Molecular Profile of *Trichophyton mentagrophytes* and *Microsporum canis* based on PCR-RFLP of Internal Transcribed Spacer

Endrawati D, Kusumaningtyas E

Indonesian Research Institute for Veterinary Science, Jl RE Martadinata 30 Bogor, Indonesia
E-mail: enikusuma@yahoo.com

(received 11-08-2020; revised 04-02-2021; accepted 05-01-2021)

ABSTRAK

Endrawati D, Kusumaningtys E. 2020. Profil molekuler *Trichophyton mentagrophytes* dan *Microsporum canis* berdasarkan PCR-RFLP dari *internal transcribed spacer*. JITV 26(1): 10-21. DOI: http://dx.doi.org/10.14334/jitv.v26i1.2546.

Trichophyton mentagrophytes dan *Microsporum canis* merupakan kapang dermatofit yang biasa menginfeksi hewan maupun manusia. Metode konvensional dan molekuler telah digunakan untuk mengidentifikasi kapang tersebut. Daerah *internal transcribed spacer* (ITS) mempunyai peluang besar untuk digunakan sebagai dasar dalam mengidentifikasi fungi. PCR-RFLP dilaporkan sebagai metode yang berguna untuk membedakan kapang dermatofit. Tujuan dari penelitian ini adalah membandingkan profil molekuler *T. mentagrophytes* dan *M. canis* berdasarkan hasil digesti fragmen ITS dengan enzim restriksi Dde I, Hinf I dan Mva I. Kapang diisolasi dari kerokan kulit 18 ekor hewan yang menunjukkan lesi dermatofitosis. Kapang ditumbuhkan pada media agar selama 14 hari pada suhu 37^{o}C kemudian diidentifikasi secara morfologi makro dan mikroskopik. Amplifikasi gen c*hitin synthase* digunakan untuk mengkonfirmasi dan memisahkan kapang dermatofit dari kapang-kapang yang lain. Fragmen ITS diamplifikasi dan selanjutnya dipotong menggunakan enzim restriksi Dde I, Hinf I dan Mva I. Hasil menunjukkan bahwa produk digesti fragmen ITS dari *T. mentagrophytes* dan *M. canis* berbeda. Fragmen 159 bp dari Dde I, 374 bp dari Hinf I dan 89 bp dari Mva I ada pada *T. mentagrophytes* tetapi tidak ditemukan di *M. canis*. Berdasarkan hasil tersebut, profil spesifik RFLP dari digesti daerah ITS oleh Dde I, Hinf I dan Mva I dapat digunakan sebagai *marker* spesifik untuk spesies dari fungi dermatofit.

Kata Kunci: Dermatofit, *Internal transcribed spacer*, PCR-RFLP

ABSTRACT

Endrawati D, Kusumaningtys E. 2020. Molecular profile of *Trichophyton mentagrophytes* and *Microsporum canis* based on PCR-RFLP of internal transcribed spacer. JITV 26(1): 10-21. DOI: http://dx.doi.org/10.14334/jitv.v26i1.2546.

Trichophyton mentagrophytes and *Microsporum canis* are dermatophytes fungi which commonly infect animal and human. Conventional and molecular methods were used for identification of the fungus. The region of internal transcribed spacer (ITS) has a high probability for fungal identification. PCR-RFLP was reported as a useful method to differentiate dermatophytes fungi. The objective of the study was to compare molecular profile of *T. mentagrophytes* and *M. canis* based on the result of ITS fragment digestion using Dde I, Hinf I and Mva I. The molds were isolated from skin scrapping of 18 animals which showed dermatophytosis lesion. The isolated molds were grown on agar plate for 14 days of incubation at 37^{o}C and then identified based on macro and microscopic morphologies. Amplification of chitin synthase gene was used for confirmation and separation of dermatophytes from other fungi. ITS fragment was amplified and then digested using restriction enzymes Dde I, Hinf I and Mva I. The result showed that digestion products from ITS fragment of *T. mentagrophytes* and *M. canis* were different. The fragment 159 bp from Dde I, 374 bp from Hinf I and 89 bp from Mva I were present in *T. mentagrophytes* but absent in *M. canis*. Based on these results, specific RFLP profile of digestion ITS region by Dde I, Hinf I and Mva I can be used as a specific marker for species of dermatophytes fungi.

Key Words: Dermatophytes, Internal transcribed spacer, PCR RFLP

INTRODUCTION

Dermatophytes are the fungus commonly invading *stratum corneum* of epidermis and keratinized tissues such as skin nails and hair of humans and animals. Cats and dogs are natural hosts which most infected by the fungus (Pasquetti et al. 2017). The fungus is commonly transmitted to human and cause *tinea capitis* and *tinea corporis* (Brillowska-Dabrowska et al. 2013). As dermatophytes transmitted from animal to animal, from animal to human and from human to human, identification and differentiation of the related species is important from an epidemiological point of view (Rezaei-Matehkolaei et al. 2012).

In the conventional identification methods, long incubation (7-14) is needed for characteristic traits to appear making the fungi difficult to be identified. Microscopic examination is limited because of the absence of macro or microconidia and the production of hyphae with prominent cross-walls. Identification was

difficult because of similarities among colonies of variant *Microsporum canis* (Rezaei-Matehkolaei et al. 2012). In addition, clinical isolates with similar geographical conditions of nature may show different phenotypes making identification even more complicated (Brillowska-Dabrowska et al. 2013; Katiraee et al. 2016).

A variety of molecular techniques, such as polymerase chain reaction (PCR) need to be considered. Other methods, such as mitochondrial DNA restriction fragment length polymorphism (RFLP) pattern and Chitin synthase 1 nucleotide sequence analysis has reported an as simple, fast and accurate method for identification (Jung et al. 2014). This research used ITS primers (ITS1 and ITS4) because the primers are universal and allow selective amplification of fungal sequences. ITS region is in a ribosomal cistron. The nuclear rRNA cistron has been used for fungal diagnostic and phylogenetics for more than 20 years (Begerow et al. (2010). The eukaryotic rRNA cistron consists of the 18S, 5.8S and 28S rRNA genes transcribed as a unit RNA polymerase I. Posttranscriptional processes split the cistron, removing two internal transcribed spacers. These spacers are the 5.8S which is referred as internal transcribed spacer (ITS) and the 18S nuclear ribosomal small subunit rRNA gene (SSU).

