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FULL PAPER

Common dermatophytes and *in vitro* anti-fungal susceptibility testing in patients attending the Dermatological Clinic at the Hospital for Tropical Medicine, Bangkok

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SUMMARY

Dermatophytes comprising the genera *Trichophyton*, *Microsporum,* and *Epidermophyton* are important causes of superficial mycoses. The number of infected patients and the distribution of species of these organisms in our hospital were unknown. We therefore aimed to investigate the clinical pattern of dermatophyte infections and to identify the species of these dermatophytes at the Dermatological Clinic of the Hospital for Tropical Medicine, Faculty of Tropical Medicine, Mahidol University, Bangkok in a 1-year period. Twenty-six patients who had typical dermatophytosis lesions were recruited (27 specimens); 17 were female (65.38%) and 9 (34.62%) were male. The age range of the patients was 16-92 years. In total, nine dermatophyte isolates were identified by macroscopic and microscopic morphological characteristics. We found *Microsporum canis* (four isolates), *Trichophyton mentagrophytes* complex (one), *Trichophyton rubrum* (two), *Trichophyton verrucosum* (one), and *Trichophyton tonsurans* (one). The *in vitro* susceptibility profiles of seven antifungal agents against the nine dermatophytes were as follows (minimum inhibitory concentration ranges in µg/ml): The results were as follows (MIC ranges in µg/ml): ciclopirox, ≤0.06-0.5, griseofulvin ≤0.06-0.5, itraconazole ≤0.002-0.06, posaconazole ≤0.015-0.03, voriconazole ≤0.02-≥1, fluconazole ≤0.08-8, and terbinafine ≤0.01-0.125. This study represents the current state of dermatophyte infections in a metropolitan area of Bangkok, Thailand.

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INTRODUCTION

Dermatophytosis (tinea infection) is an infection of the skin caused by fungi that can invade human skin and use keratin as a nutrient source. There are several predisposing factors involved such as age, sex, economic status, and climate (Wiegand *et al*., 2016). These fungi are classified as dermatophytes and comprise *Trichophyton* spp., *Microsporum* spp., and *Epidermophyton* spp. Species distribution is different in each area of the world. Therefore, dermatophytosis is still a public health problem. In Asia, Mohd Nizam *et al*. reported seven strains of *Trichophyton* spp. from 11 dermatophyte infections patients at Universiti Kebangsaan Malaysia Medical Centre (Mohd *et al.*, 2016). In Korea, *T. rubrum* is the most common species (Lee *et al.*, 2015). Cai *et al*. also reported finding that *T. rubrum* has the highest prevalence of dermatophyte isolates (Cai *et al.*, 2014). In our country, Bunyaratavej and colleagues showed that 75.1% of onychomycosis patients in the largest University Hospital of Thailand were infected with dermatophytes, i.e., *T. mentagrophytes* (46.8) and *T. rubrum* (28.3) (Bunyaratavej *et al.,* 2015).

Treatment of dermatophyte infections is usually based on clinical presentation, fungal element detection, and the identification of causative agents. Only a few reports have addressed drug resistance mechanism in dermatophytes, and most of these were described for *T. rubrum,* such as modifications of target enzymes in squalene epoxidase leading to resistance to terbinafine (an antifungal drug used in the treatment of skin, nail, and hair infections) (Robertson *et al.,* 2014). For the multidrug efflux transporter mechanisms, the *T. rubrum TruMDR1* gene was identified, which encodes an adenosine triphosphate (ATP)-binding cassette (ABC) transporter. This gene is over-expressed in the presence of various nonstructural related drugs, suggesting its participation in drug efflux events (Cervelatti *et al*., 2006). In addition, the *T. rubrum TruMDR2* gene, which encodes another ABC transporter, had an increased level of transcription when the fungus was exposed to griseofulvin, tioconazole, and various other antifungal drugs (Fachin *et al.*, 2006). Thus, susceptibility profiles of dermatophytes are very important for ensuring appropriate treatment is administered.

