การสร้างไบโอฟิล์มจากการอยู่ร่วมกันระหว่างเชื้อ*มาลาสสิเซีย พาไคเดอมาติส*กับ

*แคนดิด้า พาราฟซิโลซิส*และค่าความไวรับต่อสารต้านเชื้อรา

นางสาวกอบแก้ว บำรุงไทย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

สาขาวิชาพยาธิชีววิทยาทางสัตวแพทย์ ภาควิชาพยาธิวิทยา

คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ปีการศึกษา 2556

BIOFILM FORMATION FROM CO-EXISTENCE BETWEEN *MALASSEZIA PACHYDERMATIS* AND *CANDIDA PARAPSILOSIS* AND THEIR ANTIFUNGAL SUSCEPTIBILITIES

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Veterinary Pathobiology
Department of Veterinary Pathology
Faculty of Veterinary Science
Chulalongkorn University
Academic Year 2013

Thesis Title	BIOFILM FORMATION FROM CO-EXISTENCE BETWEEN *MALASSEZIA PACHYDERMATIS* AND *CANDIDA PARAPSILOSIS* AND THEIR ANTIFUNGAL SUSCEPTIBILITIES
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กอบแก้ว บำรุงไทย : การสร้างไบโอฟิล์มจากการอยู่ร่วมกันระหว่างเชื้อ*มาลาสสิเซีย พาไคเดอมาติส*กับ *แคนดิด้า พาราฟซิโลซิส*และค่าความไวรับต่อสารต้านเชื้อรา. (BIOFILM FORMATION FROM CO-EXISTENCE BETWEEN *MALASSEZIA PACHYDERMATIS* AND *CANDIDA PARAPSILOSIS* AND THEIR ANTIFUNGAL SUSCEPTIBILITIES) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. น.สพ. ดร.ณุวีร์ ประภัสระกูล , 87 หน้า.

การเพิ่มจำนวนของ เชื้อ*มาลาสซิเซีย พาไคเดอร์มาติส* ร่วมกับ เชื้อ*แคนดิด้า พาราซิโลซิส* บนผิวหนังของสุนัขเกี่ยวข้องกับภาวะผื่นไขมันอักเสบแบบเรื้อรังและเฉียบพลัน อย่างไรก็ตาม การตอบสนองจากการอยู่ร่วมกันระหว่างเชื้อยีสต์สองชนิดนี้ยังไม่เป็นที่เข้าใจในบทบาทของการอยู่ร่วมกัน และการตอบสนองต่อยาต้านเชื้อรา การศึกษาในครั้งนี้มีวัตถุประสงค์เพื่อแสดงถึงการสร้างไบโอฟิล์มของเชื้อ*มาลาสซิเซีย พาไคเดอร์มาติส*ร่วมกับเชื้อ*แคนดิด้า พาราฟซิโลซิส* และเปรียบเทียบคุณสมบัติของไบโอฟิล์มที่เกิดจากเชื้อเดียว และไบโอฟิล์มที่เกิดจากการอยู่ร่วมกันจากเชื้อจำนวน 40 เชื้อประกอบด้วย *มาลาสซิเซีย พาไคเดอร์มาติส* กับ *แคนดิด้า พาราฟซิโลซิส* จำนวนอย่างละ 20 เชื้อ โดยคัดเลือกมาจากผิวหนังสุนัขปกติจำนวน 10 ตัว และสุนัขที่มีรอยโรคผื่นไขมันอักเสบบนผิวหนังจำนวน 10 ตัว ทำการยืนยันจีโนไทป์ และระดับการสร้างเอนไซม์ฟอสโฟไลเปส ด้วยวิธีหาลำดับดีเอนเอที่บริเวณ IGS1 และ การสร้าง phospholipase ด้วยวิธี semi-quantitative egg-yolk plate ตรวจสอบคุณลักษณะการสร้างไบโอฟิล์มด้วยวิธีการย้อมสีคริสตัลไวโอเลท และภาพถ่ายจากกล้องจุลทรรศน์อิเล็กตรอน และทำการทดสอบความไวรับต่อยาต้านเชื้อราในภาวะที่มีการสร้างไบโอฟิล์มและเชื้อราอิสระด้วยวิธี broth dilution เชื้อ*มาลาสซิเซีย พาไคเดอร์มาติส* ถูกจัดให้อยู่ในกลุ่มจีโนไทป์ 1A จำนวน 19 ตัวและให้อยู่ในกลุ่ม 3D 1ตัว ยีสต์ทุกตัวอย่างมีความสามารถในการสร้างเอนไซม์ phospholipase ในระดับสูง เชื้อทุกตัวสามารถเริ่มสร้างไบโอฟิล์มได้ภายใน 24 ชั่วโมงและแสดงค่าการสร้างสูงสุดที่ระยะเวลา 72 ชั่วโมง จากการเจริญเติบโตร่วมกันของเชื้อทั้งสองชนิดทำให้มีการสร้างไบโอฟิล์มที่มากกว่าเชื้อเดี่ยว (Pair t-test, $P<0.05$) ไม่พบความแตกต่างของค่าความไวรับ ระหว่างกรณีที่มีการสร้างไบโอฟิล์มจากยีสต์เดี่ยวกับกรณีที่ยีสต์ 2 ชนิดเจริญร่วมกัน แต่ค่าความไวรับของเชื้อที่มีการสร้างไบโอฟิล์มสูงกว่าเชื้อที่ไม่มีการสร้างไบโอฟิล์มถึง 530 เท่า จากผลการทดลองยืนยันการดื้อยาของเชื้อที่สร้างไบโอฟิล์มในระดับสูงและแสดงการส่งเสริมการสร้างไบโอฟิล์ม ในกรณีที่เชื้อยีสต์ทั้งสองชนิดเจริญเติบโตร่วมกันในห้องปฏิบัติการ

ภาควิชา พยาธิวิทยา ลายมือชื่อนิสิต .....

สาขาวิชา พยาธิชีววิทยาทางสัตวแพทย์ ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2556

5575302831 : MAJOR VETERINARY PATHOBIOLOGY
KEYWORDS: BIOFILM / MALASSEZIA PACHYDERMATIS / CANDIDA PARAPSILOSIS / CO-COLONIZATION / ANTIFUNGL SUSCEPTIBILITY TEST

KOBKAEW BUMROONGTHAI: BIOFILM FORMATION FROM CO-EXISTENCE BETWEEN *MALASSEZIA PACHYDERMATIS* AND *CANDIDA PARAPSILOSIS* AND THEIR ANTIFUNGAL SUSCEPTIBILITIES. ADVISOR: ASST. PROF. NUVEE PRAPASARAKUL, Ph.D., 87 pp.

The co-existence and increasing of yeast populations; *Malassezia pachydermatis* and *Candida parapsilosis* on dog skin was associated with acute and chronic seborrhea dermatitis (SD). However, consequences of co-existence on dog skin are still unclear in term of symbiosis role and antifungal response. This study aimed to demonstrate biofilm formation of *M. pachydermatis* together with that of *C. parapsilosis* and to compare between single and co-colonization. A total of 40 isolates comprised of 20 isolates of *M. pachydermatis* and 20 isolates of *C. parapsilosis* were selected from 10 healthy and 10 SD dogs. All was confirm their genotype and phospholipase activity were confirmed by partial DNA sequencing at IGS1 region and a semi-quantitative egg-yolk plate, respectively. Yeast morphology and characterizations were examined by crystal violet assay and scanning electron microscope. The antifungal susceptibilities among sessile and planktonic cells were determined by a broth microdilution method. Nineteen of 20 *M. pachydermatis* were grouped in genotype 1A and another was in genotype 3D. All tested yeasts had a high level of phospholipase activity. Overall, biofilm formation could be observed within 24 hour incubation and gave the highest reacted value within 72 hr. Biofilm belonging to co-colonization was higher than that of single colonization (Pair t-test, $P<0.05$). There was no significant difference of susceptibility values between single or co-colonized growth with biofilm. Nevertheless, the susceptibility value derived from the cells with biofilm was higher than that without biofilm at least 530 times. This finding confirmed a diametrical resistance of yeasts beneath biofilm production and demonstrated mutualizing on biofilm formation in the in vitro co-colonization.

Department: Veterinary Pathology Student's Signature .....

Field of Study: Veterinary Pathobiology Advisor's Signature .....

Academic Year: 2013

ACKNOWLEDGEMENTS

This research would not have been possible without the support from many people. I would like to express my gratitude to my dearest thesis advisor Associate Professor Dr. Nuvee Prapasarakul for giving me the opportunity to complete this research, his useful statement, supports, inspiration and encouragement through the learning process of master thesis. Furthermore I would like to thank both Mrs. Waree Niyomtham and Miss Chompoonek Yurayart for providing the knowledge, assistance and support in laboratory work. Also, I like to thank Miss Prukasawan Chetanachan for providing the technique of electron microscope.

I would like to acknowledge the staffs and graduate students at the Department of Veterinary Microbiology, Faculty of veterinary Science, Chulalongkorn University who helped me through the process.

I am thankful to the 72nd Birthday Anniversary of His Majesty the King's Scholarship Grants Commission, Chulalongkorn University for the financial support.

Lastly, I would like to thank my dear family for their support and encouragement for me to reach my goal.

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LIST OF ABBREVIATIONS

ANOVA	=	Analysis of variance
MIC	=	Minimal inhibitory concentration
SMIC	=	Sessile minimal inhibitory concentration
SD	=	Seborrhea dermatitis
AD	=	Atopic dermatitis
BP	=	Base pair

CHAPTER I
INTRODUCTION

1.1. IMPORTANCE AND RATIONALE

Biofilms are the communities of single or more species of microbial cells which are able to tightly attach to the surface of biotic or non-biotic materials (Mace et al., 2008). This structure is triggered by microorganism chemist via their specific auto-inducer that is habitable among microbes and protects from extracellular matrix impacts (Hoiby et al., 2010). To date, biofilm formation inducing pathogenicity during chronic infection has been confirmed by many infectious models such as cystic fibrosis, otitis media, congestive valve endocarditis and periodontitis (Donlan and Costerton, 2002). Moreover, microbes living in biofilms can survive in the environment enriched with antimicrobial and disinfectant agents at over 2000 times of the recommended doses more than planktonic cells (free living cell) (Chandra et al., 2001; Mah and O'Toole, 2001). Attachment of microorganism via biofilms structure onto medical devices is a major cause of persistent infection and may be associated with hospital-acquired transmission especially in case of central line-associated bloodstream infection (CLABSI) via intravenous devices. *Candida albicans* and *Staphylococcus aureus* from patient skin and mucosa were found as the predominant microbes producing biofilms at intravenous catheter (Donlan, 2001).

Malassezia pachydermatis, a major microflora yeast, is a commensal on dog skin but can also act as a pathogen predisposing by host immune deviation and certain inappropriate environmental factors (Matousek et al., 2003). Atopic dermatitis is the major etiology of dog skin disorders, consisting seborrhea dermatitis, furunculosis, otitis externa, dandruff. The disease is closely related to over growth of

yeasts on the lesion area that is the cause of seborrheic dermatitis (Cafarchia et al., 2005). Not only inducing canine dermatitis, but *M. pachydermatis* were also associated with fungemia in infants at a neonatal intensive care nursery unit (Chang et al., 1998; Guillot and Bond, 1999; Chryssanthou et al., 2001), of which the evidence was likely originated from the medical staffs who kept pets in their household. More recently, *M. pachydermatis* and *Candida parapsilosis* have been trusted as commensal microorganism on dog skin and simultaneously became pathogens associated seborrhea dermatitis. Increasing of yeast population at over 100-1,000 times on healthy dog skin was confirmed and co-existence of *M. pachydermatis* and *C. parapsilosis* at the lesion area was firstly discovered (Yurayart et al., 2011). *In vitro*, planktonic form of these yeasts was mostly susceptible to azole agents that were the drug of choice for canine seborrhea dermatitis. However, biofilms act as the organic boundary, may obstruct drug exposure and lead to treatment failure. Thus, the previous interpretation might not be reflected for prediction, *in vivo (Figueredo et al., 2013a)*. Despite the fact that the studies of biofilms are major on mono species, the information of interaction in mixed species of yeast is still scarce. Understanding of microorganism interspecific interaction on dog skin in terms of pathogenic factor such as biofilm formation and antifungal response may be useful for clarifying the microbial relationship. This study aimed to characterize biofilm formation of *M. pachydermatis* and *C. parapsilosis* and to compare between single and co-colonization biofilm formation by mean of crystal violet assay and scanning electron microscope. The antifungal activities of co-colonization of yeast producing biofilms were also determined and compared to that of single species and the yeast without biofilm production.

1.2. OBJECTIVE OF STUDY

1.2.1. To characterize the biofilm productions of single *M. pachydermatis* and co – colonization *of M. pachydermatis* and *C. parapsilosis*.

1.2.2. To determine the sessile minimal inhibitory concentration (SMIC) of single yeast with biofilms, the co-colonized yeasts with biofilms and planktonic cells against ketoconazole and itraconazole.

1.3. HYPOTHESIS

1.3.1. *M. pachydermatis* and *C. parapsilosis* synergistically produce biofilms, *in vitro*.

1.3.2. The SMIC levels of co-colonizing sessile are much higher than those planktonic cells, but are in high resistant level as single biofilms.

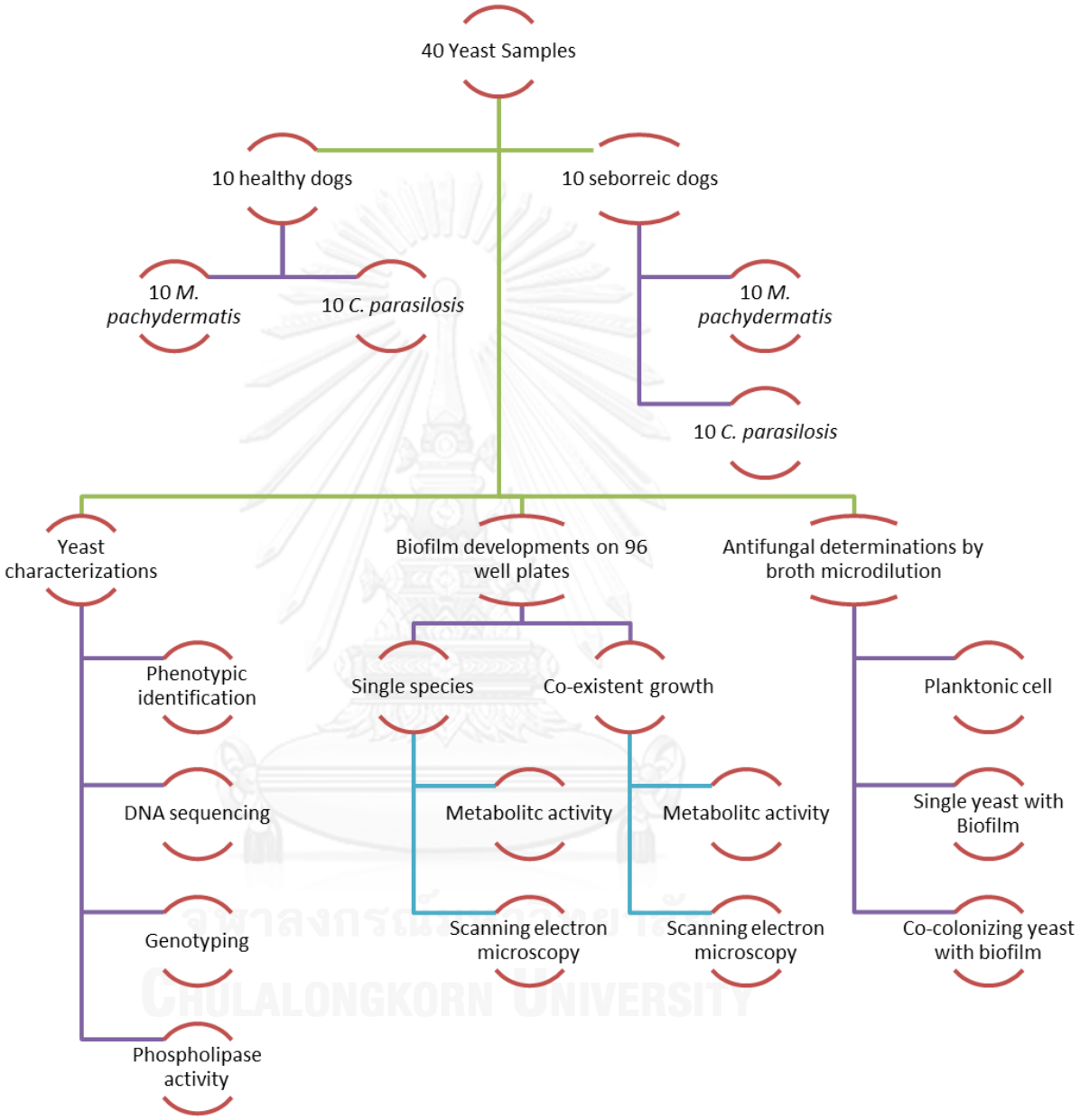
1.4. CONCEPTUAL FRAMEWORK

1.4.1. Evaluation of biofilm quantity by crystal violet staining method (CVS).

1.4.2. Demonstrate Biofilm production by Scanning Electron Microscope (SEM).

1.4.3. Determination of antifungal susceptibility of single and mixed species biofilm.

1.5. RESEARCH PLAN



CHAPTER II
LITERATURE REVIEW

2.1. Canine Skin Diseases Associated Yeasts

Skin infections are commonly found in humans and dogs presenting abnormal skin conditions such as atopic dermatitis (AD) and seborrhea dermatitis (SD). The relationship between skin infection and clinical sign of AD and its pathogenesis is still unclear. The infections are consequences after alteration of skin condition by impaired immunity or immune-deviation status resulting AD and other allergic conditions (DeBoer and Marsella, 2001; Schneider et al., 2013). SD is characterized by fault in cornification and keratinization leading to induce scale production, production of skin grease and hair loss together with secondary bacterial infection. Breed predisposing associated SD include American Cocker Spaniel, Basset Hound, Golden Retriever, West Highland white terrier and German shepherd. Canine SD can be localized or generalized inflammation. Clinical signs vary from mild itching and dandruff to severe condition, consisted exudative erythema, crust with malodor. The symptom and clinical lesion are mostly located on peri-ocular, axillary, neck, chest, groin, interdigitate areas and all skin folds.