The ITS region has the highest probability of accurate identification for fungi. ITS was referred to a candidate of fungal barcode (Schoch et al. 2012). However, amplification of the internal transcribed spacer (ITS) region representing organism diversity was still unsatisfactory as the sequences of *T mentagrophytes*, *T tonsurans*, *T rubrum* and *Microsporum gypseum* are very similar (Jung et al. 2014). PCR-RFLP of ITS fragment is a method that combines PCR and enzymatic digestion of the PCR products. The method was reported to be a rapid and accurate technique for fungal identification by generating band patterns on agarose gel electrophoresis, which takes only 5 hours to be carried out (Mohammadi et al. 2015). ITS PCR and RFLP have also been used for differentiation of brewing yeast and brewery wild yeast contaminant (Pham et al. 2011). Mirzahoseini et al. (2009) reported that PCR-RFLP was a reliable tool to identify dermatophytes from a clinical specimen.

Application of the Mva I and the Dde I restriction enzyme to the ITS amplicon resulting good, stable and reproducible in the identification of the dermatophytes species (Elavarashi et al. 2013). Previously, it used one or two restriction enzymes to compare molecular profile of dermatophytes fungi. This research used three enzymes to produce fragments of profile from digestion products. It was hoped that application of more enzymes produces more specific molecular profile. In addition, the data would provide information about the most suitable enzyme which used for species identification. Therefore, differentiation among dermatophytes species are more accurate. As dermatophytes fungi infect human and animals such as pets, wild and livestock, the samples were taken from cat and dog which represent pet animals and cattle which represent livestock. This research was conducted to compare molecular profile of *Microsporum canis* and *Trichophyton mentagrophytes* based on the result of ITS fragment digestion using Dde I, Hinf I and Mva I. *T. mentagrophytes* and *M. canis* produced different digestion product which can be used to distinguished both species

MATERIALS AND METHODS

Clinical isolate

Scrapping skin sample was collected from infected cat and dog patients which came to animal hospital around Bogor, Jakarta and Sukabumi, Indonesia. The scrap was inoculated in Sabouraud dextrose agar (SDA) with chloramphenicol 0.05 mg/mL and cycloheximide 0.5 mg/mL (Pal & Dave 2013), to inhibit bacteria and spreading mold. The plates were incubated at 37^{o}C for 7-14 days. Dermatophytes fungi were purified by picking selected single colony and inoculated in new agar plate.

Conventional identification

The fungus was identified by colonies observation and microscopic direct examination using KOH 10%. The scraping skin was put in object-glass, KOH dropped in surface, press using cover glass. Fixation was done by trough the glass up the flame. Microscopic morphology was examined under microscope. Identification was performed based on mycelia and conidia form.

DNA extraction

DNA extraction was conducted according to White et al. (1990) with some modification. Mycelium of dermatophyte fungi was placed into microtube 1.5 mL. Two grams of mycelia were ground using micro pestle to form small particles. Amount of 500 µL sodium deodecyl sulphate (SDS) was added, then incubated at 65^{o}C for 30 minutes. The mixture was let until cold, added with 500 µl chloroform isoamyl (CI 24:1) and centrifuged at 10.000 *x g* for 20 minutes. Supernatant was placed into a new tube and 500 µL phenol-chloroform isoamyl (PCI 25:24:1) was added and centrifuged at 10.000 *x g* for 10 minutes. The supernatant was placed into new tube and 100 µl Na acetate 2 M (pH 5,2) and 500 µl ethanol 100% were

added. The mixture was frozen at -20°C for 8 hours and then centrifuged at 10,000 *x g* for 30 minutes. Supernatant was discarded and pellet was dried using vacuum concentrate plus (Eppendorf) for 30 minutes. The dried pellet was added with *nuclease free water* and 5 µL RNase then incubated at 37°C for 10 minutes continued with additional incubation at 70°C for 10 minutes (for RNAse inactivation). Purity and percentage of DNA were measured using NanoDrop spectrophotometer at λ 260/280.

Polymerase chain reaction and electrophoresis

Primer *Chytin Synthase* (*CHS1*: forward 5′-GAA GAA GAT TGT CGT TTG CAT CGT CTC-3′ dan reverse 5′-CTC GAG GTC AAA AGC ACG CCA GAG-3′) (Putty *et al*. 2018) were used to amplify dermatophytes specific sequence gene from mold. Primer ITS 1 (forward: (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS 4 (reverse: 5′-TCC TCC GCT TAT TGA TAT GC-3′) were used to amplify ribosomal DNA (rDNA) and produced 600– 700 bp (Zhang et al. 2011; Tartor et al. 2016). Amplification was performed using HotStarTaq@ Master Mix Kit (Invitrogen). Total volume was 50 µl (25 µl HotStarTaq@ Master Mix, 1 µl for each primer (10 µM), 10 µl platinum™ GC enhancer, 10 µl DNA template (10 ng) and Nuclease free water until 50 µl). Polymerase chain reaction (PCR) process was conducted in pre denaturation at 95°C, 5 minutes, denaturation 95°C for 3 seconds, annealing 56°C for 60 seconds, extension 3 seconds, followed by final extension 72°C for 5 minutes. The PCR product was kept at -20°C until used. Electrophoresis for PCR product was performed using agarose 1,5 % and SYBR™ safe staining, run at voltage 100 Volt. The bands were visualized using UV transilluminator.

DNA sequencing and analysis

PCR products from amplification of ITS region were sequenced and identified. The PCR product was sent to First Base Laboratories Sdn Bhd All Right Reserved, Selangor, Malaysia for sequencing. DNA sequences were analyzed using Bioedit and Mega-X and aligned with Gene Bank database using BLAST program (www.ncbi.nlm.nih.gov) and clustalw2 (https://www.ebi.ac.uk/Tools/msa/clustalo/). Open Reading Frame was determined using https://www.ncbi.nlm.nih.gov/orffinder/

Polymerase Chain Reaction-Restriction fragment length polymorphism (PCR-RFLP)

