Phenotypic Classification of *Malassezia* Species Isolated from Pityriasis Versicolor Patients Based on Conventional Methods

การจำแนกลักษณะทางฟีโนไทป์ของเชื้อมาลาสซีเซียสปีชีส์ที่แยกได้จากผู้ป่วยโรคเกลื้อนโดยวิธีการดั้งเดิมที่ใช้กันทั่วไป

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ABSTRACT

Pityriasis versicolor (PV) is skin disease caused by *Malassezia* spp. There are only few studies and updated references in Thailand. The aim of this study was to identify *Malassezia* spp. in patients with PV. Identification was based on phenotypic characteristics by testing the pigment production, an assimilation of tween, β-glucosidase and catalase production. From 71 PV patients, 28 yeasts were isolated and identified as *M. furfur* (29%), *M. sympodialis* (25%), *M. slooffiae* (7%), *M. pachydermatis* (4%) and 36% of isolates were other yeasts. From 200 healthy subjects, 59 isolated yeasts were *M. sympodialis* (20%), *M. furfur* (12%), *M. globosa* (2%), *M. restricta* (2%), and 64% of isolates were other yeasts. These results indicated that *M. furfur* is a major causative agent of PV in Thailand.

บทคัดย่อ

โรคเกลื้อนเป็นโรคผิวหนังชนิดหนึ่งมีสาเหตุมาจากยีสต์สกุลมาลาสซีเซีย (*Malassezia*) มีการศึกษาและข้อมูลอ้างอิงที่ทันสมัยไม่มากนักในประเทศไทย วัตถุประสงค์ของการศึกษานี้เพื่อวินิจฉัยเชื้อดังกล่าวในผู้ป่วยโรคเกลื้อนจากลักษณะทางฟีโนไทป์ โดยทดสอบการสร้างเม็ดสี ความสามารถในการเจริญโดยการใช้สาร Tween ความสามารถในการสร้างเอนไซม์ β-glucosidase และ catalase ซึ่งพบว่าเชื้อยีสต์ 28 สายพันธุ์ที่แยกได้จากผู้ป่วยโรคเกลื้อน 71 คน สามารถจำแนกเป็น *M. furfur* (29%) *M. sympodialis* (25%) *M. slooffiae* (7%) *M. pachydermatis* (4%) และ 36% เป็นยีสต์อื่นๆ จากบุคคลสุขภาพดี 200 คน เชื้อยีสต์ 59 สายพันธุ์ที่แยกได้เป็น *M. sympodialis* (20%) *M. furfur* (12%) *M. globosa* (2%) และ *M. restricta* (2%) และ 64% เป็นยีสต์อื่นๆ ดังนั้นเชื้อสาเหตุหลักที่ทำให้เกิดโรคเกลื้อนในประเทศไทยคือ *M. furfur*

Key Words: *Malassezia*, Pityriasis versicolor, Phenotype
คำสำคัญ: เชื้อรามาลาสซีเซีย โรคเกลื้อน ลักษณะฟีโนไทป์

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Introduction

Pityriasis versicolor (PV) is a superficial fungal infection, characterized by scaly hypopigmented or hyperpigmented macules. The metabolic products by fungus interfere the melanocyte production in epidermis (Krisanty et al. 2009, Gupta et al. 2003). PV can be found on the surface of the body, including the upper trunk, neck or upper arm (Gupta et al. 2003). The causative organism is yeast of the genus *Malassezia*, a yeast-like lipophilic fungus that can be found as a normal flora of the skin (Sharma et al. 2012). The most important risk factors and predisposing factor of PV are people with naturally oily or excessively sweaty skin, immunosuppression and weakened immune system, HIV patient, heat, humidity, the use of oily tanning lotions or creams, and corticosteroids (Bonifaz et al. 2010, Gupta et al. 2002, Arenas et al. 2001). PV is one of the most common dermatomycosis, a review conducted in the United States between 1971 and 1974 reported a prevalence of PV was 0.8% in a population of 28,000 people. A study conducted in Sweden in 1979 reported a prevalence of PV was 1.1% in a total of 20,296 patients (Kranning and Odland 1979, Faergemann and Fredriksson 1979). Currently, PV occurs worldwide with prevalence as high as 30-40% in populations in tropical area (Krisanty et al. 2009). In Japan, the most commonly detected species in PV was *M. globosa*, found in 97% of the cases (Nakabayashi 2002) and in Spain, *M. globosa* was the main species as 90% followed by *M. sympodialis* as 41% (Aspiroz et al. 2002). In India, Iran, and Tunisia, *M. globosa* was reported as the predominant species followed by *M. furfur*. In contrast, in other studies from Japan, Brazil, and Indonesia, *M. furfur* was reported as the main species (Krisanty et al. 2009). Thailand is in tropical climates and PV is most commonly found. Previous data before species reclassification era, *M. furfur* was reported as a sole predominant species in Thailand. Fourteen species of *Malassezia* were reclassified up to date (Ashbee 2007). As many reports were published before the adoption of the new taxonomic proposals, it was possible that other species were also involved but were not recognized at the time. There are only few studies and recent references in Thailand. Thus, this study aims to phenotypically identify *Malassezia* spp. from PV patients based on morphological and biochemical characteristics.

