คุณลักษณะของเชื้อยีสต์บนผิวหนังสุนัข ความไวรับต่อยาต้านเชื้อรา และการตอบสนองทางภูมิคุ้มกันของสุนัขต่อเชื้อยีสต์ที่แยกได้จากผิวหนังสุนัขที่มีอาการผื่นไขมันอักเสบ

นางสาวชมพูเนกข์ ยุรญาติ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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CHARACTERIZATION OF CANINE SKIN YEASTS, ANTIFUNGAL SUSCEPTIBILITY AND IMMUNOLOGICAL RESPONSES TO THE YEASTS ISOLATED FROM CANINE SEBORRHEIC DERMATITIS

Miss Chompoonek Yurayart

A Dissertation Submitted in Partial Fulfillment of the Requirements
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Thesis Title	CHARACTERIZATION OF CANINE SKIN YEASTS, ANTIFUNGAL SUSCEPTIBILITY AND IMMUNOLOGICAL RESPONSES TO THE YEASTS ISOLATED FROM CANINE SEBORRHEIC DERMATITIS
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โรคผื่นไขมันอักเสบในสุนัขคือการอักเสบของผิวหนังที่เรื้อรังและซ้ำซากที่พบได้เป็นประจำในสุนัข ซึ่งมีสาเหตุโน้มนำจากระบบภูมิคุ้มกันที่ผิดปกติของโฮสต์และการติดเชื้อยีสต์ฉวยโอกาส การจำแนกความหลากหลายของเชื้อยีสต์บนผิวหนังสุนัข, การประเมินความไวรับต่อยาต้านเชื้อรา และการเปิดเผยการตอบสนองทางภูมิคุ้มกันที่ซ่อนอยู่ในการดำเนินไปของโรคผื่นไขมันอักเสบจะทำให้เข้าใจพยาธิกำเนิดของโรคผื่นไขมันอักเสบในสุนัขได้

ความหลากหลายและขนาดประชากรของเชื้อยีสต์บนผิวหนังสุนัขได้จำแนกตามลักษณะของผิวหนังและระยะของโรคแบ่งเป็นเชื้อยีสต์บนผิวหนังสุนัขปกติ, สุนัขผื่นไขมันปฐมภูมิ และสุนัขผื่นไขมันทุติยภูมิ เชื้อมาลาสซิเซีย พาไคเดอร์มาติส และ แคนดิดา พาราซิโลซิส เป็นเชื้อประจำถิ่นหลักที่พบบนผิวหนังสุนัขปกติ การอยู่อาศัยร่วมกันของเชื้อยีสต์ทั้งสองทั้งในแง่ความถี่และจำนวนประชากรที่มากนั้นมีความเกี่ยวข้องกับระยะของโรคผื่นไขมันอักเสบ แต่ยังไม่พบความแตกต่างอย่างมีนัยสำคัญของอัตราการดื้อยาต้านเชื้อราระหว่างเชื้อยีสต์ที่เพาะแยกจากสุนัขป่วยและสุนัขปกติ ยาคีโตโคนาโซลและไอตราโคนาโซลคงมีประสิทธิภาพสูงในการกำจัดเชื้อมาลาสซิเซีย พาไคเดอร์มาติส แต่อัตราการดื้อต่อยาคีโตโคนาโซลของเชื้อแคนดิดา พาราซิโลซิสเพิ่มสูงขึ้น การศึกษาอิมมูโนกลอบูลิน จี ซับคลาสสามารถใช้แยกแยะรูปแบบการตอบสนองทางภูมิคุ้มกันต่อโปรตีนเชื้อยีสต์ ในแต่ละระยะของสุนัขที่มีอาการโรคผื่นไขมันอักเสบได้ และได้มีการระบุโปรตีนที่เป็นแอนติเจนหลักที่มีการตอบสนองด้วยอิมมูโนกลอบูลิน จี ซับคลาสซึ่งอาจจะสะท้อนถึงการเป็นแอนติเจนสำคัญในการเหนี่ยวนำการเกิดหรือการควบคุมโรคได้

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CHOMPOONEK YURAYART: CHARACTERIZATION OF CANINE SKIN YEASTS, ANTIFUNGAL SUSCEPTIBILITY AND IMMUNOLOGICAL RESPONSES TO THE YEASTS ISOLATED FROM CANINE SEBORRHEIC DERMATITIS. THESIS ADVISOR: ASST. PROF. NUVEE PRAPASARAKUL, D.V. M., Ph.D., THESIS CO-ADVISOR: ASSOC. PROF. SANIPA SURADHAT, D.V. M., Ph.D., PROF. SUSUMU KAJIWARA, Ph. D., 94 pp.

Canine seborrheic dermatitis (SD) is the common chronic relapsing inflammatory skin condition; triggered by susceptible host-mediated immune responses and opportunistic yeast infection. Characterization of canine skin yeast diversity, antifungal susceptibility determination and uncovering of underlying immune responses on SD progression may take a step forward in pathogenesis of canine SD.

Diversity and population size of canine skin yeasts were characterized corresponding to skin conditions and stages of SD; healthy, primary SD (PSD) and secondary SD (SSD). *Malassezia pachydermatis* and *Candida parapsilosis* were majority population of canine skin yeast flora. The co-colonization of these yeasts in large amount and frequency associated with stages of SD. There were no significant differences in the yeasts resistant rates between SD and healthy dogs. Ketoconazole and itraconazole were still efficacious for *M. pachydermatis* but a high rate of KTZ resistant was reported in *C. parapsilosis*. Immunoglobulin G subclass could differentiate the responsive patterns on each progression of disease and immunodominant antigens to IgG subclasses were identified and may reflect the important components inducing or control of the disease.

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LIST OF ABBREVIATIONS

5-FC	=	5-fluorocytosine
ANOVA	=	Analysis of Variance
BMD	=	broth microdilution method
CFU	=	Colony forming unit
CLSI	=	Clinical Laboratory Standard Institute
DD	=	Disk diffusion method
IgG	=	Immunoglobulin G
IgE	=	Immunoglobulin E
ISO	=	International organization for standardization
ITZ	=	Itraconazole
KTZ	=	Ketoconazole
MFC	=	Minimal fungicidal concentration
MIC	=	Minimal inhibitory concentration
NYS	=	Nystatin
PCR-RFLP	=	Polymerase chain reaction-restricted fragment length polymorphism
PSD	=	Primary seborrheic dermatitis
SAS	=	Statistical Analysis System
SD	=	Seborrheic dermatitis
SEM	=	Standard error of the mean
SSD	=	Secondary seborrheic dermatitis
TERB	=	Terbinafine

CHAPTER 1

1.1. INTRODUCTION

Seborrheic dermatitis (SD) is the chronic relapsing inflammatory skin condition associated with seborrhea, immune reaction and opportunistic infection. SD is very common canine skin disease but none of yeast distributions and frequencies have been reported due to lacking of validated criteria for diagnosis or grading of clinical signs (Naldi and Rebora, 2009). Despite of the high prevalence, the etiology of yeasts associated canine dermatitis is still not elucidated. Routinely, the diagnostic criteria are based on the history, clinical examination and methylene blue staining. The predisposing cause of disease are associated with weakness of skin condition including skin damage, ectoparasite infestation, imbalance of skin flora microenvironment causing by antimicrobial or corticosteroid therapy and immune deviation such as a hypersensitivity. Generally, the severity of symptoms are variable ranged from mild skin scaling to marked acute stage; erythematous, seborrhea, dandruff, mal-odor, itching and chronic stage; hyperpigmentation, lichenification and self-induced alopecia. Up to date, there is no available specific treatment. Dogs usually show good improvement after administration of antimycotics and anti-inflammation treatments. Worsening of post-treatment is not only a recurrent of SD but also side effect from long standing treatment and costly remedy. (Batra, et al., 2005; Chen and Hill, 2005; Peano and Gallo, 2008). However, need of understanding of pathogenesis will advance the development of more suitable treatments and prevention in canine SD. Drugs in azoles group; ketoconazole and itraconazole are favorite choice of SD treatment with the recommendation of 5-10 mg/kg daily for at least 3 weeks long (Negre, et al., 2009). Due to the recommendation of long-term antifungal drug treatment and relapsing clinical aspects of SD, there is a risk of antifungal drug resistant development. Several previous studies could be able to detect the occurrence of *Malassezia* and *Candida* yeast strains isolated from dogs that resisted to few of available antifungal drugs (Brito, et al., 2007; Eichenberg, et al., 2003; Nijima, et al., 2010). Testing of antifungal drug efficacy against clinical yeast isolates from Thai dogs will provide a proper use of veterinary practitioner.

The skin yeast flora is also act as an opportunistic pathogen since triggering by those of the influential predisposing factors leading to eliciting of SD. (Gupta, et al., 2004). Previously, *Malassezia pachydermatis* were known as canine skin commensals (Kennis, et al., 1996; Uchida, et al., 1992). Recently, *M. pachydermatis* is not sole yeast associated dermatitis, otitis externa and atopic dermatitis (Chen and Hill, 2005) but also other yeasts including *C. parapsilosis, C. tropicalis, C. albicans, Saccharomyces cerevisiae* and *Rhodotorula* spp. Interestingly, these can be recovered from both diseased and healthy dogs (Brito, et al., 2009). On the other hand, several species of *Candida* spp. are also involved in canine dermatomycosis and candidal cystitis (Kano, et al., 2002; Moretti, et al., 2006; Mueller, et al., 2002). Moreover, there was a report showing a recurrent infection of *C. albicans and M. pachydermatis* in lethal acrodermatitis and might concerned with T-cell dysfunction dogs (McEwan, 2001). Therefore, thought of recurrent infection with *M. pachydermatits* and other yeasts associating canine dermatitis may aid successful of dermatoses treatment and prevention (Peano and Gallo, 2008). Nowadays, there has not been a conclusion of types and population size of yeasts on dog healthy and diseased skin. Thus, understanding of microbial environment on dog skin will be advanced for primarily discovering the pathogenesis explanation.

The interaction between yeasts and a susceptible host is an influential factor leading to skin abnormality and is used for an explanation of severity for SD. The SD and skin yeast are majorly correlated with host-mediated immune responses to yeast allergens (Brakhage, et al., 2008). SD and atopic dermatitis with yeast proliferation dogs is frequently found to develop significantly higher IgE titers against crude *M. pachydermatis* extract comparing to that of healthy (Bond, et al., 1998; Bond and Lloyd, 2002; Chen, et al., 2002). Several studies have been successful in uncovering of *M. pachydermatis* components as major allergens and have been implied the significant roles in atopic dermatitis (Bond and Lloyd, 2002; Chen, et al., 2002). Role of IgG is still controversial; few studies in dogs suggested the development of serum specific-IgG and cellular immunity against only *Malassezia* antigen in normal subjects and took an increasing level in the diseased dogs (Bond, et al., 1998; Bond, et al., 2004; Bond, et al., 2006), thus the specific IgG may be only a non specific response and accelerates in yeast overgrowth condition. Nevertheless, there was no report of *Candida* or other yeast species inducing an improper immune responses in canine

SD. Whereas, IgG titer level between to *Malassezia* patient with SD and controls was not differed (Ashbee, et al., 1994; Bergbrant, et al., 1991; Parry and Sharpe, 1998) and many researches had focused on the role of both *C. albicans* and *Malassezia* spp. allergens that frequently observed among atopic eczema dermatitis syndrome (Ishiguro, et al., 1992; Kosonen, et al., 2005; Savolainen, et al., 2001; Tanaka, et al., 1994). In addition, *M. furfur*, the causative of Pityriasis versicolor, was associated with Th2 over response, while *C. albicans* was associated with Th1 response (Savolainen, et al., 2001). Recently, the roles of immunoglobulin G antibodies have been more interesting issue due to the better understanding of Th1 and Th2-bias immune mechanism driven in the pathogenesis of hypersensitivity disorders and the discovery of non-IgE response atopic dermatitis (patients revealing the similar clinical signs of immediate-hypersensitization with undetectable serum allergen-specific IgE) (Novak and Bieber, 2003). IgG is known to be the largest density antibodies produced by host and plays important role in protection or inflammation with specific subclasses response to antigens. Each subclass of IgG is differed in conformation and electrophoretic mobility effects on the immunological functions such as ability to bind protein or carbohydrate antigen, fix complement, opsonize for phagocytosis, and attach to receptors on mast cells or basophils (Day, 2007). Previously, the research of IgG subclasses against antigens and allergens were performed in atopic dermatitis and leishmaniosis and suggested role of specific subclasses of IgG involving in house dust mite sensitization and leishmaniosis immune responses (Day, et al., 1996; Hou, et al., 2006; Quinnell, et al., 2003). These also provided a direction of predictable immunological cascade especially during T cell switching. Up to date, the fundamental information of the yeast antigen recognition pattern via specific IgG subclasses and their immunological function associated in normal and SD dogs are still needed. Overall, clarification of yeast colonizing status on various degrees of dog skin and subclass profile of yeast antigen-specific IgG antibodies are significant to provide an enlighten information of yeast-associated canine SD.

1.2. OBJECTIVES OF STUDY

1.2.1. To investigate the diversity of yeast colonizing on canine seborrheic dermatitis compared to that of healthy subjects; in term of species, anatomical sites and population size.

1.2.2. To determine an *in vitro* efficacy of antifungal agents against clinical isolates obtained from Thai seborrheic dermatitis dogs.

1.2.3. To demonstrate the subclass of IgG antibodies specific to the yeast isolates and immunodominant proteins of yeasts associated SD.

1.3. HYPOTHESIS

1.3.1. There are the types, anatomical frequency and population size differences of yeasts colonizing on skin between normal and seborrheic dermatitis dogs.

1.3.2. Various susceptibility levels to antifungal drugs can be found in skin yeast isolated from Thai dogs.

1.3.3. Subclass of IgG can help an explanation of host immune response to yeast- associated SD pathogenesis.

1.4. CONCEPTUAL FRAMEWORK

1.4.1. Determine the diversity and population size of yeast colonizing on skin of healthy and SD dogs using qualitative and quantitative statistical analysis.

1.4.2. Evaluate the efficacy of antifungal drugs against clinical yeasts isolated from Thai dogs.

1.4.3. Evaluate patterns of humoral immunity response to yeast antigens via immunoglobulin G subclasses associated in SD pathogenesis.

1.4.4. Identify the immunodominant yeast antigens recognized by immunoglobulin G subclasses associated in SD pathogenesis

1.5. LITERATURE REVIEW

Seborrheic dermatitis (SD) is defined for the clinical signs of scaling and inflammation mostly occurred in areas of the body rich in sebaceous glands, such as face, ventral neck, ear pinnae, axillae, ventral abdomen, skin folds, interdigital and perianal areas (Chen and Hill, 2005). SD is very common conditions of dermatosis observing by dermatologist and veterinary practitioner. In human, the incidence of SD is around 1-3% in the normal population, but the incidence of SD is much higher, ranging from 30-83% in population of patients who is facing with immunocompromise status (Naldi and Rebora, 2009). The epidemiology of SD is

hard to define in animals due to lacking of validated criteria for diagnosis or grading of severity. SD can be occurred in dogs of any age, sex and breed. SD in dog is mostly observes in the early ages (1-3 years of age) with some predisposing breeds; West Highland white terriers, Basset hounds, dachshunds, cocker spaniels, Shih Tzus and English setters (Plant, et al., 1992). SD may appear localized or generalized; the symptoms can range from mild dandruff to severe acute erythematous, greasy exudation, crusty and varying degrees of scaling. In chronic stage, dogs commonly develop marked lesions of hyperpigmentation and lichenification. Pruritus is mentionable in all stages of SD with variation from mild to extreme (Chen and Hill, 2005). Despite of the high incidence, the etiology and cascade of pathogenesis of SD is remained unclear. Many reports have confirmed the predisposing factors concerning a pathogenesis of SD through the human and murine models. SD is described into two main types as primary heritable SD and secondary acquired SD. Primary heritable SD is less common with the predisposing of genetical disorders conferring cornification; keratinization and epidermal hyperproliferation. On the other hand, secondary acquired SD is mentioned to be more interesting issue due to higher incidence, chance of developing of primary to secondary SD, complexity of pathogenesis, and chance of developing of new diagnosis, treatment and prevention. In the human reports, the etiology of secondary acquired SD is highly controversial since the several factors associated to the conditions such as hormone levels, fungal types, nutritional deficits and neurogenic factors are concerned (Schwartz, et al., 2006). There is the chance of hormonal signal that initiates on and off the abnormalities in infancy. Especially in HIV positives patients, SD is commonly observed and the cellular immunity defect becomes a significant key. In case of the reports in dog, predisposing factors have been poorly characterized. Secondary SD is mainly concerned with skin injuries such as physical trauma, infectious agents, irritation from allergen responses that promotes skin barrier disruption leading to opportunistic bacterial and yeast infection. The common disorders caused by skin barrier damage, SD are a consequence of skin hypersensitization (especially atopic dermatitis, flea-bite allergy, food allergy, contact dermatitis), and dermatophytosis (Chen and Hill, 2005). Initial studies of SD have been started with notice of clinical improvement and reducing in number of yeast organisms after antifungal therapeutic treatment (Gupta, et al., 2004).Then, researchers have been pointed to the

identification of yeast in qualitative and quantitative analysis. Previously *M. pachydermatis* was suggested as a normal flora of dog skin and and *Candida* spp. was known as a commensal lining on mucosa at digestive and urogenital tracts (Bond, et al., 1995; Kennis, et al., 1996). Recently, Brito and colleagues (2009) demonstrated the various types of commensal yeasts on dog skin ranging by the degree of frequency; *M. pachydematis*, *C. parapsilosis*, *C. tropicalis*, *C. albicans*, *Saccharomyces cerevisiae* and *Rhodotorula* spp., respectively. (Brito, et al., 2009). *M. pachydermatis* was believed to be the sole species of yeast and acted as an aggregating factor in cutaneous dermatoses including SD, otitis externa and ophthalmic disease (Chen and Hill, 2005). Interestingly, *M. pachydermatis* and other un-identified yeasts could be recovered with either significant or non-significant higher in areas of the lesion skins (Cabanes, et al., 2005; Cafarchia, et al., 2005; Cafarchia, et al., 2005; Girao, et al., 2006; Nardoni, et al., 2007). These might be varied from difference of subject criterias, sample collection techniques, cultivation and identification methods (Nardoni, et al., 2007). It was also difficult to allow all yeasts grown on the certain appropriate media because negative detection was found on some healthy or diseased dogs, despite being commensal yeast (Nardoni, et al., 2008; Prado, et al., 2008). Up to date, the yeast colonizing status in term of frequency and population size especially associated with degree of skin conditions such as healthy, primary acute and secondary chronic SD had not been clarified. Even the overgrowth of yeast is considered as an aggregating factor in SD but the interaction between yeast and host is believed to be more important for inducing the pathogenicity. In certain dog breeds such as Basset hounds and West Highland white terriers are commonly found to yield the high population of yeasts without skin lesion (Bond and Lloyd, 1997), which are well-known as the predisposing breeds.

Since SD is an suffered episode relating with host mediated immune response to skin flora, the deviation of cell-mediated responses have been confirmed in human patient with yeast-associated SD (Sohnle and Collins-Lech, 1982). As head and neck areas (HND) are preferentially yeast affected, there were the evidences of immediate and delayed hypersensitivity responses to *Malassezia* antigens in those areas of atopy. Regarding to humoral-mediated immune response, the specific IgE was found in all of AD patients with HND and developed the higher levels of *Malassezia*-specific IgE compared with the patients with AD without HND.

Thus, a potential role of *Malassezia* antigen in triggering of type I hypersensitivity responses may cause the clinical severity (Darabi, et al., 2009; Devos and van der Valk, 2000). Similarly, seborrheic dermatitis and atopic dermatitis in dogs were also confirmed to develop the significant higher of IgE level against crude *M. pachydermatis* extract compared to that of healthy (Bond, et al., 1998; Bond and Lloyd, 2002; Chen, et al., 2002). *M. pachydermatis* has been believed as the major allergens in atopic dermatitis pathogenesis (Bond and Lloyd, 2002; Chen, et al., 2002).

Role of IgG antibodies in canine hypersensitivity disorders has been less well defined. Only few reports have been revealed the development of serum specific-IgG and cellular immunity against *Malassezia* antigen in normal and slightly increasing IgG titers in diseased dogs (Bond, et al., 1998; Bond, et al., 2004; Bond, et al., 2006). These might be affected from the antigen preparation, groups of testing dogs or heterogeneity of IgG antibodies population. In a preliminary study of IgG subclasses performed in human and experimental animals revealed that the different physical, chemical, biological properties of IgG were found to be related to the states of disease consequence. The subclass of IgG is the representative of the type of T helper cell responsive in hypersensitivity disorders to allergen in non-IgE response atopic dermatitis (Novak and Bieber, 2003). The immune mechanism response to antigens or allergens is shown in figure 1.1 and described in the paragraph below.

Normally, after allergens expose or penetrate to skin, they are captured by an antigen-presenting cell (APC). When APC presents the antigens to naïve T lymphocytes, subset of T cell differentiation is chosen beneath the influence of the suitable cytokines. Interleukin (IL) 4 stimulates a differentiation of T helper 2 (Th2) cells that provides a help in the development of B cell committing to the production of IgE and IgG1 (in mice). Consequently, IgE-binding mast cells with Th2 cytokines lead to severe inflammation and clinical sign of type I hypersensitivity reactions. On the other hand, under the influence of interferon-gammar (IFN-gamma) and IL-12, Th1 cells are developed and secret various cytokines that induce different subclasses of IgG responses (IgG1 and IgG3 in human and IgG2 in mice). These IgG subclass antibodies may be a protective tool by pathogen clearance or activate complement, provoking inflammation (Chen and Hill, 2005; Day, 2007). IgG is a heterogenous molecules dividing into four subclasses and classify by differences in gamma-chain

sequence and electrophoretic mobility (Shakib and Stanworth, 1980). IgG subclasses were nomenclature according to their decreasing mean serum concentrations as IgG1, IgG2, IgG3 and IgG4. The role of IgG subclasses in human hypersensitivity disorders is still controversial. Allergen-specific responses are predominately by IgG1 and IgG4 subclasses. In humans, the higher levels of IgG4 were found in atopic eczema patients with existence of IgE reactivity to crude *M. sympodialis* extracts (Johansson, et al., 2004). In allergen immunotherapy (hyposensitization), patients had predominantly yielded IgG1 in early stage and switched to high IgG4 production in the late stage. This evidence suggested the down regulation of the Th2 and their cytokines as the effect of hyposensitization (Day, et al., 1996). In dogs, four IgG subclasses are also identified with the similar nomenclature. The mean serum concentration of each IgG subclasses in normal dogs has been reported; IgG1 (8.17 mg/ml), IgG2 (8.15 mg/ml), IgG4 (0.95 mg/ml) and IgG3 (0.36 mg/ml) (Mazza, et al., 1993). IgG subclasses in canine were performed only in atopic dermatitis and leishmaniosis models. Especially, the specific IgG1 and IgG4 were involved in cascade of AD pathogenesis depending on types of allergens (Day, et al., 1996). In addition, the slight higher levels of IgG1 and IgG4 in atopic dogs against house dust mite antigens *(Dermatophagoides farina)* were also demonstrated, but not significant difference in that of healthy group. This suggested that there might be the other IgG subclasses responsible for *D. farina* antigens (Hou, et al., 2006). In leishmaniosis, the disease outcomes are individual variable, based on nature of host immune status. Both Th1 and Th2 were found to up-regulate reflected the different of clinical signs. Th2 up-regulation is predominantly found during acute stage and caused the severe symptoms. The levels of IgE and some subclasses of IgG (IgG1, IgG3, and IgG4 in dogs) were found to be increased and related to the severity. On the other hand, the protective phenotypes as self-healing or asymptomatic were related to Th1-bias of both humoral (some of IgG subclasses; IgG2, IgG1) and cell-mediated immunity. Previous studies were not demonstrated only the role of IgE, but dramatically suggested the role of IgG and their subclasses in cascade of pathogenesis of hypersensitivity diseases (Hou, et al., 2005; Quinnell, et al., 2003). Up to date, nothing is known about the IgG subclasses and their immunological function against yeast antigens involved in SD consequence.

Treatment of SD should be concerned in reducing of yeast proliferation and enhancing of skin strength. Antifungal agents are available in both topical and systemic preparations such as nystatin, amphotericin B, fluorocytocine, terbinafine and azoles. Drugs in azoles group; ketoconazole and itraconazole are favorite choice of SD treatment in dogs with the recommendation of 5-10 mg/kg daily for at least 3 weeks long (Negre, et al., 2009). The recommendation of long-term antifungal drug treatment and prolong of drug exposure as a result of relapsing clinical aspects of SD, there is a risk of antifungal drug resistant development as reported in previous studies. Antifungal drug susceptibility tests against *Malassezia* yeasts isolated from Brazilian otitis external dogs using broth microdilution method were performed by Eichenberg and colleagues in 2003. They suggested the excellent susceptible level of itraconazole and a few isolates of *Malassezia* yeasts being resistant to fluconazole and ketoconazole (Eichenberg, et al., 2003). While study by Nijima and colleagues in 2010 found the first isolate of *M. pachydermatitis* from SD dog that resisted to both ketoconazole and itraconazole (Nijima, et al., 2010). Antifungal drugs susceptibility tests against *Malassezia* and *Candida* yeasts isolated from healthy, otitis external and SD dogs were also reported by Brito and colleagues (2007). They suggested *Candida* yeasts were much more resistant to azole antifungal agents than *Malassezia* yeasts (Brito, et al., 2007)**.**

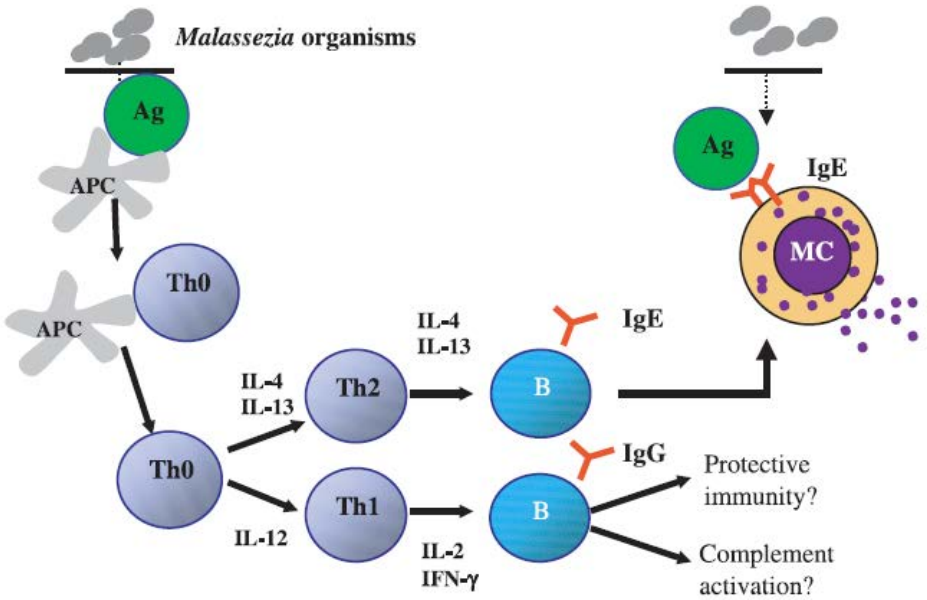


Figure 1.1 The possible pathway of development and function of major CD4+ T cell subsets that response to yeast organisms (Chen and Hill, 2005).