Restriction fragment length polymorphism (RFLPs) for PCR products were performed following Mohammadi et al. (2015) using enzymes Dde I, Hinf I and Mva I (Thermo Fisher Scientific Inc). The procedure for enzymes treatment was conducted according to the protocol of each enzyme from the company. Ten µl of ITS PCR product were mixed with 18 µl nuclease-free water (NFW), 2 µl 10x Tango buffer (composed by 33 mM Tris-acetate pH 7.9, 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/mL BSA) and 1 µl Dde I (10U/µL) (Thermo Fisher Scientific Inc). The mixture was incubated at 37°C for 1 hours. The reaction was stopped by incubation in 65°C water bath for 20 minutes. For Hinf I, ITS PCR product 10 µl, was mixed with 17 µl NFW, 2 µl 10x green buffer and 1 µl Hinf I (Thermo Fisher Scientific Inc) then incubated at 37°C for 5 minutes. The reaction was stopped by incubation in 65°C water bath for 20 minutes. For Mva I, ITS PCR product 10 µl, was mixed with 17 µl NFW, 2 µl 10x green buffer and 1 µl Mva I (Thermo Fisher Scientific Inc) then incubated at 37°C for 5 minutes. The reaction was stopped by incubation in 65°C water bath for 20 minutes. All digestion products were stored at -20°C until used. Electrophoresis for PCR digested product was performed using agarose 1.5 % and SYBR™ safe staining, run at voltage 100 Volt. The bands were visualized using UV transilluminator.

RESULTS AND DISCUSSION

Isolation of dermatophytes fungi

Dermatophytes fungi were isolated from cat, dog and cattle which came to animal hospital, pet clinics and animal husbandry around Jakarta, Bogor and Sukabumi city. There was a total of 18 patients which showed clinical signs of dermatophytosis such as itchy, red, scaly, circular rush and some hair loss as showed in Figure 1. The fungi infect certain organs or even around the body.

Table 1 provides information regarding the animal and the organ which had suspected dermatophytosis in this study. The patients were dominated by cats. It may due to less dog population compare to cat. Besides cats and dogs, dermatophytes such as *Microsporum canis* and *Trichophyton mentagrophytes* were also infected calves (Pal & Dave 2013). In this research, only one from 100 examined cattle were infected. Intensive husbandry with good sanitation reduced the possibility to be infected by the dermatophytes fungi. As shown in table 1, there was no organ or breeds preference. Age ≤ 12 months more frequently infected by the fungi. Aneke et al. (2018) reported that in dogs and cats, male and young individuals develop more frequently clinical lesions. Ilhan et al. (2016) found no significant association between genders in cats. The most likely risk factor for dermatophytes infection were seasons and age of animals. Winter and spring are the

(a) **(b)**

Figure 1. Dermatophytosis lesi detected in cat (a) and dog (b)

Tabel 1. Frequency of clinical sign of *dermatophytosis* based on the animal, age, infected organ and breeds

No	Animal	Breeds	Age (month)	Infected organ	Origin of sample
1	Cat	Local	36	back	Sindangbarang, Bogor
2	Cat	Local	24	Back, neck	Sindangbarang, Bogor
3	Cat	Local	24	almost all body	Loji, Bogor
4	Cat	Persia	24	tail, neck	Darmaga, Bogor
5	Cat	Local	4	tail	Sindangbarang, Bogor
6	Cat	Local	18	tail base	Darmaga, Bogor
7	Cat	Persia	9	abdomen, elbow, tail	Pasirkuda, Bogor
8	Dog	Golden retreiver	>24	back, neck	Pasirkuda, Bogor
9	Cat	Local	12	neck, tail	Gunungbatu, Bogor
10	Dog	Local	8	tail	Gunungbatu, Bogor
11	Cat	Local	12	tail	Ciomas, Bogor
12	Cat	Local	12	abdomen	Darmaga, Bogor
13	Cat	Local	12	neck	Sindangbarang, Bogor
14	Cat	Local	12	tail base	Darmaga, Bogor
15	Cat	Local	6	neck, head	Sindangbarang, Bogor
16	Cattle	FH	±15	Face, neck, leg	Sukabumi
17	Cat	Local	18	Tail	Animal hospital, Jakarta
18	Cat	Local	24	Head	Animal hospital, Jakarta

seasons when cases of dermatophytes were higher. *Microsporum canis* is the most common dermatophyte in cat (90-100%) globally (Torres-Guerrero et al. 2016).

Table 1 was only performed on the animal which showed the clinical sign of dermatophytoses, but the fungi had not been yet identified. In some cases, the sign leads to dermatophytoses, but the dermatophytes fungi failed to be isolated in culture and not detected in native preparation or molecular identification. The scrapping of infected skins was then observed under a microscope using KOH 10% and some were inoculated in agar medium. Colonies and microscopic of dermatophytes fungi are shown in Fig 2. Identification was performed based on the macro and microscopic morphology and confirmed by molecular identification.

As shown in Figure 2, colony of *Microsporum canis* is coarsely fluffy, furrier on top and darker in the underside of the growth medium than that of

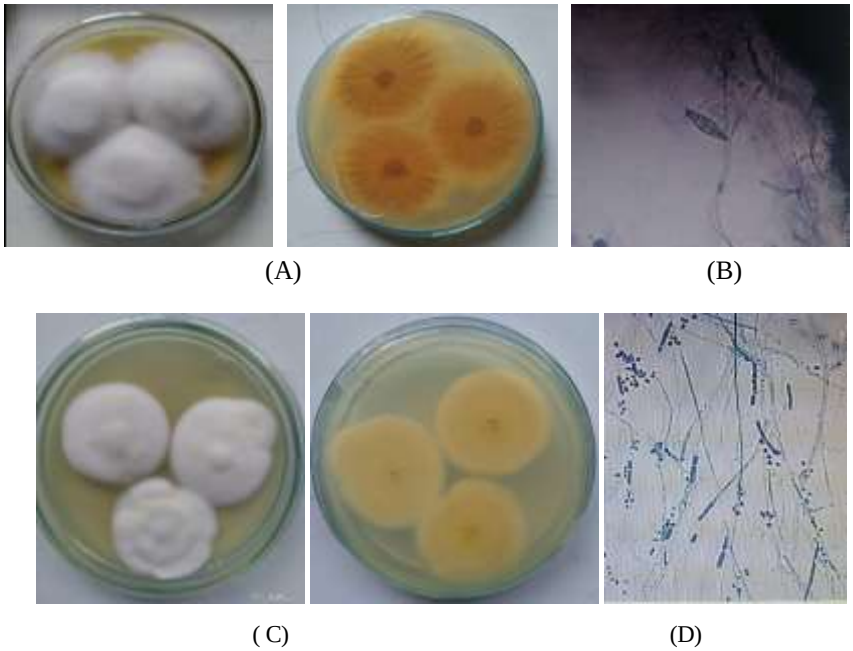

Figure 2. Colonies and microscopic morphologies of *Microsporum canis* (A, B) and *Trichophyton mentagrophytes* (C, D) at 37^{o}C for 14 days incubation

Trichopyton mentagrophytes. The dark yellow pigment was absent in some strains of *M canis due* to failure to develop macroconidia and retardation of colony growth. Macroconidia divided into compartments which are separated by coss-wall. Microconidia *M. canis* also resemble other dermatophytes therefore it is not useful for diagnostic or identification

Spora of *Trichophyton mentagrophytes* was more abundant therefore easily recognized. On the contrary for *Microsporum canis*, even with prolonged 14 days incubation, the conidia were still hardly present. As consequence, molecular identification is a necessity.