Objective of the study

The aim of this study was to identify species of *Malassezia* isolated from pityriasis versicolor patients based on phenotypic characteristics.

Methodology

Clinical specimens and organisms' isolation

The study groups included 200 normal healthy subjects and 100 patients with PV. Sample from the body surface of healthy people and patients were collected with an adhesive tape (Takiwaki et al. 2003). Then, all samples were inoculated on Dixon medium spread with 2% sterile olive oil. Plates were incubated at 32 ºC for 5-7 day.

Morphological characteristics

All isolates were incubated on Dixon medium with 2% sterile olive oil at 32 ºC for 7 days. Then, characteristic features of colony were examined, and the organism was further examined

microscopically from the culture by lactophenol cotton blue staining.

Biochemical characteristics

Although, there are morphological differences among many species of *Malassezia*, their identification usually needs to be supported by physiological and biological characteristics (Table 1).

Catalase test

Catalase test was performed to demonstrate catalase enzyme production via slide testing using catalase-reagent (H_2O_2) (Raabe et al. 1998, Guillot et al. 1996). Each of yeast isolate was incubated on Dixon medium with 2% sterile olive oil at 32 ºC for 3-5 days. Then, catalase activity was determined by adding a drop of hydrogen peroxide onto a culture smear on glass slide. A positive reaction is shown as gas bubbles (Gué ho-Kellermann et al. 2010), caused by the liberation of free oxygen. *M. restricta* is the only *Malassezia* species that exhibits no catalase activity (Raabe et al. 1998).

Trytophan test for pigment production

All yeast isolates were investigated for their ability to produce pigment. Each of isolates was inoculated on tryptophan medium and the pigment production was evaluated after 6 days of incubation at 32 ºC. The formation of brownish pigment diffused into the tryptophan medium, when tryptophan was the sole nitrogen source, was recently considered to be indicative of *M. furfur* (Raabe et al. 1998, Mayser et al. 2004, Mayser et al. 2009).

Assimilations of tween 20, tween 40, tween 60 and tween 80

Assimilations of tween 20, tween 40, tween 60 and tween 80 were examined by the agar diffusion test. Colonies of each isolate were suspended in 1 ml of sterile distilled water (1 loop ml^{-1}) and added to Sabourand dextrose agar (SDA). Once the medium had solidified, nine holes were made by means of sterile 6 mm punch and filled with 50 µl of each emulsifier (two holes for each substrate and center hole for control with sterile distilled water). The plates were incubated at 32 ºC for 10 days. Then, the growth around an individual well was assessed after 2, 4, 6, 8 and 10 days of incubation (Raabe et al. 1998).

Detection of β-Glucosidase activity using an esculin medium

Each of *Malassezia* isolates was incubated in esculin medium by stabbing into the esculin medium using a platinum inoculating needle. Then, plates were incubated at 37 ºC for 5 days and examined daily for 5 days. A positive reaction is indicated by blackening of medium and the absence of blackening sign indicates the lack of β-glucosidase activity. Certain *Malassezia* species possess a β-glucosidase are able to hydrolyse the glucosidic bond of esculin, thus liberating glucose and esculetin. The phenol moiety reacts with the iron to give a black color (Guého-Kellermann et al. 2010)

Table 1 Phenotypic characteristics of the 14 currently recognized species of *Malassezia*.

Species	Morphology	SDA (32 ºC)	TDT using Tween				Catalase	Tryptophan	β-glucosidase	Growth on Dixon's agar at		
			20	40	60	80				32 ºC	37 ºC	40 ºC
M. furfur	G E C	-	+[-]	+[-]	+[-]	+[-]	+[-]	+	+ or ±	+	+	+
M. obtusa	E C	-	-	-	-	-	+	-	+	+	+ or ±	-
M. globosa	G	-	-	-	-	-	+	-	-	+	+ or ±	-
M. slooffiae	E C	-	+ or ±[-]	+	+	-	+	-	-	+	+	+
M. sympodialis	E	-	+ or ±	+	+	+	+	-	+	+	+	+
M. restricta	G E	-	-	-	-	-	-	-	-	+	+ or –	-
M. dermatis	G E	-	+	+	+	+	+	?	-	+	+	+
M. japonica	G	-	-	±	+	-	+	?	?	+	+	-
M. nana	E	-	v	+	+	±	+	?	-	+	+	+ or –
M. yamatoensis	E	-	+	+	+	+	+	?	?	+	+	-
M. equine	G E	-	±	+	+	+	+	?	-[+]	+	±	-
M. caprae	G E	-	-	+	+	+[-]	+	?	+[-]	+	- or ±	-
M. cuniculi	G	-	-	-	-	-	+	?	+	+ or ±	+	-
M. pachydermatis	E	+ or ±	+	+	+	+	+ or ±	-	+[-]	+	+	+