CHAPTER 2

Comparative analysis of the frequency, distribution and population sizes of yeasts associated with canine seborrheic dermatitis and healthy skin

2.1. Introduction

Canine seborrheic dermatitis and otitis externa are common skin diseases in dogs and cats in Thailand. These abnormalities are often a result of atopic dermatitis and the other type 1 hypersensitivities associated with the epidemic of *Malassezia pachydermatis* (Hillier and Griffin, 2001). The quantity of *M. pachydermatis* of atopic dogs was dominant at the sites that rich in sebum as ears, interdigital areas, and groin with no significant manifestation on lesion-free sites (Kennis et al., 1996; Nardoni et al., 2007).

The roles of *M. pachydermatis* and *Candida* spp. have been well-known as commensal and opportunistic yeasts on dog skin (Kennis et al., 1996; Uchida et al., 1992). Beside *M. pachydermatis*, healthy skin was a fundamental niche of *C. parapsilosis, C. tropicalis, C. albicans, Saccharomyces cerevisiae* and *Rhodotorula* spp. (Brito et al., 2009). More recently, *M. pachydermatis* was believed to be the sole species of yeast to colonize on dog skin, as an aggregating factor in cutaneous dermatoses. On the other hand, several species of *Candida* spp. have been implicated in canine dermatomycosis and candidal cystits (Muller et al., 2002; Kano et al., 2002). However, it may be difficult to allow all yeasts grown on certain appropriate media because negative detection was found on some healthy or diseased dogs (Nardoni et al., 2008; Prado et al., 2008).

Understanding of the microbial environment on dog skin may be helpful for uncovering the cascade of pathogenesis triggered by host-mediated response (Leung and Bieber, 2003). There were a few reports of the significant relationship of yeasts, especially *Malassezia* and *Candida*, in significant amounts on healthy and diseased skin; but the role of yeasts associated with stage of skin infection is still unclear. To clarify, we attempted to compare and analyze for the association between stages of seborrhea and healthy skin with appearance of yeast species and quantity.

2.2. Materials and methods

2.2.1. Animals and sample collection

Skin swabs from 57 dogs were collected from small animal hospitals, Bangkok, Thailand. The subjects were divided into three groups; 17 healthy dogs, 20 primary lesions of seborrheic dermatitis (PSD) dogs and 20 secondary lesions of SD (SSD) dogs during 2008-2009. Subjects included in the study were of different breed, sex and habitat. Dogs with normal clinical signs were assigned to the healthy group. They were age less than six months, not atopic predisposing breeds, free from ectoparasites and skin lesions. General health condition and being untreated by steroids in the prior 3 months were included as part of the selection criteria. PSD was defined by SD with moist, erythema, mal-odor and severe pruritus, while SSD was defined by appearance of hyperpigmentation, lichenification and self-induced alopecia and itching (Griffin and DeBoer, 2001).

The yeasts were collected by sterile cotton swab soaked in sterile 0.85% normal saline solution (NSS) at approximately 1 cm^2 area in 5 different anatomical sites consisting of internal ear pinna to auricular cannel, ventral neck, interdigital area, perianal area and right groin. Samples were preserved at room temperature (Nardoni et al., 2004). Client's consent was obtained for each examined dog before the procedure.

2.2.2. Culture and enumeration

The homogenate sample was prepared by vigorous mixing of cotton swab and saline for 60 sec, and serially 10-fold dilution from 10^0 to 10^{-3}. The suspensions were cultured onto Sabouraud's Dextrose Agar (SDA) (Oxoid CM41, USA.) containing cephalexin 40 mg/ml (Pfizer, USA.), chloramphenicol 20 mg/ml (Sigma, USA.) with and without 2% sterile olive oil (Bond and Lloyd, 1996; Wikler et al., 1988). All inocula were incubated at 32°C for up to 7 days with 24 hour interval observation. The colonies were distinguished by growth duration, size, color, shape, surface appearance under stereomicroscopy (Olympus, USA.). The yeast colonies were enumerated in units of CFU/cm^2 on 4 and 7 day post-inoculation (ISO 4833). The yeast colonies were collected at least 1 colony/sampling site and 2-3 different colonies per dogs. All isolates were stored in a cryo-vial containing sterile deionized water and 80% glycerol, and kept at -80^{o}C.

2.2.3. Biochemical and physiological properties

Growth rate, colony morphology, cell and budding formation features were used for the primary criteriae of identification (Chen and Hill, 2005). Preliminary biochemical tests such as urea hydrolysis and catalase test were routinely performed. All isolates were also presumptively identified by the colony appearance on modified LNA-CHROMagar medium comprising with 47.7% (W/V) of CHROMagar *Candida* (Becton and Dickinson, Paris, France), 0.8% (W/V) of Ox bile (Difco), 2% olive oil and 0.05% of Tween 60 (Kaneko et al., 2005). *M. pachydermatis* was identified based on its ability to grow on lipid-free medium, and other *Malassezia* species were classified based on ability to grow on different sources of lipid media such as Tween assimilation, Cremophor EL agar (EL) (Sigma, USA) and Tween 60-esculin agar (TE) (Kaneko et al., 2007). *Candida* spp. were identified by proven methods consisting of the germination tube production test using pig serum incubated at 37°C for three hours, the chlamydoconidia and blastoconidia production on cornmeal agar plate (Difco Laboratories, USA), growth at 30°C for 3-5 days and sugar fermentation test (Oliveira Gdos et al., 2006). The reference strains; *M. furfur* CBS 1878, *M. sympodialis* CBS 7222, *M.globosa* CBS 7966, *M. pachydermatis* CBS 1879, *C. albicans* ATCC 90028, *C. tropicalis* ATCC 750, *C. parapsilosis* ATCC 22019, *C. guilliermondii* ATCC 9058 were included as the references.

2.2.4. Molecular analysis

2.2.4.1. PCR-RFLP for *Malassezia* spp. identification

DNA extraction was carried out by using Wizard® Genomic DNA Purification Kit (Promega, USA.) with grinding by glass bead (Sigma, USA.) (Yamada et al., 2002). To identify and confirm species of 75 *Malassezia* isolates, the PCR-RFLP was attended in this study. The target gene to amplified was 26S ribosomal DNA with this primer pairs: forward, 5′-TAACAAGGATTCCCCTAGTA and reverse, 5′-ATTACGCCAGCATCCTAAG. After purified the PCR products with a commercial kit (Promaga, USA), C*f*oI (Promega, USA) was used as the restriction enzyme. The restriction enzyme reaction was condition with 2 ul of 10x RE buffer, 0.2 ul of BSA and 10U of the restriction enzyme incubated with 1ug/ul of DNA at 37°C for 3 hours. After digestion, observed the patterns and DNA restriction fragments with 1.5 % agarose gel by 75 volts (Mirhendi et al., 2005).

2.2.4.2. Partial sequencing of ribosomal DNA

PCR were performed by the primer set of internal transcribed spacer (ITS) locating between ITS1 to ITS4 specific for ribosomal DNA gene: forward, 5′-TCCGTAGGTGAACCTGCGG and reverse, 5′-TCCTCCGCTTATTGATATGC (Gupta et al., 2000). The PCR products were purified by a commercial kit (Promega, USA.) and performed in DNA sequencing using BigDye® Terminator™ for capillary sequencer (Cybeles, Hong Kong). The DNA alignment and phylogenic analysis were performed using the Vector NTI Advance™ 10.3.1 and Mega4.1b3. Gene sequencing of 12 representative isolates were submitted to GenBank (accession numbers: GU373663, GU373658, GU373659, GU373660, GU373661, GU373662, GU373664, GU373653, GU373654, GU373655, GU373656, GU373657).

2.2.5. Statistical analyses

The statistical analyses were carried out by using Statistical Analysis System version 9.0 (SAS Inst. Inc., Cary, NC, USA.). The yeast population sizes were analyzed as quantitative data, presented as means±SEM. The frequency of isolation and the number of anatomical distribution of yeast isolates were analyzed as qualitative data and reported as a percentage. The quantitative data were analyzed by using multiple analyses of variance (ANOVA) by general linear mixed model (MIXED) procedure of SAS. The statistical models included location of the lesions; ear canal, groin, neck, perianal area and interdigital area and score lesion; no lesion, PSD and SSD as fixed effects, and included dog nested within lesion score as a random effect. Normal distribution of the data was tested using the UNIVARIATE procedure option NORMAL of SAS. Skewness, Kurtosis and Kolmogorov-Smirnov D statistic of all variables were evaluated. Due to the high variation of the dependent variables analyzed, a $\log^{10}$ transformation was applied to the data. Residuals from the statistical models were evaluated for normality under the general linear model procedure (GLM). Least-squares means were obtained and were compared by using least significant different test. The proportional data were compared between groups by using Chi-squared test (PROC FREQ). A 2x2 table was conducted for each pair of the variables. A probability value of $P<0.05$ was regarded to be statistically significant.

2.3. Results

2.3.1. Yeast isolation and classification

A total of 112 isolates were recovered from the 40 diseased dogs and 17 healthy dogs. On the basis of morphological and biochemical properties, four yeast types were classified as shown in Table 2.1 and were distinguished by color appearance using modified CHROMagar. Seventy-four isolates were identified as *M. pachydermatis*, 34 were identified as *C. parapsilosis*, 3 and 1 were identified as *C. tropicalis* and *M. furfur* respectively.

By molecular identification with 26S rDNA PCR-RFLP method, the DNA from all *Malassezia* isolates were successfully amplified with PCR products approximately sized 580 bp. And after digested with C*f*oI, all 74 isolates of non-lipid dependent *Malassezia* spp. had consistent restriction fragments with *M. pachydermatis* (3 fragment length ~ 100 bp, 230 bp and 250 bp). And one lipid-dependent *Malassezia* spp. had similar restriction pattern to *M. furfur* (2 fragment length ~ 100 bp and 250 bp). Confirmation by the partial sequencing of rDNA region with primer sets of ITS, the phylogenic analysis of 12 random isolates; representative of type A (5), type B (1), type C (5) and type D (1), were clustered within *M. pachydermatis*, *M. furfur*, *C. parapsilosis* and *C. tropicalis*, respectively as shown in Figure 2.4. Numbers of the four species cultured from skins; healthy, PSD and SSD are shown in Table 2.2.

2.3.2. Prevalence and quantitative analysis

The collection, classification and quantitative analysis for yeast were analyzed for all isolates derived from 40 seborrheic dermatitis and 17 healthy dogs. Our results revealed that the two major yeast species colonizing on dog skin were *M. pachydermatis* and *C. parapsilosis*, while *C. tropicalis* and *M. furfur* could be found occasionally. The frequency of yeast population on the skin of three different groups is shown in Table 2.3. Dog skin with SSD had more frequency of exhibiting *M. pachydermatis* (71.43%) when compared with those of healthy ($P < 0.05$) and PSD dogs ($P < 0.01$). The single infection with *C. parapsilosis* could be found significantly more on the skin with PSD than that of the secondary lesion dogs ($P < 0.01$) but there was no different frequency between PSD and the healthy group. The frequencies of the co-colonization of *M. pachydermatis* and *C. parapsilosis* were similar in all groups (14-24%).

The distribution on different anatomical sites is shown in Table 2.3. Single-colonization of *C. parapsilosis* was found at the perianal area, while there was no *M. pachydermatis* single-colonization in this area ($P< 0.01$). The frequency of isolation was higher from the groin, neck, interdigital areas and ear canal for *M. pachydermatis*, but the highest frequency of *C. parasilosis* was discovered in the perianal area ($P< 0.05$) and followed by interdigital area, ear canal, groin and neck, respectively. The frequency of the co-colonization was higher from ear, followed by perianal area, neck and interdigital area but there was only single colonization of *C. parasilosis* at the groin.

2.3.3. Correlation between yeasts and skin conditions

For quantitative data, the quantity of *M. pachydermatis* and *C. parapsilosis* was statistically compared for the 5 anatomical sites. The population sizes of both *M. pachydermatis* and *C. parapsilosis* manifest on the skin of diseased dogs were significantly greater than that of healthy dogs (ANOVA, $P < 0.05$). The population sizes and anatomical distribution were presented as mean CFU/cm^2±SEM (Table 2.4). In the healthy group, the population size of *M. pachydermatis* ranged from 0-150 CFU/cm^2 (mean 26.24±7.11 CFU/cm^2) with the highest mean distribution size at the interdigital area, followed by neck and ear canal respectively. The population size of *C. parapsilosis* was lower in healthy dogs ranged from 0-125 CFU/cm^2 (mean 6.79±3.92 CFU/cm^2).

In the PSD group, the population size of *M. pachydermatis* and *C. parapsilosis* significantly increased. The population size of *M. pachydermatis* ranged from 0-47750 CFU/ cm^2 (mean 1959.09 ± 1491.64 CFU/cm^2) with the mean distribution size, ranked from greater to lesser, for the ear, neck, interdigital area, groin and perianal area. Additionally, the number of *C. parapsilosis* ranged from 0-80000 CFU/cm^2 (mean 5154.50 ± 3271.88 CFU/cm^2) with the mean distribution size, ranked from greater to lesser, for the interdigital area, ear canal, perianal area, neck and groin.

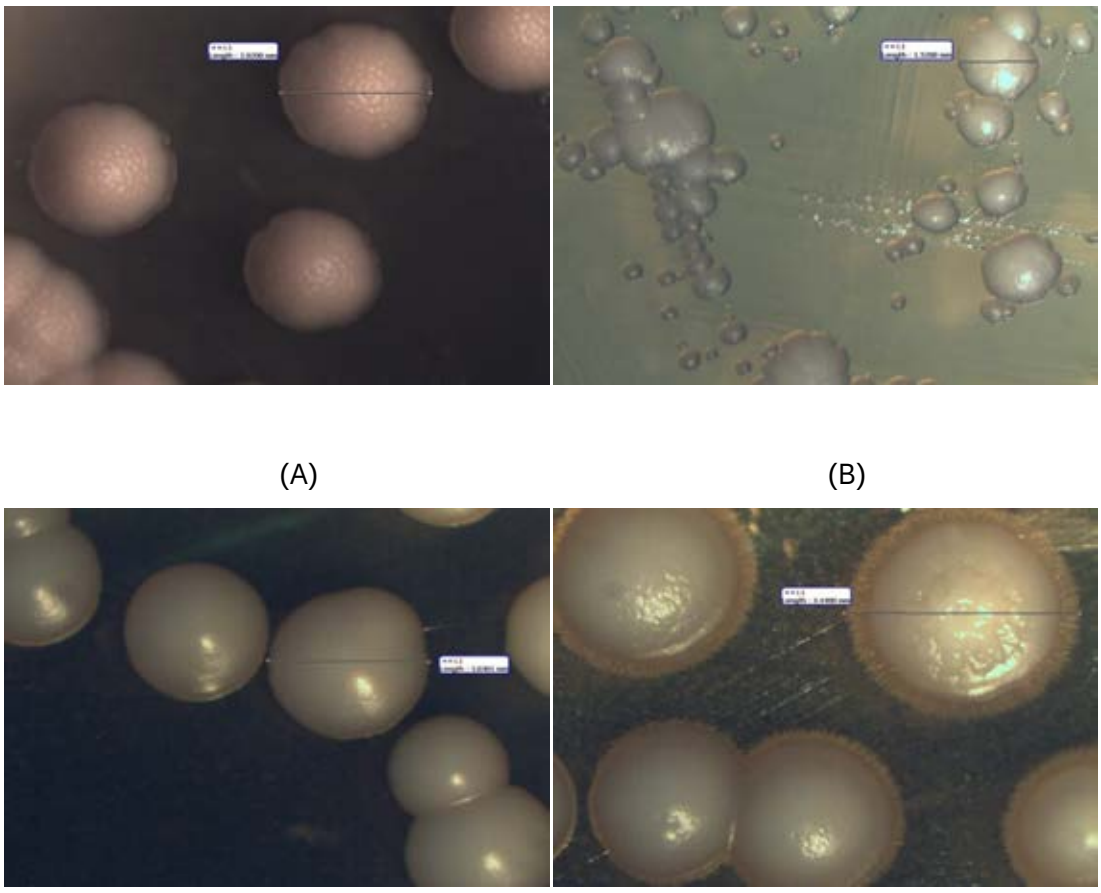
In SSD dogs, the population size of *M. pachydermatis* was dramatically increased compared with PSD ($P = 0.0014$) and healthy dogs ($P< 0.0001$). The number of *M. pachydermatis* ranged from 0-67000 CFU/cm^2 (mean 3441.67 ± 1612.91 CFU/cm^2) with the mean distribution size, ranked from greater to lesser, for

the ear canal, neck, interdigital area, perianal area and groin. Nevertheless, the number of *C. parapsilosis* was slightly decreased in the SSD compared with PSD dogs (P = 0.33) but the number was still higher than healthy dogs especially in ear canal, interdigital area and neck (P = 0.18).

Table 2.1 Physiological differentiations of 4 yeast types isolated from dog skins.

Types	Growth durations (hr.)	Colony formations[a]	Diameter sizes[a] (mm.)	Lipid dependence	Microscopic findings		CHROM agar[a]	Tween assimilation				EL	TE	Urease test	Species
					Shape	Pseudohyphae		20	40	60	80				
A	48-72	WC/S/D	2-4	-	BB	-	Cream	+	+	+	+	+	+	+	*M. pachydermatis*
B	48-72	W/S/D	1.5-2	+	Oval	-	Light cream	+	+	+	+	+	+	+	*M. furfur*
C	24-48	WC/S/Sh	3-5	-	Oval	+	White	ND	ND	ND	ND	ND	ND	-	*C. parapsilosis*
D	24-48	WC/R/Sh	3-6	-	Oval	+	Blue	ND	ND	ND	ND	ND	ND	-	*C. tropicalis*

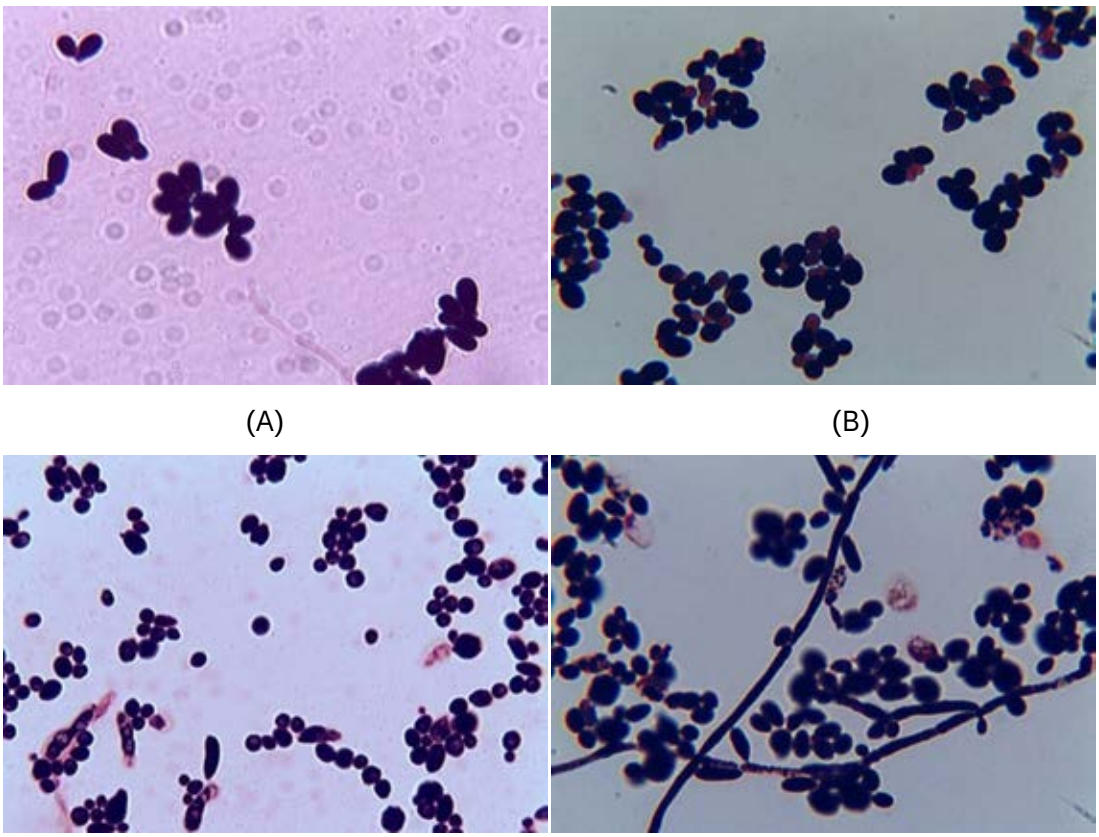
[a] Incubation at 32°C 5-7 days; +, positive result; -, negative result; ND, not determined; WC/S/D, white to cream color, smooth and dry surface colony; W/S/D, white color, smooth and dry surface colony; WC/S/Sh, white to cream color, smooth with shiny surface colony; WC/R/Sh, white to cream color, rough with shiny surface colony; BB, broad-based budding yeast; EL, cremophor EL agar and TE, Tween 60-esculin agar.



(A) (B)

(C) (D)

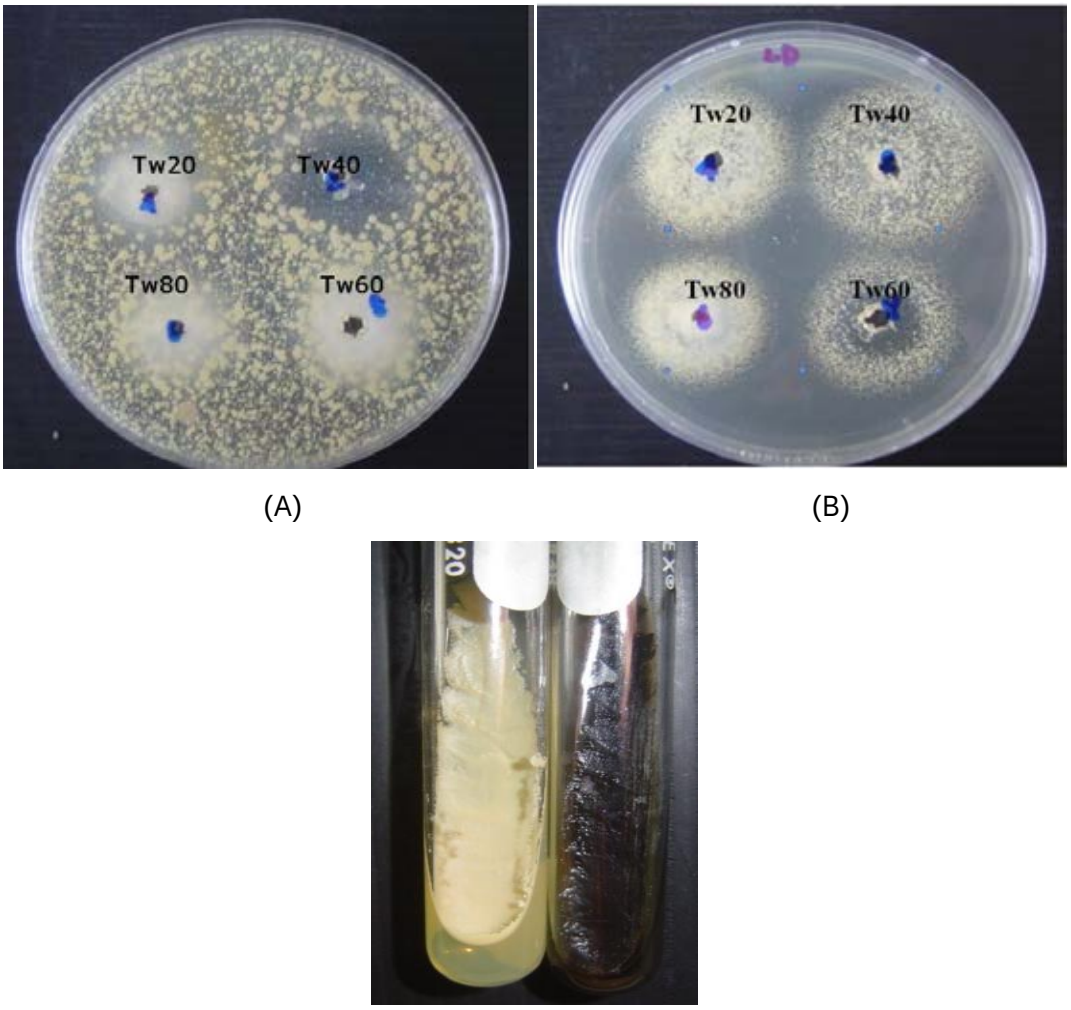
Figure 2.1 Colony of canine skin yeasts: *Malassezia pachydermatis* (A); WC/S/D, white to cream color, smooth and dry surface colony, *Malassezia furfur* (B); W/S/D, white color, smooth and dry surface colony, *Candida parapsilosis* (C); WC/S/Sh, white to cream color, smooth with shiny surface colony, and *Candida tropicalis* (D); WC/R/Sh, white to cream color, rough with shiny surface colony.



(A) (B)

(C) (D)

Figure 2.2 Microscopic features of canine skin yeasts; *Malassezia pachydermatis* (A) and *Malassezia furfur* (B) have the similar microscopic features as 3x5-7 µm., thick cell wall, oval shape with broad-based daughter cells budding (bottle-shaped) cell. *Candida parapsilosis* (C) show the round to oval shaped with irregular daughter cells and pseudohyphal formation are observed after inoculation 72-96 hour at room temperature. *Candida tropicalis* (D) show differently in the fast rate of pseudohyphal formation with the large 5x6-7 µm. (Gram's stain (x400).