Canine SD can be divided upon etiology into 2 types including primary and secondary SD. Primary SD is an inherited skin condition caused by genetic disorders characterized by defective keratinization and cornification of epidermis cell, epithelium and hair follicle. The skin disorder begins at young age (under 2 years old) and persists throughout animal's life. Secondary SD is more common than primary SD. Underlying causes of SD are systemic diseases associated endocrine abnormalities

such as diabetes mellitus and hypothyroid with high exposure to allergens (food proteins, mites, yeasts) (Berk and Scheinfeld, 2010). *M. pachydermatis* is commensal yeast colonizing on dog skin and mucosa, which is believed as an important causative agent of canine SD. Recently; *Candida parapsilosis* was also confirmed as commensal yeast on dog skin and concurrently colonized together with *M. pachydermatis* during an episode of SD. The over population of *M. pachydermatis* and *C. parapsilosis* were associated with progression of seborrhea dermatitis and their co-colonization on skin reflected stage of SD leading to strategic treatment (Yurayart et al., 2011). Moreover, the underlying causes of yeast infestation on skin may be associated with impair of skin barrier, inappropriate skin environmental such as too high moisture and alteration of temperature, alkalinity or microorganism community (Gaitanis et al., 2012), that can induce to higher susceptible stage against surrounding allergens (Aspres and Anderson, 2004). Blockage of eccrine glands; sweat and sebum, by microbial extracellular-polymeric substance (EPC) is also one of the predisposing factors for SD (Allen and Mueller, 2011). Thus, existence of commensal microbe is an important factor accelerating skin disorders especially in case of susceptible animals.

2.2. *Malassezia pachydermatis*

Kingdom: Fungi
Division: *Basidiomycota*
Subdivision: *Ustilaginomycotina*
Class: *Exobasidiomycetes*
Order: *Malasseziales*
Family: *Malasseziaceae*
Genus: *Malassezia*
Species: *M. pachydermatis*

Nowadays, there are 14 species members in genus *Malassezia*. Thirteen of them are lipid-dependent yeast and are commonly recovered from warm-blooded vertebrates such as humans, horses and ruminants. Only *M. pachydermatis* is a non-lipid dependent yeast and commonly isolated from cats and dogs (Cabanes et al., 2011). *M. pachydermatis* was first discovered in 1925 by Weidman from scales of rhinoceros (*Rhinoceros unicornis*) with skin lesion. Because of their morphologies that look similar to the causative agent of human "*Pityrosporum ovale",* Weidman proposed it named *P. pachydermatis.* In 1955, Gustafson recovered bottle shaped yeast from ear cavity of dogs with otitis externa. The isolates were classified as *Pityrosporum* according to their type of budding and cell shape and *P. canis* was proposed base on host. The name of *P. canis* has been officially replaced as *Malassezia pachydermatis* in 1984. Overall, their common morphologies can be defined by an oblong-ellipsoidal to cylindrical yeast cell (peanut shape) with 2-7μm at size, dimorphism (yeast to mycelial phase) under microaerophilic condition (Faergemann and Bernander, 1981). Still, *M. pachydermatis* may not be considered as filamentous producing yeast owing to the fact that only very shorten filaments were found (Gueho et al., 1996). The microorganism reproduces by asexual budding on unipolar site and shows distinct collarets in the middle. On Sabouraud's dextrose agar (SDA), *M. pachydermatis* colony is convex, round, white to yellowish with smooth and dry surface (Guillot and Bond, 1999). This species is very easy to distinguish from the other species of *Malassezia* because they are able to grow even without lipid supplement. By CHROM agar, *M. pachydermatis* is presented as large, pale-smooth pink colonies with precipitation zone. By biochemical profile, this species can hydrolyze urea, delay-positive to catalase test, grow on Cremophor EL

agar and produces black zone on Tween-60 Esculin agar containing ferric ammonium citrate (Kiss et al., 1996). For Tween utilization, *M. pachydermatis* is able to utilized Tween 20, 40, 60, 80 that can distinguish from the other species such as *M. globosa*, non-assimilate Tween (Kaneko et al., 2007). *M. pachydermatis* grows at 37°C for 3-7 day. Furthermore, it can tolerate wide range of temperature ranging from 25-41°C in many conditions such as aerobic, capneic and microaerophilic condition but not in anaerobic condition. This species is commonly recovered from healthy skin and ear canal of dogs and also can be associated with skin disorders such as seborrhea dermatitis and otitis externa (Cafarchia et al., 2011). Moreover, *M. pachydermatis* also caused dermatitis on bears, ferrets, sea lions and foxes located on facial folds, ear canals, lips, chins, interdigital area and anal sacs (Akerstedt and Vollset, 1996; Guillot et al., 1998). The dog breeds with floppy eye or skin fold such as Basset hounds, Cocker spaniels, Poodle and West highland white terriers are at risk of yeast infestation (Bond et al., 1996). Dogs with *M. pachydermatis* infestation may show allergic signs consisting pruritus, malodor, macules, erythematous papules, scaling with hyperpigmentation and alopecia, crusting with greasy skin and hair markedly at toe web and skin fold, which can lead to lichenification (skin thickening) in chronic cases (Chang et al., 1998). Dogs suffering from otitis externa present yellow to brownish cerumen with erythematous pinnae and ear canal. In chronic case, stenosis of ear canal is very common, this leads to concurrent infection between *Staphylococcus* and yeast infections or progressive otitis media (Bond, 2010).

The diagnosis of *Malassezia* sp. is depended on clinical-based; an increasing numbers of yeasts on skin together with mycologic and clinical response to therapy. In veterinary practice, cytological examination is a screening test at dermatological

unit because of its simplicity and reliability. It includes, for example, cellophane tape technique (Scotch tape technique): press adhesive tape on skin lesion, to observe superficial microorganism and stratum corneum by Wright's staining (Bond, 2010). For molecular identification, PCR-based identification is a suitable choice for species identification. Detection of genetic markers such as chitin synthase-2 (*chs*-2), ribosomal DNA (rDNA), internal transcribed spacer (ITS), large subunit of nuclear rDNA and intergenic spacer-1 (IGS-1), can be respectable markers for species identification. Sequencing of IGS1 has been used for classification of *M. globosa*, *M. restricta* and *M. pachydermatis* and also is useful to classify in term of genotype for *M. pachydermatis,* at present.

Yeast overgrowth on SD dogs is promoted by various unusual environmental factors and host defect as previously described (Matousek et al., 2003). On the microbe side, enzymatic production i.e. phospholipase and protease, is believed as the major chemical compounds inducing pathogenesis. Phospholipases, a heterogeneous group of enzymes, has the capacity to hydrolyze ester linkage in glycerol phospholipids which usually acts as the main component of cell membrane (Ghannoum, 2000). Thus, phospholipase is speculated as an important factor for host cells invasion by enchanting cell penetration, destroying cell membrane and inducing host immune response of animals and human (Gaitanis et al., 2012). At the first stage of colonization, phospholipase directly triggers inflammatory responses, which recruits aggregation of white blood cells, plasma protein, and releasing inflammatory mediators resulting cutaneous inflammation (Plotkin et al.,1998). More recently, Figueredo and colleagues reported that phospholipase and biofilm production of *M.*

pachydermatis might exert synergistic activity on promoting the skin lesions (Figueredo et al., 2012).

For public health concern, *M. pachydermatis* dramatically cause nosocomial infections and life-threatening fungemia in immunocompromised patients and infants in intensive care units. This was suspected that healthcare workers and medical staff who had dogs in their household, might have been a major source of yeast contamination in hospitals. Most of nosocomial caused by *M. pachydermatis* is contaminated via intravenous catheterization. The infant patients usually have much more severity due to premature birth such as respiratory distress syndrome (RDS) with very low birth weight (Chryssanthou et al., 2001). They needed to be hospitalized and treated with antifungals and parental lipid infusion for several weeks via intravenous (IV) catheter but the treatment may be failure in condition of biofilm produced yeast (Guillot and Bond, 1999).

2.3. *Candida parapsilosis*

Kingdom: Fungi

Phylum: *Ascomycota*

Subphylum: *Saccharomycotina*

Class: *Saccharomycetes*

Order: *Saccharomycetales*

Family: *Saccharomycetaceae*

Genus: *Candida*

Species: *C. parapsilosis*

C. parapsilosis was firstly isolated by Asford in 1928 that is a commensal flora on skin and mucosa of humans and animals (van Asbeck et al., 2009). *Candida* spp. also acts as opportunistic yeast infested on their usual habitat. *C. parapsilosis* colonizes on many different anatomical sites such as perianal area, interdigitale, ear canal, groin and it can be concurrently found with *M. pachydermatis* in terms of co-colonization (Yurayart et al., 2011). The predisposing factors associated with yeast overgrowth and colonization are related to prolong antibiotic usage, malignancy, indwelling medical instruments, systemic disorders such as diabetes, aging and obesity (van Asbeck et al., 2009). Recently, *C. parapsilosis* was revealed as an etiologic of canine seborrhea dermatitis, cutaneous candidiasis, otitis externa (Mueller et al., 2002). Moreover, it was also associated with fungemia leading to uncommon infections such as endocarditis, peritonitis, pancreatitis, meningitis and other systemic infections (van Asbeck et al., 2009).

On SDA agar, *C. parapsilosis* colony is oval or round-shaped, white to creamy and grown within 24-48 hr. Under microscopic finding, yeast and pseudohyphae structure can be observed defining as dimorphism but not true hypha. The biochemical and physiological properties are used for routine screening identification. Using CHROM agar is the easiest and user friendly method. In addition, the observation of germinating production using pig serum and production of blastoconidia and chlamydoconidia on cornmeal agar are also utilized for confirmation criteria (Oliveira Gdos et al., 2006). The virulence factors are quite resemble in comparison to those of *C. albicans* including polymorphism, ability to adhere endothelial and epithelial, biofilm formation, phenotypic switching, productions of proteinase, phospholipase and lipase that provides viability during

phagocytosis by macrophage (van Asbeck et al., 2009). Regarding public health concern, since it generally harbors on skin and mucosal barriers, it can easily be transferred via direct contact to other immunocompromised hosts that possibly causes infection and death. In Asia and Latin America, there was high in *C. parapsilosis* is still a major causative of candidiasis via bloodstream and emerging of *C. parapsilosis* fungemia has been raised in immunocompromised patients and infants since 1990s (van Asbeck et al., 2007). However, the related source of yeast to patients has not been elucidated and pet may be a suspect, undoubtedly.

2.4. Biofilm

In late 1600s, a Dutch scientist Antonie van Leeuwenhoek reported a group of living microorganisms from his teeth called “animalcules”. This was the first evidence of “Biofilm” (Slavkin, 1997). Two centuries later, Heukelekian and colleagues claimed that “Surface enable bacteria to develop in substrates otherwise too dilute for growth” (Heukelekian and Heller, 1940). Nowadays, this situation of sessile cells is called “biofilm”. Biofilm is the community of living microbial cells which is able to tightly attach to the surface of biotic or non-biotic materials such as living tissue, wood, plastic, glass, medical implant material and food products. Biofilm is a consequence of microorganism communication via signaling system called “Quorum Sensing: QS”, which triggers a cascade of biofilm-forming gene expression and supports microbe to colonize and embed themselves within self-produced extracellular matrix (Sutherland, 2001a). For example, *C. albicans* can produce isoprenoid farnesol as QS inducer that can regulate filamentation (Albuquerque and Casadevall, 2012). The autoinducer, farnesol, is a chemotactic substance during

biofilm formation but facilitates for cell competence during filamentation. On the other hand, the chemotactic inducer named tyrosol takes responsibility to prolong log phase of growth and induces germ tube formation and biofilm establishment. The sessile cells significantly produced higher tyrosol than free living planktonic cells. At biofilm initiation, tyrosol enhances development of cells and prolongs life span of cells (Cremer et al., 1999). These indicate that farnesol can act as retarded reaction, and tyrosol can act as progressive reaction in regulatory system of *C. albicans* (Ramage et al., 2009). Biofilm provides the advantages to microbes' survival strategy including protection from extreme environment, resistance to chemical or mechanical removal of cells and host immune system.

In general, biofilm consists of two components; the first component is established from 10% of dry mass and another 90% is extracellular matrix (Flemming and Wingender, 2010). The later matrix is also known as extracellular polymeric substances (EPS) composed of polysaccharides, lipids, nuclei acid and proteins, which support stability of biofilm, straighten attachment to surface, and immobilize cells (Renner and Weibel, 2011). EPS is the physical barrier hydrogel, which encapsulate and protect the cells from environment. EPS components provide as the source of nutrient including carbon, nitrogen and phosphorus to maintain their niche community (Sutherland, 2001b). Most of EPS are heteropolysaccharide consisting of organic and inorganic substances which involving in biological and physiological properties such as fructose and glucans (Flemming and Wingender, 2010). The other component of EPS is extracellular proteins including enzyme and structural proteins. Many kinds of enzymes detected in biofilm can autogenously degenerate biopolymers into energy source. The substrate of these enzymes consist of

polysaccharide, nucleic acid and protein (water soluble compound) and lipid, cellulose and chitin (water insoluble compound) and some organic particles stuck on biofilm (Flemming and Wingender, 2001). Certain enzymes including hydrolase and lyases involve in alteration of EPS. Because these enzymes can degenerate EPS during starvation and last step of biofilm formation that induce dispersion of cells. Structural protein base compound such as lectins plays a role in stabilization and formation of EPS network and straighten a link between EPS and bacterial surface. Moreover, flagella, pilli and fimbrea also act as skeletal element by connecting with the other EPS integral biofilm (Flemming and Wingender, 2010). The biofilm structure is complemented by surrounding nutrient, intercellular community, hydrodynamic condition, and EPS members that make a variation of morphology, surface, color and texture beneath macrocolonies. The living cells in biofilm are shortly immobilize and persist in environment for quite a while (Flemming and Wingender, 2010).

Development of biofilm formation includes 3 steps. The first step is initialized by attachment to surface by electrostatic and hydrophobic interaction (Van Mellaert et al., 2012). In this step, microbial cells are able to communicate through cell to cell communication or quorum sensing. Intracellular signaling between cells is accomplished by bacterial self-products, which is able to diffuse from one cell and reach to other cells (Watnick and Kolter, 2000). The attachment is tight via *van der Waals* force between the cell surface and the substratum. The microorganism use extracellular proteins for signaling and organelles for adhering to surface, for example fimbrea, villi, flagella, pili and outer membrane protein (Bullitt and Makowski, 1995). After the cells adhere irreversibly to surface area, they secrete EPS to accelerate the adhesion networking between cells and surfaces (Renner and Weibel, 2011).

Adhesive cells grow and duplicate into the groups of ten or hundred micron of cells diameter which so called microcolonies. For accumulation and maturation phase after attaching to surface, biofilms are developed via intercellular aggregation and distribution to the first adhered cells and then expand their clone under EPS. The mature biofilm contains macro-colonies and water based envelop with permeability characteristic. Finally, the dispersion step is started during nutrient become shortage, environmental change or rapid available of food, the sessile cells can individual disperse from group or clump. The cells in biofilms are scattered through shedding active daughter cells to new environment (Watnick and Kolter, 2000). In dispersion step, some enzymes facilitate in degradation of architecture of EPS to improve separation of organism from biofilm.