In this research, internal transcribed spacer and chitin synthase were amplified for fungal identification and characterization. According to Cafarchia et al. (2013), the first and second internal transcribed spacers (ITS1 and ITS2) of nuclear ribosomal DNA and the part the *chitin cynthase* gene (*p*chs1) have shown promise as markers for specific identification of dermatophytes.

Internal Transcribed Spacer (ITS)

ITS primers were used for amplification DNA region specific for fungi. The ITS bands were detected in fungi such as *Candida sp*., *Fusarium sp*. and dermatophytes but absent in bacteria (Elavarashi et al. 2013). Therefore, the ITS amplification products can be used as a fungal marker. This research use primers ITS 1 and ITS 4 to amplify both of marker specific and conserved sequence. These primer pairs are universal primers and are commonly used for fungal molecular diagnostic and identification (Ferrer et al. 2001; Aala 2012). The length sizes were various among genus and species. Amplification of the region using primer ITS 1 and ITS 4 in some dermatophytes from the previous research indicated that the region was conserved among dermatophytes fungi. PCR product using ITS1 and ITS4 primers is shown in Figure 3. PCR products were then sequenced for identification.

As shown in Figure 3 some of the fungi sequenced identified as dermatophytes fungi. One isolate was identified as *Trichophyton mentagrophytes* (Tm) and 7 isolates were *Microsporum canis* (Mc). However, not all fungi isolated from skin sent for sequencing. Mostly the fungi which had been confirmed as dermatophytes as the chitin synthase amplicon was detected (Figure 3 and 4), or the fungi which genus identified from macro and microscopic morphology.

PCR product using ITS 1 and ITS 4 primer was reported producing 690-720 bp for *T. mentagrophytes* and *M. canis* (Abdel-Fatah et al. 2013). ITS amplicon of *Microsporum* genus was also reported varied in size from ~851 bp in *Microsporum gypseum* to ~922 bp in *Microsporum canis* and ITS region of *M. canis* being ~50 bp longer than that of other dermatophytes (Cafarchia et al. 2013).

In this research, both *T. mentagrophytes* and *M. canis* produced 686-739 bp. Using the same primers pairs, the *M. canis* amplicon shorter than that reported by Zhang et al. (2019) (760 bp). Confirmable result was reported by Dhieb et al. (2014), 700bp. Elavarashi et al. (2013) reported that *T. mentagrophytes* ATCC 9533

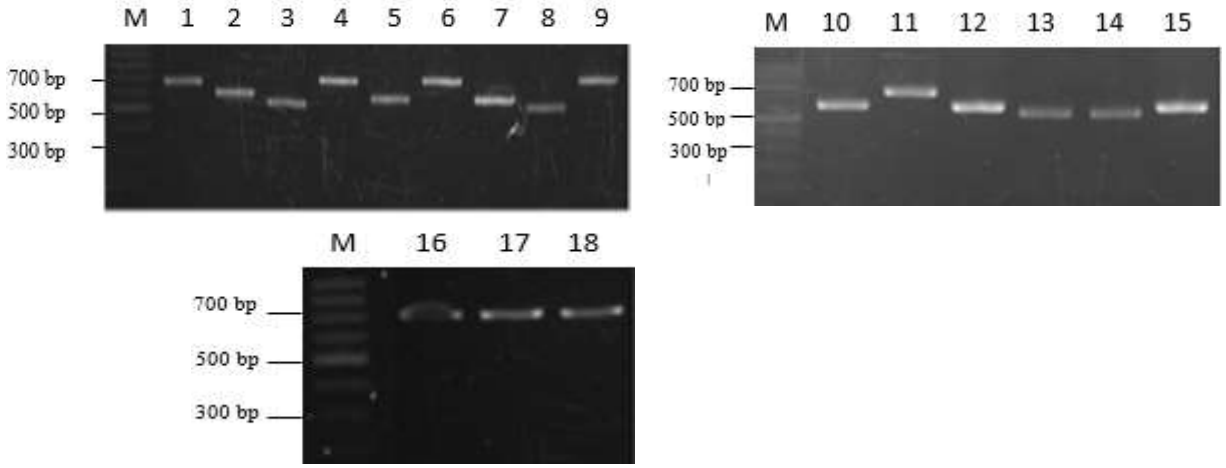

Figure 3. Internal transcribed spacer (ITS) gene of fungi isolated from skin scrapping. Dermatophytes fungi M: molecular mass DNA marker, 1, 4, 6, 9, 11, 17, 18 : *Micropsorum canis* (650 bp) and 16: *Trichopyton mentagrophytes* (650bp), 2, 3, 5, 7, 8, 10, 12, 13, 14, 15: other fungi which was isolated from skin scrapping samples.