(A) (B)

(C)

Figure 2.3 The phenotypic characterization of canine skin yeasts on different source of lipid media; (A) Tween assimilation of *Malassezia pachydermatis* show the non-lipid dependent and the utilization of Tween20, 40, 60 and 80 properties. (B) Tween assimilation of *Malassezia furfur* show the lipid-dependent, the Tween20,40,60 and 80 utilization and (C) both *Malassezia* spp. have ability to utilize polyethoxylated castor oil (EL slant; left) and ability to hydrolyze esculin and utilize Tween60 on (TE slant; right).

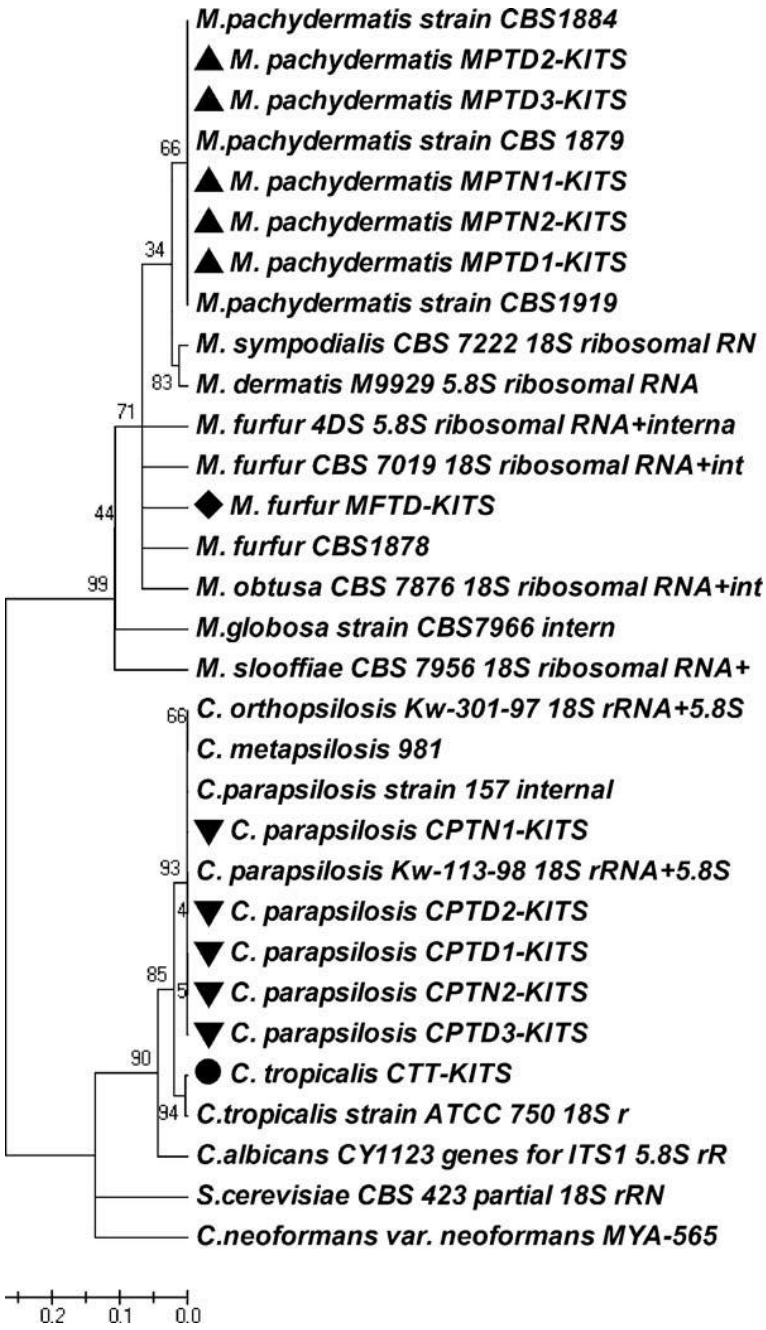


Figure 2.4 Phylogenic analysis based on DNA sequences of ribosomal DNA gene and internal transcribed spacer region of the 12 representative isolated in this study indicating by the symbols in front of each isolate dividing: type A, *M. pachydermatis* (▲);B, *M. furfur* yeast(◆);C, *C. parapsilosis*(▼); and D, *C. tropicalis* (●). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

Table 2.2 Number of yeast isolates recovered from each group of dogs.

Skin Conditions	No. of dogs	No of isolates			
		M. pachydermatis	*C. parapsilosis*	*C. tropicalis*	*M. furfur*
Normal	17	22	12	3	0
PSD	20	16	14	0	0
SSD	20	36	8	0	1
Total	57	74	34	3	1

Abbreviation: PSD, primary seborrheic dermatitis; SSD: secondary seborrheic dermatitis.

Table 2.3 Frequency of yeasts colonizing on different dog skin lesions and anatomical distribution.

		Number of isolates		
		Single *M. pachydermatis*	Single *C. parapsilosis*	Co-colonization
Skin Conditions	Normal	14/34 (41.18%) [a]	4/34 (11.76%)	7/34 (20.59%)
	PSD	11/32 (34.38%) [b]	9/32 (28.13%) [c]	5/32 (15.63%)
	SSD	30/42 (71.43%) [ab]	2/42 (4.76%) [c]	6/42 (14.29%)
Anatomical sites	Ear canal	15/28 (53.57%) [a]	5/28 (17.86%) [g]	8/28 (28.57%)
	Neck	18/25 (72.00%)[e]	1/25(4.00%) [h]	6/25 (24.00%)
	Interdigital area	16/25 (64.00%) [b]	5/25(20.00%) [c]	4/25 (16.00%)
	Groin	7/8 (87.50%) [f]	1/8 (12.50%) [d]	0/8 (0.00%)
	Perianal area	0/4(0.00%) [abef]	3/4 (75.00%)[cdgh]	1/4 (25.00%)

Frequency indicates proportion of positive isolation/total number of sampling.[a-h] indicates statistically significant differences among dog groups by χ^2 test at $P< 0.05$.Number in parenthesis indicates proportion of positive number/total of swab number.

Abbreviation: PSD, primary seborrheic dermatitis; SSD: secondary seborrheic dermatitis.

Table 2.4 Summary of frequency and population size of yeasts isolated from different dog skin conditions by body parts and distribution.

Skin condition	Number of dogs	*M. pachydermatis*											
		Overall mean (SEM)		Mean (SEM)									
				Ear canal		Neck		Interdigital area		Groin		Perianal area	
Healthy	17	22[a]	26.24[†] (7.11)	7	14.82 (7.46)	9	30.45 (13.25)	6	33.45 (15.32)	ND		ND	
PSD	20	16	1959.09[†] (1491.64)	7	4656.25 (3930.95)	5	812.50 (665.32)	3	46.33 (40.93)	1	38	0	
SSD	20	36	3441.67[†] (1612.91)	9	7977.33 (5481.55)	10	3118.64 (1347.69)	10	1363.91 (1140.18)	6	357 (338.35)	1	102

Number in parenthesis beside the mean (CFU/cm^2) is the standard error of the mean (SEM).The comparison was made vertically by overall mean (CFU/cm^2), not characterized in different body parts. ND, not determined.

† Statistically significant differences by ANOVA ($P \leq 0.05$).

[a] Numbers in the front of Mean (SD) indicates frequency of isolates.

Table 2.4 Summary of frequency and population size of yeasts isolated from different dog skin conditions by body parts and distribution (Continued).

Skin condition	Number of dogs	*C. parapsilosis*											
		Overall mean (SEM)		Mean (SEM)									
				Ear canal		Neck		Interdigital area		Groin		Perianal area	
Healthy	17	12[a]	6.79[†] (3.92)	4	12.18 (11.29)	4	3.73 (1.68)	4	4.45 (3.58)	ND	ND		
PSD	20	14	5154.50[†] (3271.88)	6	7807.33 (6650.25)	2	54.75 (46.41)	3	11691.67 (11661.69)	0		3	222.67 (176.34)
SSD	20	8	2931.47 (1316.25)	3	4133.33 (2981.31)	1	30000	2	4181.82 (3056.49)	1	3	1	450

Number in parenthesis beside the mean (CFU/cm^2) is the standard error of the mean (SEM). The comparison was made vertically by overall mean (CFU/cm^2), not characterized in different body parts. ND, not determined.

† Statistically significant differences by ANOVA ($P \leq 0.05$).

[a] Numbers in the front of Mean (SD) indicates frequency of isolates.

2.4. Discussion

Features of morphological and biochemical properties were routinely used as tools for identification in several studies (Brito et al., 2009; Prado et al., 2008; Prohic and Kasumagic-Halilovic, 2009). Given the variety of colony types, the molecular analysis was successfully performed for species confirmation and was rather consistent with those by biochemical profile. Therefore, enumeration of colonies for 3-4 days and 5-7 days of incubation periods could be used as the specific feature of *Candida* and *Malassezia* species, respectively.

Only a few isolates of *M. furfur* (lipid dependence) and *C. tropicalis* were cultured. Therefore the role of these species on dog skin is still suspected. More recently, *C. tropicalis* was recognized as a member of skin commensal together with *M. pachydermatis*, *C. parapsilosis*, *C. albicans*, *Saccharomyces cerevisiae* and *Rhodotorula* spp. All examined dogs yielded either *M. pachydermatis* or *C. parapsilosis* from at least one skin area. The results strongly confirm that *M. pachydermatis* and *C. parapsilosis* were present as common skin yeast (Brito et al., 2009) and existence of low frequency and population size of the other species might act as a transient or nomadic role on the surface.

With regard to frequency and anatomical site, the perianal area was highly colonized with *C. parapsilosis* in this study, whereas the groin was the most frequent area of *M. pachydermatis* as well as being reported in Sphynx cats and domestic short hair cats (Ahman and Bergstrom, 2009). In contrast, Prado et al., (2008) demonstrated only *M. pachydermatis* as cutaneous carriage at the ear, followed by skin and eye. The highest quantity of *M. pachydermatis* was also found at the interdigital areas, followed by ears, nail folds, mouth, groin, axillae, perianal area, and perianal glands (Nardoni et al., 2007). In our prior investigation of frequency and distribution in each anatomical site, we did distinguish the verity of colony types on the selective media. Nevertheless, animal factors associated with frequency and population size should be included in the analysis.

Generally, no significant frequency difference was found between male and female (Nardoni et al., 2004) or age of dogs (Plant et al., 1992) but high prevalence of *M. pachydermatis* was predominant for certain breeds such as poodle with otitis or

German shepherd without otitis (Girao et al., 2006). There was no apparent difference between the frequency between auricular area with and without otitis externa (seborrheic dermatitis at ear pinna to auricular canal) in this study; only quantity of yeast was confirmed to be associated with otitis externa. On the other hand, co-colonization between *M. pachydermatis* and *C. parapsilosis* was first demonstrated in multiple sites detected at the ear, perianal area, groin and interdigital area, but the microenvironmental factors of their symbiosis has not been understood. Previously, a relationship of *M. pachydermatis* and *Leishmanis infantum* on canine skin was postulated in view of the fact that phospholipase activity is an important factor in their symbiosis without skin lesions (Cafarchia et al., 2008). Therefore, symbiosis of the microorganisms may be exclusively controlled by specific energy sources and their metabolisms.

Many previous studies attempted to enumerate the yeast population size on skin, but the method and results were quite variable. For example,the mean population size for *M. pachydermatis* (26.24 CFU/cm^2) on healthy dogs was apparently higher than the quantity of *M. pachydermatis* detected from atopic dermatitis (6.98± 3.48 CFU/swab) (Nardoni et al., 2007). The accuracy of the enumeration method was augmented by rapid culturing and serial dilution with proper countable number. The fluctuation of population size might be associated with criteriae of subject selections, sampling technique and duration of growth. In this study, *Malassezia* spp. and *Candida* spp.were separately countable bycolony typing using a stereomicroscopy. The interdigital area was a difficult site for measurement, but at least approximately 1 cm^2 of the area could be swabbed, and for which the results might also be assumed to be a CFU/swab as in the previous reports (Cafarchia et al., 2005; Nardoni et al., 2007). Additionally, in our preliminary study, sample collection by contact plate was a problemmatic method for deriving an exact number in animals (data not shown) since hair coat might be an important barrier.

Moreover, the correlation between yeast amount and stage of infection (PSD and SSD) was statistically analyzed in the current study. The results reveal that the most common yeasts exhibited on healthy and diseased dogs were *M.*

pachydermatis and *C. parapsilosis*. Therefore, the theory of *M. pachydermatis* as a sole species for canine dermatitis needs to be reconsidered (Nardoni et al., 2007; Nardoni et al., 2004; Prado et al., 2008). Ears with otitis externa yielded the largest number of *M. pachydermatis* and *C. parapsilosis*, and the results closely resembled previous data (Cafarchia et al., 2005; Prado et al., 2008). Additionally, the high frequency of *C. parapsilosis* in multiple sites of the body in healthy and diseased dogs implies that this species clearly acts as commensal skin (Brito et al., 2009) and might also become a pathogen in certain circumstance. This speculation was rationally supported by the increase in population size in all examined areas especially during a PSD episode, followed by a mild decrease during SSD.

Normally, *Candida* spp. serves as a commensal at the alimentary, upper respiratory and genital mucosa of humans and animals. *C. albicans* and *C. parapsilosis* are the most commonly isolated species of canine mucocutaneous areas and cause the opportunistic infections in canine skin diseases (Mueller et al., 2002; Moretti et al., 2006). Seborrheic dermatitis is a subset of clinical signs of atopic dermatitis associated with yeast, but the pathogenesis remains unclear (Ashbee, 2007). The mycotic amount from PSD and SSD was apparently higher than that of healthy dogs. The quantitative analysis reveals that *M. pachydermatis* was dramatically increased in all stages of disease whereas that of *C. parasilosis* was decreasingly fluctuating during the chronic phase. The quantitative score of *M. pachydermatis* and *C. parapsilosis* may be related to clinical appearance during PSD and SSD. This could be explained by the theory of competitive interference whenever 2 microorganisms inhabit the same area with limited source of carbon and energy or biofilm formation of *M. pachydermatis*, which may be its protective factor (Cannizzo et al., 2007). The evidence implies that co-colonizing with large quantities potentially triggered type 1 hypersensitivity during the acute stage.

In conclusion, the results demonstrate the role of concurrent infection between *C. parapsiloisis* and *M. pachydermatis* causing canine seborrheic dermatitis at multiple sites and stage of disease. Therefore, diagnostic procedures and therapy of yeast-associated skin diseases should consider not only *M. pachydermatis* but also *C. parapsilosis*.

CHAPTER 3

Antifungal agent susceptibilities and interpretation of *Malassezia pachydermatis* and *Candida parapsilosis* isolated from dogs with and without seborrheic dermatitis skin

3.1. Introduction

Yeast-associated seborrheic dermatitis (SD) and otitis externa are commonly found in atopic dogs with overgrowth of *Malassezia pachydermatis* and *Candida parapsilosis*.In general, skin yeasts reversibly act as commensals and can be transient depending on the underlining factors that lead to SD (Gupta, et al., 2004). *M. pachydermatis* was originally thought to be the sole yeast that caused canine skin diseases (Kennis, et al., 1996; Uchida, et al., 1992). However, recently, over five yeast species were recognized as skin commensals (Brito, et al., 2009), and *C. parapsilosis* was identified as a causative yeast concurrent with *M. pachydermatis* in SD dogs (Yurayart, et al., 2011).

In animals with severe and widespread lesions, systemic ketoconazole (KTZ) and itraconazole (ITZ) are the most commonly used drugs with recommended doses of 5-10 mg/kg daily for up to 4 weeks (Akerstedt and Vollset, 1996; Carlotti, 2009; Peano and Gallo, 2008). Due to the recurring disease caused by SD, routine antifungal administration may possibly induce acquired resistance, leading to treatment failure. In addition, SD dogs can potentially be a route for zoonosis and nosocomial infection in circumstances where health-care workers have transmitted the drug-resistant yeasts shared to human patients (Chang, et al., 1998; Morris, 2005). However, the absence of standardized methodology and interpretative values for animal yeasts have limited the available data on antifungal susceptibility (Robson). The purposes of this study were: (i) to evaluate the *in vitro* KTZ and ITZ susceptibility of *M. pachydermatis* and *C. parapsilosis* from dogs with and without SD using the CLSI M27-A3 broth microdilution method (BMD) and CLSI M44-A disk diffusion method (DD); (ii) to compare the antifungal susceptibility levels between dogs with and without SD; and (iii) to assess the correlation of reference between BMD and DD using an agreement analysis.

3.2. Materials and methods

3.2.1. Yeast strains

A total of 73 yeast strains were obtained from 45 dogs that had been examined by private small animal hospitals in Bangkok, Thailand from 2008-2011. Forty-five strains of *M. pachydermatis* isolated from dogs without SD (n=12) and with SD (n=33) and 28 strains of *C. parapsilosis* isolated from dogs without SD (n=13) and with SD (n=15) were used. Dogs without SD were defined from those who were less than six months of age, not atopic predisposing breeds, lack of from ectoparasites and clinical sign with normal skin and hair coat and untreated by steroids for at least 3 months prior to collection. SD lesions were defined based on clinical signs, including pruritus, moistness, erythema, mal-odor or hyperpigmentation, lichenification and self-induced alopecia (Chen and Hill, 2005). Signalments and histories of all dogs are shown in Table 3.1.

M. pachydermatis isolates were confirmed based on morphology, lipid-independent properties, and the restriction patterns by PCR-RFLP (Kaneko, et al., 2007; Mirhendi, et al., 2005). *C. parapsilosis* isolates were characterized based on their morphological features, including germ tube production, chlamydoconidia and blastoconidia production on corn-meal agar and the sugar fermentation test (Oliveira Gdos, et al., 2006). The identifications were confirmed by partial sequencing of ribosomal DNA using a primer set for the internal transcribed spacer (ITS) located between ITS1 and ITS4 (Gupta, et al., 2000). The DNA alignment and phylogenic analysis were performed as previously described (Yurayart, et al., 2011).

3.2.2. Screening for antifungal susceptibility

The disk diffusion method (DD) was performed according to the guidelines of CLSI M44-A(CLSI, 2002). Mueller Hinton agar (Difco^{TM}, USA) supplemented with 2% dextrose and 0.5 mg/L methylene blue was used as the basic media. Commercial antifungal disks (Neo-Sensitabs, Rosco, Denmark) comprising itraconazole (ITZ), ketoconazole (KTZ), nystatin (NYS), terbinafine (TERB) and 5-fluorocytosine (5-FC) at concentrations of 8, 15, 50, 30 and 10 µg/disk, respectively, were used. The inocula included *C. parapsilosis* after one day of growth and *M. pachydermatis* after three days of growth on SDA and were adjusted to 0.5 McFarland standard absorption, which is equal to approximately 1-5x10^6 CFU/ml. The results were

determined based on the inhibition zone diameter after incubating at 35°C for 24-48 h (*Candida* species) and 48-72 h (*M. pachydermatis*) (Testore, et al., 2004). The antifungal susceptibility levels were interpreted according to the manufacturer's breakpoint guidelines (Carrillo-Munoz, et al., 1999).

3.2.3. Minimum inhibition concentration (MIC) determination

The MIC values against itraconazole (Sigma-Aldrich, USA) and ketoconazole (Sigma-Aldrich, USA) were determined by the broth microdilution method (BMD) according to the CLSI M27-A3 guidelines(CLSI, 2007). The antifungals were dissolved in dimethyl sulfoxide (DMSO) (Merck Sharp & Dohme Ltd., UK) and diluted with standard RPMI-1640 medium with L-glutamine but without sodium bicarbonate (Sigma Chemical Co., USA) that was supplemented with 2% dextrose and buffered at pH 7.0 with 0.165 morpholinepropanesulfonic acid (MOPS) (Sigma Chemical Co., USA). The solutions were serially diluted two-fold from 0.06 to 32 µg/ml. The inocula included *C. parapsilosis* after one day of growth and *M. pachydermatis* after three days of growth on SDA. The inoculum sizes were adjusted to a final concentration of approximately 2-3x10^3 CFU/ml using a spectrophotometer at 530 nm and appeared 95% translucent when diluted with RPMI media (Garau, et al., 2003). BMD was performed in 96-well microtiter plates (Nunc, USA). Positive and negative controls of yeast growth, including no antifungal drug and sterile media, respectively, were included on each tested plate. After incubating at 35°C for 48 h (*Candida* species) and 48-72 h (*M. pachydermatis*), the MIC values were determined by the unaided eye. The MICs and MIC_{90} were analyzed using WHONET version 5.5. The BMD breakpoints were interpreted according to the CLSI M44A. (Kiraz, et al., 2010; Prado, et al., 2008; Velegraki, et al., 2004).The minimum fungicidal concentrations (MFCs) were determined using a described standard (Brito, et al., 2007).

3.2.4. Quality control

Both DD and BMD methods were validated at all steps using the quality control strains *C. parapsilosis* ATCC 22019 and *C. albicans* ATCC 90028 and also quantitatively determined by cultivating on SDA after unaided eye observation.

3.2.5. Statistical analysis and interpretation of results

Differences among the means of inhibition zone diameter, MIC and MFC for *M. pachydermatis* and *C. parapsilosis* between groups of dogs with and without SD were analyzed using the Student's t-test. The Chi-square test was used to compare the rates of susceptible (S), intermediate (I) or susceptible dose-dependent (SDD) and resistant (R) stains between these groups of dogs. $P < 0.05$ was considered statistically significant. To assess the correlation between the DD and BMD methods, the diameters of the inhibition zone were plotted against the respective BMD. MICs and statistical analysis were performed by linear regression. The results were also analyzed in terms of the agreement categorization as described by Vandenbossche et al., 2002. The accuracy of determinations was assessed using very major errors (VME; the number of susceptible strains by DD that were considered resistant by BMD in which the number of resistant strains according to BMD was the denominator), major errors (ME; the number of resistant strains by DD that was judged as susceptible by BMD in which the number of susceptible strains according to BMD was the denominator) and minor errors (M; the number of susceptible strains according to DD that were evaluated as susceptible dose-dependent by BMD in which the total number of tested strains was the denominator) (Vandenbossche, et al., 2002).

3.3. Results

Using Mueller Hinton agar supplemented with 2% dextrose and 0.5 mg/L methylene blue, an inhibition zone could be observed and verified based on the type of *Candida* strain. All tested *M. pachydermatis* isolates were susceptible to ITZ, KTZ, NYS and TERB but resistant to 5-FC (100%). By contrast, *C. parapsilosis* exhibited intermediate and resistant percentages for KTZ (32% I, 7% R), TERB (14% I, 7% R) and 5-FC (36% I, 50% R) but all tested *C. parapsilosis* isolates were sensitive to ITZ and NYS. The results of the DD test, including the susceptibility levels and mean of inhibition zone diameter, are shown in Table 3.2 and 3.3.

By BMD, the MIC endpoint for *C. parapsilosis* could be observed within 48 h post incubation, while that of *M. pachydermatis* was observed at 72 h. All *M. pachydermatis* isolates from dogs without SD were susceptible to KTZ and ITZ (MICs <0.03 µg/ml). SD-origin *M. pachydermatis* wassusceptible with an MIC that ranged

from <0.03-0.12 µg/ml. In addition, one and two isolates derived from the SD group exhibited an intermediate and susceptible dose-dependent level against KTZ and ITZ (MIC = 0.25 µg/ml), respectively.

C. parapsilosis from both groups showed a variation in the MIC values against KTZ and ITZ. In dogs without SD, eight KTZ-resistant strains (MIC ranging from 0.5-1 µg/ml) were detected and two of them showed cross-resistance for both KTZ and ITZ. The resistant rates or mean MIC values for KTZ and ITZ were not significantly different between dog groups with and without SD. Based on the BMD test, the susceptibility levels and mean MICs were determined and are summarized in Table 3.2 and 3.3.

The MIC_{90} of all tested *M. pachydermatis* isolates was similar to the MFC and thus could be used interchangeably. The *M. pachydermatis* isolates from dogs with SD had higher MFC ranges than those obtained from dogs without SD at <0.03-0.5 µg/ml and <0.03 µg/ml, respectively. There were three intermediate and one resistant *M. pachydermatis* isolate in dogs with SD and six susceptible dose-dependent strains for KTZ and ITZ, respectively. Nevertheless, the MIC_{90} of *C. parapsilosis* was lower than the MFC but was still in the range of resistance for KTZ and ITZ. The MICs and MFCs for *M. pachydermatis* and *C. parapsilosis* are presented in Table 3.3.

The correlation between the DD and BMD results were analyzed based on the values of the correlation coefficient *r* and are shown in Figure 3.1 and 3.2. By assessing the agreement between the methods, the minor errors for *M. pachydermatis* were only 2.2% and 4.4% for KTZ and ITZ, respectively, while there were no VME or ME. For *C. parapsilosis*, only 1% and 0.2% VME occurred for KTZ and ITZ, respectively. The susceptibility levels to azoles together with the error percentage between DD and BMD are shown in Table 3.4.

Table 3.1 Signalments and histories of all tested dogs.

Gr. of dogs	Breed	Age (years)				Lesions	Anatomical sites of lesions	History of drug usage		Concurrent bacterial infection
		<1	1-5	5-10	>10			KTZ	ITZ	
Healthy (13)	Mixed breed (10) Poodle (3)	13	-	-	-	No	No	No	No	No
PSD (12)	Shih tzu (4) Poodle (4) Basset hound (1) Beagle (1) Mixed breed (2)	-	1	10	1	Recurrent moderate to severe moist, erythema, mal-odor, itch	Generalize; ears, muzzle, neck, interdigital areas, groin, ventral abdomen, perianal areas	12	10	*Staphylococcus pseudintermedius* (7)
SSD (21)	Shih tzu (5) Poodle (5) Basset hound (2) Beagle (1) English cocker spaniel (2) Mixed breed (4) Pug (2)	-	5	13	3	Recurrent moderate to severe moist, hyperpigmentation, lichenification, alopecia, mal-odor, itch	Generalize; ears, muzzle, neck, interdigital areas, groin, ventral abdomen, perianal areas	21	18	*Staphylococcus pseudintermedius* (5)

Abbreviation: PSD, primary seborrheic dermatitis; SSD, secondary seborrheic dermatitis; KTZ, ketoconazole; ITZ, itraconazole.

Table 3.2 Summary of the ketoconazole (KTZ) and itraconazole (ITZ) susceptibility levels by the disk diffusion and broth microdilution methods for clinical yeast isolates from Thai dogs.