For *in vitro* biofilm measurement and examination, crystal violet staining (CV) has been used as gold standard for determination (Cremet et al., 2013). This method can determine a quantity of biofilm biomass. CV assay works by staining the negative charged molecule such as bacteria and polysaccharide of EPS with crystal violet dye. After the cell is bound with dye, solvents such as ethanol or acetic acid are added to elute the non-adsorbed CV. A remained quantity of biofilms bind with CV will revealed the higher value by the optical absorbance (Jacques et al., 2010). This method is inexpensive and easy to set up, but there is some limitation such as the method cannot differentiate between alive or dead cells.

Segregation between live and dead cells requires a technique based on metabolic activity of living cells. Many kinds of viability stains associate with the use of tetrazolium salts, for instance, Cyano ditolyl Tetrazolium Chloride (CTC) and 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium inner

salt (XTT) and Resazurin assay (known as Alamar Blue assay) (Peeters et al., 2008). The principal of metabolic assay such as Alamar Blue assay based on the resazurin, can be reduced by metabolically living cells into water-soluble resorufin, which is fluorescent and pink in color (O'Brien et al., 2000). Quantifying of viable cells can be measured by measurement of fluorescent from resazurin assay. Metabolic assays are a good method to study biofilm drug susceptibility test since they are able to determine viability of the sample within intact complexity of biofilm.

To demonstrate ultrastructure of biofilm formation is a consensual criteria providing overall of biofilm biomass extracellular matrix or other extracellular components. Scanning electron microscope (SEM) is a technique for examination surface structure using electron beam that can provide the evidence of morphology and quantity of biofilm leading to relationship of multispecies microbial biofilm system (Somayaji et al., 2010). In addition, imaging by SEM can demonstrate co-existence of 2 microorganisms embedded in biofilm such as *Candida albicans* and *Staphylococcus aureus* (Harriott and Noverr, 2009; Bandara et al., 2010; Pammi et al., 2013). SEM has such higher resolution of imaging biofilm than other techniques and can be applied to investigate and evaluate the biofilm structure in three dimensions. However, a disadvantage of SEM is the complexity in preparation of high vacuum condition. Environmental scanning electron microscope (ESEM) can overcome this problem, but the magnification is lower than that of the conventional SEM.

According to WHO's report, biofilm is associated with many infectious diseases. Amount 65% of nosocomial infection caused by biofilm is present (Mah and O'Toole, 2001). Within biofilm, the microorganism can survive in surrounding enriched with high dosage of antimicrobials that results treatment failure to infection and

remains daughter cells at infective sites (Francolini and Donelli, 2010). Human chronic diseases such as cystic fibrosis, otitis media, congestive valve endocarditis, periodontitis and delay wound healing are mostly concerned to biofilm production (Mah and O'Toole, 2001; Martinez and Casadevall, 2006a; Schierle et al., 2009). Biofilm interrupts wound healing by EPS and extracellular enzyme and prolonging inflammatory response (Mah and O'Toole, 2001). Production of biofilm at indwelling biomedical devices seriously life-threatens for the patients who are manipulated by all implanted medical instruments such as intravenous catheter, urinary catheter, orthopedic devices, prosthetic heart valves (Elias and Banin, 2012). Biofilm on indwelling medical instruments may be contaminated with various kinds of microorganisms including Gram-negative bacteria, Gram-positive bacteria, fungi and yeast (Donlan and Costerton, 2002). The sources of microbes usually derive from patient skin, healthcare person, or environmental sources. Biomass can be composed of both mono or multispecies on the devices, depending on site of insertion, duration of use and risk of exposure (Donlan, 2001). *C. parapsilosis* was reported as the causative agent of fungemia after medical management by implantable intravenous catheter in bone marrow transplant and oncology patients (Levin et al., 1998). The evidence of multispecies biofilm was found in urinary catheter by co-existence with yeasts; *C. glabrata*, *C. parapsilosis*, *Saccharomyces cerevisiae* or bacteria; *Proteus mirabilis*, *E. coli* and *Klebsiella pneumonia* (Macleod and Stickler, 2007; Hola et al., 2010; Ruzicka et al., 2012). Forming of biofilm can turn antimicrobial susceptible to high resistant situation leading to chronic disease and treatment failure. The antimicrobial susceptibility level will be increased from 10 to over 1000 times against the sessile cells (Mah and O'Toole, 2001). The immune evasion under

biofilm condition was explained by a cystic fibrosis model that directly interfered the immune signaling and phagocytosis (Donlan and Costerton, 2002). Macrophage phagocytic activity was ineffective against extracellular slime producing by *S. epidermidis* (Shiau and Wu, 1998) as well as persistence of *Cryptococcus neoformans* sessile cell during antifungal treatment (Martinez and Casadevall, 2006a).

2.5. Treatment of Yeast Infestation

Treatment of yeast dermatitis in pet requires long treatment and high dose of antifungal drugs (Negre et al., 2009). It is very important to analyze on the interaction among host competent, microbial infectivity and predisposing factor based on environment. In veterinary practice, topical and systemic therapies are effective methods to reduce yeast infection. Topical therapies, for example, antifungal shampoo, cream and spray, can kill yeast and retard yeast colonization via changing the environment of cutaneous surface (e.g. alteration alkalinity or degreasing) (Bond, 2010). Systemic antifungal therapy is considered in severe and generalized cases or in case of topical treatment failure.

Azole antifungal drugs were discovered in 1970's and are broad spectrum antifungal drug. Ketoconazole and itraconazole are commonly used for treatment of systemic or superficial fungal infections, such as candidiasis, aspergillosis, cryptococcosis and malassezia skin infection. The target of azole drug is cytochrome P 450 dependent enzyme14-α-demethylase enzyme resulting in inhibition of ergosterol synthesis since ergosterol is the main component of fungal cell wall, resulting minimization of cell wall integrity and cell disruption (Rajendran et al., 2011). Moreover, azole can cause abnormalities at cytoplasmic vacuoles, cell wall

and plasma membrane inducing impair of cell division and growth of hypha (Borgers and Van de Ven, 1989). To date, use of ketoconazole is still popular for veterinary use in case of fungal skin infection because it can excrete via eccrine glands and sebum. Ketoconazole is also effective for systemic treatment of generalized fungal dermatitis, blastomycosis, coccidioidomycosis, cryptococcosis and aspergillosis (Fortun, 2011). For oral application, ketoconazole can well-absorb in gastric acidic condition with food. Dose administration for canine fungal infection varies by type of diseases and sites of infection. For blastomycosis, cryptococcosis and histoplasmosis, the recommended dose is 10-20 mg/kg, two times a day, for at least 2 months. For candidiasis, the recommended dose is 10 mg/kg/day for 6-8 weeks. For *Malassezia* sp. infection, only 5-10 mg/kg/day are recommended (Chermette et al., 2008). Long term use of ketoconazole can cause adverse effects including vomiting, anorexia, weight loss, nausea and elevated serum hepatic enzyme with increasing serum bilirubin concentration associated with hepatic injury. Despite the side effects, ketoconazole is still largely used in veterinary practice because of cheapness, empiric, efficacy, and proper dosing program (Hector, 2005). Itraconazole is a lipophilic compound drug that is also a broad spectrum against many important fungal diseases in animals. For circulating form, 99% of drug is bound to protein which helps to distribute and accumulate in many organs such as lung, kidney, liver, muscle, skin and nail. Due to the delivery reaching to skin via sebum, this drug can reach skin and nail in high concentration with long period. The recommended dose for dogs is orally 5-10 mg/kg/day, for 2-4 weeks (Hector, 2005). This azole drug has atinophilic and lipophilic properties; it prolongs the remaining on skin in animals. In general, itraconazole is a more preferable choice than ketoconazole because of its

lower side effect and higher activity. The advantage of itraconazole is higher affinity for fungal cell membrane rather than mammalian cytochrome P 450 enzyme resulting in a better safety profile (Sheehan et al., 1999). Hepatic metabolism and biliary secretion are the primary eliminated routes for itraconazole. The half-life of these drugs in dogs is 24-30 hour and can persist longer in the tissue than in the plasma (Andes, 2003). According to *in vitro* study, *M. pachydermatis* and *Candida* sp. were still sensitive to both itraconazole and ketoconazole (Yurayart et al., 2013). However, *M. pachydermatis* azole-resistant isolates can be detected from clinical lesion (Nijima et al., 2011), and *C. parapsilosis* resistance to azole drug was found from the canine lesion suffering from seborrhea dermatitis (Yurayart et al., 2013). More recently, biofilm-forming *M. pachydermatis* were categorized as resistant to azole drug (Figueredo et al., 2013a). Nevertheless, the information about biofilm drug susceptibility of co-culture biofilm is still doubted and the progression of resistance is very challenging to access by *in vivo*.

2.6. Antifungal Susceptibility Test for Biofilm

The most antimicrobial susceptibility tests have commonly determined for pure planktonic cells. Nevertheless, in natural microbial ecological system, microorganisms can appear as adhered communities of mixed species embedded within biofilm and barely exist as planktonic cells (Donlan and Costerton, 2002). Recently, biofilm has been referred to monitoring life-threatened marker in human and veterinary medicines due to the resistant abilities to antimicrobial drugs (Donlan, 2001). However, the information about biofilm susceptibility of co-colonization and their progression of resistance is still scarce both *in vitro* and *in vivo*

(Figueredo et al., 2013a). Regarding the susceptibility method from Clinical and Laboratory Standards Institute (CLSI) are recommended for determination among planktonic cells, of which the results are unable to indicate of susceptibility level for biofilm, *in vivo* (Pierce et al., 2008). There are various models for biofilm evaluation including sterile glass, plastic, CDC biofilm reactor, perfused biofilm fermenter and Calgary biofilm device (Coenye and Nelis, 2010). They are time-consuming, costly, and unhandy. On the other hand, use of 96 well microtiter plate has been more usable for the evaluation in *C. albicans, M. pachydermatis*, *Cryptococcus neoformans* and other *Candida* spp. (Martinez and Casadevall, 2006b; Martinez and Casadevall, 2007; Figueredo et al., 2012; Nweze et al., 2012), The procedure of this model begins with biofilm producing microorganism at the bottom of well, followed by using metabolic assay to evaluate the metabolic activity of cells within biofilm. The advantages of this technique are quick, user-friendly, inexpensiveness and accurate reproducibility since various type of 96 well plates are easily accessible (Martinez and Casadevall, 2006b; Pierce et al., 2008; Nweze et al., 2012; Figueredo et al., 2013a).

CHAPTER III

MATERIALS AND METHODS

3.1. Yeasts and Culture

A total of 40 yeasts divided as 20 of *M. pachydermatis* and 20 of *C. parapsilosis* isolates were obtained from 10 dogs with seborrheic dermatitis and 10 healthy. All isolates were derived from the previous study of Yurayart and colleagues

(2011). Identifications of *M. pachydermatis* were confirmed on the basis of colony morphology, ability to grow on lipid free medium and restriction patterns by PCR-RFLP as described previously (Yurayart et al., 2011). *C. parapsilosis* were confirmed by morphological appearance, such as assimilation of sugar, producing blastoconidia and chlarmydoconidia on corn-meal agar and germ tube production (Yurayart et al., 2011). All isolates were finally identified by partial sequencing of ribosomal DNA using a primer set for the internal transcribed spacer (ITS) which located between ITS-1and ITS-4 (Gupta et al., 2000). Analysis of phylogenetic relationship and DNA alignment was performed as formerly described (Yurayart et al., 2011). All yeasts were grown on routine Sabouraud's Dextrose Agar (SDA) (Oxoid CM41, USA) for 2 days for *C. parapsilosis* and 3-4 days for *M. pachydermatis* at 32-37˚C (Yurayart et al., 2011).

3.2. Genotypic Identification of *Malassezia pachydermatis*

A total of 20 *M. pachydermatis* isolates were cultured on SDA at 32˚C for 4 days. After incubation, the grown cells were collected by a sterile cotton swab and suspended into the 1000 ml of PBS solution. The DNA isolation was carried out using Genomic DNA purification kits (Promega, USA). The cell was washed with 100 ml of PBS solution, 3 times, the solutions was spun down to collect the pellets. Next, the glass bead at 425-600 µm size was added at approximately 1:1 ratio of pellet volume, and 300 µl of cell lysis buffer was added. The tube was then kept in -80ºC for 15 minutes then vortexed for 45 minutes. This step was repeated for three times to completely break the cell membrane of yeast (Yamada et al., 2002). To confirm the digested yeast structure, the suspension was stained with a crystal violet before observed under a microscope. The procedure of DNA extraction was carried out

according to recommendation of the manufacturer (Macherey, Germany). The quantity of DNA was confirmed using a spectrophotometer machine (Nanodrop, Thermoscientific, USA). Gel electrophoresis was finally used to demonstrate DNA quality.

Genotyping of *M. pachydermatis* by IGS1 region analysis was performed following the method of Kobayashi *et al*. (2011). A volume of 25 µl PCR mixture (12.5 µl of 2X GoTag Green Master Mix (Promega, Medison, WI, USA), 1.75 µl of Forward and reverse primers, 10 µl of DNase-free water and 1 µl of DNA template (200 ng/µl). DNA product was amplified by a thermal cycle machine (Bio-rad, USA) as follows: 94˚C for 3 minutes, followed by 35 cycles of PCR amplification; for denaturation at 94˚C for 30 seconds, annealing at 59˚C for 30 seconds, and extension at 72˚C for 1 minute. Oligonucleotide bases of the used primers are showing in Table 1. PCR products were purified by a commercial product (Nucleospin® Extract II, Macherey-Nagel, Germany) and illustrated by 1.5% agarose gel electrophoresis at 100 voltage for 30 minutes. The results were observed by using a gel documentation machine (Bio-rad, USA). PCR products were submitted for DNA sequencing using the BigDye Terminator™ (QIAGEN, USA) via a commercial service (First BASE, Malaysia). At least two nucleotide fragments amplified by 26s-F and Mala-R primers, was analyzed using Contig Express version 10.30 (Invitrogen, USA). The phylogenetic tree was constructed using Neighbor-Joining method by MEGA4 program (Tamura et al., 2007).

Table 1. Primer sequences for genotyping analysis by IGS-1 region

Primers	Nucleotide Sequences	Reference
26S-F	5-ATCCTTTGCAGACGACTTGA	(Kobayashi et al., 2011)
Mala-R	5-TGCTTAACTTCGCAGATCGG	(Kobayashi et al., 2011)

3.3. Phospholipase Activity Assays

The evaluation of phospholipase activities was performed using the semi-quantitative egg-yolk plate based method as described by Price et al. (1982). Base medium was composed of 6.5% w/v of SDA, 0.55% w/v of $CaCl_2$, 5.8% w/v of NaCl in 1000 ml distilled water. For preparation, fresh egg-yolk was centrifuged at 500 x g. for 15 minutes. The 10% v/v of egg-yolk supernatants was added to an empty sterile petri dish before poured the base medium at 45-50°C. After that, 10 µl ($1x10^6$ cell/ml) of 3-4 day-old yeast suspension, were spotted onto egg-yolk plates and incubated at 32°C. The precipitation zone around the spotted colony was daily observed from day 7^{th}-12^{th} after incubation. The zone was representing a phospholipase activity. This method was conducted in duplication for each *M. pachydermatis* strain. The activities of phospholipase (Pz) were calculated as a ratio of colony diameter, divided with colony diameter and precipitation zone. The clear zones for *M. pachydermatis* were interpreted into 3 levels: very high (Pz < 0.64), high (Pz ≥ 0.64 and<1), and null (Pz = 1). The clear zones for *C. parapsilosis* were interpreted into 3 levels: high (Pz < 0.70), moderate (Pz = 0.7-0.89) and null (Pz = 1) as shown in the formula below.

$$Pz = \frac{\text{colony diameter}}{\text{Colony diameter + precipitation zone}}$$

3.4. Biofilm production

Biofilm formation of all isolates was evaluated using crystal violet staining method (CVS) as described by Figueredo et al. (Figueredo et al., 2012). Each of pure yeasts were prepared in yeast peptone dextrose (YPD) broth (1%of yeast extract, 2% of Bacto peptone and 2% of glucose) at 32˚C for 3 days in an orbital shaker (Zhcheng, China). After 3 days incubation, the concentration of inoculum was adjusted using a spectrophotometer (Bausch & Lomb, USA) to the optical density of 0.1 at 600 nm or approximately $1.0x10^6$ cells/ml. The concentration was confirmed by enumeration on SDA and performed Colony Forming Unit according to the method of (ISO 7218:1996).