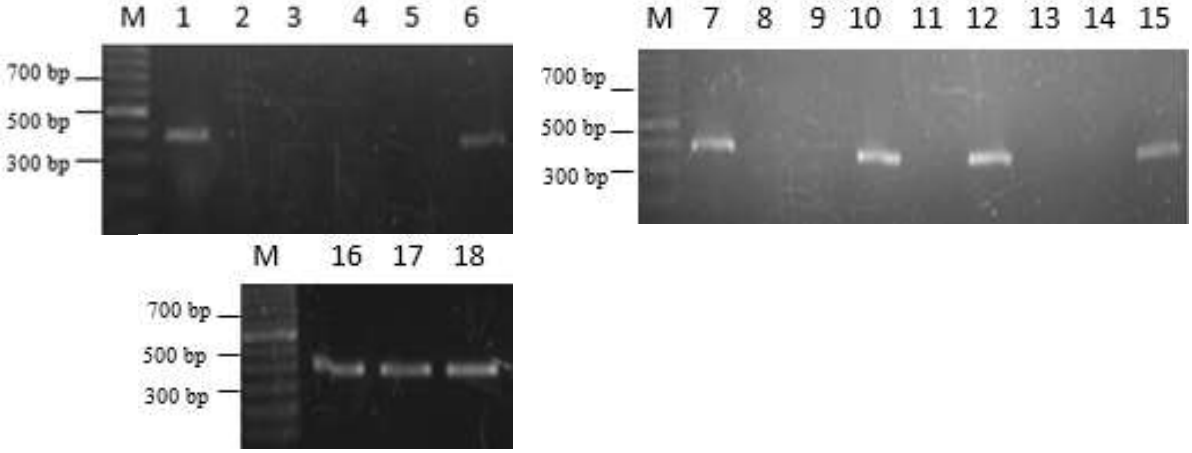

Figure 4. PCR products of chitin synthase *1* gene of fungi isolated from skin scraping. Dermatophytes fungi produced chitin synthase I band (350-400 bp) for *Microsporum canis* [6 (400bp), 7 (400 bp), 10 (350bp), 11 (350 bp), 12 (350bp), 17 (400 bp), 18 (400)] and *Trichophyton mentagrophytes* [1, 16 (400bp)]. M: marker. The bands were absent in non-dermatophytes fungi

produced almost similar amplicon, 690 bp. ITS fragments, produced by non-dermatophytes fungi (Table 2) such as *Aspergillus niger* (600bp), *Aspergillus bridgeri* (600 bp), yeast (around 500bp) and *Chaetomium pachypodiodes* (around 500 bp), were shorter than that of dermatophytes fungi. A similar result was reported by (Elavarashi et al. 2013) who revealed that ITS 1 and ITS 4 pairs primer produced around 550-600 bp PCR products in *Candida sp* and *Fusarium sp*. This result showed that PCR products of ITS 1 and ITS 4 pair primers can be used to distinguish dermatophytes and non-dermatophytes fungi.

Chitin synthase

Amplification of chitin synthase region was aimed to confirm that the isolate was dermatophytes fungi. The existence of chitin synthase band indicated a dermatophytes fungi. Saprophytic fungi isolated from skin scrapping did not produce this band. PCR for chitin synthase 1 gene was powerful to identify the presence of dermatophytes fungi from clinical isolate such as skin scraping and hair. Sharma et al. (2017) found 10 samples that were negative on the fungal culture but were positive for dermatophytes by PCR of chitin synthase indicating that PCR was more sensitive than culture.

Putty et al. (2018) reported that amplification of chitin synthase I gene resulting in 288 bp product size. They added that amplification of the gene was able to be considered as a rapid test for dermatophytosis to decided appropriate antifungal therapy. In this research, the same primer pairs produced longer PCR products, around 400 bp. According to Emam & Abd El-salam (2016) PCR products may be varied among the dermatophyte genus and amplicon size 288 bp was

Table 2. Similarity of dermatophytes in this research with database in GeneBank

Dermatophyte fungi in this research	Percent similarity	Acession number
Microsporum canis (Mc-1)	99.54%	*Microsporum canis* MT487816.1
	99.54%	*Microsporum canis* MT423728.1
Microsporum canis (Mc-2)	99.35%	*Microsporum canis* MT487816.1
	99.35%	*Microsporum canis* MT423728.1
Microsporum canis (Mc-3)	99.40%	*Microsporum canis* MT487816.1
	99.40%	*Microsporum canis* MT423731.1
Microsporum canis (Mc-4)	100%	*Microsporum canis* MT633048.1
	100%	*Microsporum canis* MT632638.1
Microsporum canis (Mc-5)	99.68%	*Microsporum canis* MT487816.1
	99.68%	*Microsporum canis* MT423728.1
Microsporum canis (Mc-6)	99.86%	*Microsporum canis* MT361863.1
	99.29%	*Microsporum canis* KF733019.1
Microsporum canis (Mc-7)	99.59%	*Microsporum canis* MT423731.1
	99.59%	*Microsporum canis* MT423730.1
Trichophyton mentagrophytes (Tm)	99.56%	*Trichophyton mentagrophytes* LC317435.1
	99.56%	*Trichophyton mentagrophytes* LC317440.1

found mostly in genus *Trichopyton*. However, as shown in Figure 4, both *Trichophyton mentagrophytes* and *Microsporum canis* produced amplicon around 400 bp. Hryncewicz-Gwóźdź et al. (2011) use the same primer to amplify chitin synthase gene of dermatophytes fungi. The result showed that both *Trichophyton tonsurans* and *T. mentagrophytes* produced 366 bp, almost similar to the PCR product in this research. Based on the result, amplification of the chitin synthase using primer CHS 1 was powerfull to differenciate dermatophytes and non dermatophytes but did not able to distinguish among genera within dermatophytes. This result also indicates that primer CHS 1 can be used for determination of dermatophytes fungi from clinical samples such as skin scraping from the animals suspected suffer from dermatophytosis.

Identification of dermatophytes fungi

Sequence analysis of PCR product of ITS genes showed that they were confirmed as *Trichophyton mentagrophytes* and *Microsporum canis*. The similarity percentage of both fungi with sequence database in GeneBank is more than 99% (Table 2).

Seven dermatophytes fungi were identified based on the characteristic colonies, microscopic morphologies and their nucleotide sequences of ITS PCR product. The fungi were identified as *M. canis and T. mentagrophytes*. Sequencing result of ITS 1 to ITS 4 regions of Mc1-7 showed that they had similarity almost 100% with ITS regions of *M. canis* from GeneBank. Conventional identification using macro dan microscopic morphology of *T. mentagrophytes* was also confirmed by sequencing result of the ITS region. Identification of dermatophytes and non-dermatophytes fungi isolated from cats, dogs and cattle suspected dermatophytosis as displayed in Table 3.

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP)

For further molecular profile, PCR products of ITS fragment were then digested using restriction enzymes Dde I, Hinf I and Mva I. The enzymes cut the ITS sequence in their cleavage site. ITS sequence affects the cleavage site position resulting difference in fragment size of digestion products. The digestion product was presented at Figure 5. *T. mentagrophytes* (Tm) and *M. canis* (Mc) were digested by Dde I produced different fragments. Fragment 159 bp in Tm and 201 bp in Mc was able to differentiate both genera. *Microsporum canis* (Mc) 1-5 isolated from Bogor has a different pattern with Mc 6-7 which was isolated from Jakarta. This different pattern may represent different strains circulating between both regions, although it needs further examination to prove it.