Organisms	Gr. of dogs	Antifungal agent	Disk diffusion method			Minimum inhibitory concentration (µg/ml)			MIC_{90}	Minimum fungicidal concentration (µg/ml)
			Mean inhibition zone diameter (mm.)							
			S	I	R	S	I	R		
Breakpoints		KTZ	≤30	29-23	≥22	≤0.12	0.13-0.4	≥0.5		
		ITZ	≤16	15-11	≥10	≤0.12	0.13-0.4	≥1		
Candida albicans ATCC 90028	Quality controls	KTZ	40	-	-	0.06	-	-		
		ITZ	22	-	-	0.06	-	-		
Candida parapsilosis ATCC 22019		KTZ	44	-	-	0.06	-	-		
		ITZ	24	-	-	0.12	-	-		

Abbreviation: S, susceptible; I, intermediate susceptible; R, resistant; $MIC_{90,}$ MIC at which 90% of isolates were inhibited.

Table 3.2 Summary of the KTZ and ITZ susceptibility levels by the DD and BMD methods for clinical yeast isolates from Thai dogs (Continued)

Organisms	Gr. of dogs	Antifungal agent	Disk diffusion method			Minimum inhibitory concentration (µg/ml)			MIC_{90}	Minimum fungicidal concentration (µg/ml)
			Mean inhibition zone diameter (mm.)							
			S	I	R	S	I	R		
M. pachydermatis (45)	H (12)	KTZ	72.4 (12)	0	0	<0.03 (12)	0	0	< 0.03	< 0.03 (12)
		ITZ	55.9 (12)	0	0	<0.03 (12)	0	0	< 0.03	< 0.03 (12)
	SD (33)	KTZ	48.3 (33)	0	0	<0.03 (29), 0.12 (3)	0.25 (1)	0	< 0.03-0.5	< 0.03 (29), 0.25 (3), 0.5 (1)
		ITZ	52.6 (33)	0	0	<0.03 (26), 0.06 (1), 0.12 (4)	0.25 (2)	0	< 0.03-0.5	< 0.03 (26), 0.12 (1), 0.25 (4), 0.5 (2)
C. parapsilosis (28)	H (13)	KTZ	39 (7)	25 (5)	24 (1)	<0.03 (4), 0.03 (1), 0.06 (1)	0.25 (1)	0.5 (3), 1(3)	1	<0.03 (4), 0.12 (1), 0.25 (1), 0.5 (3), 1(1), 2 (1), 16 (2),
		ITZ	23 (13)	0	0	<0.03 (4) 0.06 (1), 0.12 (5)	0.25 (1)	1 (2)	1	<0.03 (3), 0.12 (3), 0.25 (5), 16 (2)
	SD (15)	KTZ	36 (10)	25 (4)	21 (1)	<0.03 (3), 0.03 (2), 0.12 (1)	0.25 (7)	0.5 (1), 1 (1)	1	<0.03 (1), 0.03 (2), 0.06 (1), 0.12 (1), 0.25 (2), 0.5 (5), 1(3)
		ITZ	24 (14)	15 (1)	0	<0.03 (3), 0.12 (9)	0.25 (3)	0	0.5	<0.03 (2), 0.03 (1), 0.06 (1), 0.12 (3), 0.25 (6), 0.5 (1), 16 (1)

Abbreviation: S, susceptible; I, intermediate susceptible; R, resistant; $MIC_{90,}$ MIC at which 90% of isolates were inhibited.

Table 3.3 Mean values for the inhibition zone diameter, MICs and MFCs for antifungal agents against clinical yeast isolates from healthy and SD dogs.

Organisms	Gr. of dogs	Mean values of inhibition zone diameter (mm) (SD)					Mean values of Minimum inhibitory concentration (µg/ml)		Mean values of Minimum fungicidal concentration (µg/ml)	
		Antifungal agent					Antifungal agent		Antifungal agent	
		5-FC	TERB	NYS	KTZ	ITZ	KTZ	ITZ	KTZ	ITZ
M. pachydermatis (45)	Healthy (12)	0	130.4[a] (11.1)	31.5 (3.7)	72.4[b] (15.6)	56.3 (12.7)	0.03[c]	0.03[d]	0.03[e]	0.03[f]
	SD (33)	0	113.3[a] (15.0)	32.5 (4.3)	48.3[b] (14.1)	52.6 (15.5)	0.045[c] (0.045)	0.055[d] (0.058)	0.064[e] (0.101)	0.088[f] (0.130)
C. parapsilosis (28)	Healthy (13)	12.8[g] (13.0)	23.5 (10.0)	24.1 (2.6)	31.8 (7.8)	22.6 (4.2)	0.382 (0.401)	0.233 (0.346)	2.845 (5.86)	2.592 (5.95)
	SD (15)	24.8[g] (7.6)	24.3 (9.8)	24.4 (1.4)	33.7 (9.0)	24.3 (9.2)	0.235 (0.251)	0.128 (0.072)	0.418 (0.36)	1.234 (4.09)

SD, seborrheic dermatitis; 5-FC, 5-fluorocytosine; TERB, terbinafine; NYS, nystatin; KTZ, ketoconazole and ITZ, itraconazole.

[a, b, c, d, e, f and g] Statistically significant differences in the mean±SD for the inhibition zone diameter, MICs and MFCs among the dog groups by Student's t-test at $P < 0.01$. The same letters indicate statistically significant differences.

Table 3.4 Summary of azole susceptibility levels and percentages of error detected between DD and BMD in clinical yeast isolates from Thai dogs.

Antifungal agent	Organism	Test method[a]	% of results in each category[b] (no. of strains)			% Error[c]		
			S	SDD	R	VME	ME	M
ITZ	*M. pachydermatis*	DD	100(45/45)	0	0	0	0	4.4(2/45)
		MIC	95.6(43/45)	4.4(2/45)	0			
	C. parapsilosis	DD	96.9(31/32)	3.1(1/32)	0	1(1/1)	0	21.9(7/32)
		MIC	78.1(25/32)	18.8(6/32)	1			
KTZ	*M. pachydermatis*	DD	100(45/45)	0	0	0	0	2.2(1/45)
		MIC	97.8(44/45)	2.2(1/45)	0			
	C. parapsilosis	DD	46.9(15/32)	46.9(15/32)	6.2(2/32)	0.2(2/10)	0	25 (8/32)
		MIC	40.6(13/32)	28.1(9/32)	31.3(10/32)			

[a] Itraconazole and ketoconazole disk diffusion testing was performed according to CLSI method M44-A, and the broth microdilution method MIC testing was performed according to CLSI method M27-A2.[b] The itraconazole susceptibility categories are as follows: S, susceptible; SDD, susceptible dose-dependent; R, resistant.[c] Percentage of error detection was calculated and described as follows: VME, very major errors; ME, major errors; M, minor errors.

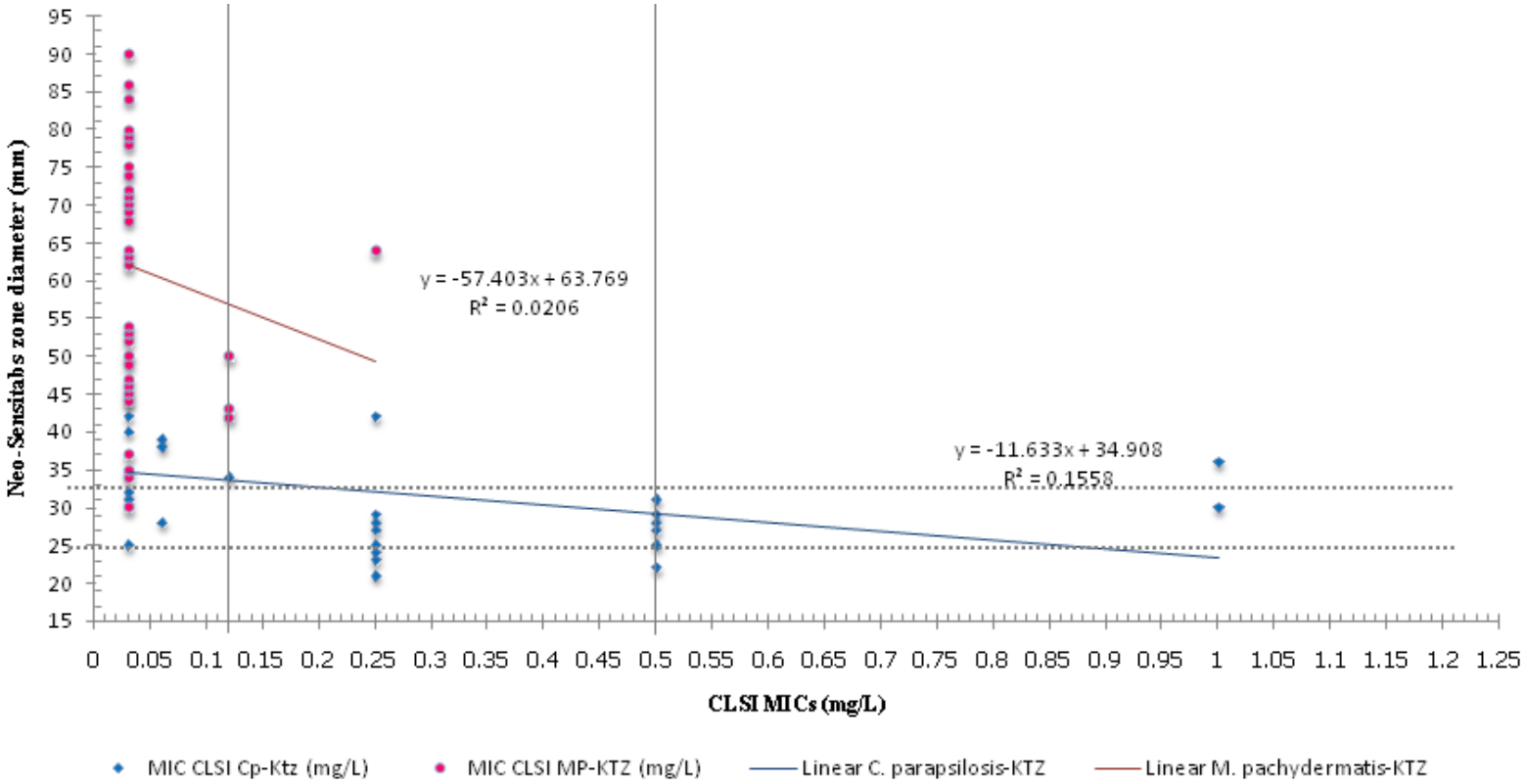


Figure 3.1 Scatter diagram of CLSI MICs for ketoconazole, and the zone diameters obtained with this antifungal disk for *Candida parapsilosis* and *Malassezia pachydermatis*. The dots represent the susceptibility value for each tested strain and each line indicates the regression line with correlation coefficient *r* (R^2). Gray dotted and solid lines are the breakpoints for the disk diffusion (DD) and broth microdilution (BMD) MICs according to CLSI, respectively.

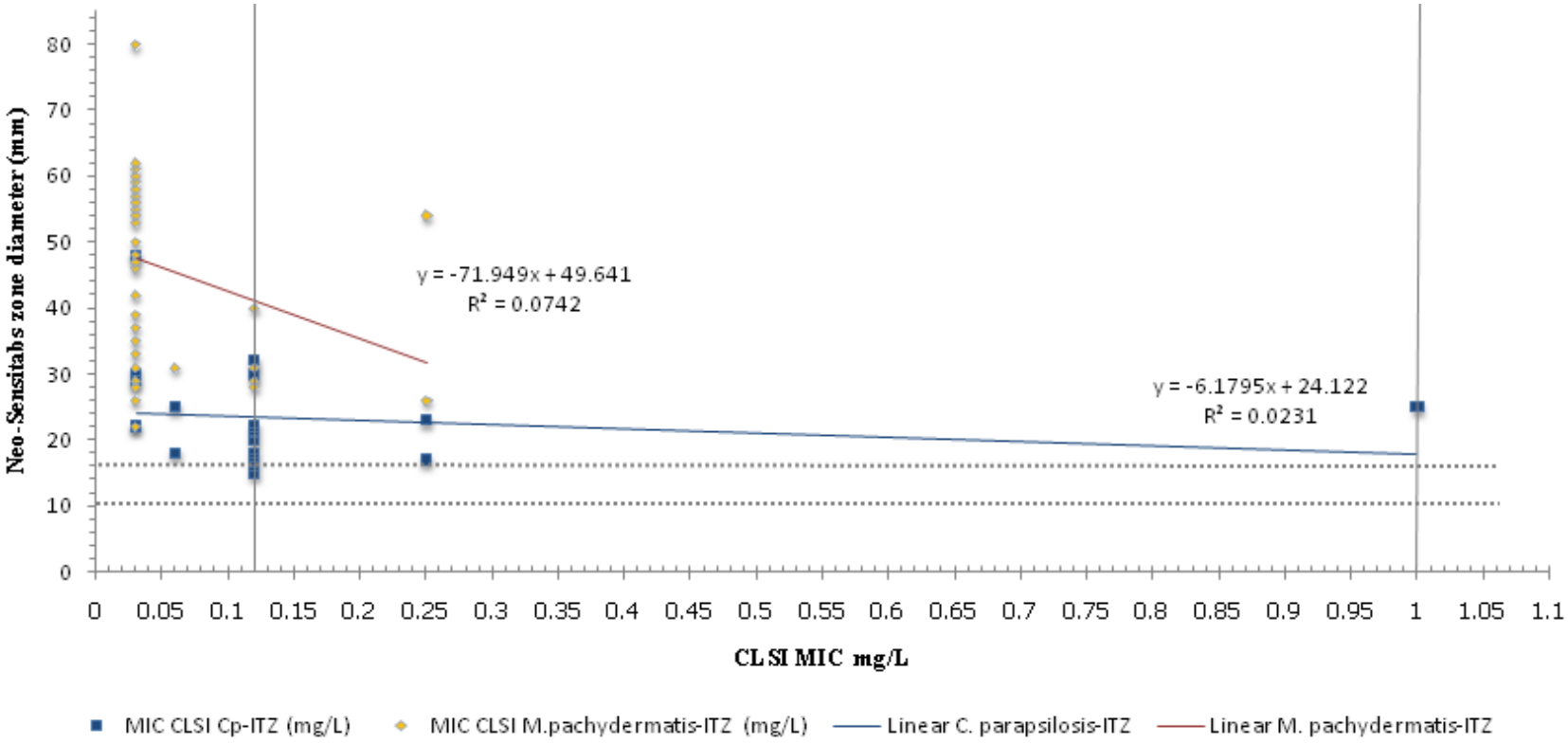


Figure 3.2 Scatter diagram of CLSI MICs for itraconazole, and the zone diameters obtained with this antifungal disk for *Candida parapsilosis* and *Malassezia pachydermatis*. The dots represent the susceptibility value for each tested strain and each line indicates the regression line with correlation coefficient *r* (R^2). Gray dotted and solid lines are the breakpoints for the disk diffusion (DD) and broth microdilution (BMD) MICs according to CLSI, respectively.

3.4. Discussion

The present study expanded our previous study on the frequency and distribution of canine skin yeasts at different stages of seborrheic dermatitis (Yurayart, et al., 2011). To treat mycoses, it is nece ssary to correctly identify the pathogen(s) and subsequently choose the proper antifungal drugs. Despite many available data on human yeast susceptibilities, the availability of susceptibility data on animal isolates is scant.

Since 1983, the CLSI or formerly the 'National Committee for Clinical Laboratory Standards (NCCLS) has published documentation for the standard reference microdilution methodologies for yeasts (CLSI, M27-A3) and molds (CLSI, M38-A). Agar-based diffusion methods such as DD (M44-A) and the E-test (ET) were also developed as a functional and simpler method and were consistent with the CLSI standard reference (Hazen, et al., 2003; Matar, et al., 2003; Pfaller, et al., 2004). However, these document guidelines are only relevant for *Candida* species and *Cryptococcus neoformans*, but also apply to *Malassezia* species and other emerging yeasts (Garau, et al., 2003; Gupta, et al., 2000; Velegraki, et al., 2004).

Mueller Hinton agar supplemented with 2% dextrose and 0.5 mg/L methylene blue was used for DD to allow growth of *C. parapsilosis* and *M. pachydermatis* and clearly presented an inhibition zone, except for KTZ and ITZ. As mentioned in the manufacturer's guidelines, the zones of tested yeast growth could be partially inhibited by these azoles disks. Our DD procedure was proposed to perform susceptibility screening for *M. pachydermatis* and *C. parapsilosis* in dogs.

In order to establish the susceptibility method for *Malassezia* yeasts, various media were tested to replace the standard RPMI 1640 recommended by the CLSI protocol, such as modified RPMI 1640 with lipid supplementation, modified Christensen's urea broth, Sabouraud dextrose broth with 1% Tween 80 and modified Dixon broth (Cafarchia, et al., 2012; Nijima, et al., 2011; Rincon, et al., 2006; Velegraki, et al., 2004). The BMD methodology used to test *M. pachydermatis* against various azoles followed that described by Jesus and colleagues together with the guidelines of the CLSI, M27-A3 and used the breakpoints for *Candida* species (Jesus, et al., 2011). All lipid-free media allowed sufficient growth and susceptibility observation within 48-72 h, and the validity of the methods was assessed in parallel

using reference controls. Since the age and concentration of the *M. pachydermatis* inocula were different in various studies, we confirmed that *M. pachydermatis* growth on day 3, equal to 2-3 x10^3 CFU/ml, was the proper inoculum (Brito, et al., 2007; Gupta, et al., 2000; Velegraki, et al., 2004). A higher density (1-5 x 10^6 CFU/ml) should be recommended for lipid-dependent *Malassezia* species (Rincon, et al., 2006). Therefore, an overly dense *M. pachydermatis* inoculum might yield an over incidence of antifungal resistance (Cafarchia, et al., 2012; Cafarchia, et al., 2012; Hensel, et al., 2009; Peano, et al., 2012).

In this study, the scatter diagrams and the statistical analysis using linear regression could not explain the correlation between the CLSI MICs and inhibition zone diameters due to a lack of diversity among the susceptibility levels of *M. pachydermatis*. Therefore, a detection of errors or percentages of agreement were more appropriate, as recommended by the CLSI M23-A2 and a previous study (Sims, et al., 2006). Regarding the FDA criteria for acceptable categorical agreement, there was an excellent correlation between BMD and DD for *M. pachydermatis* and *C. parapsilosis* with ≤ 1.5% VME and ≤3% ME (Alexander, et al., 2007). Thus, DD may be useful for clinical screening of antifungal susceptibility against *M. pachydermatis* and *C. parapsilosis* in animals. Regarding the DD results for MICs and MFCs, there was 99% agreement, which highlights the concordance and relationship between these methods. Herein, the CLSI document guidelines of *Candida* species could be employed and assessed for *M. pachydermatis* but an extended incubation time was essential (72 h). The *Candida* species breakpoints were used to interpret the *M. pachydermatis* results with good agreement.

M. pachydermatis isolates were highly sensitive to all tested antifungal drugs, except 5-FC. These results are consistent with previous studies where 5-FC resistance was shown to occur due to the absence of cytosine permease and deaminase and therefore is not recommended as a monotherapy to treat *Malassezia* infections (Johnson and Perfect, 2010; Sanglard, 2002). Even though the resistant rates between groups of dogs did not exist, *M. pachydermatis* strains that originated from dogs with SD had a significantly reduced mean inhibition zone diameter against KTZ (48.3 mm.) and TERB (113.3 mm) compared to those of dogs without SD (72.4 and 129.5 mm., respectively) ($P< 0.001$). The high resistant rates of canine *C.*

parapsilosis for KTZ, TERB and 5-FC were detected by DD. Our results were consistent with those in human isolates, but there are no available data on animal-origin *Candida* species for comparison (Zomorodian, et al., 2011).

Generally, KTZ and ITZ are the routine antifungal drugs for canine seborrheic dermatitis, and their MIC values could be reliably used rather than values obtained by DD. Most *M. pachydermatis* isolates were highly susceptible to KTZ and ITZ at MICs <0.03 µg/ml, which is consistent with previous reports (Brito, et al., 2009; Cafarchia, et al., 2012; Garau, et al., 2003; Gupta, et al., 2000; Jesus, et al., 2011; Nijima, et al., 2011; Prado, et al., 2008). The MIC values of *Candida* species and *M. pachydermatis* from dogs with SD were significantly higher than those in dogs without SD. To date, only one strain of *M. pachydermatis* that was resistant to KTZ and ITZ was isolated from a dog with SD (KTZ MIC = 1 µg/ml, ITZ MIC = 2 µg/ml) (Nijima, et al., 2011). Since the breakpoints for *Malassezia* species have not been established, the MIC_{50} and MIC_{90} of*M. pachydermatis*represent a reliable level instead of the breakpoint criteria. In a previous study, KTZ- and ITZ-resistant canine *M. pachydermatis* was defined for isolates with values over the MIC_{90} or MIC_{50} and was detected in both groups of dogs with a higher number of resistant strains among dogs with lesions (12.5% for KTZ-resistant and 9.4% for ITZ-resistant). However, the incidence was possibly higher than expected when compared to CLSI interpretative breakpoints (KTZ; MIC_{50} = 0.016 µg/ml, MIC_{90} = 0.03 µg/ml, ITZ; MIC_{50}< 0.008 µg/ml, MIC_{90}<0.008 µg/ml) (Cafarchia, et al., 2012).

Regarding zoonotic transmission, resistant strains of non-albicans *Candida* species, *Trichosporon* species, *Malasseizia* species and *Rhodotorula* species are the important causes of nosocomial infection(Miceli, et al., 2011). Over 50% of strains with intermediate and KTZ/ITZ-resistance and an MIC ranging from 0.25-1 µg/ml and MFC ranging from 0.25-16 µg/ml were detected among the tested *C. parapsilosis* strains from dogswith and without SD. The results suggest a source of infection because 6-23% of the KTZ- and ITZ-resistant *C. parapsilosis* strains were recovered from both groups of dogs.

Because of insignificant *in vitro* resistant levels between dogs with and without SD, the evolution of resistance is difficult to access by *in vivo* and needed a monitoring of yeast population over time in patients undergoing antifungal therapy

(Anderson, 2005). However, the history of antifungal use in each group was not recorded, and thus this hypothesis should be further examined in future follow-up studies. In addition, resistant progression was suggested the continuing exposure to sub-therapeutic concentration of antifungal drugs associated in resistant development in *M. pachydermatis* (Nakano, et al., 2005).

Our previous study clearly demonstrated that at the stage of primary lesions in seborrheic dermatitis, dogs were coexisting infected with over 1,000 times of *C. parapsilosis* and 100 times of *M. pachydermatis* number (Yurayart, et al., 2011). Therefore, the diagnosis and treatment of yeast-associated canine dermatitis should be reconsidered. In cases of primary lesion SD, itraconazole may be a more appropriate antifungal choice because of the emergence of KTZ-resistant *C. parapsilosis*.

In conclusion, the clinical canine *M. pachydermatis* strains isolated from dogs with and without SD demonstrated *in vitro* susceptibility to all antifungal drugs tested except 5-FC whereas KTZ- and ITZ-resistant *C. parapsilosis* were found by BMD. However, the effectiveness of clinical therapeutic decisions based on these data would best be assessed by follow-up after treatment. There was very good agreement between CLSI DD and BMD, indicating the reliability of these methods for antifungal susceptibility in dog-origin yeasts.

CHAPTER 4

Serological profile markers and immunodominant antigens of *Malassezia pachydermatis* cell wall on progression of canine seborrheic dermatitis

4.1. Introduction

Malassezia pachydermatis are well known as a skin commensal on dog skins and also associated with seborrheic dermatitis (SD) in dog prone to atopic condition. SD is defined for skin allergic condition caused by type-1 hypersensitivity with *M. pachydermatis* infestation (Chen and Hill, 2005). Recently, we proposed concurrent over growth of *Candida parapsilosis* and *M. pachydermatis* in canine SD and their proportion and frequency depended on stage of SD (Yurayart, et al., 2011). However, underlying mechanism and immune response leading these yeasts convert from commensals toward causative agents remained disputative.

The roles of yeasts involving in skin abnormalities are wildly discussed; induction of host immune responses to yeast antigens, irritating free fatty acid generated by fungal lipase, irritating lipid metabolites generated by lipid peroxidase and toxin (DeAngelis, et al., 2007; Saunders, et al., 2012). In humans, immune profile was indicated by IgE-specific to *M. globosa* and *M. sympodialis* allergens where located on cell wall and secretory proteome (Gioti, et al., 2013; Ishibashi, et al., 2009). In dogs, the high levels of IgE and IgG against crude *M. pachydermatis* extracts were reported in SD and atopic dermatitis (AD) and major allergens were assumed (Bond, et al., 1998; Bond and Lloyd, 2002). Because of short half-life of serum IgE and unclassified crude components, the immunodominant profiles might not be fully demonstrated (Schroeder and Cavacini, 2010).

Molecules of IgG subclass have been widely mentioned in the immunological cascade of various allergic diseases. The higher IgG4 levels to crude *M. sympodialis* were used for determination of IgE existence in atopic eczema patients (Johansson, et al., 2004) and the proper proteins were selected for candidate in desensitization (Griffin and Hillier, 2001). On the other hand, studies of canine IgG subclass have been performed only in house dust mite sensitized-AD and leishmaniasis models. The specificity of IgG subclass could demonstrate in cascade of the AD pathogenesis depending on types of allergens (Day, et al., 1996) and could be reflected defect of T helper cell during disease progression (Barbosa, et al., 2011; Oliveira, et al., 2009).

To reveal recognition patterns, sera derived from each stage of SD dogs responding to cell wall components may be a very useful protein markers for overview determination and their progress prediction. The aim of this study was to determine patterns of IgG subclasses responded to extracted cell wall antigens of *C. parapsilosis* and *M. pachydermatis* mediated during the progression of SD.

4.2. Materials and methods

4.2.1. Yeast strains and cultivation

Cell wall proteins were prepared from canine skin yeasts; *C. parapsilosis* ATCC 22019, *M. pachydermatis* CBS 17879, and each of *C. parapsilosis* and *M. pachydermatis* isolated from healthy, primary and secondary seborrheic dermatitis (GenBank accession numbers: GU373653, GU373654, GU373656, GU373658, GU373660 and GU373661). *C. parapsilosis* and *M. pachydermatis* were grown at 32°C for 12 h in YPD broth (1% yeast extract, 1% peptone and 2% glucose) and Leeming and Notman broth (LNB; 1% peptone, 1% glucose, 0.2% yeast extract, 0.8% ox bile, 1% glycerol, 0.05% glycerol monostearate, 0.5% Tween 60 and 2% olive oil), respectively. Yeast density were measured and adjusted into the identical fresh medium by optical density at 600 nm (OD_{600}) of 1. One ml of yeast suspension was transferred to 100 ml of fresh broth medium and grown at 32°C for 48 h in a shaking incubator. In addition, *C. albicans* ATCC 10261 and *C. utilis* IFO 0988 were used as internal controls for enzymatic digestion of cell wall.

