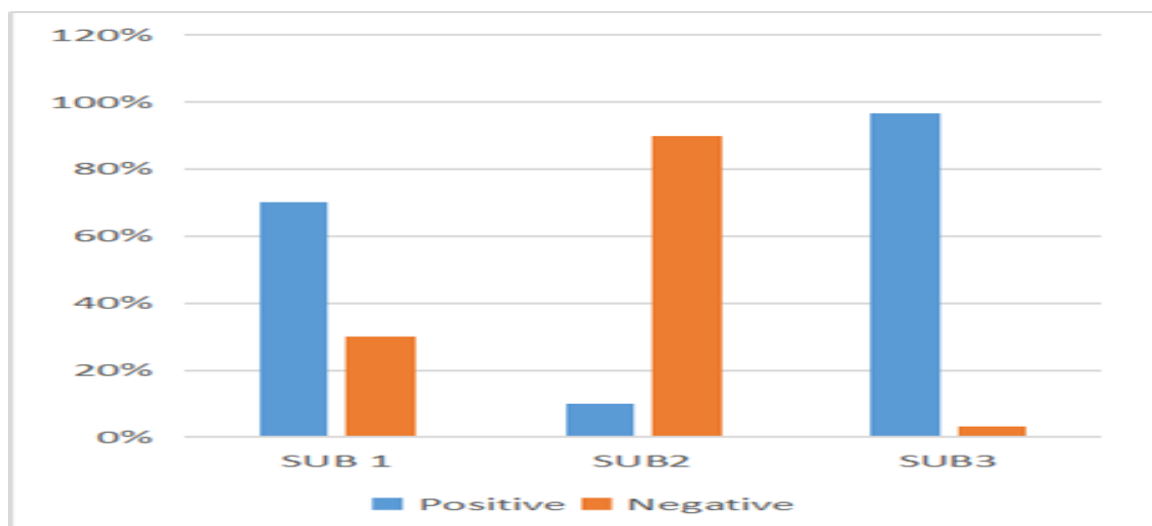


Figure 4. Percentage of *SUB* (1-3) genes in *M.Canis* isolates



Discussion

Microsporum canis is one of the important pathogenic and most common dermatophytes that may cause tinea capitis and tinea corporis in humans [1]. In our study, *M.canis* isolates were the most common dermatophytes isolated as they were 38.5% , a similar percentage (42.9%) was detected in Tehran during the period from June 2014 till March 2015 [14]. On the other hand, **González de Cossío et al.** observed a higher incidence of *M.canis* infections (75.5%) and it was the most common isolated dermatophytes in their study [15]. Lower prevalence of *M.canis* was detected Crete and Greece [16]. Our results can be explained as *M.canis*

natural hosts are cats and dogs, and these animals are prevalent in our community, as well as poor environmental sanitation especially in rural areas may be a fungal reserve. Arthroconidia may remain for months or years, particularly when embedded in sofas, beds, chairs, furniture, on floors, walls, and objects associated with grooming, transportation, and housing of animals [17]. The high prevalence in males may be attributed to as more keeping of pets like dogs and contact with stray animal.

Since dermatophytes is a major health problem in Egypt, a rapid and specific identification for them should be available [18]. In our study we compared the detection of *M.canis* using the

traditional primers of *Mc* gene that detected 93.3% of isolates positive and the new primers of *velB* gene which detected 100% of the isolates. This finding concurred with Ciesielska and Stączek that found the Mc-VelB primers very specific in detection *M. canis* species (100%) and did not detect any other dermatophyte [19]. This is due to, the nucleotide sequence of the *velB* gene contains a unique microsatellite motif (CAGCAC)₈ for identification of *M. canis*. These species-specific primers can distinguish *M. canis* from the other phylogenetically closely related species that can cause similar clinical manifestations [19].

The production of subtilisin proteases is an important virulence factor for dermatophytosis and the serine protease SUBs inhibitor significantly inhibits the adherence of *M. canis* arthroconidia to the stratum corneum [20]. Our results showed high percentage of *SUB 1* and *SUB 3* among the isolates with a percentage of 70% and 96.7% respectively. On the other side *SUB 2* was found only in 10% of isolates. Two isolates only (6.7%) were negative for the three *SUB* genes. A study by **Khalili et al.** [20] detected high percentage of *SUB 1,2* and *3* as they were 80%, 90% and 70% respectively. Other study by **Karami et al.** [8] detected *SUB 1* and *SUB 3* with percentage of 80% and 70% respectively. On the other hand, Lemsaddek et al. reported that *SUB2* (82%) and *SUB1*(79%) were detected in a high percentage of *M. canis* isolates [21]. *SUB3* expression in *M. canis* DNA is important in initial contact [3]. We reported that *SUB3* gene was observed in a high percentage, **Lemsaddek et al.** reported that *SUB3* were detected in a low percentage (68%) of *M. canis* isolates [21]. These results suggested that the expression of *SUBs* may be crucial to both adherence and early invasion and are important in the occurrence of dermatophytosis.

Conclusion and recommendations

Dermatophytosis is one of the important health problems, great efforts by the medical and veterinary services together with health education are needed to prevent the spread of *M. canis* infection. Different diagnostic methods and the knowledge of the virulence factors should be further studied. In our study we conclude that the new primer Mc-VelB showed high specific results in detection of *M. canis* isolates and could be a good alternative for the diagnosis. We recommend that further studies assess the specificity of the primer for detection of *M. canis* from direct patients samples.

Also, we have concluded that Subtilisin genes 1 and 3 were detected with high percentage in our study which may have a major role in the infection by *M. canis* and should be further studied. By knowing the virulence genes, we will be able to take effective steps in drug and vaccination research in the future.

Conflict of interest

We declare that we have no conflict of interest.

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