For mono-species producing biofilms, amount 150 µl of yeast suspension was transferred into a 96-well plate (Thermo scientific Nunc, Roskilde, Denmark). For co-colonizing biofilm production, the criteria used to select the isolates in this study were as follows: 3 pairs of both *M. pachydermatis* and *C. parapsilosis* collected from healthy dog skins, 3 pairs of both *M. pachydermatis* and *C. parapsilosis* collected from SD dog skins, 4 pairs of both *M. pachydermatis* and *C. parapsilosis* collected from healthy and SD dog skins. A total of 150 µl derived from 75 µl of *M. pachydermatis* and 75 µl of *C. parapsilosis,* were added into each well. Thereafter, the plates were incubated for 24 hours at 32˚C in an orbital shaker at 75 rpm to obtain adhering phase of biofilm formation. Then, planktonic cells were gently removed by double washing with 150 µl of phosphate buffered saline solution (PBS,

pH7.2). After the rinsing step, 200 µl of YPD broth were replaced into each well and the microtiter plates were re-incubated for 3 days at 32˚C in an orbital shaker at 75 rpm and the media were changed daily. Prior to evaluation, the plates were doubly washed with 200 µL of PBS and left until completely dry. For crystal violet staining, the dry plates were added with 0.5%crystal violet solution for 45 minutes and washed with 200 µL of sterile distilled water, and destained with 95%ethanol for 200 µL for 45 minutes. A total of 100 µL from each well was transferred to a new microtiter plate and measured an optical density (OD.) by an ELISA reader (Labsystem Multiskan Ms., Finland) at 620 nm. The isolates were performed in triplicate and measured the average OD. The evaluation of biofilms quantity directly represented by the value of OD. The measurement was separately performed on 1, 2, 3 and 4 days after incubation. The medium without yeast was used as control wells and the average OD were subtracted from the average OD of samples. The evaluation of OD from each strain was conducted in quartet fashion (Figueredo et al., 2012).

3.5. Structure of Biofilm at Scanning Electron Microscope (SEM)

The selected 3 *M. pachydermatis* (MP) [genotype A1 (n= 2) and D3 (n=1)] produced biofilm and the randomized 3 *C. parapsilosis* (CP) produced biofilm was, in turn, performed on scanning electron microscope**.** A total of 4 pairs of the co-colonization produced biofilms comprising MP-A1 + CP a healthy (MPH and CPH), MP-A1 + CP from SD dogs (MPD + CPD), MPD-D3 + CPD, MPD-A1+CPH, were the selected experimental groups representing a variety of genotype and source of isolates.

All 72-hour-old yeasts from the solid were cultured in YPD broth at 32ºC for 3 days. The suspension concentration was adjusted to $1x10^6$ CFU/ml by measuring the optical density of 0.1 at 600 nm. A total of 1 ml of adjusted solution was transferred into a 24 well cell culture plate (Corning Incorporated, NY, USA) containing 1 cm polystyrene of IV catheter (Becton Dickson Medical Pte. Ltd., Singapore). After incubation at 32ºC for 4 days in an orbital shaker, the IV catheters were fixed in 2.5% glutaraldehyde for 2 hours at 4 ºC, then triply washed with phosphate buffer (pH 7.3) for 20 minutes at 4 ºC. Thereafter, the specimens were rehydrated in ethanol alcohol panel, which were serially 30%, 50%, 70%, 80%, 90% and 95% at 20 minutes interval of each. For final rehydration, the catheters were triply submerged in 100% ethanol for 20 minutes of each. For drying step, the specimens were reached to critical point dried (CPD) condition by CO_2 at approximately 1-2 hours. Lastly, the catheters were mounted on the stub and coated with gold-palladium in an ion sputter. The samples were examined using Scanning Electron Microscope (*JEOL* Ltd., Tokyo, Japan). All samples were performed in duplication.

3.6. Antifungal Susceptibility Test

The MIC values to itraconazole (Sigma-Aldrich, USA) and ketoconazole (Sigma-Aldrich, USA) of biofilm produced yeasts and planktonic yeasts were determined as the previous recommendations (Pierce et al., 2008; Figueredo et al., 2013b). The antifungals were dissolved in dimethyl sulfoxide (DMSO) (Merck Sharp & Dohme Ltd, Herts, Uk) and 2-fold-serially diluted from 0.06-32 µg/ml. For planktonic cell condition, the MIC values were determined using broth microdilution method reference CLSI M27-A2 with modification (Cafarchia et al., 2012a; Cafarchia et al.,

2012b). The inoculum included 3 days old of *M. pachydermatis* and 2 days old of *C. parapsilosis* on SDA. The growth medium in this study was Sabouraud Dextrose broth (SDB) (Oxoid CM41, USA) with 1% (v/v) of Tween 80. The final concentration was adjusted to $1x10^6$ CFU/ml using spectrophotometer. A total 100 µl of the inoculum was added into the 96 well plates containing 100 µl of serial dilution of antifungal drugs. After incubation at 32°C for 48h, optical density was measured by an ELISA reader (Labsystem Multiskan Ms., Finland) at 620 nm. For interpretation, the wells presented 50% lower absorbance value compared to that of positive control was interpreted as MIC50 (Peano et al., 2012).

For mono and co-cultured biofilm condition, the biofilm were prepared as described above. After biofilms production setting on the 96 well plates, the supernatants were discarded and triply washed with 200 µl PBS solution. A volume of 100 µl of YPD broth was added into all wells, and 100 µl of each serial antifungal dilution was added to the related wells as shown in Fig 1. The positive wells in the last column were contained 200 µl of YPD media without antifungal (column 11) and negative control was composed of the media and antifungal without biofilm produced yeasts (column 12).

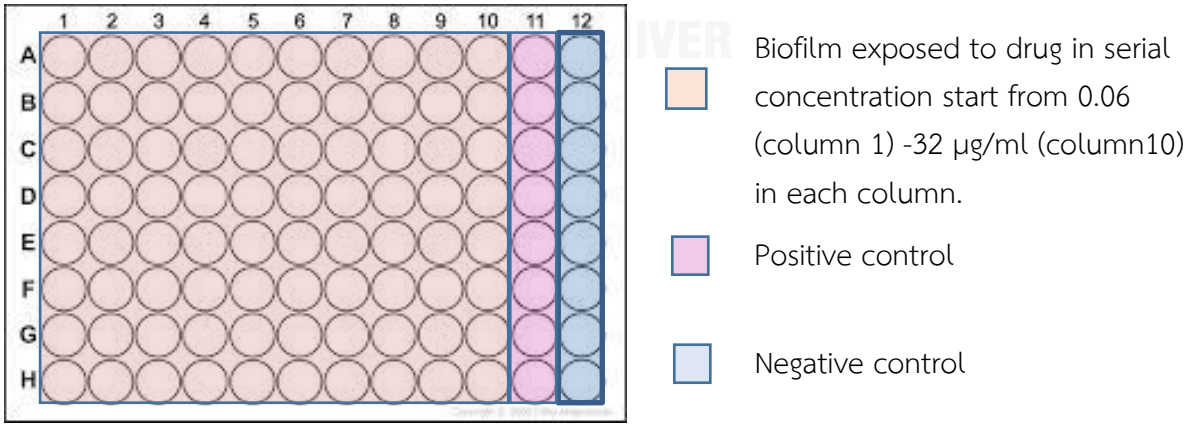


Figure 1. Drug serial dilution in a 96 well plate containing biofilms

After incubation at 32ºC for 48h for biofilm condition, 20 µl of 10X Alamar Blue® (Invitrogen, USA) was added to each well to evaluate density of cell viability. The plates were incubated in darkness at 37ºC for 2-3 hours, then 100 µl of suspension was transferred a new 96-well plates for measuring by an ELISA reader at 570 nm. For interpretation, the wells presented 50% lower absorbance value compared to that of positive control was interpreted as SMIC50 (Ruzicka et al., 2007; Pierce et al., 2008; Figueredo et al., 2013a).

3.7. Statistical Analysis

The difference of biofilm production from crystal violet assay was assessed in term of origin and genotype variants by student *t*-test. Additionally, the biofilm forming duration and quantity of biofilm derived from *M. pachydermatis* or *C. parapsilosis* and their co-colonization were analyzed by ANOVA (multiple comparisons). Statistical analyses were performed using SPSS version 17.0 (IBM, USA). A value of $P \leq 0.05$ was considered as statistical significance. The differences of MIC value between each of experimental groups were analyzed using pair *t*-test.

CHAPTER IV
RESULTS

4.1. Yeast Isolates

The colonies of *M. pachydermatis* were white to creamy, round convex at 2-3 mm diameter with slightly smooth or rough surface on SDA. The isolates were re-identified with the approved biochemical tests. All isolates were able to utilize Tween 20, 40, 60, 80. The biochemical test results such as TE and EL slants are

shown in the Table 2. By light microscope observation, *M. pachydermatis* morphology was round cell-budding with peanut shape in the size of 3-7μm. under 100x (Fig 2).

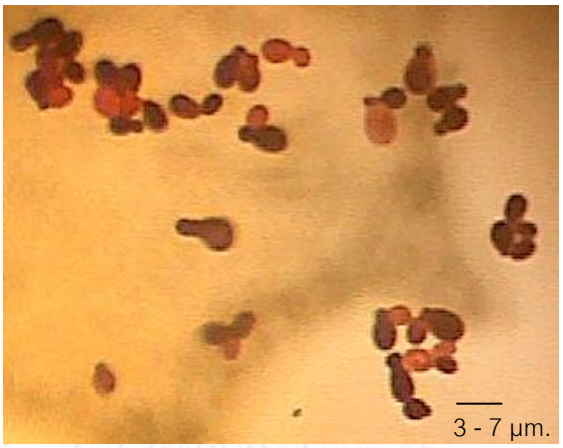


Figure 2. Microscopic feature of *M. pachydermatis* shows the peanut-shape yeast cells in the size of 3-7 μm

Table 2. Biological profiles of the selected 20 *M. pachydermatis* isolates

Order	Growth on	Utilization of [a]				EL	TE	Catalase
	SDA	Tween 20	Tween 40	Tween 60	Tween 80	slant[a]	slant[a]	reaction
1	+	+	+	+	+	+	GB[b]	+
2	+	+	+	+	+	+	GB	+
3	+	+	+	+	+	+	GB	+
4	+	+	+	+	+	+	GB	+
5	+	+	+	+	+	+	GB	+
6	+	+	+	+	+	+	GB	+
7	+	+	+	+	+	+	GB	+

8	+	+	+	+	+	+	GB	+
9	+	+	+	+	+	+	GB	+
10	+	+	+	+	+	+	GB	+
11	+	+	+	+	+	+	GB	+
12	+	+	+	+	+	+	GB	+
13	+	+	+	+	+	+	GB	+
14	+	+	+	+	+	+	GB	+
15	+	+	+	+	+	+	GB	+
16	+	+	+	+	+	+	GB	+
17	+	+	+	+	+	+	GB	+
18	+	+	+	+	+	+	GB	+
19	+	+	+	+	+	+	GB	+
20	+	+	+	+	+	+	GB	+

[a] Incubated at 32ºC for 4-7 days

[b] GB, Growth and produced black zone

For *C. parapsilosis*, the colonies were white to creamy, round convex at 3-5 mm diameter with shiny surface. After incubation for 2-3 days, the isolates were observed under a regular light microscope and appeared as round to oval cell-shaped (Fig 3).

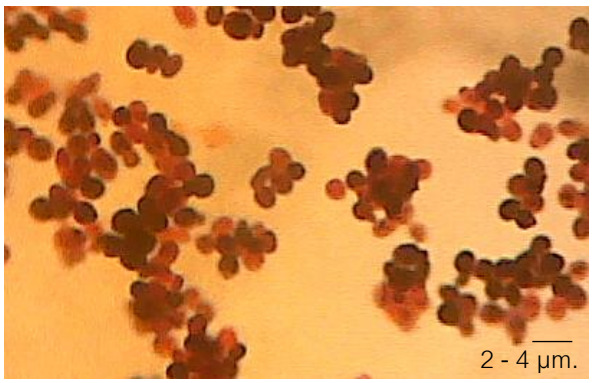


Figure 3. Microscopic feature of *C. parapsilosis* shows the round to ovoid budding yeast in the size of 2-4 µm.

4.2. Phospholipase Activity Assays

Using the semi-quantitative egg-yolk plate, the precipitation zones were shown around the colonies indicated as phospholipase production (Fig.4). The phospholipase activities (Pz) from each isolate were calculated as a ratio of colony diameter and precipitation zone. The results of phospholipase enzyme activities for *M. pachydermatis* and *C. parapsilosis* are shown in Table 3 and 4, respectively.

There was no significant difference in phospholipase enzyme activities between the isolates derived from SD and healthy sources in both *M. pachydermatis* (0.4 ± 0.095, 0.419 ± 0.019) and *C. parapsilosis* (0.405 ± 0.013, 0.405 ± 0.019) (Student *t*-test, P=0.543 and 0.989 respectively).

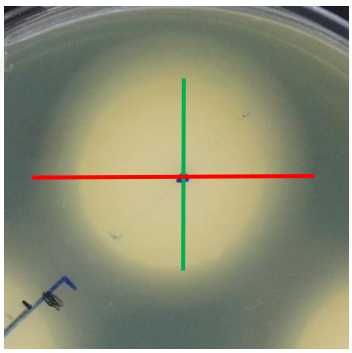


Figure 4. Presence of the length of precipitation zone (red line) and colony (green line) for phospholipase activity calculation.

Table 3. The values and interpretation of phospholipase enzyme activities of *M. pachydermatis*, categorized according to Pz value

Sources of yeast	No. of isolates in activity / total no. in groups (%)		
	Very high (Pz < 0.64)	High (Pz ≥ o.64 < 1)	Null (Pz = 1)
Healthy	10/10 (100)	- (0)	- (0)
SD	10/10 (100)	- (0)	- (0)
Total	20/20 (100)	- (0)	- (0)

Table 4. The values and interpretation of phospholipase enzyme activities of *C. parapsilosis*, categorized according to Pz value

Sources of yeast	No. of isolates in activity / total no. in groups (%)		
	High (Pz < 0.70)	Moderate (Pz = 0.7-0.89)	Null (Pz = 1)
Healthy	10/10 (100)	- (0)	- (0)
SD	10/10 (100)	- (0)	- (0)
Total	20/20 (100)	- (0)	- (0)

4.3. Genotypic identification of *Malassezia pachydermatis*

By genotyping of *M. pachydermatis* using IGS1 region analysis, nineteen samples showed molecular weight at 900 base pair (bp) and only one sample showed product size of 700 bp (Fig. 4).

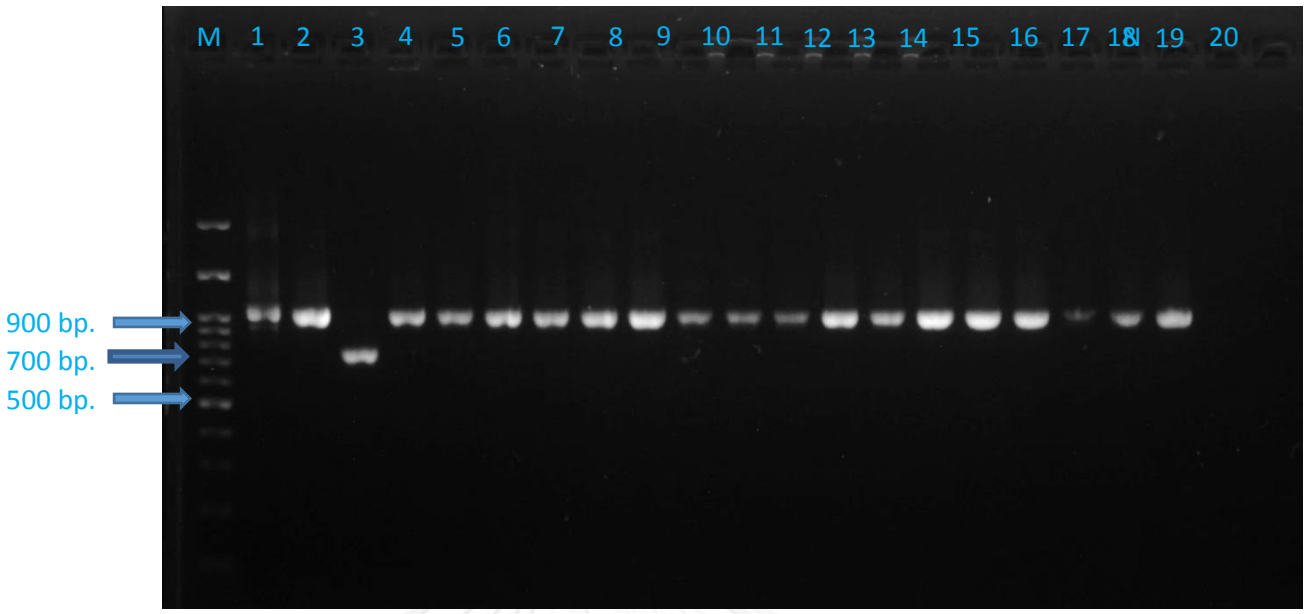


Figure 5. PCR products from IGS1 region amplification of *M. pachydermatis.* M; DNA marker, 1-10; *M. pachydermatis* isolated from SD skin, 11-20; *M. pachydermatis* isolated from normal skin, N; DNase free water as negative control.