Globose (G); ellipsoidal (E); cylindrical (C); Sabouraud dextrose agar (SDA); weakly positive (±); rare deviation from usual pattern ([-]); unknown (?). Tween diffusion test (TDT); Tryptophan consumption; Catalase; Tryptophan; β-glucosidase (Cafarchia et al. 2011)

Results

The number of yeasts isolated from healthy people and pityriasis versicolor patients

Samples from skin lesion of patient or skin of the neck and arm of healthy person were collected with adhesive tape and all samples were inoculated on Dixon medium with 2 % olive oil at 32°C, for 7 days. From 200 healthy people, about 59 isolates of yeast were obtained and 28 isolates of yeast were obtained from 71 pityriasis versicolor patients. All of these isolates were tested for their phenotypic characteristics.

Morphological characteristics

After samples from skin of patient or healthy person were inoculated on Dixon medium with 2 % olive oil. Colonies on Dixon medium at day 7 were convex, somewhat shiny, white and pale yellowish-cream. Cells shapes were unipolar, ellipsoidal to short, ovoid or globose and bud on a broad or narrow base depending on species (table 1).

The species of *Malassezia* isolated from healthy people and pityriasis versicolor patients classified by biochemical tests

A total of 59 yeasts from healthy people and 28 yeasts from PV patients were isolated in the study. By biochemical analysis, the species of *Malassezia* were identified as shown in table 2. The number of *Malassezia* yeasts from healthy cases were 12 (20%), 7 (12%), 1 (2%), 1 (2%) for *M. sympodialis*, *M. furfur*, *M. globosa*, and *M. restricta* , respectively. While 38 (64%) isolates were other yeasts. In the others hand, yeast isolates from PV patients were *M. sympodialis* 7 (25%) isolates, *M. furfur* 8 (29%), *M. slooffiae* 2 (7%), *M. pachydermatis* 1 (4%) and other yeasts 10 (36%) isolates.

Table 2. The species of *Malassezia* yeasts isolated from pityriasis versicolor patients compared to healthy people isolates based on phenotypic characteristics

Species	Patient isolates (n = 28)	Healthy people isolates (n = 59)
Malassezia sympodialis	7 (25%)	12 (20%)
M. furfur	8 (29%)	7 (12%)
M. slooffiae	2 (7%)	0 (0%)
M. globosa	0 (0%)	1 (2%)
M. restricta	0 (0%)	1 (2%)
M. pachydermatis	1 (4%)	0 (0%)
Other yeasts	10 (36%)	38 (64%)

Discussion

By phenotypic evaluation, *M. furfur* was a major causative agent of PV which was different from reports in Europe, and Japan which *M. globosa* was predominantly found (Aspiroz et al. 2002, Nakabayashi et al. 2000). The different species may relate to the area or the climate in particular place. This finding also showed that not only *M. furfur* but also other *Malassezia* such as *M. sympodialis*, *M. slooffiae* and *M. pachydermatis* caused such a skin disease. However, fungal pathogen could not be isolated from skin scraping from some PV patient since they possibly have prior topical antifungal appliance. The skin discolorization can persist for several months even pathogen has gone. There are still some other yeasts which are not *Malassezia* can grow on Dixon medium or even *Malassezia* yeasts which cannot be identified into species by just only phenotypic methods. So these isolates should be further evaluated as a microbiom, normal flora of the skin or need more advance method assisting in species classification.

Although the conventional methods based on phenotypic evaluation are more accessible to every laboratory, it is important note that the isolated yeasts should be further confirmed by molecular techniques considered as fast and more accurate methods to identify *Malassezia* spp.

Conclusion

PV is a superficial mycosis, caused by lipophilic yeasts in the genus *Malassezia*. Recently, this disease is considered as high prevalence in tropical climates. In Thailand, *M. furfur* was previously reported as a sole predominant species. However, by conventional methods based on phenotypic classification demonstrated that *M. sympodialis* was predominating in healthy people while *M. furfur* was still the most common causative agent of PV but not a sole causative agent. The second most common was *M. sympodialis* followed by *M. slooffiae* and *M. pachydermatis*, respectively.

Acknowledgements

This research was supported by the Research and diagnostic center for emerging infectious diseases and invitation research grant No. I57303 from faculty

of medicine, Khon Kaen University. The author is indebted to Asst. Prof. Dr. Sirida Youngchim for providing a reference strains.

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