Digestion ITS sequence using Hinf I showed that there was almost no difference pattern among *M. canis*. *T. mentagrophytes* revealed 374 bp at Hinf I and 89 bp at Mva I digestion products which were absent in

Tabel 3. Isolated fungi from skin scrapping of animal suspected dermatophytosis

No	Animal	Fungi
1.	Cat	Yeast
2.	Cat	White colony mold
3.	Cat	Yeast, *Aspergillus* sp
4.	Cat	*Aspergillus niger*
5.	Cat	Yeast
6 .	Cat	Yeast, *Microsporum canis* (Mc-1)
7.	Cat	White colony mold
8.	Dog	White colony mold, *Microsporum canis* (Mc-2)
9.	Cat	Yeast
10.	Dog	*Aspergillus bridgeri*, *Microsporum canis* (Mc-3)
11.	Cat	White colony mold
12.	Cat	Yeast, *Microsporum canis* (Mc-4)
13.	Cat	*Microsporum* (Mc-5)
14.	Cat	Yeast
15.	Cat	White colony mold
16.	Cattle	*Trichophyton mentagrophytes*
17.	Cat	*Chaetomium pachypodiodes*, *Microsporum canis* (Mc-6)
18.	Cat	*Aspergillus bridgerii*, *Microsporum canis* (Mc-7)

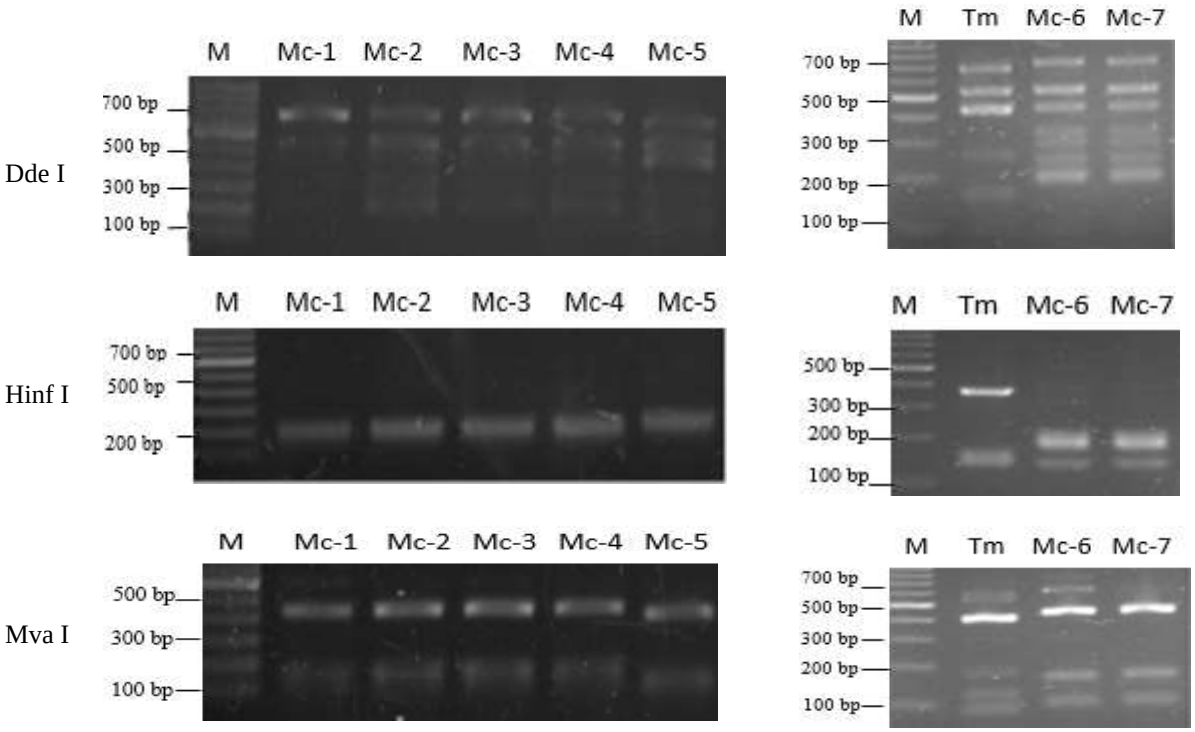

Figure 5. ITS fragment PCR product after digestion with Dde I, Hinf I and Mva I. M: molecular marker, Mc: *Microsporum canis*, Tm: *Trichophyton mentagrophytes*. Specific fragments for Tm are 159 bp at Dde I, 374 bp at Hinf I and 89 bp at Mva I digestions

Table 4. In silico analysis of ITS sequence digested using Dde I, Hinf I and Mva I restriction enzymes

Dermatophytes species	ITS size	Dde I (CTNAG) (bp)		Hinf I (GANTC) (bp)		Mva I (CCWGG) (bp)	
	(bp)	Cut site	In silico of RFLP fragments	Cut site	In silico of RFLP fragments	Cut site	In silico of RFLP fragments
T. mentagrophytes	686	159/160 260/261	**100,159,260, 425, 526**	374/375, 382/383, 540/541	7, 145, **157**, 165,303,311, **374**,382, 540	407/409, 531/533, 581/583, 595/597	12, 48, 62, **89**,103,**122**, 153,172,**186,**277 ,**407**,531,581, **595**
M. canis (Mc-1)	738	205/206, 296/297,5 42/543	90,**195**,205, 245,296,**336**, **441**,532, **542**.	213/214, 407/408, 415/416, 595/596	7, 142, 179, 187,193,**201**213, 322,330,381,407 ,415, 524, 595	440/442, 605/607, 633/635	26,103,131, **163**,191,296, **440**,605,633
M. canis (Mc-2)	738	207/208, 298/299, 544/545	90,**193**,207, 245,298,**336, 440**,530, **544**	215/216, 409/410, 417/418, 597/598	7, 142, 179, 187,193,**201,**213 ,322,330,381, 407,415, 524, 595	442/444, 607/609, 635/637	26,101,129, **163**,191,294,**442** ,607,635
M. canis (Mc-3)	738	205/206, 296/297, 542/543	90,**195**, 205, 245,296,**336, 441**,532,**542**	213/214, 407/408, 415/416, 595/596	7, 144, 179, 187,193,**201,** 213,322,330,381 ,407,415,524, 595	440/442, 605/607, 633/635	26,103,131, **163**,191,296,**440** ,605,633
M. canis (Mc-4)	739	205/206, 296/297, 542/543	89,**196**, 205, 245,295,**336, 442**,533,**542**	213/214, 407/408, 415/416, 595/596	7, 143, 179, 187,193,**201,**213 ,323,331,381, 407,415,525, 595.	440/442, 605/607, 633/635	26,104, 132, **163**,191,297,**440** ,605,633
M. canis (Mc-5	739	207/208, 298/299, 543/544	90,**195**,207, 244,298**,335, 440**,531,**543**	215/216, 338/339, 408/409, 416/417, 596/597	7, 69, 77, 122,142,179,187 ,192,200,**215**,25 7,322,330,338, 380,400,408,416 523, 596	441/443, 606/608, 634/636	26,**103**, 131, 163,191,296,**441** ,606,634
M. canis (Mc-6)	738	205/206, 296/297, 542/543	90,195, **205**, 245,**296**,336, **441**,**532**,542	213/214, 407/408, 415/416, 594/595	7, **143**, 178, 186,193,**201,**213 ,322,330,380, 407,415,524, 594	440/442, 604/606, 632/634	26,**104**, 132, **162**,190,296,**440** ,**604**,632
M. canis (Mc-7)	738	206/207, 297/298, 543/544	90,194, **206**, **245**,**297**,336, **440**, **531**	214/215, 408/409, 416/417, 596/597	7, **141**, 179, 187,**201**,214,193 ,321,329,381, 408,416, 523, 596	441/443, 606/608, 634/636	26,**102**, 130, **163**,191,295,**441** ,606,634