After visualized by gel electrophoresis, PCR products were purified using DNA purification kit (Nucleospin® Extract II) (Macherey-Nagel, Germany) and sequenced using the BigDye Terminator™ (QIAGEN, USA). The results revealed that 19 samples were similar to subtype 1A and 1 isolate was similar to subtype 3D. The numbers of IGS1 subtype compared to the origin of isolate are shown in Table 5. The details are shown in appendix A.

Table 5. Summary of IGS 1 subtype of *M. pachydermatis* from different origins

Sources of yeast	IGS 1 subtype (Number of strain)
Healthy skin dogs	1A(10)
SD skin dogs	1A(9)
	3D(1)

4.4. Biofilm formation

The ability of *M. pachydermatis* to form biofilms were determined by crystal violet assay on 96 micro titer plate. Figure 6 shows the OD value of each strain. All strains (n= 20) were able to produce biofilm. There was no statistically significant difference between isolates from SD dogs and those from dogs with healthy skin based on the *T*-test (*P* = 0.47).

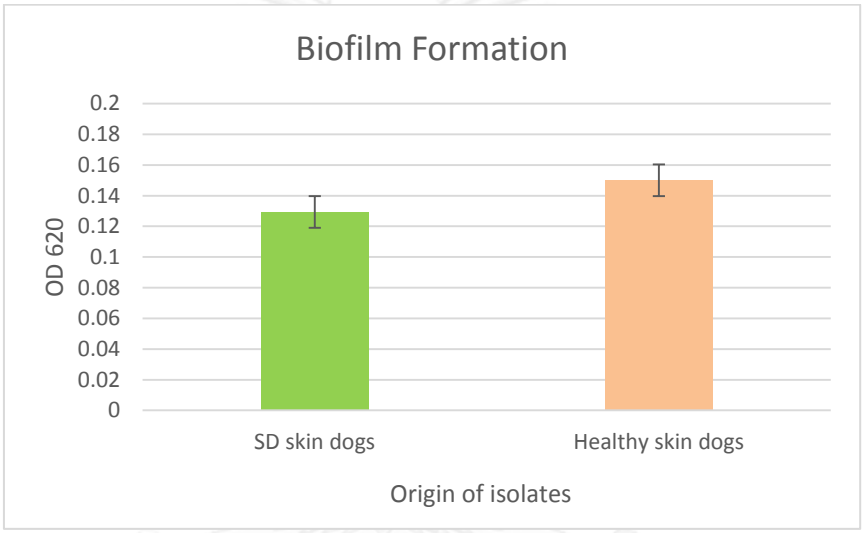


Figure 6. Detection of biomass OD during biofilm production at 72 hour on a 96 well plate by *M. pachydermatis* from SD and healthy skin dogs with no significant difference.

For *C. parapsilosis,* all tested isolates had the ability to form biofilm. There was no significant difference between the isolates from SD or healthy groups by *T*-test analysis (*P* =0.446) (Fig 7).

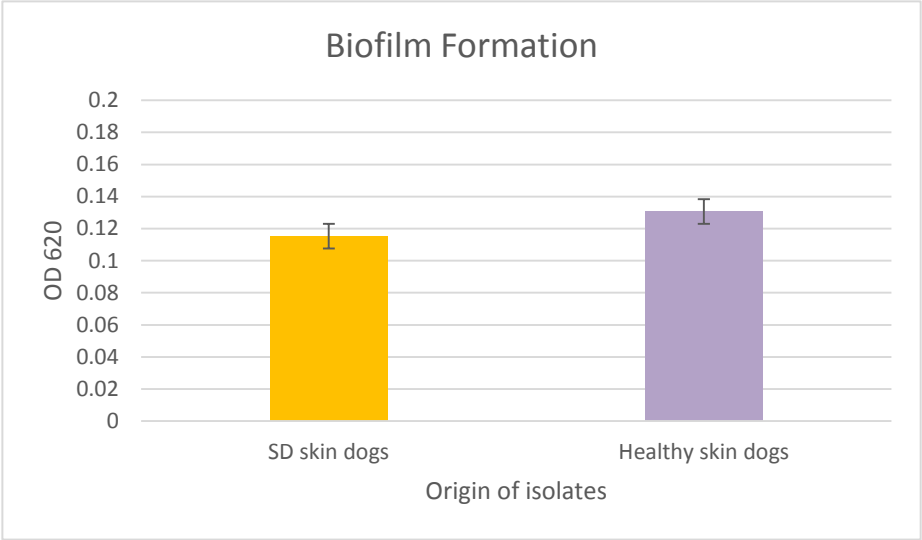


Figure 7. Detection of biomass OD during Biofilm production at 48 hour on a 96 well plate by *C. parapsilosis* isolated from SD and healthy skin dogs without significant difference.

The biomass value at OD620 from single species of *M. pachydermatis* or *C. parapsilosis* and their co-colonization throughout the observation periods are presented in Figure 8. The results showed that the colonization of dual species gave the higher value of biomass production in comparison to mono species. The biomass value from co-colonization groups was decreased at 96 hour while *C. parapsilosis* serially showed increase of biomass production from 24-96 hour and only slight decrease was found in *M. pachydermatis* in 96 hour.

There was no significant difference between single species of *M. pachydermatis* or *C. parapsilosis* and co-cultured biofilm formation at 24h (P = 0.896 and 0.314) and 48h (P = 0.198 and 0.889). Although, biomass from co-colonization had significantly the higher value than that of single colonization at 72 and 96h, ($p < 0.05$) (Multiple Comparison, ANOVA test).

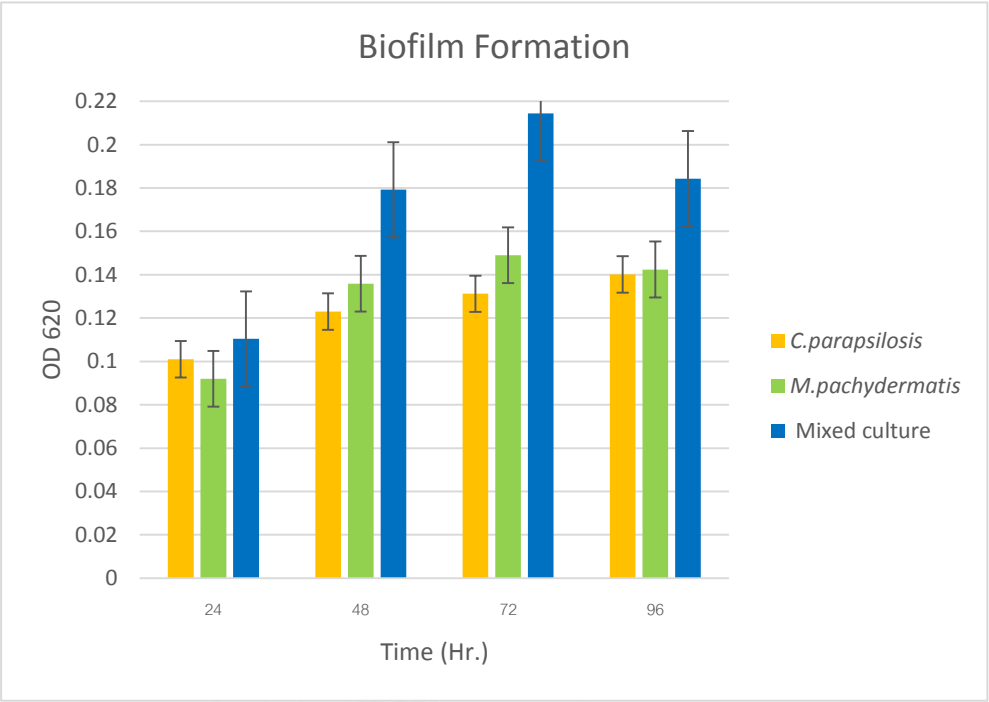


Figure 8. Comparison between mono-species (*M. pachydermatis and C. parapsilosis)* with mixed culture biofilm on 96 well plate by crystal violet assay under different time (24-96h.). OD value of each single colonization and each co-colonization groups were presented by mean values with standard deviation (SD) by error bars.

4.5. Ultrastructural Observation by Scanning Electron Microscope (SEM)

Biofilms were developed on polyethylene IV catheter as a vehicle. At 24 hour of colonization, free living cell was presented without biomass sheath and an initial connection by extracellular matrix (EPS) was observed among outer membranes (Fig. 9). At 48-96 hour, the extracellular matrix constructed adhesion was serially thickened and covered over all colonizing communication as presents in three dimensional structures (Fig. 10).

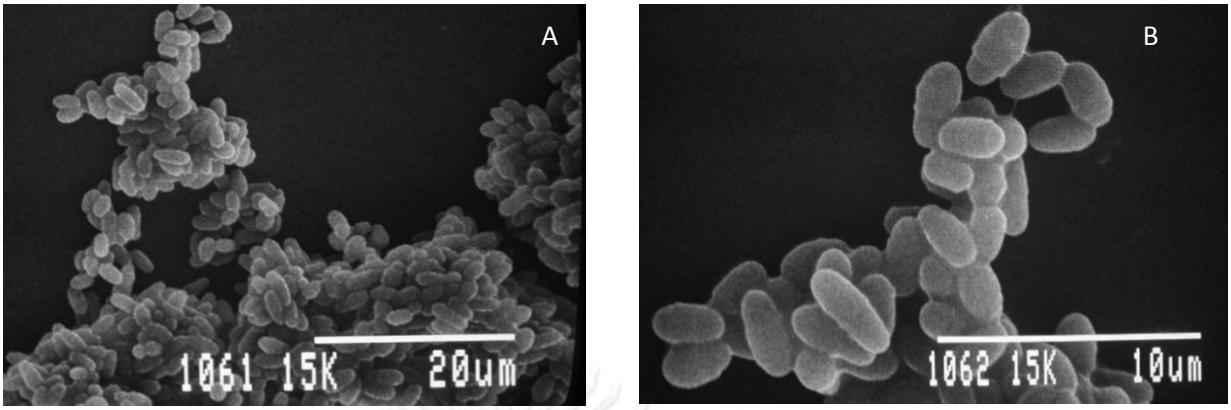


Figure 9. Biofilm productions of M. pachydermatis at 24h (A) and (B) visualized by SEM, Biofilm were constructed in thin layer of collarets yeast cells at 5,000 times magnification.

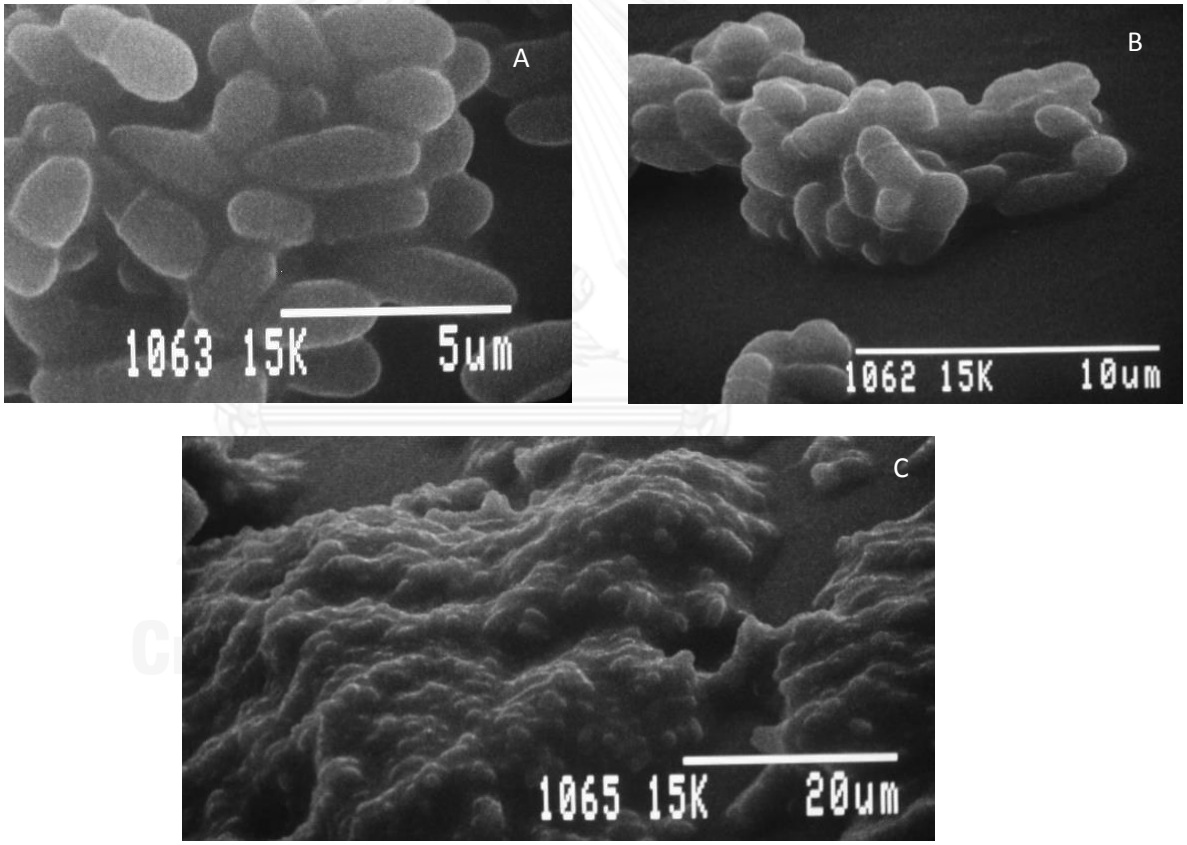


Figure 10. Biofilm production of *M. pachydermatis* at 48h (A) and 72h (B), showed cell covered with thin layer biomass. Biofilm production of *M. pachydermatis* at 96h (C) is constructed in EPS multi-layer at 5,000 times magnification.

For *C. parapsilosis,* a number of oval cells and budding blastoconidia with and without thin EPS layers was demonstrated by SEM at 24 hour (Figure11).

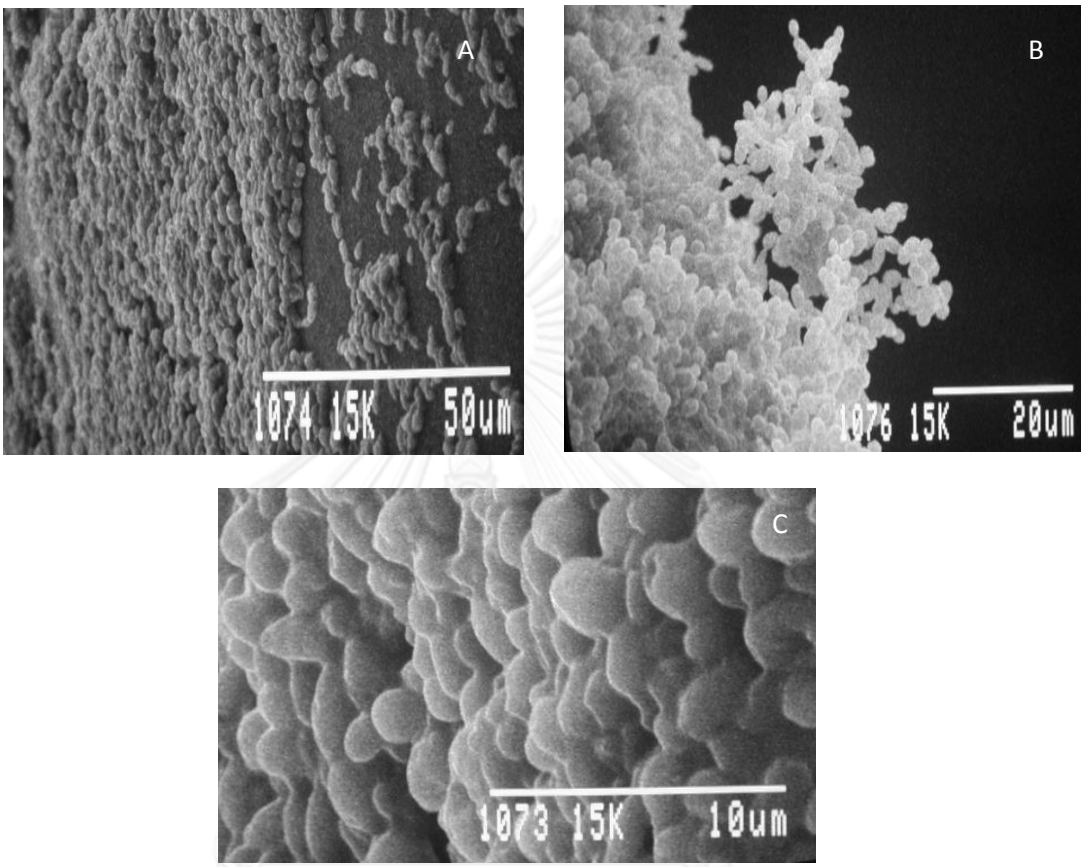


Figure 11. Presence of initial growth of *C. parapsilosis* at 24 hour containing free living cell (Planktonic) (A) with budding characteristic (B) and the cell covered by Thin EPS layer at a condense area (C) by SEM at 5,000 times magnification.