4.2.2. Dog sera

All the animal experiment involving in blood sampling procedure was approved by the Institutional Animal Care and Use Committee (IACUC) at Chulalongkorn University, Thailand (approval number 11310018). Dog sera were collected from healthy dogs (n=4), primary seborrheic dermatitis (PSD, n= 4) and secondary seborrheic dermatitis (SSD, n= 11). All sera were collected together with yeast isolation as described in our previous reports (Yurayart, et al., 2011; Yurayart, et al., 2013). In brief, healthy group was age less than six months, not atopic predisposing breeds, free from external parasites and skin lesions. In addition, general health condition and being untreated by steroids in the prior 3 months were included as part of the selection criteria. PSD was defined by SD with moist,

erythema, mal-odor and severe pruritus, while SSD was defined by appearance of hyperpigmentation, lichenification, self-induced alopecia and itching Serum samples were collected from cephalic vein in a volume of 3 ml per dog. All sera were stored at -20°C until use.

4.2.3. Cell wall protein extraction

The extraction of cell wall was carried out based on β-glucanase enzymatic digestion of cell wall using the recommended protocol (Ishikawa, et al., 2013; Thomas, et al., 2008). Briefly, yeast cells were treated by 10% Triton X-100 in phosphate buffer saline (PBS) for 1 h at room temperature, following by washing 3 times with PBS and once with Tris-EDTA (pH 8.0). Protease inhibitor cocktail (Nacalaitesque, Japan) was added prior addition of digestion enzyme. Yeast cells were suspended in 0.5%Westase™ (Takara, Japan) dissolved in McIlvain buffer (pH 6.0) and 0.5 M sodium tartate as an osmotic stabilizer. After treated with cell wall lytic enzyme, the yeast suspension was reciprocally shaken at 30°C and evaluated for time optimization of protoplast preparation at 2h, 4h, 6h, 12h and 24h. Cell wall proteins were collected as supernatants by centrifugation at 12000 x g, 5 min and concentrated by ultrafiltration using a pore-size of 3000 Da (Amicon, USA) to the final volume of 1 ml. Protein quantitation was performed by the Bradford assay.

4.2.4. Protein separation by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method of Laemmli (Laemmli, 1970) using 12% Tris-glycine polyacrylamide (Fisher Scientific, UK) separating gel and 5% stacking gel in a discontinuous buffer system. The concentration of proteins in all samples was adjusted to 1 mg/ml by diluting with an equal volume of dye buffer containing of 125 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol (Fisher Scientific, UK) and 0.005% bromophenol blue (Fisher Scientific, UK). The samples were heated at 100°C for 5 minutes before loaded 10 µl of samples and 5 µl of MagicMark™ XP western protein standard (Invitrogen, USA) and PageRuler™ Prestained protein ladder (Thermo Pierce, USA) into each broad well of the gel and the electrophoresis run at 200 V for 2.30 h (ATTO, Japan). The

protein patterns were confirmed by gel staining with 0.025% Coomassie brilliant blue R-250.

4.2.5. Immunoblotting technique

The cell wall proteins derived from SDS-PAGE were transferred to nitrocellulose membrane (Amersham Hybond™ ECL; GE Healthcare, UK) by a semi-dry electrophoretic transfer cell (Trans-Blot® SD, Bio-Rad). The blotted membranes were blocked in blocking reagent (for Can Get Signal®; Toyobo, Japan) for 1 h. The membranes were primarily reacted 12 h with each group of dogs' sera at different dilution ranged from 1:100-1:50,000 diluted with the immunoreactions enhancer solution (Can Get Signal®, Toyobo, Japan). The membranes were washed for 10 minutes three time with washing buffer (PBS, pH 7.5, 0.1% Tween 20) and secondly reacted with polyclonal goat anti-dog IgG1 or IgG2 conjugated with horseradish peroxidase (HRP) (AbDSerotec, UK) at a 1:25,000 diluted with the immunoreactions enhancer solution (Can Get Signal®, Toyobo, Japan) at room temperature for 1 h. The membranes were washed three times with washing buffer for 10 minutes. Chemiluminescent signals were illustrated by incubation with the luminol substrate ECL (ECL™ Prime, Amersham Pharmacia Biotech, UK) for 5 minutes and images were obtained with a charge coupled device (CCD) camera (Kodak Digital Science™ Image Station 2200, Kodak, USA) for 1 minute and 10 minutes exposure.

4.2.6. Recognition patterns analysis

Kodak Digital Science™ 1D Image analysis software was used to determine the signal magnitude of individual bands and their molecular weights by analyzing positions relative to the molecular weight standards. Student t-test at $p<0.05$ was used to analyze the difference of means of band number reacted to each IgG subclass between dog groups. Number and type of protein patterns recognized by each of IgG subclass were simplified by using bar graph in the unit of molecular weight (kDa) dividing by disease progression. Frequency of IgG recognition was calculated from positive band detection by percentage to each of IgG subclass.

4.3. Results

4.3.1. Cell wall protein profiles of *C. parapsilosis* and *M. pachydermatis*

By SDS-PAGE, cell wall protein profiles of *C. parapsilosis* and *M. pachydermatis* were quantitatively and qualitatively unique within their species and there was no different pattern among the strains isolated from healthy, PSD or SSD and the reference strains (*M. pachydermatis* CBS 1879 and *C. parasilosis* ATCC 22019, respectively).

The proteins of *C. parapsilosis* revealed approximately 13-16 bands ranging from 96-23kDa (Figure4.1, A) and *M. pachydermatis* revealed approximately 30-34 bands ranging from 15-189 kDa (Figure 4.2, A). Common proteins between these two yeast species were highly detected at the molecular weights of 34-48, 55-69 and 76-82 kDa. Then, *M. pachydermatis*and *C. parapsilosis* isolated from SSD was chosen for immunodominant determination by immunoblotting.

4.3.2. Patterns of *C. parapsilosis* cell wall proteins recognition by IgG subclass

All sera did not present antigen-specific IgG1 reacted to *C. parapsilosis* cell wall proteins at the titer < 1:10. While, IgG2 reactivity was detected at the serum titer 1:100 (in healthy and PSD dogs) and 1:1000 (in SSD dogs). All of IgG-2 reacted bands were commonly detected by all groups of dogs with the molecular weights of 30, 34, 40, 42, 44, 48, 69 and 82 kDa (Figure 4.1, B).

4.3.3. Patterns of *M. pachydermatis* cell wall proteins recognition by IgG subclass

By using specific IgG subclass, dog sera in each group showed unique patterns recognized to cell wall proteins of *M. pachydermatis*. The sera derived from SSD demonstrated the higher titers of IgG1 and IgG2 specific to *M. pachydermatis* at 1:1,000-50,000, whereas those of healthy and PSD dogs had undetectable band at the serum titer 1:100.

In healthy, approximately 29±4 recognized bands from cell wall proteins were recognized by IgG2 in greater proportion than those of compared to IgG1 (only 4±3 bands). In all tested dog sera, the common antigens recognized by both IgG subclass were 42, 47, 50, 53-54, 56-57 and 64 kDa. In PSD, the15±0.8 bands proteins were mainly recognized by IgG2 which the number of recognized band was lower than that of healthy (p= 0.01), whereas IgG1-reacted proteins were

occasionally observed (5±3.4 bands). In SSD, multiple bands of proteins became recognize by both IgG1 and IgG2 with higher titer than those of healthy and PSD (IgG1; 19±9 and IgG2; 15±2.8 reactive bands). IgG subclasses reacted to *M. pachydermatis* cell wall proteins and comparison of means of protein band recognition among dog groups are shown in Figure 4.2-4.4 and 4.7, respectively.

The digested data reviewed *M. pachydermatis* protein recognized by IgG subclasses are shown in Figure 4.5-4.6, which are shown by bar graph presenting molecular weights of recognized bands and frequency of IgG subclass detection (%) among dog groups. According to IgG2-binding proteins in healthy dog sera, four proteins with molecular weights of 20, 114-115, 138 and 150-152 kDa were uniquely detected (Figure 4.5). On the other hand, IgG1-binding protein pattern were resemble to those of IgG2 when reacted with the sera derived from SSD. Interestingly, among those remarkable IgG1-binding proteins, the particular six bands; 18, 58-59, 67-68, 86 and 154 kDa were exclusively recognized by only SSD dogs (Figure 4.6).

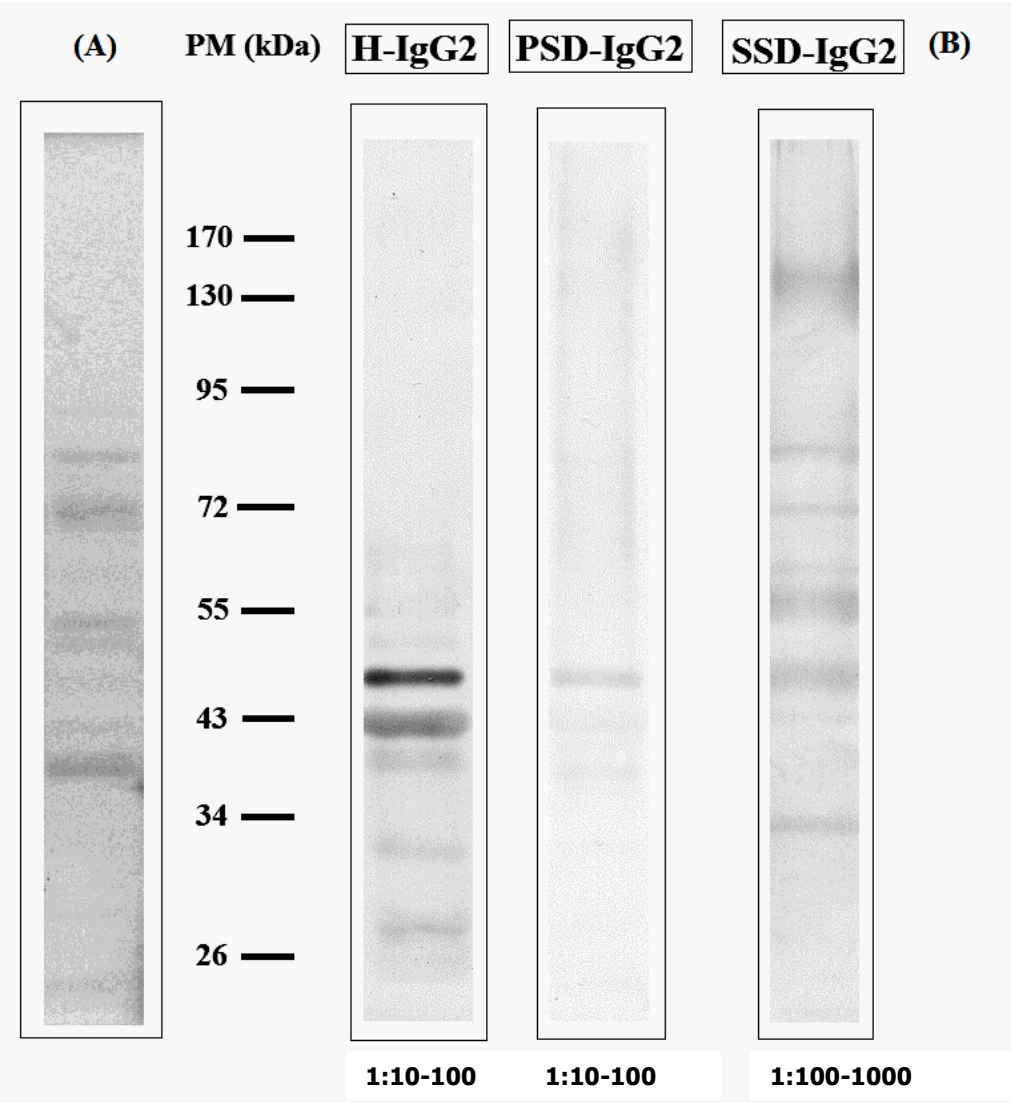


Figure 4.1 Protein pattern of *C. parapsilosis* cell wall by using SDS-PAGE (A) and patterns of IgG2 recognition in healthy (H-IgG2), primary seborrheic dermatitis (PSD-IgG2) and secondary seborrheic dermatitis (SSD-IgG2) dog sera.PM; protein marker (B).

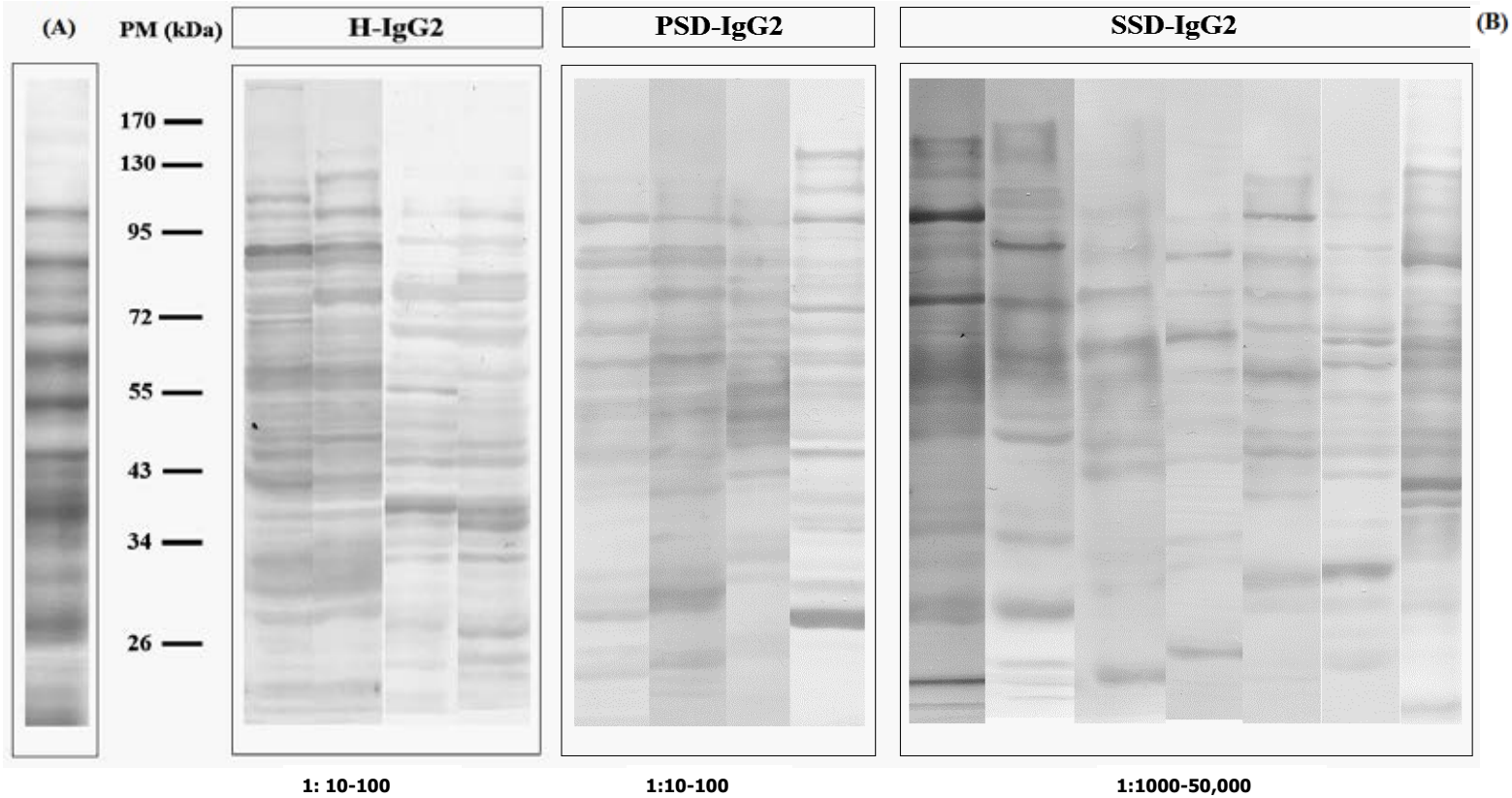


Figure 4.2 Protein profile of *M. pachydermatis* cell wall by using SDS-PAGE (A) and patterns of IgG2 recognition (B) compared among healthy (H-IgG2), primary seborrheic dermatitis (PSD-IgG2) and secondary seborrheic dermatitis (SSD-IgG2) dog sera.
PM; protein marker.

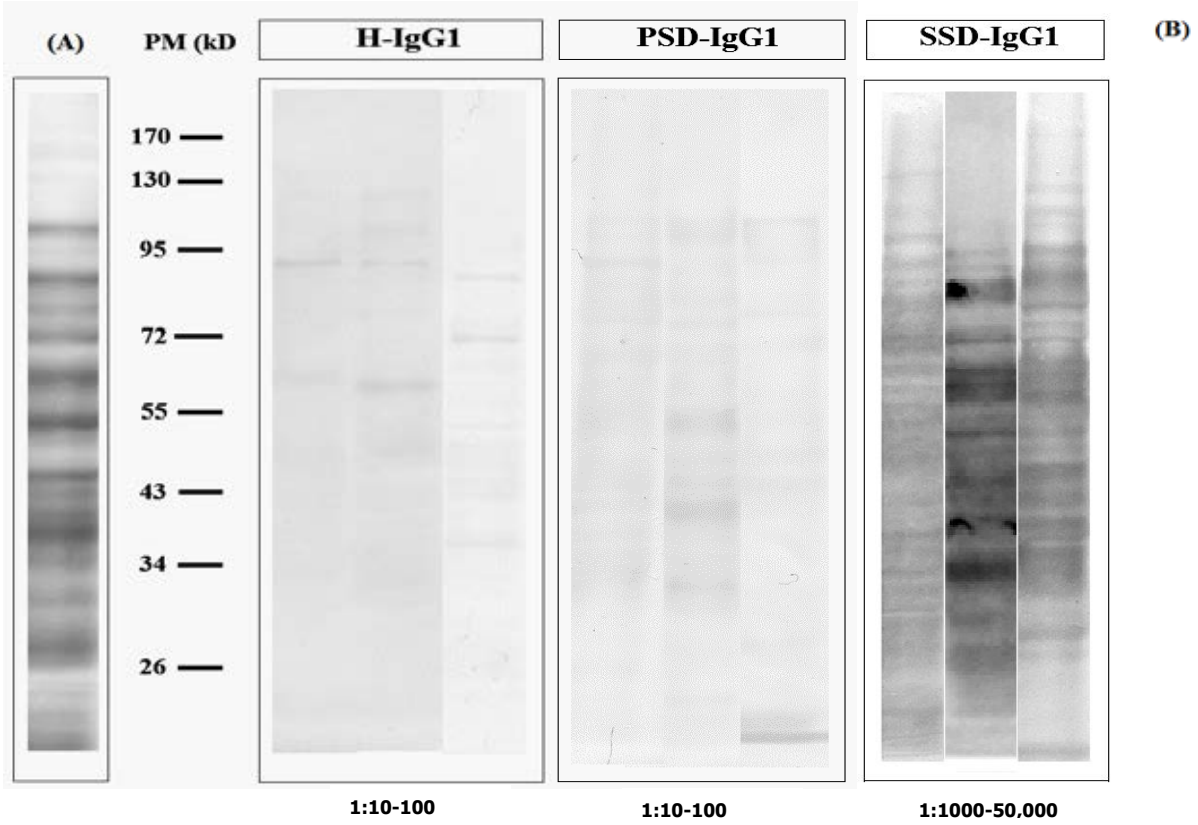


Figure 4.3 Protein pattern of *M. pachydermatis* cell wall by using SDS-PAGE (A) and patterns of IgG1 recognition (B) compared among healthy (H-IgG1), primary seborrheic dermatitis (PSD-IgG1) and secondary seborrheic dermatitis (SSD-IgG1) dog sera.
PM; protein marker.

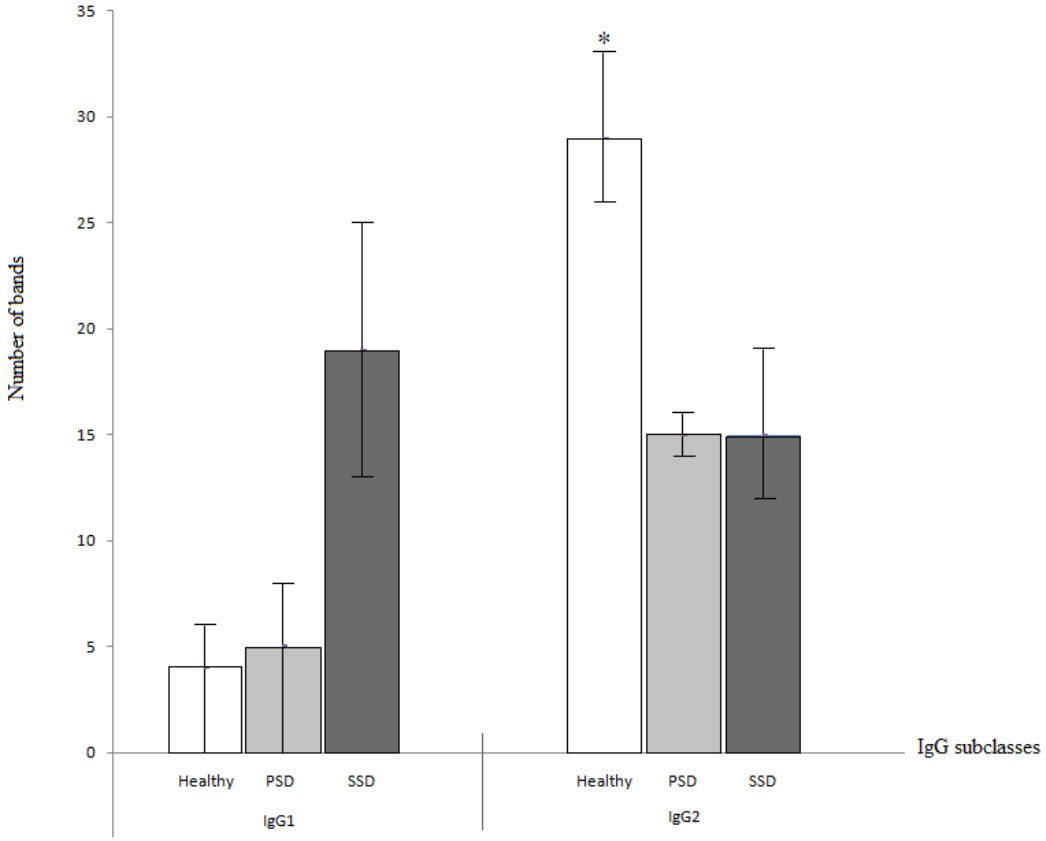


Figure 4.4 Comparison of protein number classified by IgG subclass among dog groups. Error bars represent standard errors.* $p \leq 0.05$.

PSD; primary seborrheic dermatitis dogs, SSD; secondary seborrheic dermatitis dogs.

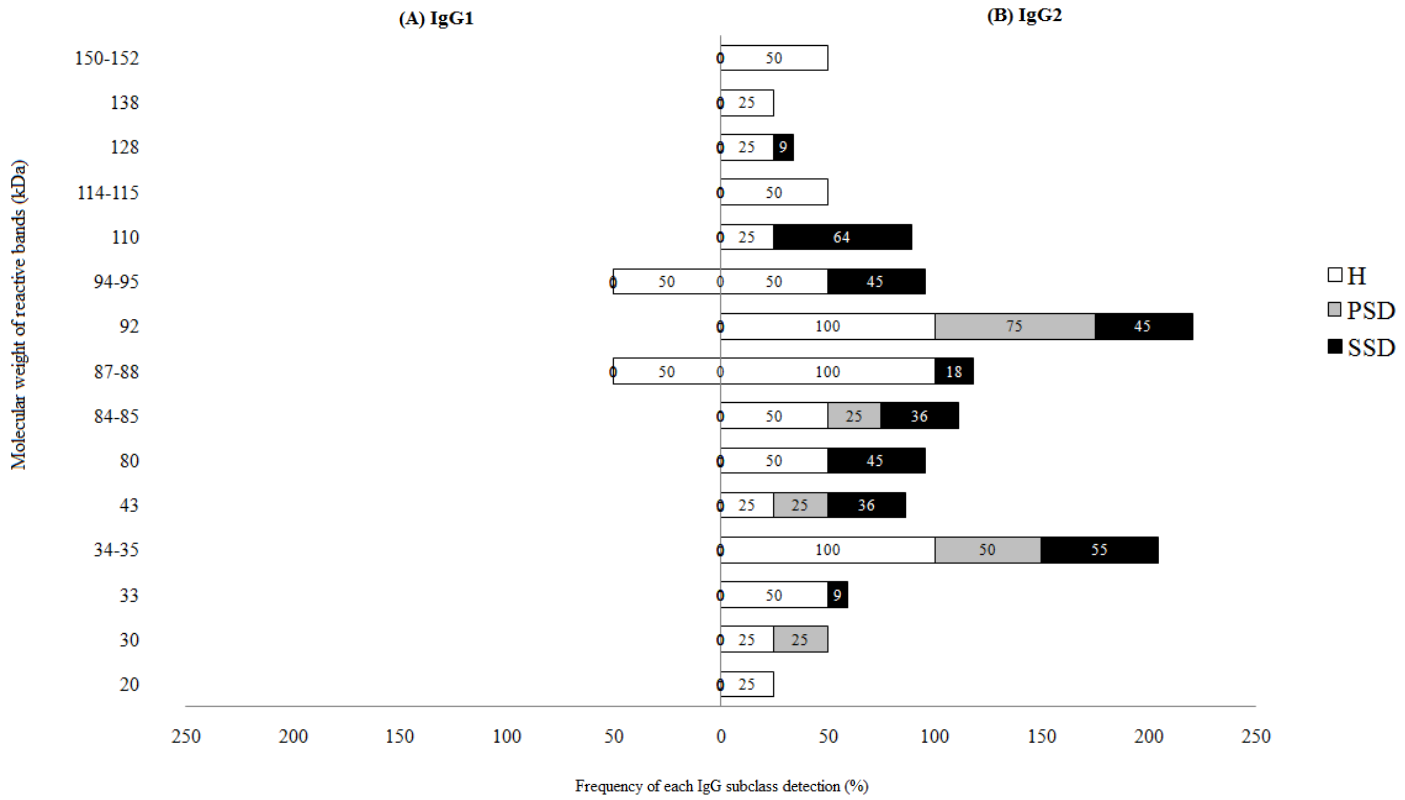


Figure 4.5 The digested data derived from *M. pachydermatis* proteins that lightly reacted with IgG1 in healthy and frequencies of (A) IgG1 and (B) IgG2 recognition in dog sera presenting by percentages (in the middle of each bar).