The epidemiology of dermatophytosis, the numbers of infected patients, and the species distributions and susceptibility profiles of these organisms in our hospital were not known. We therefore aimed to investigate the clinical pattern of dermatophyte infections and to identify the spe-

Key words:
Dermatophytes, *Trichophyton* spp., *Epidermophyton* spp., *Microsporum* spp., Susceptibility testing.

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cies of these organisms at the Dermatological Clinic of the Hospital for Tropical Medicine, Faculty of Tropical Medicine, Mahidol University, Bangkok in a 1-year period.

MATERIALS AND METHODS

Patients

This study was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University (MUTM 2015-060-01). Patients were randomly selected from outpatients admitted to the Dermatological Clinic of the Hospital for Tropical Medicine, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand from 12 November 2015 to 11 November 2016. All patients who agreed to participate in this study read a participant information sheet (document FTM ECF-020-02) and signed a written informed consent form (document FTM ECF-021-04).

Patients of both sexes over the age of 18 years, with typical dermatophytosis lesions as diagnosed by a dermatologist, were eligible to participate in this study. The exclusion criteria were as follows: use of antifungal therapy (oral as well as topical) within the 2-3 months prior to the commencement of the study, and the presence of serious underlying systemic conditions judged as inappropriate by the dermatologist. Moreover, patients with other complications such as bacterial infections, and disturbances of the skin folds and nails including paronychia, chronic eczema, and cellulitis were excluded.

To sample the areas of skin abnormality, they were cleaned with 70% alcohol, and skin scrapings or nail clippings were collected and sent in sterile containers to the Medical Mycology Laboratory, Department of Immunology and Microbiology, Faculty of Tropical Medicine, Mahidol University.

Mycology laboratory procedures

Microscopic examination for the presence of fungal elements was performed after treatment with 15% potassium hydroxide (KOH). All specimens were inoculated in Sabouraud Dextrose Agar tubes and Dermasel Agar tubes (Oxoid, England). These tubes were incubated at 25°C with daily observation for fungal colonies. After 30 days, tubes with no visible fungal colonies were discarded as negative. Identification of genera and species of the fungal colonies was performed by observing macroscopic and microscopic morphology by lactophenol cotton blue staining. If *Trichophyton* was suspected, a urease test was performed. If *Microsporum* was suspected, a rice grain test was performed.

For *Candida* identification, Chromogenic Candida Agar (CLINAG, Thailand) was used as it produces different colony colors which are specific for each species; a green colony denotes *C. albicans,* a blue colony denotes *C. tropicalis,* and dry irregular pink or brown colonies denote *C. krusei*.

For dematiaceous fungi identification, polymerase chain reaction (PCR) and sequencing were performed. In brief, DNA was extracted with an E.Z.N.A. Fungal DNA Mini Kit (Omega Bio-tek). DNA samples were quantified with a NanoDrop 2000 spectrophotometer (Thermo Fisher, Wilmington, DE, USA) and stored at -20°C until further use. PCR amplification was carried out for the region between the internal transcribed spacers (ITS) 1 and 2 and the 5.8S gene of the rRNA with an ITS5 primer (5'-GGAAGTAAAAGTCGTAACAAGG-3') and an ITS4 primer (5'-TCCTCCGCTTATTGATATGC-3'). Each 25-µl reaction mixture contained KAPA 2G Fast HS ReadyMix with loading dye (KAPA Biosystems, USA), 0.5 µM of each primer, nuclease-free water, and DNA template. PCR amplifications were carried out in a T100 Thermal Cycler (Bio-Rad) according to the following protocol: preheating at 96°C for 6 min, followed by 35 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 45 s, and a final extension step at 72°C for 10 min. Five microliters of the resulting PCR products were electrophoretically separated on a 1.5% agarose gel in 1× Tris/borate/EDTA (TBE) buffer containing SERVA DNA Stain G (SERVA Electrophoresis GmbH, Germany) and photographed using a Gel Doc XR+ system (Bio-Rad). PCR products were purified with a FavorPrepTM GEL/PCR Purification Mini Kit (Favorgen Biotech Corporation, Taiwan) and bidirectionally sequenced by AITbiotech Pte Ltd (Singapore). The retrieved sequence files were edited and subjected to pairwise alignment using BioEdit software (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Edited sequences were compared with existing sequences in GenBank using BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Antifungal susceptibility testing