ITS size was determined from sequence between 5' forward and 3' reverse primer annealing position in this study. Bold: the fragments present in electrophoresis gel

M. canis. Therefore, 374 bp Hinf I and 89 bp Mva I digestion product of ITS were as a marker for *T. mentagrophytes* which can be used to distinguish it from *M. canis*. The different pattern between *M. canis* from Bogor and Jakarta was also showed by digestion using Mva I. *M. canis* isolated from Jakarta (Mc 6,7) produce more bands compared to that from Bogor. The pattern of *M. canis* isolated from Jakarta was almost similar to what was reported by Rezaei-Matehkolaei et al. (2012) (Table 5).

As shown in Table 4 and 5, not all fragments in silico analysis present on an electrophoresis gel. The absent fragments concentration might be very low, and they were not detected in electrophoresis gel. It was also still leaving uncut fragments 686-738 bp in Dde I digestion which may be caused by the condition or

Table 5. Size of PCR and RFLP products of dermatophytes fungi present on electrophoresis gel

Dermatophytes species	PCR product	ITS fragments after digested by enzyme (bp)		
	ITS	Dde I	Hinf I	Mva I
T. mentagrophytes	686	159, 260, 425, 526, 686	157, 374	89, 122, 186, 407, 595
M. canis (Mc-1)	738	195, 336, 441, 542, 738	201	163, 440
M. canis (Mc-2)	738	193, 336, 440, 544, 738	201	162, 442
M. canis (Mc-3)	738	195, 336, 441, 542, 738	201	163, 440
M. canis (Mc-4)	739	196, 336, 442, 542, 739	201	163, 440
M. canis (Mc-5	739	195, 335, 440, 543, 739	215	103, 441
M. canis (Mc-6)	738	205, 245, 296, 441, 532, 738	143, 201	104, 162, 440, 604
M. canis (Mc-7)	738	206, 245, 297, 440, 531, 738	141, 201	102, 163, 441
T. mentagrophytes (Abdel-Fatah et al 2013)	680	240, 200, 190, 90	375, 158, 81, 65, 8	-
M. canis (Dhieb et al 2014)	700	-	140, 200, 240, 260	-
M. canis (Rezaei-Matehkolaei et al 2011)	737	-	-	441, 165, 103, 28
M. canis (Didehdar et al 2016)	737	-	-	441, 165, 103
M.canis (Abdel-Fatah et al 2013)	720	No cutting pattern	-	-

digestion time was not optimum. Ratio enzyme and DNA might not suitable as the concentration of PCR product was not measured. As in enzyme protocol mentioned the reaction is placed 1-16 hours. It is possible that the digestion processes needed further optimization. In another case, Abdel-Fatah B, et al. (2013) also reported that no cutting pattern in ITS fragments digested by Dde I.

As shown in Table 3, 4, 5 and Figure 5, digestion products from Dde I, Hinf I and Mva I has similar RFLP profile among *M. canis* from cats and dogs. The digestion profile from the three enzymes was able to differentiate between *T. mentagrophytes* and *M. canis* and also *M. canis* from Jakarta and Bogor. Based on these results, it is possible that specific RFLP profile of digestion ITS region using Dde I, Hinf I and Mva I are used as a specific marker, especially to distinguish among species and strain. However, it still needs further research to compare more samples isolated from more region. Recently, PCR RFLP is commonly used to generate species-specific DNA which used for dermatophytes identification (Mohammadi et al. 2015). Amplification of ITS region and digestion using Mva I and Dde I was also reported equally good for RFLP analysis and identification of dermatophytes directly from clinical material (Elavarashi et al. 2013).

CONCLUSION

Molecular profil from PCR_RFLP using Dde I, Hinf I and Mva I was different between *Trichophyton mentagrophytes* and *Microsporum canis.* Digestion product 159 bp from Dde I, 374 bp from Hinf I and 89 bp from Mva I were present in *T. mentagrophytes* but absent in *M. canis*. Based on these differences, it is possible that specific RFLP profile of digestion ITS region using Dde I, Hinf I and Mva I are used as a specific marker to differentiate among species, especially between *T. mentagrophytes* and *M. canis* local isolate from Indonesia.

ACKNOWLEDGEMENT

This work was supported by Grant from the Indonesian Agency for Agricultural Research and Development, Ministry of Agriculture APBN 2019 No 1806.202.052.B.

AUTHOR CONTRIBUTIONS

Endrawati D and Kusumaningtyas E had full access to all data in this study and contributed equally to this work.

REFERENCES

Aala F. 2012. Conventional and molecular characterization of *Trichophyton rubrum*. African J Microbiol Res. 6:6502–6516.

Abdel-Fatah B, Ahmad M, Moharam M, El-Din A, Moubasher AH A-RM. 2013. Genetic relationships and isozyme profile of dermatophytes and Candida strain from Egypt and Libya. Am J Biochem Mol Biol. 3:271–292.