At 48-96 hour, *C. parapsilosis* showed amorphous structure with cell liked protrusion covered by thick EPS layers (Fig.12).

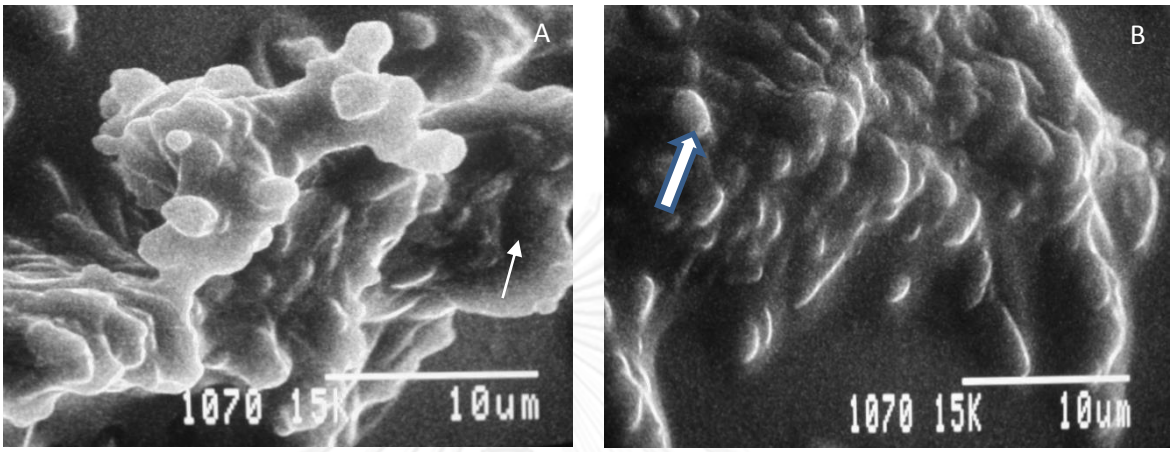


Figure 12. Biofilm production of *C. parapsilosis* at 48 hour (A) and 96 hour (B) constructed in thick layers of cells with abundant EPS at 5,000 power magnification. The thin and thick arrows indicate extracellular material and blastoconidia, respectively.

For co-cultured biofilm, there were a variety of shape among community consisting oval, round *(C. parapsilosis)*, bottle shape (*M. pachydermatis*) and budding between mother and daughter cells at 24 hour and also a mild connecting fibrillar extracellular materials was observed (Fig.13). At 48 hour, biofilms from co-colonization apparently presented thicker layers of EPS over abundant cells which could not be distinguished by cell-size and shape anymore. At 72h, both *M. pachydermatis* and *C. parapsilosis* were bedded under the highly produced extracellular material with amorphous architecture (Fig.14).

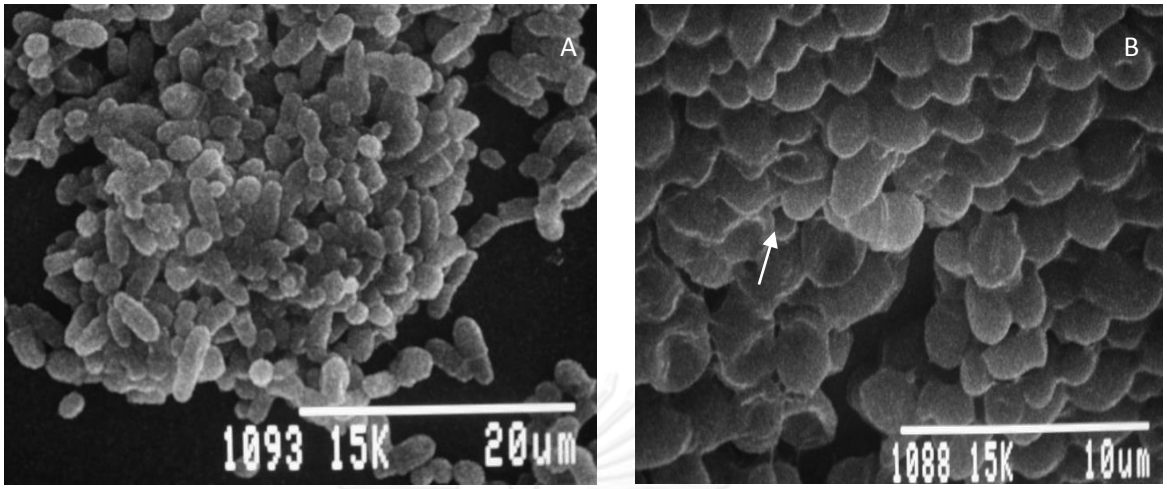


Figure 13. Biofilm produced by co-colonization of *M. pachydermatis* (yeast with collarets) with *C. parapsilosis* (round cell yeast) at 24h under SEM at 5,000 power magnification. The thin arrow indicates the fibrillar extracellular material.

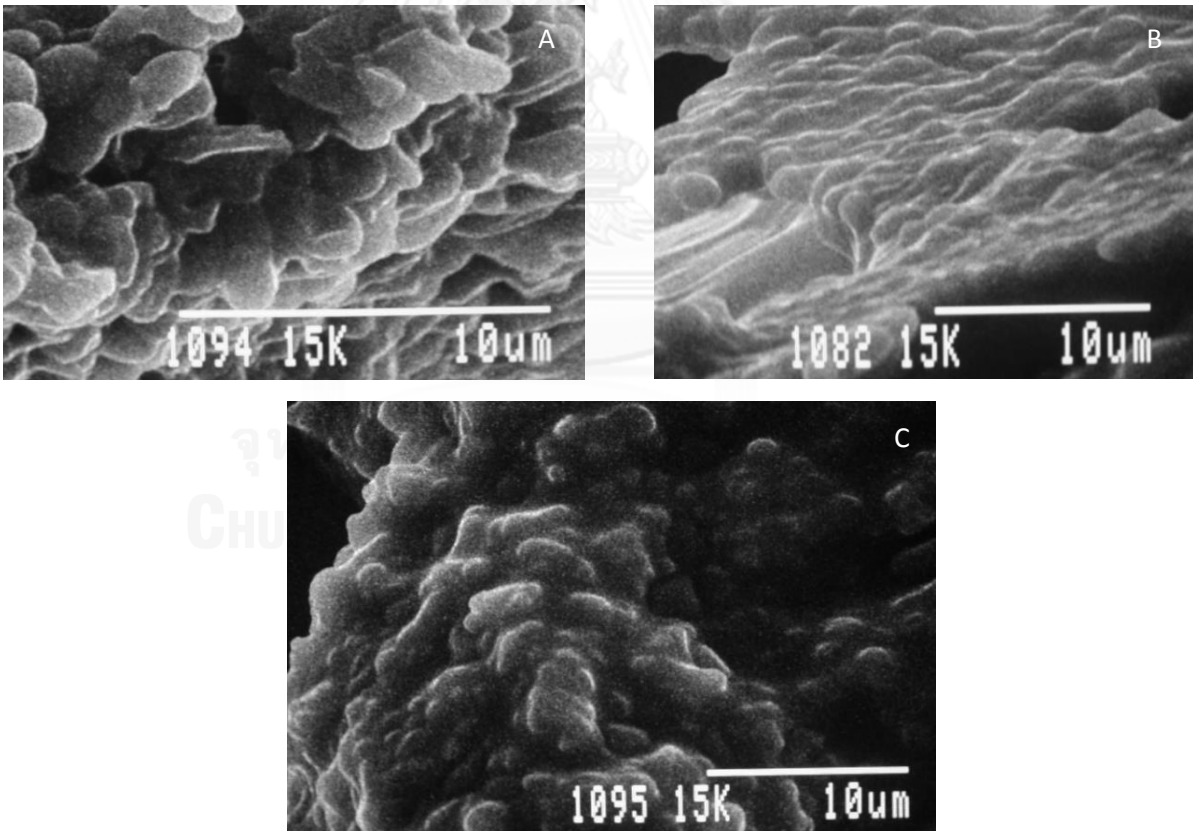


Figure 14. Biomass production of co-cultured biofilm at 48 (A), 72 (B) and 96 hours (C) constructed in multi-layers EPS over embedded abundant cells at 5,000 power magnification.

4.6. Antifungal Drug Susceptibility Test of Biofilm

The MIC values of biofilm of *M. pachydermatis* and *C. parapsilosis* to itraconazole and ketoconazole were determined as described by Figueredo and colleagues (2013). Amount of alive cells after exposed to antifungals were presented by reduction of 50% colorimetric value compared to the biofilm control without antifungal exposure (SMIC50). For free-living or planktonic form, all isolates were highly susceptible to itraconazole and ketoconazole at 0.03 µg/ml. By contrast, all sessile form in biofilm of either *M. pachydermatis* or *C. parapsilosis* were interpreted in resistant range (>16 µg/ml.) (Table 6).

Table 6. Comparison of the MIC values of *M. pachydermatis* and *C. parapsilosis* between planktonic and sessile forms against ketoconazole and itraconazole.

Yeast species	Cell characteristic	MIC50 (µg/ml.)	
		Ketoconazole	Itraconazole
M. pachydermatis	Planktonic form	0.03	0.03
	Sessile form	> 16	> 16
C. parapsilosis	Planktonic form	0.03	0.03
	Sessile form	> 16	> 16

There was no difference of MIC levels determining between *M. pachydermatis* and *C. parapsilosis,* or between source of isolation (SD dogs and healthy). Comparing between biofilm produced co-colonization and single colonization, there was on difference of the values. Most of groups were in high resistance MIC at >16 µg/ml but only group 4 at 2 µg/ml. However, the end point

over 16 µg/ml of SMIC50 was not continually detected (Table 7). The details are shown in appendix B

Table 7. The MIC values of co-cultured *M. pachydermatis* and *C. parapsilosis* biofilm collected from healthy and seborrhea dermatitis skin dog against itraconazole and ketoconazole.

Groups	Origin of isolates	Itraconazole	Ketoconazole
		SMIC 50	SMIC 50
1	MH10.1P+CH10.1E	>16	>16
2	MH11.1C+CH2.4i	>16	>16
3	MH1.2E+CH9.1P	>16	>16
4	MSD9.4Ab+CH12.5i	2	2
5	MSD7/1+CH2.4Pi	>16	>16
6	MH11.1C+CSD1.1E	>16	>16
7	MH10.1P+CSD1/1	>16	>16
8	MSD4/1+CSD2/1	>16	>16
9	MSD1/3+CSD1.1E	>16	>16
10	MSD2/1+CSD3/1	>16	>16

MH; *M. pachydermatis* collected from healthy skin dog., MSD; *M. pachydermatis* collected from seborrhea dermatitis skin dog., CH; *C. parapsilosis* collected from healthy skin dog., CSD; *C. parapsilosis* collected from seborrhea dermatitis skin dog.

CHAPTER V
DISCUSSION

Seborrhea dermatitis is a clinical symptom following allergic skin disease of dogs, which is associated with a complexity of reaction consisting among impaired host immunity, microbial infectivity, allergen and inappropriate environments (Yurayart et al., 2011). On the basis of microbial, microenvironment on skin lesion had a major co-existing between *M. pachydermatis* and *C, parapsilosis* community in 1,000 times higher number than in normal condition (Yurayart et al., 2011). Their relationships affecting pathogenesis, such as ability of biofilm production and antifungal resistant trait were fulfilled in our study.

All tested yeasts was initially confirmed their basic characteristics in term of physiological properties and pathogenesis. The results suggested that such a low genetic variation among the *M. pachydermatis* either derived from healthy or diseased dogs was found (1A; n=19, 3D; n = 1) but both *M. pachydermatis* and *C. parapsilosis* could highly produce phospholipase in the same level. Thus, all had a pathogenic potential on dog skin and could be the representative for our further study. However, the strain belonged to genotype 3D was firstly confirmed in our country, which ever found in canine skin lesions in Japan and Taiwan (Kobayashi et al., 2011). Because of major detection of 1A genotype, it differed from a major genotype distribution (1B) in the previous report, which reflected in different geographic origin and pet management (Machado et al., 2010). It was the reason why 3D strain was chosen for all experiments in comparison to the results from the selected 1A strains. Regarding a high phospholipase activity, the enzyme seemed to be an essential protein for basic living on host skin, while a high production of

phospholipase was ever found in the isolates derived from diseased dog (Cafarchia and Otranto, 2004). To confirm this hypothesis, there is needed to determine in a higher number together with simultaneous detection to other enzymatic assays.

Use of polyethylene catheter as vehicle for SEM manipulation successfully illustrated ultrastructure of cell producing biofilm and attaching to catheter mimicked an adhesive model in medical devices (Yasuda et al., 1999). We clearly demonstrated that *M. pachydermatis* and *C. parapsilosis* were capable to produce biofilm with various architectural structure and biomass quantity. All selected *M. pachydermatis* and *C. parapsilosis* collected from both healthy and SD dogs had the ability to form biofilm, similarly. Thus, the capability to biofilm production is more likely to be an intrinsic characteristic (Silva et al., 2011; Figueredo et al., 2012). Moreover, biofilm has been reported as the causative agent of human atopic dermatitis since it was proposed to be the cause of eccrine gland obstruction (Allen and Mueller, 2011). EPS and microorganisms were able to occlude in eccrine gland secretion resulting in the inflammation and skin pruritus (Allen et al., 2014). Since *M. pachydermatis* showed the highest biofilm formation at 72 hour and decreased at 96 hour by biomass measurement. It is likely that the values were serially raised from lag phase to exponential phase and reduced at stationary phase in associate with growing period (Mireles et al., 2001). As well as the faster growth rate of *C. parapsilosis*, their biomass at adhesion phase were detected earlier than that of *M. pachydermatis* (Silva et al., 2011). On the basic of growth phase, our new finding could support that evidence of higher *C. parapsilosis* population in primary (acute) SD and then replaced with *M. pachydermatis* in secondary (chronic) SD (Yurayart et al., 2011).

This study was firstly demonstrated the co-colonization between *M. pachydermatis* and *C. parapsilosis* with mutual biofilm production. The synergistic effect producing biofilm of other yeast species were similar to the previous evidences tested by clinical isolates (Filoche et al., 2004; Yamada et al., 2005; Burmolle et al., 2006; Elias and Banin, 2012). In co-colonization, no difference of biofilm produced isolates from both SD lesions or normal skin confirmed a higher influence of host defect induced SD lesion than yeast factor (Figueredo et al., 2012). The symbiotic effect during co-colonization was speculated by the capability among different species largely depended on cell surface component providing the adhesion area of one microorganism to another. In case of bacterial co-colonization, *Porphyromonas gingivalis* was ever used as a model of synergistic co-colonization with *Treponema denticola* in oral cavity (Yamada et al., 2005). In case of yeast co-colonization, *C. albicans* increased biomass production when inoculated with *C. glabrata* (Pathak et al., 2012). Gene up-regulation the first one enhanced the cell wall integrity, production of extracellular material, and recruitment of another microorganism into biofilm community due to adhesive and intercellular signaling properties (Kuboniwa et al., 2006). This hypothesis could be supported by the SEM pictures that *C. parapsilosis* was the base of biofilm mass within 24 hour, *M. pachydermatis* was recruited at biofilm mass, *in situ*, within 48-72 hour. Eventually, co-existence of the yeasts were observed as sessile structure within biofilm house. There was no antagonistic effect among the experimental groups, thus mutualism between *M. pachydermatis* and *C. parapsilosis* might be explained in their symbiosis, *in vitro*. However, demonstration of ultrastructure could not be numerically analyzed the level of biomass in biofilms. Use of crystal violet staining could reveal

the level by the colorimeter tool, the result clearly confirmed by the higher biomass production from co-colonized *M. pachydermatis* and *C. parapsilosis* in comparison to single colonization. A disadvantage of the staining assay was undifferentiating between livings or dead cells at time of observation. However, detection of biofilm product at adhesive materials was paid attention rather than cell viability.