H; healthy dogs, PSD; primary seborrheic dermatitis dogs, SSD; secondary seborrheic dermatitis dogs.

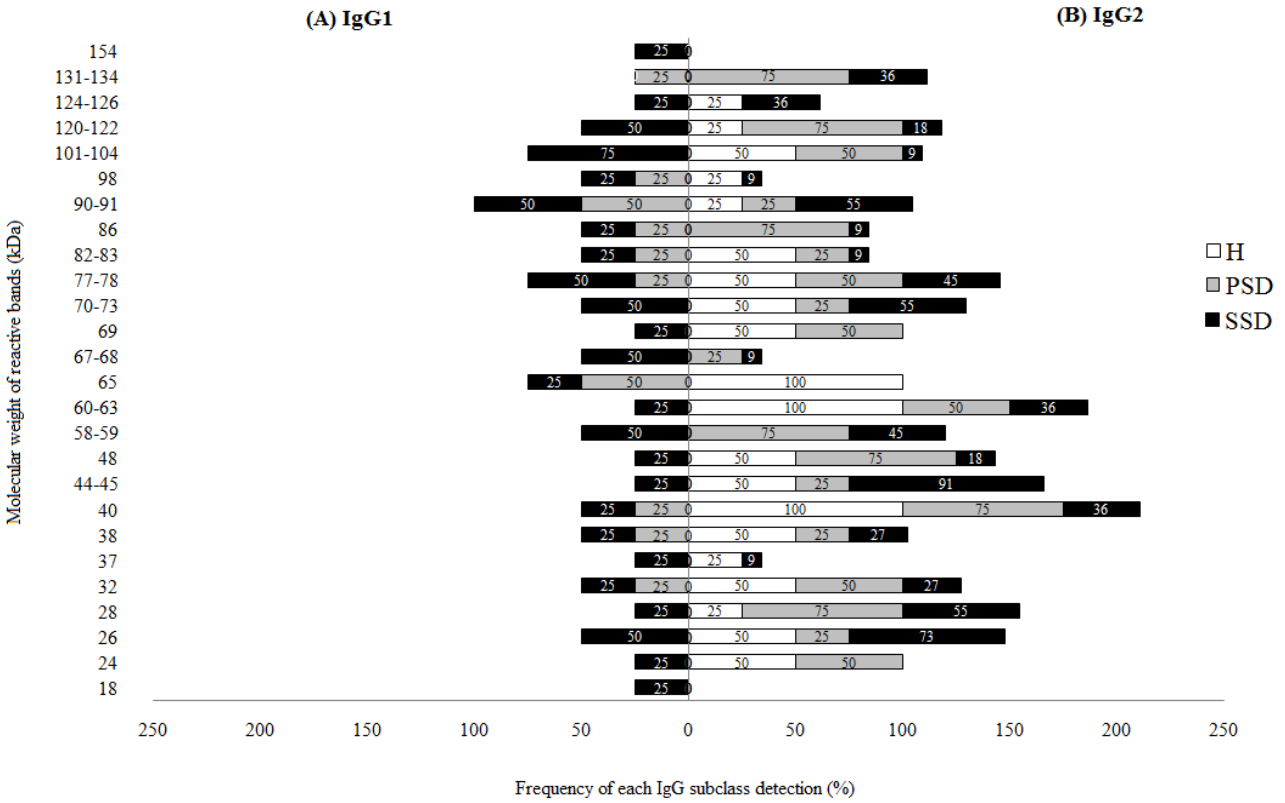
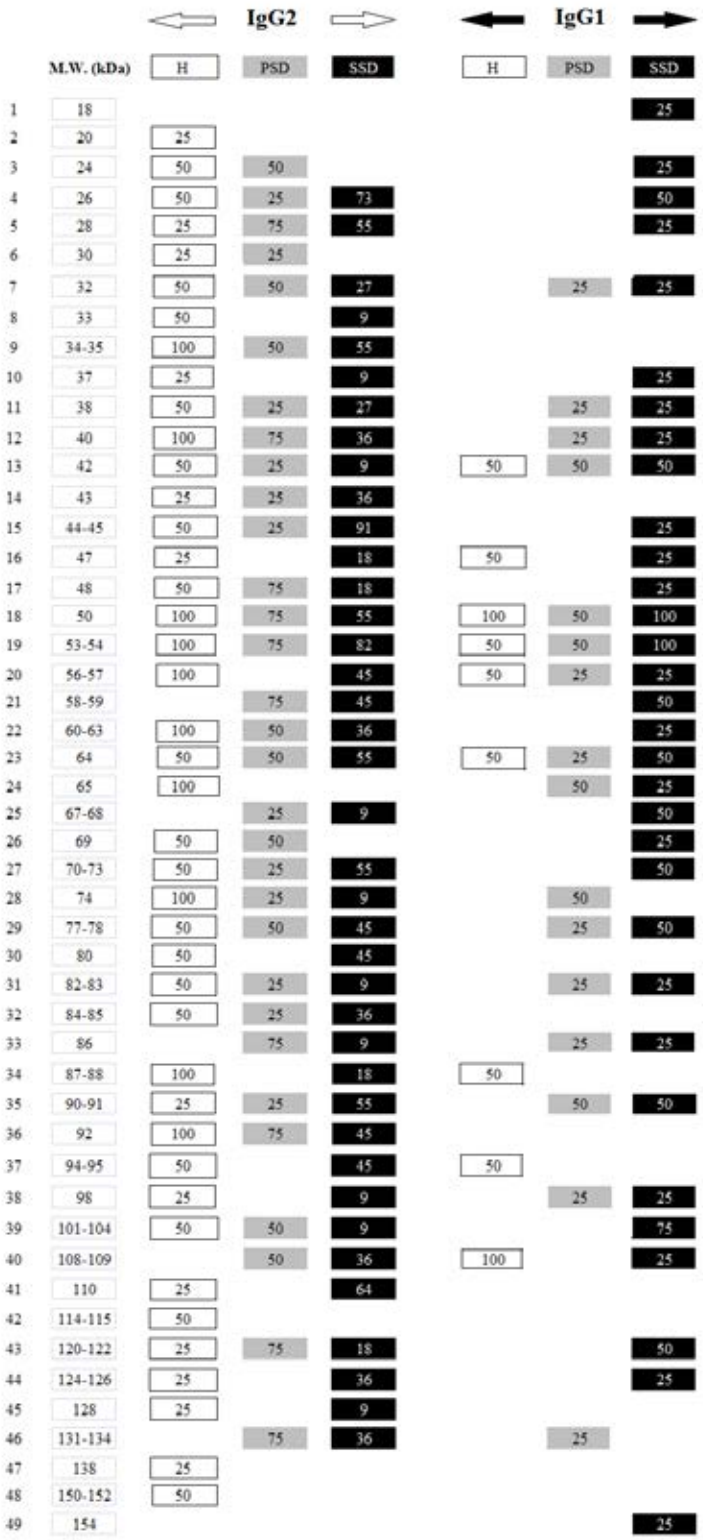


Figure 4.6 The digested data derived from *M. pachydermatis* proteins that highly reacted with IgG1 in diseased dogs and frequency of IgG1 (A) and IgG2 recognition (B) in dog sera presenting by percentages (in the middle of each bar).

H; healthy dogs, PSD; primary seborrheic dermatitis dogs, SSD; secondary seborrheic dermatitis dogs.



	M.W. (kDa)	IgG2 H	IgG2 PSD	IgG2 SSD	IgG1 H	IgG1 PSD	IgG1 SSD
1	18						25
2	20	25					
3	24	50	50				25
4	26	50	25	73			50
5	28	25	75	55			25
6	30	25	25				
7	32	50	50	27		25	25
8	33	50		9			
9	34-35	100	50	55			
10	37	25		9			25
11	38	50	25	27		25	25
12	40	100	75	36		25	25
13	42	50	25	9	50	50	50
14	43	25	25	36			
15	44-45	50	25	91			25
16	47	25		18	50		25
17	48	50	75	18			25
18	50	100	75	55	100	50	100
19	53-54	100	75	82	50	50	100
20	56-57	100		45	50	25	25
21	58-59		75	45			50
22	60-63	100	50	36			25
23	64	50	50	55	50	25	50
24	65	100				50	25
25	67-68		25	9			50
26	69	50	50				25
27	70-73	50	25	55			50
28	74	100	25	9		50	
29	77-78	50	50	45		25	50
30	80	50		45			
31	82-83	50	25	9		25	25
32	84-85	50	25	36			
33	86		75	9		25	25
34	87-88	100		18	50		
35	90-91	25	25	55		50	50
36	92	100	75	45			
37	94-95	50		45	50		
38	98	25		9		25	25
39	101-104	50	50	9			75
40	108-109		50	36	100		25
41	110	25		64			
42	114-115	50					
43	120-122	25	75	18			50
44	124-126	25		36			25
45	128	25		9			
46	131-134		75	36		25	
47	138	25					
48	150-152	50					
49	154						25

Figure 4.7 Demonstration of the overall proteins of *M. pachydermatis* recognized by each IgG subclasses reacted with dog sera obtained from health (H), primary seborrheic (PSD) and secondary seborrheic dogs (SSD).

4.4. Discussion

Varieties of protein profiles were influential by fungal growth phase, culture conditions, and methods of protein extraction (Habibah, et al., 2005). In this study, the extracted cell wall proteins of *C. parapsilosis* and *M. pachydermatis* derived from different dog skin conditions shown a perfect similar pattern in their species. These results were similar to previous studies of whole cell extracts by the mechanical disruption; glass bead-beating or homogenizer of cell membrane (Coutinho, et al., 1997; Kim, et al., 2010). Therefore, we presumed that yeast protein structure was steadfast in any role of symbiosis and a representative could reflect antigenic structures. The cell wall proteins are believed to be the first target to host immune system due to their immunogenic epitopes (Heilmann, et al., 2012). All sera were collected together with yeast isolation from healthy and the clinical dogs since 2009-2010 and strongly revealed the correlation between frequency/distribution of yeast and the stage of SD in our previous report (Yurayart, et al., 2011). Then the sera derived from well-defined dogs were used for immunological determination in this study.

Previously, the titer level of IgE-mediated in canine atopic dermatitis were characterized by ELISA (Farver, et al., 2005) and a few *Malassezia* proteins recognized by IgE were defined by immunoblotting (Chen, et al., 2002; Nuttall and Halliwell, 2001). However, titers and specificity of *Malassezia*-specific IgE were low and gave a minor correlation between healthy and clinical dogs with *Malassezia* overgrowth; because of difference in cutaneous and circulating levels of IgE (Farver, et al., 2005). On the contrary, IgG to *M. pachydermatis* is a solid marker representing a longstanding response of host exposed to commensal yeast in both healthy and *Malassezia*-associated skin diseases (SD, otitis externa and AD) that might reflect the protective or inflammation roles (Bond, et al., 1998; Chen and Hill, 2005). Therefore, a total IgG responses against *Malassezia* antigens could not fulfill in explanation of pathogenesis cascade (Bond and Lloyd, 2002). To increase specificity of IgG1 and IgG2 were used as the representative of Th2-like and Th1-like regulate immune responses, respectively, that were able to linked between cytokine profiles correlated with canine leishmaniasis (Barbosa, et al., 2011; Oliveira, et al., 2009; Reis, et al., 2006). Thus, the available polyclonal anti-dog IgG1 and IgG2

antibodies were used to evaluate for T lymphocytes regulate humoral immune responses to the yeast antigens.

The present study expanded our previous study of canine skin yeasts colonizing status in different stages of SD (Yurayart, et al., 2011); in term of humoral immune responses to those opportunistic skin yeast cell wall antigens. Overall image by immunoblotting patterns, dog sera demonstrated the significant differences in IgG subclass responded to antigens of *C. parapsilosis* and *M. pachydermatis*. The pattern of *C. parapsilosis* recognized by IgG2 was potentially cross-reacted to those of *M. pachydermatis* antigens, whereas the undetectable antigen-specific IgG1 reactivity was observed until SSD stage. We speculated that *C. parapsilosis* antigen might not play a pathogenic role during over growth but maintained as commensal. In human AD, the IgE specific to *Malassezia* was associated with Th2-cytokine but not to *Candida* spp. By contrast, the appearance of *Candida* antigens were related to Th1 cytokine but it seemed not trigger IgG production (Savolainen, et al., 2001). In addition, only IgE cross-reactivity among these two fungal antigens was mentioned but the IgE specific to *Candida* antigens was not importance in sensitization to *Malassezia* spp. (Huang, et al., 1995).

Our results showed the different immunological patterns between IgG1 and IgG2 responses to *M. pachydermatis* antigens in each dog group. In SSD dogs, the highest titers of both IgG1 and IgG2 were detected with over 30 reacted proteins whereas those of healthy and PSD were lower in quantitative and qualitative levels. The low IgG1 titer was confirmed the role of commensal in healthy but was not relevant to the clinical sign at PSD. Thus, the unrelated between IgG1 and IgE level was noticed during primary lesion (Willemse, et al., 1985). Regarding to exposure time and on-going inflammation, strong reaction of IgG2 mediated by Th1 cell was not influential over Th2-like response but might aggregate during episode of chronic inflammation (Tomee, et al., 1996).

In normal dogs, numerous epitopes of *M. pachydermatis* antigens were commonly recognized by IgG2 in a low level, especially to 20, 114-115, 138 and 150-152 kDa. These four proteins assembled as the unique profile, might indicate balancing condition between host and yeast interaction. In PSD dogs, levels of both IgG subclasses reactivity were maintained similarly to healthy but significantly

reduced in numbers of IgG2-recognized epitopes. The profile of the absent IgG2-reacted bands in PSD (protein molecular weights of 33, 56-57, 65, 80, 87-88, 94-95 and 114-115 kDa) might be associated an impair recognition of dendritic cells or mal-conformation of any antigen presenting cells during skin abnormality (Buentke and Scheynius, 2003; Eisenbarth, et al., 2003). Interestingly, severe erythematous lesion on PSD was not related to the strong reacted to IgG1 mediated by Th2, therefore an allergic signal might up-regulate the degradation of mast cell via only Th2 mediated IgE (Welle, et al., 1999; Willemse, et al., 1985). Regarding to chronic stage (SSD), dog sera were overwhelmed with the high levels of both IgG1 and IgG2 with the higher number of IgG1-reacted protein bands. This was speculate that IgG1 provoked lesions of lichenification and hyperpigmentation by Th2 responses concurrent with healing process, whereas the high response of IgG2 mediated from Th1 could be not influent on this stage. These were agreed with the previous study which demonstrated the mixed cytokine expression profiles of Th1 (IFN-ɣ, IL-12 and TNF-ɒ) and Th2 (IL-13) in the chronic lesions of canine AD (Schlotter, et al., 2011).

The evidence of cross-reactive idiotype expression was demonstrated among IgG1, IgG2 and IgE recognized *M. pachydermatis* antigens. In this study, the common proteins; 42, 52, 56 and 63 kDa were recognized by IgG1 and IgG2 in all tested dog sera that strongly related to the major allergens recognized by IgE-specific to *M. pachydermatis* in canine AD with *Malassezia* overgrowth(Chen, et al., 2002), these proteins might not be used for pathogenicity makers. We revealed that the IgG1 and IgG2 specific to proteins; 18, 58-59, 67-68, 86 and 154 kDa were uniquely found among SSD dogs that should be a promising marker for disease determination. These proteins except 154 kDa were identical to the major antigens recognized by total serum IgG of *Malassezia*-associated SD and AD dogs in previous studies (Bond and Lloyd, 2002; Chen, et al., 2002; Kim, et al., 2010), that became immunodominant epitopes in *M. pachydermatis* cell wall recognition during the late stage.

In conclusion, only *M. pachydermatis*but not *C. parapsilosis*, were contributed on host humoral immune responses associated in canine SD. By using IgG subclass could determine the responsive patterns on healthy and each

progression of diseases and the immunodominant proteins may reflect the important components inducing or controlling of symptom.

CHAPTER 5

CONCLUSION

The characterization of canine skin yeasts described in this dissertation was conducted with three main objectives. The first objective was to investigate the diversity of yeast colonizing on canine seborrheic dermatitis compared to that of healthy subjects; in term of species, anatomical sites and population size. The second objective was to determine an *in vitro* efficacy of antifungal agents against clinical isolates obtained from Thai seborrheic dermatitis dogs. The third objective was to demonstrate the subclass of IgG antibodies specific to the yeast isolates and immunodominant proteins of yeasts associated SD. For the first objective, we demonstrated the co-colonization of *M. pachydermatis* and *C. parapsilosis* in large amounts and frequency associated with stage of disease and anatomical site based on fungal cultivation and identification. According to the chronic relapsing aspects of SD and long-term of antifungal drugs exposure, the awareness of drug resistance was concerned in the second objective of study. Together with the results from the first objective, strains of canine skin yeasts-derived from different dog skin condition were determined for antifungal susceptibility. For the third objective, we evaluated the host-pathogen interaction through the humoral immune responses; IgG subclasses recognition patterns against *M. pachydermatis* cell wall antigens.

The first objective was to investigate the diversity of yeast associated with the degree of canine seborrheic dermatitis (SD) by anatomical sites. Yeast isolation and characterization were carried out based on microscopical features and biochemical properties. DNA analysis at the internal transcribed spacer I of 26S rDNA region was utilized for species confirmation. Four species of yeast consisting *M. pachydermatis, M. furfur, C. parapsilosis* and *C. tropicalis* recovered from examined dogs. *M. pachydermatis* and *C. parapsilosis* were isolated from all dogs, but *C. tropicalis* and *M. furfur* were recovered from 3 healthy dogs and one diseased dog, respectively. The number of *M. pachydermatis* and *C. parapsilosis* in diseased dogs was higher than that of healthy specimens ($P< 0.01$). High frequency and population size of *C. parapsilosis* were closely associated to PSD, while those of *M. pachydermatis* were associated with both PSD and SSD ($P< 0.01$). *C. parapsilosis* were predominant at the perianal area. This study demonstrated the co-colonization

of *M. pachydermatis* and *C. parapsilosis* in large amounts and frequency associated with stage of disease and anatomical site.

The second objective was to determine and compare the susceptibility levels of yeasts isolated from dogs with and without seborrheic dermatitis (SD) using the disk diffusion method (DD) for itraconazole (ITZ), ketoconazole (KTZ), nystatin (NYS), terbinafine (TERB) and 5-fluorocytosine (5-FC) and the broth microdilution method (BMD) for ITZ and KTZ. The reliability between the methods was assessed using an agreement analysis and linear regression. By DD, all tested *M. pachydermatis* isolates were susceptible to ITZ, KTZ, NYS and TERB but resistant to 5-FC. Only 46-60% of the tested *C. parapsilosis* isolates were susceptible to KTZ, TERB and 5-FC, but ITZ and NYS were effective against all. By BMD, over 95% of *M. pachydermatis* isolates were susceptible to KTZ and ITZ with an MIC_{90}< 0.03 and 0.12 μg/ml, respectively. The frequency of KTZ- and ITZ-resistant *C. parapsilosis* was 29% and 7%, and the MIC_{90} values were 1 μg/ml and 0.5-1μg/ml, respectively. Regarding the agreement analysis, 2.2% of minor errors were observed in *M. pachydermatis* and 0.2-1% of very major errors occurred among *C. parapsilosis*. There were no significant differences in the yeast resistance rates between dogs with and without SD. KTZ and ITZ were still efficacious for *M. pachydermatis* but a high rate of KTZ resistant was reported in *C. parapsilosis*.

The third objective was to reveal the pattern of IgG1 and IgG2 mediated during each progression of SD, responded to cell wall of *C. parapsilosis* and *M. pachydermatis* by using immunoblotting analysis. Dog sera were obtained from three groups; healthy skin (n=4), acute moist, erythema, pruritic skin defined as primary SD (PSD, n= 4) and chronic hyperpigmentation and lichenification skin defined as secondary SD (SSD, n= 11). The proteins of *C. parapsilosis* were not responded to IgG1 and the titer of IgG2 was in low level to all stages of disease. The different immunoblotting patterns between IgG1 and IgG2 to *M. pachydermatis* antigens were demonstrated. In healthy and PSD dogs, specific-IgG2 was presented at 1:100 titers whereas IgG1 was lower. The numbers of IgG2-reacted bands were significantly highest in healthy dogs with uniquely molecular weights; 20, 114-115, 138 and 150-152 kDa. In SSD dogs, both subclasses of IgG were detected at 1:1000-1:10,000

titers and multiple bands were strongly recognized with the immunodominant proteins at18, 58-59, 67-68, 86 and 154 kDa.

In conclusion, this dissertation indicated that various yeasts live on skin of dogs and serve as commensals. Induction of host immune response to yeast antigens was contributed on skin damage and abnormalities of seborrheic dermatitis. Up to date, controlling of opportunistic yeast proliferation by commonly used antifungal drugs, especially azoles was still effective. Serological profile markers and immunodominant antigens of *M. pachydermatis* cell wall recognized by IgG subclasses could determine stage of SD progression and health status.

REFERENCES

Akerstedt J and Vollset I 1996. *Malassezia pachydermatis* with special reference to canine skin disease. Br Vet J. 152(3): 269-281.

Alexander BD, Byrne TC, Smith KL, Hanson KE, Anstrom KJ, Perfect JR and Reller LB 2007. Comparative evaluation of Etest and sensititre yeastone panels against the Clinical and Laboratory Standards Institute M27-A2 reference broth microdilution method for testing *Candida* susceptibility to seven antifungal agents. J Clin Microbiol. 45(3): 698-706.

Anderson JB 2005. Evolution of antifungal-drug resistance: mechanisms and pathogen fitness. Nat Rev Microbiol. 3(7): 547-556.

Ashbee HR, Fruin A, Holland KT, Cunliffe WJ and Ingham E 1994. Humoral immunity to *Malassezia furfur* serovars A, B and C in patients with pityriasis versicolor, seborrheic dermatitis and controls. Exp Dermatol. 3(5): 227-233.

Barbosa MAG, Alexandre-Pires G, Soares-Clemente M, Marques C, Rodrigues OR, De Brito TV, Da Fonseca IP, Alves LC and Santos-Gomes GM 2011. Cytokine Gene Expression in the Tissues of Dogs Infected by *Leishmania infantum*. J. Comp. Pathol. 145(4): 336-344.

Batra R, Boekhout T, Gueho E, Cabanes FJ, Dawson TL and Gupta AK 2005. *Malassezia* Baillon, emerging clinical yeasts. FEMS Yeast Res. 5(12): 1101-1113.

Bergbrant IM, Johansson S, Robbins D, Bengtsson K, Faergemann J, Scheynius A and Soderstrom T 1991. The evaluation of various methods and antigens for the detection of antibodies against *Pityrosporum ovale* in patients with seborrhoeic dermatitis. Clin Exp Dermatol. 16(5): 339-343.

Bond R, Elwood CM, Littler RM, Pinter L and Lloyd DH 1998. Humoral and cell-mediated responses to *Malassezia pachydermatis* in healthy dogs and dogs with *Malassezia* dermatitis. Vet Rec. 143(14): 381-384.

Bond R and Lloyd DH 1997. Skin and mucosal populations of *Malassezia pachydermatis* in healthy and seborrhoeic basset hounds. Vet Dermatol. 8: 101-106.

Bond R and Lloyd DH 2002. Immunoglobulin G responses to *Malassezia pachydermatis* in healthy dogs and dogs with *Malassezia* dermatitis. Vet Rec. 150(16): 509-512.

Bond R, Patterson-Kane JC and Lloyd DH 2004. Clinical, histopathological and immunological effects of exposure of canine skin to *Malassezia pachydermatis*. Med Mycol. 42(2): 165-175.

Bond R, Patterson-Kane JC, Perrins N and Lloyd DH 2006. Patch test responses to *Malassezia pachydermatis* in healthy basset hounds and in basset hounds with *Malassezia* dermatitis. Med Mycol. 44(5): 419-427.

Bond R, Saijonmaa-Koulumies LE and Lloyd DH 1995. Population sizes and frequency of *Malassezia pachydermatis* at skin and mucosal sites on healthy dogs. J Small Anim Pract. 36(4): 147-150.

Brakhage AA, Zipfel PF, Mayser PA, Lang SK and Hort W 2008. Pathogenicity of *Malassezia* Yeasts In: Human and Animal Relationships. K Esser (ed) Vol 6 Springer Berlin Heidelberg. 115-151.

Brito EH, Fontenelle RO, Brilhante RS, Cordeiro RA, Monteiro AJ, Sidrim JJ and Rocha MF 2009. The anatomical distribution and antimicrobial susceptibility of yeast species isolated from healthy dogs. Vet J. 182(2): 320-326.

Brito EH, Fontenelle RO, Brilhante RS, Cordeiro RA, Soares Junior FA, Monteiro AJ, Sidrim JJ and Rocha MF 2007. Phenotypic characterization and in vitro antifungal sensitivity of *Candida* spp. and *Malassezia pachydermatis* strains from dogs. Vet J. 174(1): 147-153.

Buentke E and Scheynius A 2003. Dendritic cells and fungi. APMIS. 111(7-8): 789-796.

Cabanes FJ, Hernandez JJ and Castella G 2005. Molecular analysis of *Malassezia sympodialis*-related strains from domestic animals. J Clin Microbiol. 43(1): 277-283.

Cafarchia C, Figueredo LA, Iatta R, Colao V, Montagna MT and Otranto D 2012. In vitro evaluation of *Malassezia pachydermatis* susceptibility to azole compounds using E-test and CLSI microdilution methods. Medical mycology : official publication of the International Society for Human and Animal Mycology. 50(8): 795-801.

Cafarchia C, Figueredo LA, Iatta R, Montagna MT and Otranto D 2012. In vitro antifungal susceptibility of *Malassezia pachydermatis* from dogs with and without skin lesions. Vet Microbiol. 155(2-4): 395-398.