Aneke CI, Otranto D, Cafarchia C. 2018. Therapy and antifungal susceptibility profile of *Microsporum canis.* J Fungi. 4:1–14.

Begerow D, Nilsson H, Unterseher M MW. 2010. Current state and perspectives of fungal DNA barcoding and rapid identification procedures. Appl Microbiol Biotechnol. 87:99–108.

Brillowska-Dabrowska A, MichałŁek E, Saunte DML, Sogaard Nielsen S, Arendrup MC. 2013. PCR test for *Microsporum canis* identification. Med Mycol. 51:576–579.

Cafarchia C, Gasser RB, Figueredo LA, Weigl S, Danesi P, Capelli G, Otranto D. 2013. An improved molecular diagnostic assay for canine and feline dermatophytosis. Med Mycol. 51:136–143.

Dhieb C, Essghaier B, El Euch D, Sadfi-Zouaoui N. 2014. Phenotypical and molecular characterization of *Microsporum canis* strains in North-Tunisia. Polish J Microbiol. 63:307–315.

Elavarashi E, Kindo AJ, Kalyani J. 2013. Optimization of PCR-RFLP directly from the skin and nails in cases of dermatophy-tosis, targeting the ITS and the 18s ribosomal DNA regions. J Clin Diagnostic Res. 7:646–651.

Emam SM, Abd El-salam OH. 2016. Real-time PCR: A rapid and sensitive method for diagnosis of dermatophyte induced onychomycosis, a comparative study. Alexandria J Med. 52:83–90.

Ferrer C, Colom F, Frasés S, Mulet E, Abad JL, Alió JL. 2001. Detection and identification of fungal pathogens by PCR and by ITS2 and 5.8S ribosomal DNA typing in ocular infections. J Clin Microbiol. 39:2873–2879.

Hryncewicz-Gwóźdź A, Beck-Jendroschec V, Brasch J, Kalinowska K, Jagielski T. 2011. *Tinea capitis* and *Tinea corporis* with a severe inflammatory response due to *Trichophyton tonsurans*. Acta Derm Venereol. 91:708–710.

Ilhan Z, Karaca M, Ekin IH, Solmaz H, Akkan HA, Tutuncu M. 2016. Detection of seasonal asymptomatic dermatophytes in Van cats. Brazilian J Microbiol. 47:225–230.

Jung HJ, Kim SY, Jung JW, Park HJ, Lee YW, Choe YB, Ahn KJ. 2014. Identification of dermatophytes by polymerase chain reaction-restriction fragment length polymorphism analysis of metalloproteinase-1. Ann Dermatol. 26:338–342.

Katiraee F, Asharafi Helan J, Teifoori F. 2016. Multiple Cases of Feline Dermatophytosis Due to Microsporum anis Transmitted to Their Owners. J Zoonotic Drsease. 1:24–27.

Mirzahoseini H, Omidinia E, Shams-Ghahfarokhi M, Sadeghi G, Razzaghi-Abyaneh M. 2009. Application of PCR-RFLP to rapid identification of the main pathogenic dermatophytes from clinical specimens. Iran J Public Health. 38:18–24.

Mohammadi R, Abastabar M, Mirhendi H, Badali H, Shadzi S, Chadeganipour M, Pourfathi P, Jalalizand N, Haghani I. 2015. Use of restriction fragment length polymorphism to rapidly identify dermatophyte species related to dermatophytosis. Jundishapur J Microbiol. 8:4–9.

Pal M, Dave P. 2013. Ringworm in cattle and man caused by *Microsporum canis*: Transmission from dog. Int J Livest Res. 3:100.

Pasquetti M, Min ARM, Scacchetti S, Dogliero A, Peano A. 2017. Infection by *Microsporum canis* in paediatric patients: A veterinary perspective. Vet Sci. 4:2–7.

Pham T, Wimalasena T, Box WG, Koivuranta K, Storgårds E, Smart KA, Gibson BR. 2011. Evaluation of ITS PCR and RFLP for differentiation and identification of brewing yeast and brewery "wild" yeast contaminants. J Inst Brew. 117:556–568.

Putty K, Shiva Jyothi J, Sharanya M, Srikanth Reddy M, Sai Ram Sandeep G, Abhilash M, Venkatesh Yadav J, Purushotham P, Kesavulu Naidu I, Uma Chowdhary A, et al. 2018. PCR as a rapid diagnostic tool for detection of dermatophytes. Int J Curr Microbiol Appl Sci. 7:2021–2025.

Rezaei-Matehkolaei A, Makimura K, Sybren de Hoog G, Shidfar MR, Satoh K, Najafzadeh MJ, Mirhendi H. 2012. Multilocus differentiation of the related dermatophytes *Microsporum canis*, *Microsporum ferrugineum* and *Microsporum audouinii*. J Med Microbiol. 61:57–63.

Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W, Bolchacova E, Voigt K, Crous PW, et al. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proc Natl Acad Sci U S A. 109:6241–6246.

Sharma R, Gupta S, Asati DP, Karuna T, Purwar S BD. 2017. A pilot study for the evaluation of PCR as a diagnostic tool in patients with suspected dermatophytosis. Indian Dermatol Online J. 8:176–180.

Tartor YH, El Damaty HM MY. 2016. Diagnostic performance of molecular and conventional methods for

identification of dermatophyte species from clinically infected Arabian horse in Egypt. Vet Dermatol. 27:401-e102.

Torres-Guerrero E, González de Cossío AC, Segundo ZC, Cervantes ORA, Ruiz- Esmenjaud J, Arenas R. 2016. *Microsporum canis* and other dermatophytes isolated from humans, dogs and cats in mexico city. Glob Dermatology. 3:275–278.

White TJ, Bruns TD, Lee SB, Taylor JWWhite TJ, Bruns TD, Lee SB TJ. 1990. Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. In: PCR Protoc A Guid to Methods Appl. New York (US): Academic Press; p. 315-322.

Zhang F, Tan C, Xu Y, Yang G. 2019. FSH1 regulates the phenotype and pathogenicity of the pathogenic dermatophyte *Microsporum canis*. Int J Mol Med. 44:2047–2056.

Zhang R, Ran Y, Day Y, Zhang H LY. 2011. A case of kerion celsi caused by *Microsporum gypseum* in a boy following dermatoplasty for a scalp wound from a road accident. Med Myco. 49:90–93.