In vitro antifungal susceptibility testing was performed according to the protocol described by the Clinical and Laboratory Standards Institute (CLSI) guidelines, document name M38-A2 for filamentous fungi (Rex *et al.*, 2008). All antifungal drug powders (ciclopirox, griseofulvin, itraconazole, posaconazole, voriconazole, fluconazole, and terbinafine) were purchased from Sigma-Aldrich. Stock solution of fluconazole was prepared in water and the other drugs were prepared in dimethyl sulfoxide (DMSO). Each drug (stock solution) was diluted in Roswell Park Memorial Institute (RPMI) 1640 buffer with 0.165 mmol/L of M-morpholinepropanesulfonic in twice the final concentration when combined with the working inoculum of dermatophyte isolates, in U-shaped 96-well microdilution plates. The following final concentration ranges were tested: ciclopirox 0.06-32 µg/ml, griseofulvin 0.06-32 µg/ml, itraconazole 0.002-1 µg/ml, posaconazole 0.015-8 µg/ml, voriconazole 0.002-1 µg/ml, fluconazole 0.06-32 µg/ml, and terbinafine 0.001-0.5 µg/ml.

Before testing, dermatophytes were subcultured on potato dextrose agar at 35°C for 4 days or until numerous conidia were produced. The fungal colonies were covered with 5 ml Phosphate Buffer Saline pH 7.4 with 0.2% tween 20 and a suspension was prepared by gently probing the colonies, before transferring to a new sterile collection tube. The final fungal suspensions were adjusted to 5×10^3 using a counting chamber.

In 96-well plates, 100 µl of each antifungal drug was placed into each well along with the 2× inoculum suspension. Serial dilutions were then performed to achieve the concentrations described above. All plates were incubated at 35°C for 96 h. Plates with insufficient growth were incubated for 120 h.

T. mentagrophytes ATCC MYA-3634 was chosen for quality control with ciclopirox, griseofulvin, itraconazole, posaconazole, voriconazole, and terbinafine, and *T. rubrum* MRL666 ATCC MYA-4438 was chosen for quality control with ciclopirox, fluconazole, and voriconazole, according to the CLSI M38-A2 document (Rex *et al.,* 2008).

Minimum inhibitory concentration (MIC) was defined as the lowest concentration of an antifungal drug that sub-

stantially inhibited growth of the organism, as detected visually, when testing most antifungal agents. For the conventional microdilution procedure, the growth in each well was compared with the growth of the control with the aid of a reading mirror (Rex *et al*., 2008).

RESULTS

Over the 1-year period, 26 patients with typical dermatophytosis lesions were recruited (27 specimens); 17 were female (65.38%) and 9 (34.62%) were male (*Table 1*). The age range was 16-92 years. In total, 27 specimens were processed by mycological laboratory procedures, i.e., direct examination with KOH, culture on Sabouraud Dextrose Agar and Dermasel Agar, and then genus identification by lactophenol cotton blue staining and/or specific tests for genus identification. Seven antifungal susceptibility tests were then performed.

The main body site of infection was the arm (mycologically confirmed). The distribution of dermatophytosis infection is described in *Figure 1* and *Table 2*. In 27 samples, ten samples were positive by KOH examination, and 11 samples were culture positive. Three samples were negative by KOH examination but produced colony growth on culture media, and only one sample was positive by KOH but did not produce culture growth.