Cafarchia C, Gallo S, Capelli G and Otranto D 2005. Occurrence and population size of *Malassezia* spp. in the external ear canal of dogs and cats both healthy and with otitis. Mycopathologia. 160(2): 143-149.

Cafarchia C, Gallo S, Romito D, Capelli G, Chermette R, Guillot J and Otranto D 2005. Frequency, body distribution, and population size of *Malassezia* species in healthy dogs and in dogs with localized cutaneous lesions. J Vet Diagn Invest. 17(4): 316-322.

Carlotti DN 2009. How to treat atopic dermatitis in dogs. Eur J Companion Anim Pract. 19(3): 268-275.

Carrillo-Munoz AJ, Abarca L, Quindos G, Arevalo P, Bornay F, Cabanes FJ, Casals JB, Estivill D, Gonzalez-Lama Z, Iglesias I, Hernandez-Molina JM, Linares MJ, Martin-Mazuelos E, Paya MJ, Pereiro M, Jr San Millan R and Rubio MC 1999. Multicenter evaluation of Neo-Sensitabs, a standardized diffusion method for yeast susceptibility testing. Revista iberoamericana de micologia. 16(2): 92-96.

Chang HJ, Miller HL, Watkins N, Arduino MJ, Ashford DA, Midgley G, Aguero SM, Pinto-Powell R, von Reyn CF, Edwards W, McNeil MM and Jarvis WR 1998. An epidemic of *Malassezia pachydermatis* in an intensive care nursery associated with colonization of health care workers' pet dogs. N Engl J Med. 338(11): 706-711.

Chen TA, Halliwell RE, Pemberton AD and Hill PB 2002. Identification of major allergens of *Malassezia pachydermatis* in dogs with atopic dermatitis and *Malassezia* overgrowth. Vet Dermatol. 13(3): 141-150.

Chen TA, Halliwell REW and Hill PB 2002. Immunoglobulin G responses to Malassezia pachydermatis antigens in atopic and normal dogs. In: ed Oxford Blackwell Science. 202-209.

Chen TA and Hill PB 2005. The biology of *Malassezia* organisms and their ability to induce immune responses and skin disease. Vet Dermatol. 16(1): 4-26.

CLSI 2002. Method for antifungal disk diffusion susceptibility testing in yeasts. Approved guideline, M44-A Wayne, PA: CLSI.

CLSI 2007. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeast: Approved Guideline M27-A3 Wayne, PA: CLSI.

Coutinho SD, de Souza T and Paula CR 1997. Protein profiles of *Malassezia pachydermatis* isolated from dogs. Mycopathologia. 139(3): 129-135.

Darabi K, Hostetler SG, Bechtel MA and Zirwas M 2009. The role of *Malassezia* in atopic dermatitis affecting the head and neck of adults. J Am Acad Dermatol. 60(1): 125-136.

Day MJ 2007. Immunoglobulin G subclass distribution in canine leishmaniosis: a review and analysis of pitfalls in interpretation. Vet Parasitol. 147(1-2): 2-8.

Day MJ, Corato A and Shaw SE 1996. Subclass profile of allergen-specific IgG antibodies in atopic dogs. Res Vet Sci. 61(2): 136-142.

DeAngelis YM, Saunders CW, Johnstone KR, Reeder NL, Coleman CG, Kaczvinsky JR, Jr Gale C, Walter R, Mekel M, Lacey MP, Keough TW, Fieno A, Grant RA, Begley B, Sun Y, Fuentes G, Youngquist RS, Xu J and Dawson TL Jr 2007. Isolation and expression of a *Malassezia globosa* lipase gene, LIP1. J Invest Dermatol. 127(9): 2138-2146.

Devos SA and van der Valk PG 2000. The relevance of skin prick tests for *Pityrosporum ovale* in patients with head and neck dermatitis. Allergy. 55(11): 1056-1058.

Eichenberg ML, Appelt CE, Berg V, Muschner AC, Nobre MDO, Matta DD, Alves SH and Ferreiro L 2003. Susceptibility of *Malassezia pachydermatis* to azole antifungal agents evaluated by a new broth microdilution method. Acta Sci Veterinariae. 31(2): 75-80.

Eisenbarth SC, Piggott DA and Bottomly K 2003. The master regulators of allergic inflammation: dendritic cells in Th2 sensitization. Curr Opin Immunol. 15(6): 620-626.

Farver K, Morris DO, Shofer F and Esch B 2005. Humoral measurement of type-1 hypersensitivity reactions to a commercial *Malassezia* allergen. Vet Dermatol. 16(4): 261-268.

Garau M, Pereiro M Jr and del Palacio A 2003. In vitro susceptibilities of *Malassezia* species to a new triazole, albaconazole (UR-9825), and other antifungal compounds. Antimicrob. Agents Chemother. 47(7): 2342-2344.

Gioti A, Nystedt B, Li W, Xu J, Andersson A, Averette AF, Munch K, Wang X, Kappauf C, Kingsbury JM, Kraak B, Walker LA, Johansson HJ, Holm T, Lehtio J, Stajich JE, Mieczkowski P, Kahmann R, Kennell JC, Cardenas ME, Lundeberg J, Saunders CW, Boekhout T, Dawson TL, Munro CA, de Groot PW, Butler G, Heitman J and Scheynius A 2013. Genomic insights into the atopic eczema-associated skin commensal yeast *Malassezia sympodialis*. MBio. 4(1): e00572-00512.

Girao MD, Prado MR, Brilhante RS, Cordeiro RA, Monteiro AJ, Sidrim JJ and Rocha MF 2006. *Malassezia pachydermatis* isolated from normal and diseased external ear canals in dogs: a comparative analysis. Vet J. 172(3): 544-548.

Griffin CE and Hillier A 2001. The ACVD task force on canine atopic dermatitis (XXIV): allergen-specific immunotherapy. Vet Immunol Immunopathol. 81(3–4): 363-383.

Gupta AK, Batra R, Bluhm R, Boekhout T and Dawson TL 2004. Skin diseases associated with *Malassezia* species. J Am Acad Dermatol. 51(5): 785-798.

Gupta AK, Kohli Y, Li A, Faergemann J and Summerbell RC 2000. In vitro susceptibility of the seven *Malassezia* species to ketoconazole, voriconazole, itraconazole and terbinafine. Br J Dermatol. 142(4): 758-765.

Gupta AK, Kohli Y and Summerbell RC 2000. Molecular differentiation of seven *Malassezia* species. J Clin Microbiol. 38(5): 1869-1875.

Gupta AK, Nicol K and Batra R 2004. Role of antifungal agents in the treatment of seborrheic dermatitis. Am J Clin Dermatol. 5(6): 417-422.

Habibah A, Catchpole B and Bond R 2005. Canine serum immunoreactivity to *M. pachydermatis* in vitro is influenced by the phase of yeast growth. Vet Dermatol. 16(3): 147-152.

Hazen KC, Baron EJ, Colombo AL, Girmenia C, Sanchez-Sousa A, del Palacio A, de Bedout C and Gibbs DL 2003. Comparison of the susceptibilities of *Candida* spp. to fluconazole and voriconazole in a 4-year global evaluation using disk diffusion. J Clin Microbiol. 41(12): 5623-5632.

Heilmann CJ, Sorgo AG and Klis FM 2012. News from the fungal front: wall proteome dynamics and host-pathogen interplay. PLoS Pathog. 8(12): e1003050.

Hensel P, Austel M, Wooley RE, Keys D and Ritchie BW 2009. In vitro and in vivo evaluation of a potentiated miconazole aural solution in chronic *Malassezia* otitis externa in dogs. Vet Dermatol. 20(5-6): 429-434.

Hou CC, Day MJ, Nuttall TJ and Hill PB 2006. Evaluation of IgG subclass responses against *Dermatophagoides farinae* allergens in healthy and atopic dogs. Vet Dermatol. 17(2): 103-110.

Hou CC, Pemberton A, Nuttall T and Hill PB 2005. IgG responses to antigens from *Dermatophagoides farinae* in healthy and atopic dogs. Vet Immunol Immunopathol. 106(1-2): 121-128.

Huang X, Johansson S, Zargari A and Nordvall S 1995. Allergen cross-reactivity between *Pityrosporum orbiculare* and *Candida albicans*. Allergy. 50(8): 648-656.

Ishibashi Y, Kato H, Asahi Y, Sugita T and Nishikawa A 2009. Identification of the major allergen of *Malassezia globosa* relevant for atopic dermatitis. J Dermatol Sci. 55(3): 185-192.

Ishiguro A, Homma M, Torii S and Tanaka K 1992. Identification of *Candida albicans* antigens reactive with immunoglobulin E antibody of human sera. Infect Immun. 60(4): 1550-1557.

Ishikawa T, Itoh F, Yoshida S, Saijo S, Matsuzawa T, Gonoi T, Saito T, Okawa Y, Shibata N, Miyamoto T and Yamasaki S 2013. Identification of Distinct Ligands for the C-type Lectin Receptors Mincle and Dectin-2 in the Pathogenic Fungus *Malassezia*. Cell Host Microbe. 13(4): 477-488.

Jesus FP, Lautert C, Zanette RA, Mahl DL, Azevedo MI, Machado ML, Dutra V, Botton SA, Alves SH and Santurio JM 2011. In vitro susceptibility of fluconazole-susceptible and -resistant isolates of *Malassezia pachydermatis* against azoles. Vet Microbiol. 152(1-2): 161-164.

Johansson C, Tengvall Linder M, Aalberse RC and Scheynius A 2004. Elevated levels of IgG and IgG4 to *Malassezia* allergens in atopic eczema patients with IgE reactivity to *Malassezia*. Int Arch Allergy Immunol. 135(2): 93-100.

Johnson MD and Perfect JR 2010. Use of Antifungal Combination Therapy: Agents, Order, and Timing. Curr Fungal Infect Rep. 4(2): 87-95.

Kaneko T, Makimura K, Abe M, Shiota R, Nakamura Y, Kano R, Hasegawa A, Sugita T, Shibuya S, Watanabe S, Yamaguchi H, Abe S and Okamura N 2007. Revised culture-based system for identification of *Malassezia* species. J Clin Microbiol. 45(11): 3737-3742.

Kano R, Hattori Y, Okuzumi K, Miyazaki Y, Yamauchi R, Koie H, Watari T and Hasegawa A 2002. Detection and identification of the *Candida* species by 25S ribosomal DNA analysis in the urine of candidal cystitis. J Vet Med Sci. 64(2): 115-117.

Kennis RA, Rosser EJ, Olivier NB and Walker RW 1996. Quantity and distribution of *Malassezia* organisms on the skin of clinically normal dogs. J Am Vet Med Assoc. 208(7): 1048-1051.

Kim HJ, Kim ET, Lim CY, Park C, Kang BT, Kim JW, Yoo JH and Park HM 2010. The immunoglobulin G response to *Malassezia pachydermatis* extracts in atopic and non-atopic dogs. Can Vet J. 51(8): 869-872.

Kiraz N, Dag I, Oz Y, Yamac M, Kiremitci A and Kasifoglu N 2010. Correlation between broth microdilution and disk diffusion methods for antifungal susceptibility testing of caspofungin, voriconazole, amphotericin B, itraconazole and fluconazole against *Candida glabrata*. J Microbiol Methods. 82(2): 136-140.

Kosonen J, Lintu P, Kortekangas-Savolainen O, Kalimo K, Terho EO and Savolainen J 2005. Immediate hypersensitivity to *Malassezia furfur* and *Candida albicans* mannans in vivo and in vitro. Allergy. 60(2): 238-242.

Laemmli UK 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227(5259): 680-685.

Matar MJ, Ostrosky-Zeichner L, Paetznick VL, Rodriguez JR, Chen E and Rex JH 2003. Correlation between E-test, disk diffusion, and microdilution methods for antifungal susceptibility testing of fluconazole and voriconazole. Antimicrob Agents Chemother. 47(5): 1647-1651.

Mazza G, Duffus WP, Elson CJ, Stokes CR, Wilson AD and Whiting AH 1993. The separation and identification by monoclonal antibodies of dog IgG fractions. J Immunol Methods. 161(2): 193-203.

McEwan NA 2001. *Malassezia* and *Candida* infections in bull terriers with lethal acrodermatitis. J Small Anim Pract. 42(6): 291-297.

Miceli MH, Díaz JA and Lee SA 2011. Emerging opportunistic yeast infections. Lancet Infect Dis. 11(2): 142-151.

Mirhendi H, Makimura K, Zomorodian K, Yamada T, Sugita T and Yamaguchi H 2005. A simple PCR-RFLP method for identification and differentiation of 11 *Malassezia* species. J Microbiol Methods. 61(2): 281-284.

Moretti A, Boncio L, Posteraro B, Mechelli L, Balducci M, Fadda G, La Sorda M, Di Chio M, Grelloni V and Agnetti F 2006. Co-cutaneous infection in a dog: PCR-reverse identification of *Candida tropicalis* on skin biopsy. J Med Mycol. 16(1): 30-36.

Morris DO 2005. *Malassezia pachydermatis* carriage in dog owners. Emerg Infect Dis. 11(1): 83-88.

Mueller RS, Bettenay SV and Shipstone M 2002. Cutaneous candidiasis in a dog caused by *Candida guilliermondii*. Vet Rec. 150(23): 728-730.

Nakano Y, Wada M, Tani H, Sasai K and Baba E 2005. Effects of beta-thujaplicin on anti-Malassezia pachydermatis remedy for canine otitis externa. J Vet Med Sci. 67(12): 1243-1247.

Naldi L and Rebora A 2009. Clinical practice. Seborrheic dermatitis. N Engl J Med. 360(4): 387-396.

Nardoni S, Corazza M and Mancianti F 2008. Diagnostic and clinical features of animal malasseziosis. Parassitologia. 50(1-2): 81-83.

Nardoni S, Dini M, Taccini F and Mancianti F 2007. Occurrence, distribution and population size of *Malassezia pachydermatis* on skin and mucosae of atopic dogs. Vet Microbiol. 122(1-2): 172-177.

Negre A, Bensignor E and Guillot J 2009. Evidence-based veterinary dermatology: a systematic review of interventions for *Malassezia* dermatitis in dogs. Vet Dermatol. 20(1): 1-12.

Nijima M, Kano R, Nagata M, Hasegawa A and Kamata H 2011. An azole-resistant isolate of *Malassezia pachydermatis*. Vet Microbiol. 149(1-2): 288-290.

Novak N and Bieber T 2003. Allergic and nonallergic forms of atopic diseases. J Allergy Clin Immunol. 112(2): 252-262.

Nuttall TJ and Halliwell RE 2001. Serum antibodies to *Malassezia* yeasts in canine atopic dermatitis. Vet Dermatol. 12(6): 327-332.

Oliveira Gdos S, Ribeiro ET and Baroni Fde A 2006. An evaluation of manual and mechanical methods to identify *Candida* spp. from human and animal sources. Rev Inst Med Trop Sao Paulo. 48(6): 311-315.

Oliveira TM, Mineo TW, Bason M, Day MJ and Machado RZ 2009. IgG subclass profile of serum antibodies to *Leishmania chagasi* in naturally infected and vaccinated dogs. Vet Parasitol. 162(1): 16-22.

Parry ME and Sharpe GR 1998. Seborrhoeic dermatitis is not caused by an altered immune response to *Malassezia* yeast. Br J Dermatol. 139(2): 254-263.

Peano A, Beccati M, Chiavassa E and Pasquetti M 2012. Evaluation of the antifungal susceptibility of *Malassezia pachydermatis* to clotrimazole, miconazole and thiabendazole using a modified CLSI M27-A3 microdilution method. Vet Dermatol. 23(2): 131-135, e129.

Peano A and Gallo MG 2008. Management of *Malassezia*-related diseases in the dog. Parassitologia. 50(1-2): 85-88.

Pfaller MA, Messer SA, Boyken L, Rice C, Tendolkar S, Hollis RJ and Diekema DJ 2004. Evaluation of the NCCLS M44-P disk diffusion method for determining susceptibilities of 276 clinical isolates of *Cryptococcus neoformans* to fluconazole. J Clin Microbiol. 42(1): 380-383.

Plant JD, Rosenkrantz WS and Griffin CE 1992. Factors associated with and prevalence of high *Malassezia pachydermatis* numbers on dog skin. J Am Vet Med Assoc. 201(6): 879-882.

Prado MR, Brilhante RS, Cordeiro RA, Monteiro AJ, Sidrim JJ and Rocha MF 2008. Frequency of yeasts and dermatophytes from healthy and diseased dogs. J Vet Diagn Invest. 20(2): 197-202.

Prado MR, Brito EH, Brilhante RS, Cordeiro RA, Leite JJ, Sidrim JJ and Rocha MF 2008. Subculture on potato dextrose agar as a complement to the broth

microdilution assay for *Malassezia pachydermatis*. J Microbiol Methods. 75(2): 341-343.

Quinnell RJ, Courtenay O, Garcez LM, Kaye PM, Shaw MA, Dye C and Day MJ 2003. IgG subclass responses in a longitudinal study of canine visceral leishmaniasis. Vet Immunol Immunopathol. 91(3-4): 161-168.

Reis AB, Teixeira-Carvalho A, Vale AM, Marques MJ, Giunchetti RC, Mayrink W, Guerra LL, Andrade RA, Corrêa-Oliveira R and Martins-Filho OA 2006. Isotype patterns of immunoglobulins: Hallmarks for clinical status and tissue parasite density in brazilian dogs naturally infected by *Leishmania* (*Leishmania*) *chagasi*. Vet Immunol Immunopathol. 112(3–4): 102-116.

Rincon S, Cepero de Garcia MC and Espinel-Ingroff A 2006. A modified Christensen's urea and CLSI broth microdilution method for testing susceptibilities of six *Malassezia* species to voriconazole, itraconazole, and ketoconazole. J Clin Microbiol. 44(9): 3429-3431.

Robson D 2007. *Malassezia*: Mechanisms of possible drug resistance. Proceedings of the Australia College of Veterinary Scientists Dermatology Chapter Science Week Proceedings, Gold Coast. 6-7th July 2007, p. 63-67.

Sanglard D 2002. Clinical relevance of mechanisms of antifungal drug resistance in yeasts. Enferm Infecc Microbiol Clin. 20(9): 462-469; quiz 470, 479.

Saunders CW, Scheynius A and Heitman J 2012. *Malassezia* fungi are specialized to live on skin and associated with dandruff, eczema, and other skin diseases. PLoS Pathog. 8(6): e1002701.

Savolainen J, Lintu P, Kosonen J, Kortekangas-Savolainen O, Viander M, Pene J, Kalimo K, Terho EO and Bousquet J 2001. *Pityrosporum* and *Candida* specific and non-specific humoral, cellular and cytokine responses in atopic dermatitis patients. Clin Exp Allergy. 31(1): 125-134.

Schlotter YM, Victor R, Riemers FM, Knol EF and Willemse T 2011. Lesional skin in atopic dogs shows a mixed Type-1 and Type-2 immune responsiveness. Vet Immunol Immunopathol. 143: 20-26.

Schroeder HW Jr and Cavacini L 2010. Structure and function of immunoglobulins. J Allergy Clin Immunol. 125(2 Suppl 2): S41-52.

Schwartz RA, Janusz CA and Janniger CK 2006. Seborrheic Dermatitis: An Overview. Am Fam Physician. 74(1): 125-130.

Shakib F and Stanworth DR 1980. Human IgG subclasses in health and disease. (A review). Part I. Ric Clin Lab. 10(3): 463-479.

Sims CR, Paetznick VL, Rodriguez JR, Chen E and Ostrosky-Zeichner L 2006. Correlation between microdilution, E-test, and disk diffusion methods for antifungal susceptibility testing of posaconazole against *Candida* spp. J Clin Microbiol. 44(6): 2105-2108.

Sohnle PG and Collins-Lech C 1982. Analysis of the lymphocyte transformation response to *Pityrosporum orbiculare* in patients with tinea versicolor. Clin Exp Immunol. 49(3): 559-564.

Tanaka M, Aiba S, Matsumura N, Aoyama H, Tabata N, Sekita Y and Tagami H 1994. IgE-mediated hypersensitivity and contact sensitivity to multiple environmental allergens in atopic dermatitis. Arch Dermatol. 130(11): 1393-1401.

Testore GP, Dori L, Buonomini AR, Schito GC, Soro O, Fortina G, Andreoni S, Carlone N, Tullio V and Andreoni M 2004. In vitro fluconazole susceptibility of 1565 clinical isolates of *Candida* species evaluated by the disk diffusion method performed using NCCLS M44-A guidelines. Diagn Microbiol Infect Dis. 50(3): 187-192.

Thomas DS, Ingham E, Bojar RA and Holland KT 2008. In vitro modulation of human keratinocyte pro- and anti-inflammatory cytokine production by the capsule of *Malassezia* species. FEMS Immunol Med Microbiol. 54(2): 203-214.

Tomee JF, Dubois AE, Koëter GH, Beaumont F, van der Werf TS and Kauffman HF 1996. Specific IgG4 responses during chronic and transient antigen exposure in aspergillosis. Am J Respir Crit Care Med. 153(6): 1952-1957.

Uchida Y, Mizutani M, Kubo T, Nakade T and Otomo K 1992. Otitis externa induced with *Malassezia pachydermatis* in dogs and the efficacy of pimaricin. J Vet Med Sci. 54(4): 611-614.

Vandenbossche I, Vaneechoutte M, Vandevenne M, De Baere T and Verschraegen G 2002. Susceptibility testing of fluconazole by the NCCLS broth macrodilution

method, E-test, and disk diffusion for application in the routine laboratory. J Clin Microbiol. 40(3): 918-921.

Velegraki A, Alexopoulos EC, Kritikou S and Gaitanis G 2004. Use of fatty acid RPMI 1640 media for testing susceptibilities of eight *Malassezia* species to the new triazole posaconazole and to six established antifungal agents by a modified NCCLS M27-A2 microdilution method and Etest. J Clin Microbiol. 42(8): 3589-3593.

Welle MM, Olivry T, Grimm S and Suter M 1999. Mast Cell Density and Subtypes in the Skin of Dogs with Atopic Dermatitis. J Comp Pathol. 120(2): 187-197.

Willemse A, Noordzij A, Rutten VP and Bernadina WE 1985. Induction of non-IgE anaphylactic antibodies in dogs. Clin Exp Immunol. 59(2): 351-358.

Willemse A, Noordzij A, Van den Brom WE and Rutten VP 1985. Allergen specific IgGd antibodies in dogs with atopic dermatitis as determined by the enzyme linked immunosorbent assay (ELISA). Clin Exp Immunol. 59(2): 359-363.

Yurayart C, Chindamporn A, Suradhat S, Tummaruk P, Kajiwara S and Prapasarakul N 2011. Comparative analysis of the frequency, distribution and population sizes of yeasts associated with canine seborrheic dermatitis and healthy skin. Vet Microbiol. 148(2-4): 356-362.

Yurayart C, Nuchnoul N, Moolkum P, Jirasuksiri S, Niyomtham W, Chindamporn A, Kajiwara S and Prapasarakul N 2013. Antifungal agent susceptibilities and interpretation of *Malassezia pachydermatis* and *Candida parapsilosis* isolated from dogs with and without seborrheic dermatitis skin. Med Mycol. Early Online: 1-10.

Zomorodian K, Rahimi MJ, Pakshir K, Motamedi M, Ghiasi MR and Rezashah H 2011. Determination of antifungal susceptibility patterns among the clinical isolates of *Candida* species. J Global Infect Dis. 3(4): 357-360.