For antifungal susceptibility, the result was quite consistent to many previous biofilm reports indicating an increasing of resistant level of biofilm produced sessile yeast comparing to the planktonic cells (Chandra et al., 2001; Mah and O'Toole, 2001; Burmolle et al., 2006; Hoiby et al., 2010; Figueredo et al., 2013a). The yeast harboring in biofilm would be protected from antifungals by the barrier-associated effect of EPS (Figueredo et al., 2013a) that ever reported in *Candida* spp. This barrier contains the high components of EPS composing of the complexity among protein, phosphorus and carbohydrate (Ramage et al., 2009). One of the most important carbohydrate compositions, β-1, 3 glucans, was increased during the biofilm forming process, but it could not be detected from free living cell. Structure of β-1,3 glucans was designated as "drug sponge" regarding to the antifungal sequestration ability, which leaded to the reduction of the activity of azole against *C. albicans* (Ramage et al., 2012). However, a number of sessile cells within biofilm (higher than10^6 CFU/ml), was at least 1,000 time higher than the planktonic cell number (10^6 CFU/ml), thus difference of these initial concentrations might influence to MIC values. This confounding factor could be omitted since we majorly focused on existence of biofilm in an equal unit, therefore it seemed an over control case in term of yeast counting beneath biofilm structure (Perumal et al., 2007). The mechanisms involving drug efflux pump inducing resistance was also feasible as previous described in

Candida biofilm by increasing of overexpression of membrane which impaired the activity of azole drug by inducing of the various stress response and the persistence of yeast (Ramage et al., 2012; Taff et al., 2013). In this study, MICs level of biofilm produced symbiont was in high resistant level as well as the single colonization. This evidence also supported no antagonistic hypothesis during yeast defensive under the biological habitat (Al-Fattani and Douglas, 2006). However, at least over 512 times of the MIC value from free living, was dramatically detected from all biofilms groups apart from group 4. It might implied how difficult of fungal treatment, *in vivo*, in term of time and dose administration and their ability to long-lived persistence on dog skin. Additionally, we did not found any resistant free-living yeast that was the representative of regular form in susceptibility determination (Yurayart et al., 2013). Thus, it should be a controversy of correlation between laboratory and clinical outcomes.

This study successfully revealed an *in vitro* microbial community model that might imply their symbiosis between *M. pachydermatis* and *C. parapsilosis* on clinical lesion. Colonization of symbiont generates their biological protection and antifungal invasion rather the single species and free-living cell. The evidence in this thesis can be an awareness in veterinary practice and veterinary public health, especially how to handle and challenge with the symbiont. The information also provided an impact of biomass during colonization of either single or symbiont to treatment and eradication. As *M. pachydermatis* and *C. parapsilosis* are yeasts associated with the canine SD, two of them can colonize and synergistically produce the biomass. Moreover, the appearance of maximal biofilm at 72 hour was definitely consistent to the indication of certain medical devices replacement preventing

persistent infection such as catheterization by intravenous and urinary catheter devices (O'Grady et al., 2011; Miller et al., 2012). For clinical use, we are paying attention on two viewpoints; systemic and superficial colonization. Once, there is appearance of biofilm forming in an internal device, such as artificial heart value or bone joint, it is needed to be suddenly removed because of incurable of antifungals in regular dosage. Regarding superficial infection, it is a higher possibility of symbiont forming biofilms and tends to be more difficulty to treatment by oral administration. Therefore, use of topical therapies is thought to remove the matrix of EPS and biomass by using sugar ester mixed in medical shampoo or ointment. In summary, our study demonstrated the possible relationship among commensal yeasts associated canine SD, which could be adapted for other multispecies producing biofilm modeling and provided the clarification of the mutual symbiont harboring in biofilm community. This finding also confirmed a dramatic resistance of yeasts beneath biofilm production.

Conclusion

1. The symbiont between *M. pachydermatis* and *C. parapsilosis* from dog skin was mutually produced biofilm, *in vitro.*
2. Antifungal susceptibility value to azole was tremendous increased by biofilm produced condition in both single and co-colonization.

Advantages of this study

1. Enlightening the biological relation between the dominant canine skin yeasts; *M. pachydermatis* and *C. parapsilosis* in term of mutual biofilm production.
2. Providing an awareness of yeast producing biofilm impact to treatment failure.

APPENDICES

APPENDIX A

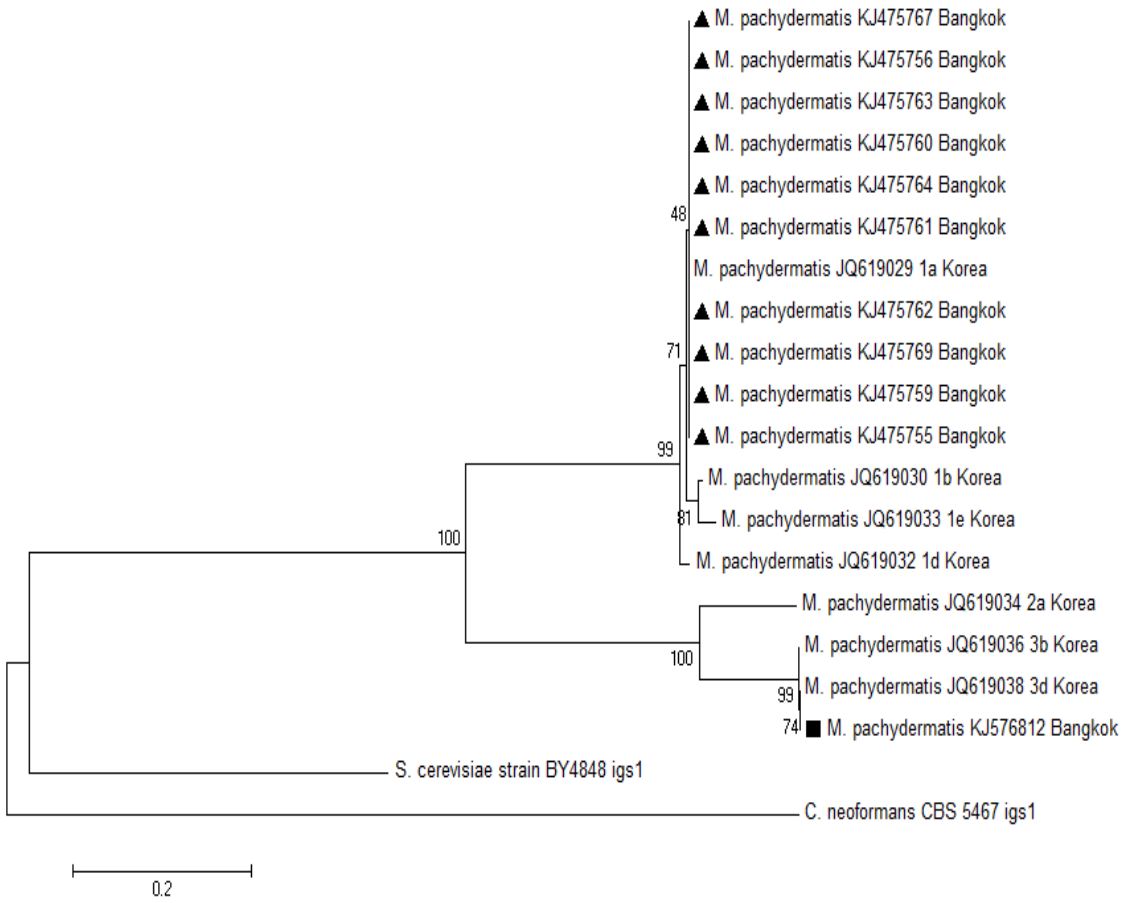


Figure 15. Phylogenetic analysis base on DNA sequences of intergenic spacer 1 (IGS1) of 11 representative strains in this experiment using the Neighbor-joining method (Kimura-2 parameter). The symbols in front of each strain indicated: *M. pachydermatis* genotype 1a (■); *M. pachydermatis* genotype 3d (▲).

APPENDIX B

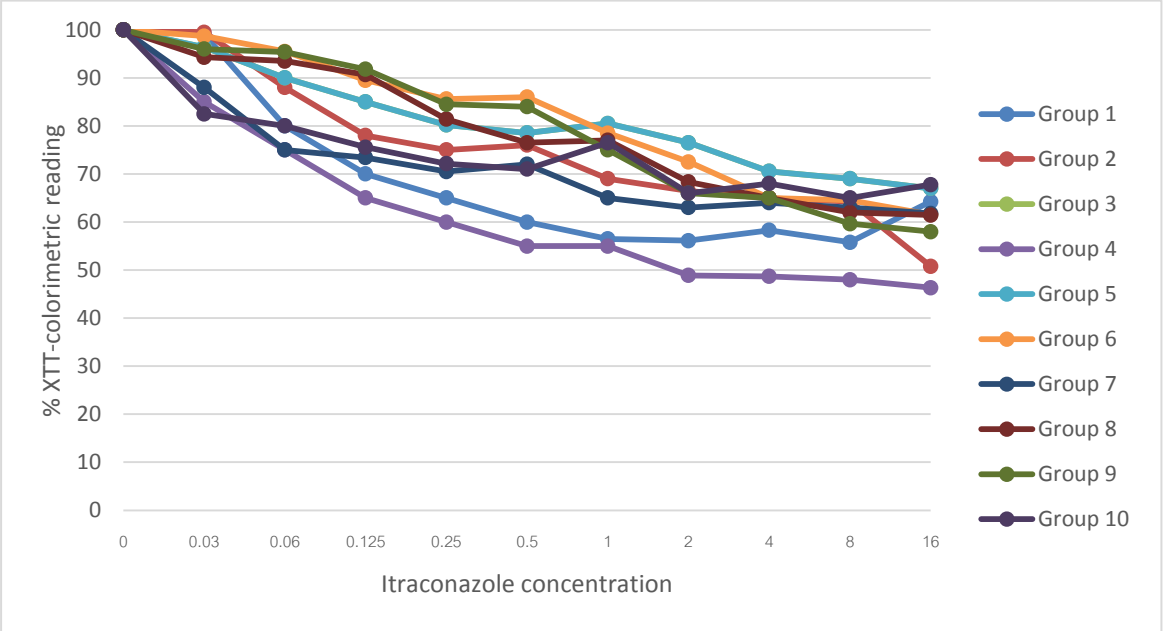


Figure 16. Activity of different itraconazole concentrations against co-cultured biofilms. Values are showed as average percent colorimetric readings for XTT-reduction assays as compared to control wells. Group 1-3 were the co-cultured of *M. pachydermatis* and *C. parapsilosis* from healthy dog skin, group 4-6 were the co-cultured of *M. pachydermatis* and *C. parapsilosis* from SD dog skin, group 7-10 were the co-cultured of *M. pachydermatis* and *C. parapsilosis* from SD and healthy skin dogs.

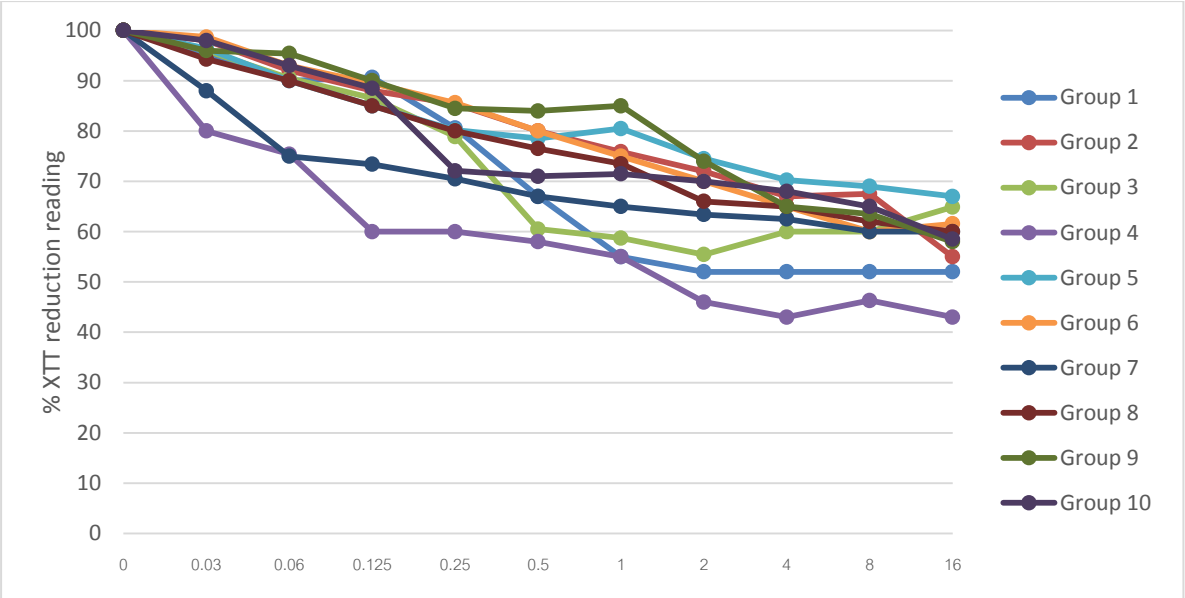


Figure 17. Activity of different ketoconazole concentrations against co-cultured biofilms. Values are showed as average percent colorimetric readings for XTT-reduction assays as compared to control wells. Group 1-3 were the co-cultured of *M. pachydermatis* and *C. parapsilosis* from healthy dog skin, group 4-6 were the co-cultured of *M. pachydermatis* and *C. parapsilosis* from SD dog skin, group 7-10 were the co-cultured of *M. pachydermatis* and *C. parapsilosis* from SD and healthy skin dogs.

APPENDIX C

Nucleotide sequences

1. *M. pachydermatis* isolate KJ475755

26S-5S ribosomal RNA intergenic spacer1, partial sequence, region 796 nucleotide; GenBank: KJ475755

TCTGTCTCCT AGGCAATGTA GGTGTGTATC TCTATGGAGG CCGTCTGCGA AGTGTGGTGT GTGTAGCAAT GTAGGTGTGT ATGTATACTA GAATAAGATC CGATTCTTCC TTGTCCTCTG ACTTGGCCAA GTCCGTGCGC GCCCTGAATA CAATAAGAAT TTTTTTTTTT TTTAGATAGT GTCACACTAG ACTGTCTAAA TCCATTTTAG ACGGACTCTA TAAAATTAGA TGACAGACCC TTGGAAAATA AAACAAATAG ATAGACCCCC CCATCCATCT AGATACCTCG ACACCCCGTC ACCCCACCGT CACCCCTTTC ACAGTACCAG CATAGTACTC ACATAGCACT ACCACATCTG TCAGCACACA TTCTCGCCAA GTACGCATGC ACAGCGACCG GGATCGATCC GATTCGCTCC TCTTCCAGCC AGGCTCCACA CGATTCGTGT GCATACAAAA AGCACCAGCA CAGCCTCGTT CCTCGTGCAG TGAGTGGGTG TGGGTGTGTC CCCTCCCACC CTAGTTGCAT GGCACAGCAT ACCAACATGC CTTTGCACCA CCACCTTGCA ACACGAGAGA GACCATACAG CATATAACAC ACAGCACAGC ACAGCATAAC ATAACACACA ACACAACACA ATACAGCACA GCATAACATA ACACACAACA CAACACAATA CAACACAGCA TAACATAACA CAACACTCAA CACAACACAC AGCACAGCAT AACATAACAC ACAACACAAC ACAATACAGC ACAGCATAAC ACAACACAAC AACACAACAC AACACA

2. *M. pachydermatis* isolate KJ475756

26S-5S ribosomal RNA intergenic spacer1, partial sequence, region 748 nucleotide; GenBank: KJ475756

CTATGGAGGC CGTCTGCGAA AGTGTGGTGT GTGTAGCAAT GTAGGTGTGT ATGTATACTA GAATAAGATC CGATTCTTCC TTGTCCTCTG ACTTGGCCAA GTCCGTGCGC GCCCTGAATA CAATAAGAAT TTTTTTTTTT TTTAGATAGT GTCACACTAG ACTGTCTAAA TCCATTTTAG ACGGACTCTA TAAAATTAGA TGACAGACCC TTGGAAAATA AAACAAATAG ATAGACCCCC CCATCCATCT AGATACCTCG ACACCCCGTC ACCCCACCGT CACCCCTTTC ACAGTACCAG CATAGTACTC ACATAGCACT ACCACATCTG TCAGCACACA TTCTCGCCAA GTACGCATGC ACAGCGACCG GGATCGATCC GATTCGCTCC TCTTCCAGCC AGGCTCCACA CGATTCGTGT GCATACAAAA AGCACCAGCA CAGCCTCGTT CCTCGTGCAG TGAGTGGGTG TGGGTGTGTC CCCTCCCACC CTAGTTGCAT GGCACAGCAT ACCAACATGC CTTTGCACCA CCACCTTGCA ACACGAGAGA GACCATACAG CATATAACAC ACAGCACAGC ACAGCATAAC ATAACACACA ACACAACACA ATACAGCACA GCATAACATA ACACACAACA CAACACAATA CAACACAGCA TAACATAACA CAACACTCAA CACAACACAC AGCACAGCAT AACATAACAC ACAACACAAC ACAATACAGC ACAGCATAAC ACAACACA