Nine dermatophyte isolates were identified by macroscopic and microscopic morphological characteristics. We found *M. canis* (four isolates), *T. mentagrophytes* complex (one; comprising *T. mentagrophytes*, *T. interdigitale*, *T. erinacei*, and *Arthroderma benhamiae* (Gräser *et al.,* 1998; Gräser *et al.,* 2008; Sun *et al.,* 2016)), *T. rubrum* (two), *T. verrucosum* (one), and *T. tonsurans* (one). Colony and microscopic pictures of the most common dermatophyte (*M. canis*) in this study are shown in *Figure 2*. Additionally, we found two isolates of *C. albicans* (*Figure 3*) and one isolate of *Coniosporium* spp. as the sole superficial causative agents (colony and conidia stained by lactophenol cotton blue are shown in *Figure 4*).

The *in vitro* susceptibility profiles of seven antifungal agents against the nine dermatophytes isolated from patients who were admitted to the Dermatological Clinic of the Hospital for Tropical Medicine, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand were assessed using a broth microdilution technique following CLSI M38-A2. The results were as follows (MIC ranges in µg/ml): ciclopirox, ≤0.06-0.5, griseofulvin ≤0.06-0.5, itraconazole ≤0.002-0.06, posaconazole ≤0.015-0.03, voriconazole ≤0.02-≥1, fluconazole ≤0.08-8, and terbinafine ≤0.01-0.125.

Table 1 - *Patients, demographics, infection sites, and mycological laboratory results.*

Patients	*Sex*	*Age*	*Sample site*	*KOH*	*Culture*
P1	Female	20	Arm	Negative	*M. canis*
P2	Female	27	Arm	Negative	No growth
P3	Female	53	Chin	Negative	No growth
P4	Female	55	Neck	Negative	No growth
P5	Female	28	Elbow	Negative	No growth
P6	Female	41	Groin	Negative	No growth
P7	Female	16	Face	Negative	No growth
P8	Female	70	Head	Negative	No growth
P9	Female	31	Arm	Positive	*M. canis*
P10	Female	68	Ankle	Positive	*Coniosporium* species
			Finger	Positive	*C. albicans*
P11	Male	92	Hip	Positive	*T. mentagrophytes* complex
P12	Male	43	Abdomen	Negative	No growth
P13	Female	38	Arm	Negative	No growth
P14	Male	39	Waist	Positive	No growth
P15	Female	44	Head	Negative	No growth
P16	Male	35	Buttock	Positive	*T. verrucosum*
P17	Female	22	Arm	Positive	*M. canis*
P18	Female	48	Chest	Negative	*M. canis*
P19	Male	37	Nail	Negative	No growth
P20	Male	42	Back	Negative	No growth
P21	Male	92	Groin	Positive	*T. tonsurans*
P22	Female	25	Toe	Negative	*T. rubrum*
P23	Male	56	Groin	Positive	*C. albicans*
P24	Female	16	Nail	Negative	No growth
P25	Female	92	Head	Negative	No growth
P26	Male	21	Groin	Positive	*T. rubrum*