APPENDICES

APPENDIX A

Buffer and reagent

Media for yeast cultivation

1. Sabouraud Dextrose Agar (SDA)

Dehydrated SDA agar	65	g
Distilled water	1	L

Sterile by autoclaving at 121°C 15 minutes

Cool to 50°C and aseptically add cephalexin 40 mg/ml and chloraphenicol 20 mg/ml and mix well

2. Sabouraud Dextrose Agar (SDAO)

Dehydrated SDA agar	65	g
Olive oil	20	mL
Distilled water	1	L

Sterile by autoclaving at 121°C 15 minutes

Cool to 50°C and aseptically add cephalexin 40 mg/ml and chloraphenicol 20 mg/ml and mix well

3. Yeast Peptone Dextrose Agar (YPD)

Bacteriological peptone	20	g
Glucose	20	g
Yeast extract	10.0	g
Agar	15.0	g
Distilled water	1	L

Sterile by autoclaving at 121°C 15 minutes

4. Modified Leeming and Notman Agar (MLNA)

Bacteriological peptone	10	g
Glucose	10	g
Yeast extract	2.0	g
Ox bile, desiccated	8.0	g

Glycerol	10	mL
Glycerol monostearate	0.5	g
Tween 60	5.0	mL
Olive oil	20	mL
Agar	15	g
Distilled water	1.0	L

Sterile by autoclaving at 121°C 15 minutes

5. Modified Leeming and Notman Agar (MLNB)

Bacteriological peptone	10	g
Glucose	10	g
Yeast extract	2.0	g
Ox bile, desiccated	8.0	g
Glycerol	10	mL
Glycerol monostearate	0.5	g
Tween 60	5.0	mL
Olive oil	20	mL
Distilled water	1.0	L

Sterile by autoclaving at 121°C 15 minutes

Media for yeast identification

6. Cremophor EL Agar (EL slant)

Dehydrated SDA agar	65	g
Cremophor EL	10	mL
Distilled water	1	L

Sterile by autoclaving at 121°C 15 minutes

7. Tween 60-esculin Agar (TE slant)

Bacteriological peptone	10	g
Glucose	10	g
Yeast extract	2.0	g
Tween 60	5	mL

Ferric ammonium citrate	0.5	g
Esculin	1	g
Agar	15	g
Distilled water	1	L

Sterile by autoclaving at 121°C 15 minutes

Media for antifungal susceptibility test

8. RPMI 1640 medium for broth microdilution method

RPMI 1640 medium, L-glutamine without sodium bicarbonate buffered	10.4 g
3-(N-morpholino) propanesulfonic acid (MOPS 0.165 mol/L)	34.53 g
Glucose	20 g
Distilled water	900 mL

Dissolve and adjust pH to 7.0, add distilled water to 1 L

Sterile by filtration through filtered membrane pore-sized 0.45 µm and store at 4°C until use

APPENDIX B

Protocol for molecular canine skin yeast identification

1. Primers used in this study

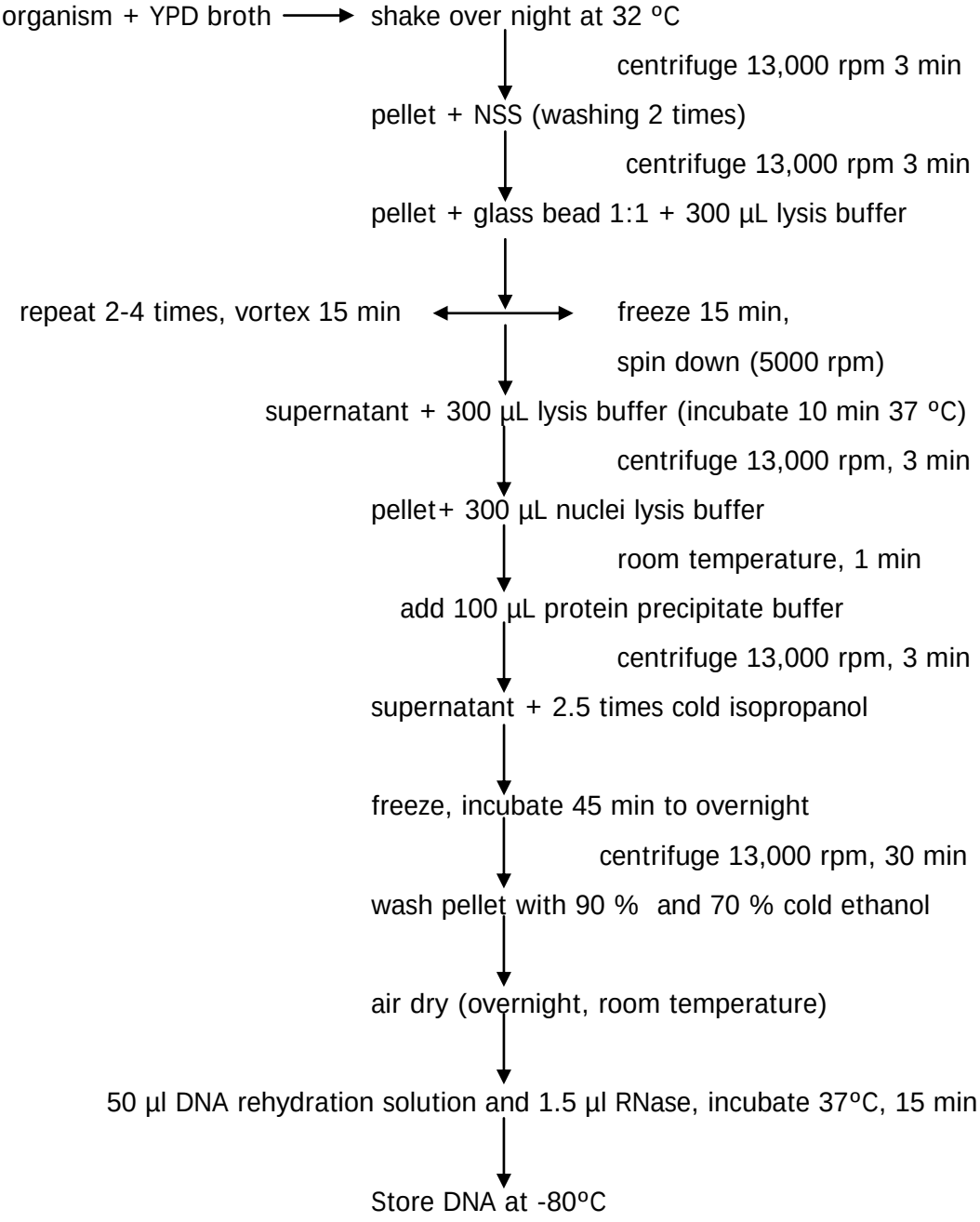
Primer name	Sequence (5´- 3´)
26S Forward	TAACAAGGATTCCCCTAGTA
26S Reverse	ATTACGCCAGCATCCTAAG
ITS1 Forward	TCCGTAGGTGAACCTGCGG
ITS4 Reverse	TCCTCCGCTTATTGATATGC

2. Restriction enzyme used in this study

   C*f*oI

3. Protocol for fungal DNA extraction

Wizard® Genomic DNA Purification Kit (Promega)



APPENDIX C

Nucleotide sequences

1. *Candida parapsilosis* isolate CPTD1-KITS

   18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 28S ribosomal RNA gene, region 538 nucleotide; GenBank: GU373653

   GAAGGCCTCCCAAAGCAAAGTTTTCAAAAAGAGAAAAACACATGTGTAAGAAAAAATG
   CAGTTAAGCACTTTTCATTCTGTAATGATCTTTGGGGGGATCGGGAGGGAATCAAACG
   TGTTTAAAGGCATTATTTCTTACACATGTGCCCTTTTTTTTCTGAAAACGGAAATTTGG
   TAGGCCTTCTATATGGGGCCCCCCCGAGATTAAACTCAACCAAATTTTATTTAATGTCA
   ACCGATTATTTAATAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGA
   AGAACGCAGCGAAATGCGATAAGTAATATGAATTGCAGATATTCGTGAATCATCGAAT
   CTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTTGAGCGTCAT
   TTCTCCCTCAAACCCTCGGGTTTGGTGTTGAGCGATACGCTGGGTTTGCTTGAAAGAA
   AGGCGGAGTATAAACTAATGGATAGGTTTTTTCCACTCATTGGTACAAACTCCAAAAC
   TCTTCCAAATTCG

2. *Candida parapsilosis* isolate CPTD2-KITS

   18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 28S ribosomal RNA gene, region 472 nucleotide; GenBank: GU373654

   ATTTTTTCTTACACATGTGTTTTTCTTTTTTTGAAAACTTTGCTTTGGTAGGCCTTCTA
   TATGGGGCCTGCCAGAGATTAAACTCAACCAAATTTTATTTAATGTCAACCGATTATTT
   AATAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGC
   GAAATGCGATAAGTAATATGAATTGCAGATATTCGTGAATCATCGAATCTTTGAACGC
   ACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTTGAGCGTCATTTCTCCCTCA
   AACCCTCGGGTTTGGTGTTGAGCGATACGCTGGGTTTGCTTGAAAGAAAGGCGGAGT
   ATAAACTAATGGATAGGTTTTTTCCACTCATTGGTACAAACTCCAAAACTTCTTCCAAA
   TTCGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGG
   AAAAAA

3. *Candida parapsilosis* isolate CPTD3-KITS

18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 28S ribosomal RNA gene, region 478 nucleotide; GenBank: GU373655

TACTGCATTTTTTCTTACACATGTGTTTTTCTTTTTTTGAAAACTTTGCTTTGGTAGGC
CTTCTATATGGGGCCTGCCAGAGATTAAACTCAACCAAATTTTATTTAATGTCAACCGA
TTATTTAATAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAAC
GCAGCGAAATGCGATAAGTAATATGAATTGCAGATATTCGTGAATCATCGAATCTTTG
AACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTTGAGCGTCATTTCTC
CCTCAAACCCTCGGGTTTGGTGTTGAGCGATACGCTGGGTTTGCTTGAAAGAAAGGC
GGAGTATAAACTAATGGATAGGTTTTTTCCACTCATTGGTACAAACTCCAAAACTTCTT
CCAAATTCGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGC
GGAGGAAAAAG

4. *Candida parapsilosis* isolate CPTN1-KITS

18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 28S ribosomal RNA gene, region 467 nucleotide; GenBank: GU373656

TTACAGAATGAAAAGTGCTTAACTGCATTTTTTCTTACACATGTGTTTTTCTTTTTTTG
AAAACTTTGCTTTGGTAGGCCTTCTATATGGGGCCTGCCAGAGATTAAACTCAACCAA
ATTTTATTTAATGTCAACCGATTATTTAATAGTCAAAACTTTCAACAACGGATCTCTTG
GTTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATATGAATTGCAGATAT
TCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATG
CCTGTTTGAGCGTCATTTCTCCCTCAAACCCTCGGGTTTGGTGTTGAGCGATACGCTG
GGTTTGCTTGAAAGAAAGGCGGAGTATAAACTAATGGATAGGTTTTTTCCACTCATTG
GTACAAACTCCAAAACTTCTTCCAAATTCGACCTCAAATCAGGTAGGACTACCCGCTGA

5. *Candida parapsilosis* isolate CPTN2-KITS

   18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 28S ribosomal RNA gene, region 496 nucleotide; GenBank: GU373657

   CTTGCCTCGTCAGGTGCTTACTGCATTTTTTCTTACACATGTGTTTTTCTTTTTTTGAA
   AACTTTGCTTTGGTAGGCCTTCTATATGGGGCCTGCCAGAGATTAAACTCAACCAAAT
   TTTATTTAATGTCAACCGATTATTTAATAGTCAAAACTTTCAACAACGGATCTCTTGGT
   TCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATATGAATTGCAGATATTC
   GTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCC
   TGTTTGAGCGTCATTTCTCCCTCAAACCCTCGGGTTTGGTGTTGAGCGATACGCTGGG
   TTTGCTTGAAAGAAAGGCGGAGTATAAACTAATGGATAGGTTTTTTCCACTCATTGGT
   ACAAACTCCAAAACTTCTTCCAAATTCGACCTCAAATCAGGTAGGACTACCCGCTGAAC
   TTAAGCATATCAATAAGCGGAGGAAAAGA

6. *Malassezia pachydermatis* isolate MPTD1-KITS

   18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 28S ribosomal RNA gene, region 728 nucleotide; GenBank: GU373658

   GATCATTAGTGAATTTGTAGGGCCTGCCATACGGATGCGCAAGCGTCTCTGGCGCCCA
   ACCCACTATACATCCACAAACCCGTGTGCACTTGTGTTGCTTTGGCCTGTATGGGCGA
   GCGCACGCATTCAAACAAACTCGTATGGTTGTATGTACGTTGTAAACGTTGGACCGTC
   ACTGGCCAACAAACTTTATACAACTTTCGACAACGGATCTCTTGGTTCTCCCATCGATG
   AAGAACGCAGCGAAACGCGATAGGTAATGTGAATTGCAGAATTCCGTGAATCATCGAA
   TCTTTGAACGCACCTTGCGCTCCATGGTATTCCGTGGAGCATGCCTGTTTGAGTGCCG
   CGAATTCTCCCACCCCAAACGGTTGCCGAAAGGTACTGTGCGGCGGAGGGGTTGGAT
   GGGTGCTACTGCCTGTGGTGGGGAAACTACAACAGGCTCGCCCGAAATGCATTAGCG
   CCTCAGGACACATTCGCTACTGCTCTACAAAAGGAAGAGCAGCGAAGCGTATGGGGG
   GGAAAACCACCACCGGTTATTCCAAACCTCGCCTGACTGTTTTTGCGCGCATGGCATG
   ATACGTCATTTGCTGTGTTGCGTAGGGGGGTGCATGAAAAAGTGGGAGTGCCTCTGG
   AGATCCTGCGGAGGAGCTCAATAATAAGCGGAGGAGCAGGTGAACAAGCTGCGGAGC
   ATCTCATTTAGCGGGCGATTCGGGCCCTCTCTCTTC

7. *Malassezia pachydermatis* isolate MPTD2-KITS

18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 28S ribosomal RNA gene, region 713 nucleotide; GenBank: GU373659

AGTGAATTTGTAGGGCCTGCCATACGGATGCGCAAGCAGTCATCAGGCGGAGGACCC
ACTATACATCCACAACCGGTGAGCACTTGTGTTGCTTTGGCCTGTATGGGCGAGCGCA
CGCATTCAAACAAACTCGTATGGTTGTATGTACGTTGTAAACGTTGGACCGTCACTGG
CCAACAAACTTTATACAACTTTCGACAACGGATCTCTTGGTTCTCCCATCGATGAAGAA
CGCAGCGAAACGCGATAGGTAATGTGAATTGCAGAATTCCGTGAATCATCGAATCTTT
GAACGCACCTTGCGCTCCATGGTATTCCGTGGAGCATGCCTGTTTGAGTGCCGCGAAT
TCTCCCACCCCAAACGGTTGCCGAAAGGTACTGTGCGGCGGAGGGGTTGGATGGGTG
CTACTGCCTGTGGTGGGGAAACTACAACAGGCTCGCCCGAAATGCATTAGCGCCTCAG
GACACATTCGCTACTGCTCTACAAAAGGAAGAGCAGCGAAGCGTATGGGGGGGAAAA
CCACCACCGGTTATTCCAAACCTCGCCTGACTGTTTTTGCGCGCATGGCATGATACGT
CATTTGCTGTGTTGCGTAGGGGGGTGCGTGAGAAAGTGGGAGTGCCTCTGGAGCTTT
GGCGGGGGAGCACAATAATAAGCGGAGGAAAAGGTGCCAAGCTGCGGAGCATCTCAA
CTCGCTTTCAATTCTGGTCTC

8. *Malassezia pachydermatis* isolate MPTD3-KITS

18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 28S ribosomal RNA gene, region 877 nucleotide; GenBank GU373660

TAGCGCCGCCGGCCGCCCGACGGTTGAACCTGCGGAGCAATTATCAATCGGAGGAGG
ATCCGTTGGTCCTGCTGAGGAGCATATTATTAAGCGGAGGATCATTTGGACGAGCGCA
CGCATTCAAACAAACTCGTATGGTTGTATGTACGTTGTAAACGTTGGACCGTCACTGG
CCAACAAACTTTATACAACTTTCGACAACGGATCTCTTGGTTCTCCCATCGATGAAGAA
CGCAGCGAAACGCGATAGGTAATGTGAATTGCAGAATTCCGTGAATCATCGAATCTTT
GAACGCACCTTGCGCTCCATGGTATTCCGTGGAGCATGCCTGTTTGAGTGCCGCGAAT
TCTCCCACCCCAAACGGTTGCCGAAAGGTACTGTGCGGCGGAGGGGTTGGATGGGTG
CTACTGCCTGTGGTGGGGAAACTACAACAGGCTCGCCCGAAATGCATTAGCGCCTCAG
GACACATTCGCTACTGCTCTACAAAAGGAAGAGCAGCGAAGCGTATGGGGGGGAAAA
CCACCACCGGTTATTCCAAACCTCGCCTGACTGTTTTTGCGCGCATGGCATGATACGT

CATTTGCTGTGTTGCGTAGGTTGGTGCGTGGGACTGTGTGAGTGCCTCTAGAGCTTT
GAGAAGTGTGTTTCGTGTGCTACGAACTGAAAAAGTACATTCCTTTTTTTCAATTCTG
GTCTCAAATCAGGTAGGATCACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAA
AAGCTTCGGTGTGCATTTGTAGGACCGCATTACGAAGCAAGCTTCTGTGGGGCCCACC
CAATATAGTCAAACCGGGGACATGCTATGAGCTGGACCGCATGACGCGCGAGACAGCC
ACAGAAAAAT

9. *Malassezia pachydermatis* isolate MPTN1-KITS

18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 28S ribosomal RNA gene, region 779 nucleotide; GenBank: GU373661

TAGGTGATGTGAGCGGTCTTATTACATATCATCCCCATCGCTCGTGTCTCTCTTTCTTT
AGAGGAGGACCCCAAAGCTTTTTTCGAAGTGTGTATAAATTGTTGTCCCAGGGGGGT
GCGTACATTTATCAAGATACACAACACACACACCCTCCTGTTGAAGGCGTGCGCTCAG
CCCCTCCTGGCCCAAGCAACACATGGCACCACGTTTGGAGGTATAGTGGGTTGGGCG
CTAGAGACGCTGCGCATCCGTATGGCCGGCCCGGACAAATACATCAATAAGCGGAGCT
TCCGTAGGTGAACCTGCGGAAGGATCATTAGTGAATTTGTAGGGCCTGCCATACGGAT
GCGCAAGCGTCTCTGGCGCCCAACCCACTATACATCCACAAACCCGTGTGCACTTGTG
TTGCTTTGGCCTGTATGGGCGAGCGCACGCATTCAAACAAACTCGTATGGTTGTATGT
ACGTTGTAAACGTTGGACCGTCACTGGCCAACAAACTTTATACAACTTTCGACAACGG
ATCTCTTGGTTCTCCCATCGATGAAGAACGCAGCGAAACGCGATAGGTAATGTGAATT
GCAGAATTCCGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCATGGTATTCCGT
GGAGCATGCCTGTTTGAGTGCCGCGAATTCTCCCACCCCAAACGGTTGCCGAAAGGTA
CTGTGCGGCGGAGGGGTTGGATGGGTGCTACTGCCTGTGGTGGGGAAACTACAACAG
GCTCGCCCGAAATGCATTAGCGCCTCA

10. *Malassezia pachydermatis* isolate MPTN2-KITS

18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 28S ribosomal RNA gene, region 831 nucleotide; GenBank: GU373662

TAACAGAGGTGAGGCGCGAGGCGGTCGAAGCTGCGGAGCAATCATTAATCGGAGGAG
CCTTCGTTGGTCAAGCGGAGGAGCACTTGTGTTGCTGAGGACTGTATGGGCGAGCGC
ACGCATTCAAACAAACTCGTATGGTTGTATGTACGTTGTAAACGTTGGACCGTCACTG
GCCAACAAACTTTATACAACTTTCGACAACGGATCTCTTGGTTCTCCCATCGATGAAGA
ACGCAGCGAAACGCGATAGGTAATGTGAATTGCAGAATTCCGTGAATCATCGAATCTT
TGAACGCACCTTGCGCTCCATGGTATTCCGTGGAGCATGCCTGTTTGAGTGCCGCGAA
TTCTCCCACCCCAAACGGTTGCCGAAAGGTACTGTGCGGCGGAGGGGTTGGATGGGT
GCTACTGCCTGTGGTGGGGAAACTACAACAGGCTCGCCCGAAATGCATTAGCGCCTCA
GGACACATTCGCTACTGCTCTACAAAAGGAAGAGCAGCGAAGCGTATGGGGGGGAAA
ACCACCACCGGTTATTCCAAACCTCGCCTGACTGTTTTTGCGCGCATGGCATGATACG
TCATTTGCTGTGTTGCGTAGGTTGGTGCGTGGGACTGTGTGAGTGCCTCTAGAGCTT
TGAGAAGTGTGTTTCGTGTGCTACGAACTGAAAAAGTACATTCCTTTTTTTCAATTCT
GGTCTCAAATCAGGTAGGATCACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAC
AACCGTAGGTGAAACCTGCGGAGCCCCCCCACAAAGAAGCGGAAGCTTCTGTGGACCA
ACTTTTGACTCTCCGACCGGGGG

11. *Malassezia furfur* isolate MFTD-KITS

18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 28S ribosomal RNA gene, region 586 nucleotide; GenBank: GU373663

GAAAGGGGGCAGGTGCGGGAGGAAGTTAATTGAGGAGTAAGCTGAGTAGGTAGCCCA
GCCTGTATTTATAAAGAGAATTCAGGGTTATTGGTGAAAGTGAGAGGGGGCGAAAAA
TACACACAGAAAGCATATATGAGTGAAAATTAGGTTAGCTAAGAATAAACGAACCAAA
AGGGAAAGAGTTCCACGAAATCTGTTGAATCAACCGGTAACGATGGCTTCACTTCAAG
GTCGTGGTTGGATAGGTGAATATTCCAAATTAGCAAGAAACAGGCTCGCCCGAAATGC
ATTAGCGCCTTTGGGACACACTCTACAAACAGCTCTGAAAGGGAGGGCGGCAGAGGG
GATGGAGGAACTCCGCCCGTCAGCTATACCAAACTTTGCCCCAGGCAAGGGCATGGCA
TGATACGTCATTTGCTGTGTGTGCGTCCTGAGGCAAGGGGCCGATGTGGTGCCTTTG
TCACTATGTGGGTGTGTTGGTGCGCTACCAATCCTGCGTGGAGGCTGGTCTGTGAGC
AAGCATAGAGCAGTTGTTGGTGAACGCAATCCATTCTACATCTCTGGTCTCAAATCAG
TAGTACCAAGC

12. *Candida tropicalis* isolate CTT-KITS

    18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 28S ribosomal RNA gene, region 475 nucleotide; GenBank: GU373664

    GGGCTTCTGATGCTTATTGCACCACATGTGTTTTTTATTGAACAAATTTCTTTGGTGG
    CGGGAGCAATCCTACCGCCAGAGGTTATAACTAAACCAAACTTTTTATTTACAGTCAAA
    CTTGATTTATTATTACAATAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATC
    GATGAAGAACGCAGCGAAATGCGATACGTAATATGAATTGCAGATATTCGTGAATCAT
    CGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTTGAGC
    GTCATTTCTCCCTCAAACCCCCGGGTTTGGTGTTGAGCAATACGCTAGGTTTGTTTGA
    AAGAATTTAACGGGGAAACTTATTTTAAGCGACTTAAGTTTATCCAAAACGCTTATTTT
    GCCAGGGGCCCCCCCAATTTATTTCAAAACTTTGACCCCAAATCCGGGAGGAATACCC
    CCTGAACT

APPENDIX D

Buffer and reagent for protein extraction and western blotting

Buffer and reagent for SDS-polyacrylamide gelelectrophoresis (SDS-PAGE)

1. 10% ammoniumpersulfate solution (w/v)

Ammoniumpersulfate	1	g
Sterile water	10	mL

   Divide to each microcentrifuge tube 250 µL

   Storeat-20°C and replacethestock solution every 2-3weeks

2. 10% SDS stock solution (w/v)

SDS	10	g
Sterile water (add to be)	100	mL

   This stock solution is stable for 6 month at room temperature.

3. 1.5 M Tris-HCl pH 8.8

Trisma base	18.2	g
Distilled water	70	mL

   Dissolve the solution and cool to 25°C. Adjust the pH to 8.8 by adding concentrated HCl and adjust the volume of the solution to 100 mL with distilled water.Sterile by autoclaving at 121°C 15 minutes.Replace the stock solution every 2-3 weeks.

4. 1M Tris-HCl pH 6.8

Trisma base	12.1	g
Distilled water	70	mL

   Dissolve the solution and cool to 25°C

   Adjust the pH to 6.8 by adding concentrated HCl and adjust the volume of the solution to 100 mL with distilled water.Sterile by autoclaving at 121°C 15 minutes.Replace the stock solution every 2-3 weeks.

5. Acrylamide : Bis-acrylamide (30:0.8)

Acrylamide	30	g
Bis-acrylamide	0.8	g
Distilled water (add to be)	100	mL

Keep in 4°C and cover with aluminium foil (light sensitive chemical reagent).

6. Saturated butanol

Butyl alcohol : distilled water	1:1

Vigerous shaking and use only the upper part

7. Tris-glycine buffer (5X stock solution)

Trisma base	15.1	g
Glycine	94	g
10% SDS	50	mL
Distilled water (add to be)	1	L

8. Solutions for preparing 5%stacking gelsfor Tris-glycine SDS-PAGE

		Volume(ml) ofcomponentsrequiredto cast gelsofindicated volumes							
Components	Gel volume	1	2	3	4	5	6	8	10
Distilled water		0.68	1.4	2.1	2.7	3.4	4.1	5.5	6.8
30% acrylamide mix		0.17	0.33	0.5	0.67	0.83	1.0	1.3	1.7
1M Tris-HCl pH 6.8		0.13	0.25	0.38	0.5	0.63	0.75	1.0	1.25
10% SDS		0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
10% APS		0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
TEMED		0.001	0.002	0.003	0.004	0.005	0.006	0.008	0.01

9. Solutions for preparing resolving gelsforTris-glycine SDS-polyacrylamide gelelectrophoresis

		Volume(ml) ofcomponentsrequiredto cast gelsofindicated volumes							
Components	Gel volume	5	10	15	20	25	30	40	50
10% gel									
Distilled water		1.9	4.0	5.9	7.9	9.9	11.9	15.9	19.8
30% acrylamide mix		1.7	3.3	5.0	6.7	8.3	10.0	13.3	16.7
1.5M Tris-HCl pH 8.8		1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS		0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
10% APS		0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED		0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
12% gel									
Distilled water		1.6	3.3	4.9	6.6	8.2	9.9	13.2	16.5
30% acrylamide mix		2.0	4.0	6.0	8.0	10.0	12.0	16.0	20.0
1.5M Tris-HCl pH 8.8		1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS		0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
10% APS		0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED		0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02

10. Coomassie Brilliant Blue staining (CBB)

Methanol	900	mL
Acetic acid	180	mL
Distilled water	900	mL
CBB (R250)	4.95	g

Mixed by mean of magnetic sterror 24 hours, extract with filter paper no. 2

11. Destaining buffer

Methanol	50	mL
Acetic acid	75	mL
Distilled water	875	mL

Buffer and reagent for western blotting

12. Phosphate buffer saline 10x (PBS) pH 7.5

NaCl	80	g
$KH_2\ PO_4$	2	g
$Na_2HPO_4 \bullet 12H_2O$	29	g
KCl	2	g
Distilled water	800	mL

Adjust the pH to 7.5 and adjust the volume to 1L

Sterile by autoclaving at 121°C 15 minutes

13. Phosphate buffer saline (1x) Tween 20

10x PBS	100	mL
Distilled water	900	mL
Tween 20	500	µL

Sterile by autoclaving at 121°C 15 minutes

14. Transfer buffer

0.2M Glycine	3.0028	g
25mM Tris	0.6055	g
Distilled water	200	mL

Preparation for western blotting per 1 time

100% methanol	20	mL
Distilled water	72	mL
Transfer buffer	8	mL

BIOGRAPHY

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EDUCATIONAL CREDENTIALS

Doctor of Philosophy (Veterinary Pathobiology, Microbiology) (2008-2013)
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ACADEMIC PUBLICATION

1. **Yurayart, C.**, Chindamporn, A., Suradhat, S., Tummaruk, P., Kajiwara, S., Prapasarakul, N. 2011. Comparative analysis of the frequency, distribution and population sizes of yeasts associated with canine seborrheic dermatitis and healthy skin. Vet. Microbiol. 148: 356-362.
2. **Yurayart, C.**, Nuchnoul, N., Moolkum, P., Jirasuksiri, S., Niyomtham, W., Chindamporn, A., Kajiwara, S., Prapasarakul, N. 2013. Antifungal agent susceptibilities and interpretation of *Malassezia pachydermatis* and *Candida parapsilosis* isolated from dogs with and without seborrheic dermatitis skin. Med. Mycol. In Press.DOI: 10.3109/13693786.2013.777165
3. **Yurayart, C.**, Niyomtham, W., Suradhat, S., Chindamporn, A., Kajiwara, S., Prapasarakul, N. Serological profile markers and immunodominant antigens of *Malassezia pachydermatis* cell wall on progression of canine seborrheic dermatitis. Manuscript in preparation and submission.