3. *M. pachydermatis* isolate KJ475759

26S-5S ribosomal RNA intergenic spacer1, partial sequence, region 787 nucleotide; GenBank: KJ475759

GTTCTATAGA TTTGTTCGAT TTATCGAACC ACTTCTCTCT CTGTCTCCTA GGCAATGTAG GTGTGTATCT CTATGGAGGC CGTCTGCGAA GTGTGGTGTG TGTAGCAATG TAGGTGTGTA TGTATACTAG AATAAGATCC GATTCTTCCT TGTCCTCTGA CTTGGCCAAG TCCGTGCGCG CCCTGAATAC AATAAGAATT TTTTTTTTTT TTAGATAGTG TCACACTAGA CTGTCTAAAT CCATTTTAGA CGGACTCTAT AAAATTAGAT GACAGACCCT TGGAAAATAA AACAAATAGA TAGACCCCCC CATCCATCTA GATACCTCGA CACCCCGTCA CCCCACCGTC ACCCCTTTCA CAGTACCAGC ATAGTACTCA CATAGCACTA CCACATCTGT CAGCACACAT TCTCGCCAAG TACGCATGCA CAGCGACCGG GATCGATCCG ATTCGCTCCT CTTCCAGCCA GGCTCCACAC GATTCGTGTG CATACAAAAA GCACCAGCAC AGCCACGTTC CTCGTGCAGT GAGTGGGTGT GGGTGTGTCC CCTCCCACCC TAGTTGCATG GCACAGCATA CCAACATGCC TTTGCACCAC CACCTTGCAA CACGAGAGAG ACCATACAGC ATATAACACA CAGCACAGCA CAGCATAACA TAACACACAA CACAACACAA TACAGCACAG CATAACATAA CACACAACAC AACACAATAC AACACAGCAT AACATAACAC AACACTCAAC ACAACACACA GCACAGCATA ACATAACACA CAACACA

4. *M. pachydermatis* isolate KJ475760

26S-5S ribosomal RNA intergenic spacer1, partial sequence, region 699 nucleotide; GenBank: KJ475760

TCCTAGGCAA TGTAGGTGTG TATCTCTATG GAGGCCGTCT GCGAAGTGTG GTGTGTGTAG CAATGTAGGT GTGTATGTAT ACTAGAATAA GATCCGATTC TTCCTTGTCC TCTGACTTGG CCAAGTCCGT GCGCGCCCTG AATACAATAA GAATTTTTTT TTTTTTAGAT AGTGTCACAC TAGACTGTCT AAATCCATTT TAGACGGACT CTATAAAATT AGATGACAGA CCCTTGGAAA ATAAAACAAA TAGATAGACC CCCCCATCCA TCTAGATACC TCGACACCCC GTCACCCCAC CGTCACCCCT TTCACAGTAC CAGCATAGTA CTCACATAGC ACTACCACAT CTGTCAGCAC ACATTCTCGC CAAGTACGCA TGCACAGCGA CCGGGATCGA TCCGATTCGC TCCTCTTCCA GCCAGGCTCC ACACGATTCG TGTGCATACA AAAAGCACCA GCACAGCCTC GTTCCTCGTG CAGTGAGTGG GTGTGGGTGT GTCCCCTCCC ACCCTAGTTG CATGGCACAG CATACCAACA TGCCTTTGCA CCACCACCTT GCAACACGAG AGAGACCATA CAGCATATAA CACACAGCAC AGCACAGCAT AACATAACAC ACAACACAAC ACAATACAGC ACAGCATAAC ATAACACACA ACACAACACA ATACAACACA GCATAACATA ACACAACAC

5. *M. pachydermatis* isolate KJ475761

26S-5S ribosomal RNA intergenic spacer1, partial sequence, region 666 nucleotide; GenBank: KJ475761

TTCCTTGTCC TCTGACTTGG CCAAGTCCGT GCGCGCCCTG AATACAATAA GAATTTTTTT TTTTTTTAGA TAGTGTCACA CTAGACTGTC TAAATCCATT TTAGACGGAC TCTATAAAAT TAGATGACAG ACCCTTGGAA AATAAAACAA ATAGATAGAC CCCCCCATCC ATCTAGATAC CTCGACACCC CGTCACCCCA CCGTCACCCC TTTCACAGTA CCAGCATAGT ACTCACATAG CACTACCACA TCTGTCAGCA CACATTCTCG CCAAGTACGC ATGCACAGCG ACCGGGATCG ATCCGATTCG CTCCTCTTCC AGCCAGGCTC CACACGATTC GTGTGCATAC AAAAAGCACC AGCACAGCCT CGTTCCTCGT GCAGTGAGTG GGTGTGGGTG TGTCCCCTCC CACCCTAGTT GCATGGCACA GCATACCAAC ATGCCTTTGC ACCACCACCT TGCAACACGA GAGAGACCAT ACAGCATATA ACACACAGCA CAGCACAGCA TAACATAACA CACAACACAA CACAATACAG CACAGCATAA CATAACACAC AACACAACAC AATACAACAC AGCATAACAT AACACAACAC TCAACACAAC ACACAGCACA GCATAACATA ACACACAACA CAACACAATA CAGCACAGCA TAACAC

6. *M. pachydermatis* isolate KJ475762

26S-5S ribosomal RNA intergenic spacer1, partial sequence, region 699 nucleotide; GenBank: KJ475762

TGTGTATGTA TACTAGAATA AGATCCGATT CTTCCTTGTC CTCTGACTTG GCCAAGTCCG TGCGCGCCCT GAATACAATA AGAATTTTTT TTTTTTTAGA TAGTGTCACA CTAGACTGTC TAAATCCATT TTAGACGGAC TCTATAAAAT TAGATGACAG ACCCTTGGAA AATAAAACAA ATAGATAGAC CCCCCCATCC ATCTAGATAC CTCGACACCC CGTCACCCCA CCGTCACCCC TTTCACAGTA CCAGCATAGT ACTCACATAG CACTACCACA TCTGTCAGCA CACATTCTCG CCAAGTACGC ATGCACAGCG ACCGGGATCG ATCCGATTCG CTCCTCTTCC AGCCAGGCTC CACACGATTC GTGTGCATAC AAAAAGCACC AGCACAGCCT CGTTCCTCGT GCAGTGAGTG GGTGTGGGTG TGTCCCCTCC CACCCTAGTT GCATGGCACA GCATACCAAC ATGCCTTTGC ACCACCACCT TGCAACACGA GAGAGACCAT ACAGCATATA ACACACAGCA CAGCACAGCA TAACATAACA CACAACACAA CACAATACAG CACAGCATAA CATAACACAC AACACAACAC AATACAACAC AGCATAACAT AACACAACAC TCAACACAAC ACACAGCACA GCATAACATA ACACACAACA CAACACAATA CAGCACAGCA TAACACAAC

7. *M. pachydermatis* isolate KJ475763

26S-5S ribosomal RNA intergenic spacer1, partial sequence, region 771 nucleotide; GenBank: KJ475763

TATAGATTTG TTCGATTTAT CGAACCACTT CTCTCTCTGT CTCCTAGGCA ATGTAGGTGT GTATCTCTAT GGAGGCCGTC TGCGAAGTGT GGTGTGTGTA GCAATGTAGG TGTGTATGTA TACTAGAATA AGATCCGATT CTTCCTTGTC CTCTGACTTG GCCAAGTCCG TGCGCGCCCT GAATACAATA AGAATTTTTT TTTTTTTTAG ATAGTGTCAC ACTAGACTGT CTAAATCCAT TTTAGACGGA CTCTATAAAA TTAGATGACA GACCCTTGGA AAATAAAACA AATAGATAGA CCCCCCCATC CATCTAGATA CCTCGACACC CCGTCACCCC ACCGTCACCC CTTTCACAGT ACCAGCATAG TACTCACATA GCACTACCAC ATCTGTCAGC ACACATTCTC GCCAAGTACG CATGCACAGC GACCGGGATC GATCCGATTC GCTCCTCTTC CAGCCAGGCT CCACACGATT CGTGTGCATA CAAAAAGCAC CAGCACAGCC TCGTTCCTCG TGCAGTGAGT GGGTGTGGGT GTGTCCCCTC CCACCCTAGT TGCATGGCAC AGCATACCAA CATGCCTTTG CACCACCACC TTGCAACACG AGAGAGACCA TACAGCATAT AACACACAGC ACAGCACAGC ATAACATAAC ACACAACACA ACACAATACA GCACAGCATA ACATAACACA CAACACAACA CAATACAACA CAGCATAACA TAACACAACA CTCAACACAA CACACAGCAC AGCATAACAT A

8. *M. pachydermatis* isolate KJ475764

26S-5S ribosomal RNA intergenic spacer1, partial sequence, region 739 nucleotide; GenBank: KJ475764

TAGGCAATGT AGGTGTGTAT CTCTATGGAG GCCGTCTGCG AAGTGTGGTG TGTGTAGCAA TGTAGGTGTG TATGTATACT AGAATAAGAT CCGATTCTTC CTTGTCCTCT GACTTGGCCA AGTCCGTGCG CGCCCTGAAT ACAATAAGAA TTTTTTTTTT TTTAGATAGT GTCACACTAG ACTGTCTAAA TCCATTTTAG ACGGACTCTA TAAAATTAGA TGACAGACCC TTGGAAAATA AAACAAATAG ATAGACCCCC CCATCCATCT AGATACCTCG ACACCCCGTC ACCCCACCGT CACCCCTTTC ACAGTACCAG CATAGTACTC ACATAGCACT ACCACATCTG TCAGCACACA TTCTCGCCAA GTACGCATGC ACAGCGACCG GGATCGATCC GATTCGCTCC TCTTCCAGCC AGGCTCCACA CGATTCGTGT GCATACAAAA AGCACCAGCA CAGCCTCGTT CCTCGTGCAG TGAGTGGGTG TGGGTGTGTC CCCTCCCACC CTAGTTGCAT GGCACAGCAT ACCAACATGC CTTTGCACCA CCACCTTGCA ACACGAGAGA GACCATACAG CATATAACAC ACAGCACAGC ACAGCATAAC ATAACACACA ACACAACACA ATACAGCACA GCATAACATA ACACACAACA CAACACAATA CAACACAGCA TAACATAACA CAACACTCAA CACAACACAC AGCACAGCAT AACATAACAC ACAACACAA

9. *M. pachydermatis* isolate KJ475767

26S-5S ribosomal RNA intergenic spacer1, partial sequence, region 680 nucleotide; GenBank: KJ475767

GTGTGGTGTG TGTAGCAATG TAGGTGTGTA TGTATACTAG AATAAGATCC GATTCTTCCT TGTCCTCTGA CTTGGCCAAG TCCGTGCGCG CCCTGAATAC AATAAGAATT TTTTTTTTTT TTAGATAGTG TCACACTAGA CTGTCTAAAT CCATTTTAGA CGGACTCTAT AAAATTAGAT GACAGACCCT TGGAAAATAA AACAAATAGA TAGACCCCCC CATCCATCTA GATACCTCGA CACCCCGTCA CCCCACCGTC ACCCCTTTCA CAGTACCAGC ATAGTACTCA CATAGCACTA CCACATCTGT CAGCACACAT TCTCGCCAAG TACGCATGCA CAGCGACCGG GATCGATCCG ATTCGCTCCT CTTCCAGCCA GGCTCCACAC GATTCGTGTG CATACAAAAA GCACCAGCAC AGCCTCGTTC CTCGTGCAGT GAGTGGGTGT GGGTGTGTCC CCTCCCACCC TAGTTGCATG GCACAGCATA CCAACATGCC TTTGCACCAC CACCTTGCAA CACGAGAGAG ACCATACAGC ATATAACACA CAGCACAGCA CAGCATAACA TAACACACAA CACAACACAA TACAGCACAG CATAACATAA CACACAACAC AACACAATAC AACACAGCAT AACATAACAC AACACTCAAC ACAACACACA GCACAGCATA

10. *M. pachydermatis* isolate KJ475769

26S-5S ribosomal RNA intergenic spacer1, partial sequence, region 784 nucleotide; GenBank: KJ475769

TAAGCCTTTG TTCTATAGAT TTGTTCGATT TATCGAACCA CTTCTCTCTC TGTCTCCTAG GCAATGTAGG TGTGTATCTC TATGGAGGCC GTCTGCGAAG TGTGGTGTGT GTAGCAATGT AGGTGTGTAT GTATACTAGA ATAAGATCCG ATTCTTCCTT GTCCTCTGAC TTGGCCAAGT CCGTGCGCGC CCTGAATACA ATAAGAATTT TTTTTTTTTT TAGATAGTGT CACACTAGAC TGTCTAAATC CATTTTAGAC GGACTCTATA AAATTAGATG ACAGACCCTT GGAAAATAAA ACAAATAGAT AGACCCCCCC ATCCATCTAG ATACCTCGAC ACCCCGTCAC CCCACCGTCA CCCCTTTCAC AGTACCAGCA TAGTACTCAC ATAGCACTAC CACATCTGTC AGCACACATT CTCGCCAAGT ACGCATGCAC AGCGACCGGG ATCGATCCGA TTCGCTCCTC TTCCAGCCAG GCTCCACACG ATTCGTGTGC ATACAAAAAG CACCAGCACA GCCACGTTCC TCGTGCAGTG AGTGGGTGTG GGTGTGTCCC CTCCCACCCT AGTTGCATGG CACAGCATAC CAACATGCCT TTGCACCACC ACCTTGCAAC ACGAGAGAGA CCATACAGCA TATAACACAC AGCACAGCAC AGCATAACAT AACACACAAC ACAACACAAT ACAGCACAGC ATAACATAAC ACACAACACA ACACAATACA ACACAGCATA ACATAACACA ACACTCAACA CAACACACAG CACAGCATAA CATA

11. *M. pachydermatis* isolate KJ576812

26S-5S ribosomal RNA intergenic spacer1, partial sequence, region 685 nucleotide; GenBank: KJ576812

AGCAGTAGAG TAGCCTCGTT GCTACGATCT GCTGAGGCTA AGCCTTTGTT CTATAGATTT GTTCGATTTA TCGAACCACT TCTCTCTCTG TATGCTACGC AATGGAGGTG TGTATCTATT CGTAACCAGA GCGCGAAGTG TGGTACTCGT AGCAATGTAG GTGTGTATGA TATCATATGC TTTGTCAAGT CCGTGCGCGC GCCCTGAATA TAATAAAATA AGTACAAGAC AGTCTCACTA AAATATACGA CTCCTATCTA GGTAGGCACA TACGTTACCT TTGCAGCATT TGTCATACCA ACATAGTACT CACATAGCGC TAGCACCTCT GTCAATACAC AATCTCGCCA AGGACCCACG CGCAGCGACC GGGATCGATC CAATTCGCTC CTCGTCCAGC CAGGCTCCAC ACGATTCGTC CGCGCGGGCG GTGCATACAA TCCAGCAGCC GGCGGCGGCC CACGCCACGG CCATAGTCAT AGTCATAGTC ACAGCACAGC ACAGCACAGC ACAGCACAGC ACAGCACAGC ACAGCACAGC ACAGCACAGC ACAGCACAGC ACCCCTACAA TGCAACGCAA CAACACAACA TCTCATATCC CACGTTACAT ATACACTCTA CCCGGCACAC ATCACCGTTA TCACCCACAC ACACACATCC TCTCCCATCT GCGGCCACAG AACCG

APPENDIX D

Media and reagent

Media for yeast cultivation

1. Sabouraud's dextrose agar (SDA)

Dehydrated SDA agar	65	g
Distilled water	1	L

Mixed well and sterile by autoclaving at 121°C for 15 minutes

2. Yeast extract peptone dextrose broth (YPDB)

Yeast extract	10	g
Bacteriological peptone	20	g
Glucose	20	g
Distilled water	1	L

Sterile by autoclaving at 121°C for 15 minutes

Media for yeast identification

3. Cremophor EL slant

Dehydrated SDA agar	65	g

Cremophor EL	10	ml
Distilled water	1	L

Sterile by autoclaving at 121°C for 15 minutes

4. Tween 60-esculin agar (TE slant)

Glucose	10	g
Yeast extract	2	g
Tween 60	5	ml
Ferric ammonium citrate	0.5	g
Esculin	1	g
Granulated agar	15	g
Distilled water	1	L

Sterile by autoclaving at 121°C for 15 minutes

Buffer solution

5. 0.85% Normal saline

Sodium chloride	0.85	g
Distilled water	1	L

6. 10X Phosphate buffered saline

Sodium chloride	40	g
Potassium chloride	1	g
Disodium hydrogen phosphate	14.5	g
Monopotassium phosphate	1	g
Distilled water	500	ml

pH 7.2 ± 0.2

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