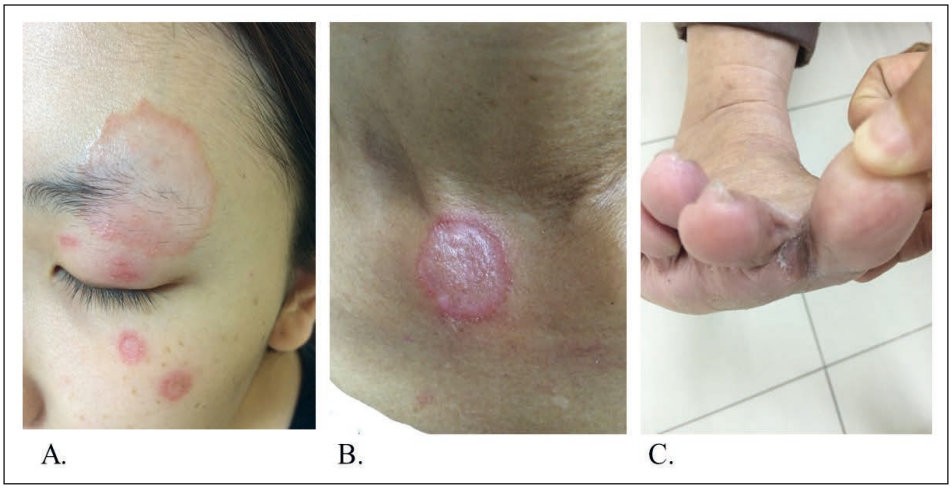

Figure 1 - *Clinical pictures of dermatophytosis infection in our study. A. Tinea faciei on Patient no. 1 (P1). B. Tinea corporis on P18. C. Tinea pedis on P22.*

Table 2 - *Distribution and results of antifungal susceptibility testing.*

Patients	Isolates	Minimum inhibitory concentrations (μg/ml)						
		Ciclopirox	*Griseofulvin*	*Itraconazole*	*Posaconazole*	*Voriconazole*	*Fluconazole*	*Terbinafine*
P1	*M. canis*	≤0.06	≤0.06	≤0.002	≤0.015	≤0.02	≤0.08	≤0.01
P9	*M. canis*	0.25	0.25	0.03	≤0.015	0.125	2	0.25
P11	*T. mentagrophytes*	0.5	0.25	0.06	0.06	0.125	8	0.06
P16	*T. verrucosum*	0.5	0.25	0.03	≤0.015	0.06	4	0.03
P17	*M. canis*	2	1	0.06	≤0.015	≥1	4	≥0.5
P18	*M. canis*	≤0.06	≤0.06	≤0.002	≤0.015	≤0.02	≤0.08	≤0.01
P21	*T. tonsurans*	0.25	0.5	0.06	≤0.015	0.125	4	0.125
P22	*T. rubrum*	0.25	0.5	0.015	≤0.015	0.06	4	0.06
P26	*T. rubrum*	0.25	0.5	0.08	≤0.015	0.015	0.5	≤0.06

Figure 2 - *Culture of M. canis on Sabouraud Dextrose Agar (A and B) and macroconidia of M. canis stained by lactophenol cotton blue (40×) (C.).*

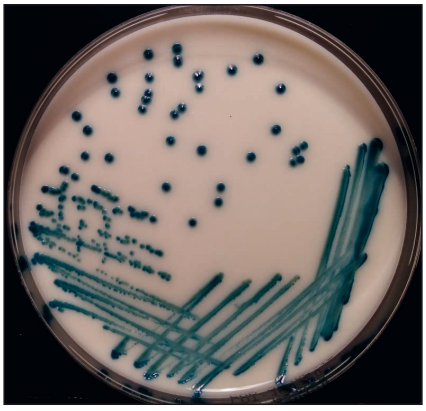

Figure 3 *Green C. albicans isolated from P10 and P23 on CHROMOGENIC Candida Agar after 48 h.*

DISCUSSION

Superficial fungal infections are an important public health problem in tropical areas such as Thailand, especially dermatophytosis infections. To date, numerous studies have reported on epidemiology and susceptibility testing of dermatophyte infections in several countries (Diaz *et al.,* 2015; Oz *et al.,* 2017; Sharifzadeh *et al.,* 2016). The natural niches of the dermatophyte genera in our hospital were unknown. Dermatophyte infections are infections of the skin, hair, and nails, which are rich in keratin (a predisposing factor and nutritional requirement of filamentous fungi in the *Trichophyton*, *Microsporum*, and *Epidermophyton* genera). The arm was the most frequently affected site of superficial infection in this study. We observed that direct examina-

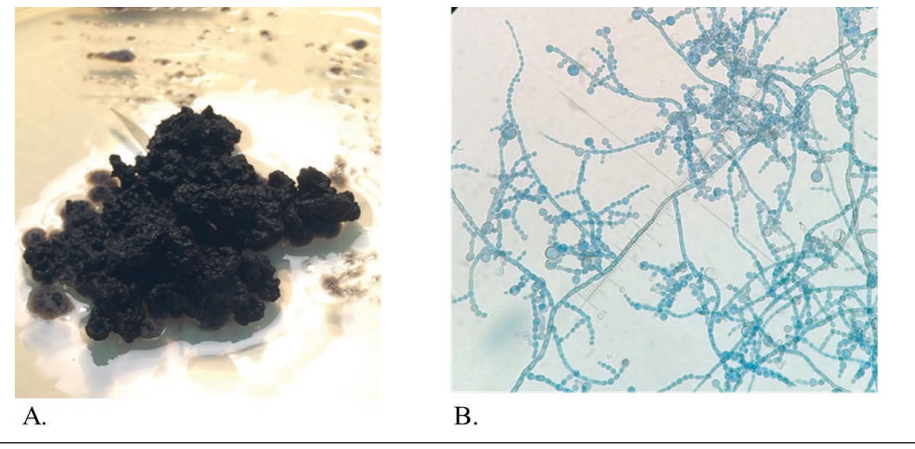

Figure 4 - *A: Colony on Sabouraud Dextrose Agar (14 days). B: Elongated and monilioid hyphae, arthroconidia, conidial chains, mature conidial chains, and large conidia with transverse septa.*

tion using KOH was still a fast and simple technique for dermatophytosis diagnosis. Moreover, KOH results corresponded with reference standard cultures, but three specimens were KOH negative but culture positive. The limitation of KOH testing is that it cannot identify the specific genus and species of the fungi. Therefore, standard culture and identification is important to confirm the etiological agents. We found 34.61% dermatophytosis cases from 26 enrolled patients (confirmed by culture positive results). *M. canis* was the most common isolate. Therefore, zoophilic dermatophytes had emerged in this period of our study. Although the number of patients was limited, we found different infection sites and variation in dermatophyte species, meaning that other predisposing factors may have roles in infection, such as patients' environments, hygiene, social economic status, and climate.

Nowadays, many molecular techniques are used to identify filamentous fungi, including those of the dermatophyte genera. Surprisingly, we also found non-dermatophyte filamentous fungi implicated in superficial infections: two *C. albicans* and one dematiaceous filamentous fungus. We carried out PCR and sequencing with ITS5 and ITS4 primers which specified the internal transcribed spacer (the standard barcoding genetic locus). The sequences showed the greatest similarity (93%) to *Coniosporium* spp. Li *et al*. previously reported that they found *Coniosporium* epidermidis sp. nov., a new species, from a superficial skin lesion with blackish discoloration in an 80-year-old Chinese patient (Li *et al.,* 2008). This is the first report of *Coniosporium* spp. in a superficial infection in Thailand.

The previous version of the M38-A standard document on susceptibility testing of filamentous fungi does not specifically address the testing of dermatophytes. In 2003, a multicenter study investigated the reproducibility of the broth microdilution method for testing the susceptibility of dermatophytes (Ghannoum *et al.,* 2006). Data from this study supported the introduction of this method for testing dermatophytes: the CLSI M38-A2 standard. Nowadays, several laboratories evaluate the *in vitro* susceptibility of dermatophyte fungi using the broth microdilution CLSI M38-A2 document (Bao *et al.,* 2013; Itoi *et al.,* 2012; Tamura *et al.,* 2014).

The ability of dermatophytes to develop resistance against a drug is an evolutionary process. An amino acid substitution (L393F) in the squalene epoxidase of a terbinafine-resistant *T. rubrum* clinical isolate was identified (Osborne *et al.,* 2005); the mutant was about 1,000-fold less susceptible to terbinafine and other squalene epoxidase inhibitors than normal strains (Mukherjee *et al.,* 2003). In our study, all the antifungal drugs tested showed good activity with the exception of fluconazole, and the results agreed with those of other previous studies.

In conclusion, our study showed the current state of dermatophyte infections in a metropolitan area, although the small number of patients enrolled is a limitation. Therefore, we will monitor the distribution of dermatophyte infections in a larger population in a further study.

Conflict of interests

None declared

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