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Correlation of allergen specific immunoglobulin to house dust mites and commensal microbes in atopic dogs and identification of major allergen in dermatophagoides farinae

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CORRELATION OF ALLERGEN SPECIFIC IMMUNOGLOBULIN TO HOUSE DUST MITES AND COMMENSAL MICROBES IN ATOPIC DOGS AND IDENTIFICATION OF MAJOR ALLERGEN IN DERMATOPHAGOIDES FARINAE

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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ความสัมพันธ์ของอิมมูโนโกลบูลินที่เฉพาะต่อสารก่อภูมิแพ้ไรฝุ่น และจุลชีพประจำถิ่นในสุนัขภูมิแพ้ผิวหนังชนิดอาโทปี และการระบุสารก่อภูมิแพ้ที่สำคัญในไรฝุ่นเดอร์มาโทฟากอยเดส ฟารินี

น.ส.ณัฐรดา คันทวี

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Field of Study	Veterinary Pathobiology
Thesis Advisor	Associate Professor Dr. NUVEE PRAPASARAKUL
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Accepted by the FACULTY OF VETERINARY SCIENCE, Chulalongkorn University in Partial Fulfillment of the Requirement for the Doctor of Philosophy

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ณัฐรดา คันทวี : ความสัมพันธ์ของอิมมูโนโกลบูลินที่เฉพาะต่อสารก่อภูมิแพ้ไรฝุ่น และจุลชีพประจำถิ่นในสุนัขภูมิแพ้ผิวหนังชนิดอาโทปี และการระบุสารก่อภูมิแพ้ที่สำคัญในไรฝุ่นเดอร์มาโทฟากอยเดส ฟารินี. (CORRELATION OF ALLERGEN SPECIFIC IMMUNOGLOBULIN TO HOUSE DUST MITES AND COMMENSAL MICROBES IN ATOPIC DOGS AND IDENTIFICATION OF MAJOR ALLERGEN IN DERMATOPHAGOIDES FARINAE) อ.ที่ปรึกษาหลัก : ร.ศ. ดร.ณุวีร์ ประภัสระกูล, อ.ที่ปรึกษาร่วม : ศ. ดร.สันนิภา สุรทัตต์,รศ. ดร.นิทัศน์ สุขรุ่ง

การตรวจยืนยันชนิดสารก่อภูมิแพ้ที่เป็นสาเหตุให้เกิดภาวะภูมิแพ้ผิวหนังชนิดอาโทปีในสุนัขสามารถทำได้โดยการตรวจหาสารก่อภูมิแพ้ผ่านทางผิวหนังหรือการตรวจทางซีรัมวิทยาเพื่อหาปริมาณอิมมูโนโกลบูลินอีที่เฉพาะกับสารก่อภูมิแพ้ ซึ่งการตรวจทางซีรัมทางวิทยาสามารถทำได้ง่าย สะดวก และปลอดภัยกว่า แต่อย่างไรก็ตามผลการตรวจทางซีรัมสามารถผันแปรได้ตามชนิดและแหล่งที่มาของสารก่อภูมิแพ้ที่ใช้ทดสอบ การทดสอบซีรัมที่มีในท้องตลาดขณะนี้ยังใช้โปรตีนสกัดหยาบมาทดสอบภาวะภูมิแพ้อยู่ และโปรตีนของจุลชีพบนผิวหนังมักไม่ถูกรวมอยู่ในชุดทดสอบด้วย ในการศึกษานี้ ทางผู้วิจัยได้พัฒนาชุดทดสอบทางซีรัมเพื่อระบุภาวะภูมิแแพ้ไรฝุ่นที่เฉพาะกับไรฝุ่นบ้านเดอร์มาโตฟาร์กอยเดส ฟารินี และเดอร์มาโตฟาร์กอยเดส พเทอร์โรนิสสินัส และวัดการตอบสนองทางภูมิคุ้มกันที่มีต่อจุลชีพบนผิวหนังของสุนัขอันได้แก่ยีสต์มาลาสซีเซีย พาไคเดอร์มาทิส และแบคทีเรียสแตปฟีโลคอคคัส ซูดอินเตอร์มิเดียส ผลการวิจัยพบว่า ระดับอิมมูโนโกลบูลินอีที่สูงขึ้นที่เฉพาะกับไรฝุ่นบ้านสามารถใช้เพื่อแยกสุนัขอาโทปีที่แพ้ไรฝุ่น กับสุนัขอาโทปีที่ไม่ได้แพ้ไรฝุ่นออกจากกันได้ และยังพบอีกว่าระดับอิมมูโนโกลบูลินจีวันซับคลาสสามารถแยกภาวะภูมิแพ้ไรฝุ่นได้เช่นกันแต่ใช้ปริมาณตัวอย่างที่น้อยกว่าการวัดระดับอิมมูโนโกลบูลินอีถึง 100 เท่า ในขณะที่การวัดระดับอิมมูโนโกลบูลินจีทั้งหมด และอิมมูโนโกลบูลินชนิดจีทูซับคลาสไม่สามารถนำมาใช้เพื่อแยกภาวะภูมิแพ้ไรฝุ่นได้ ทั้งระดับระดับอิมมูโนโกลบูลินอีและจีวันให้ผลสอดคล้องกับการตรวจหาสารก่อภูมิแพ้ผ่านทางผิวหนังในระดับเดียวกัน จากการศึกษาด้วยวิธี two-dimensional IgE blotting ทำให้พบโปรตีนของไรฝุ่นที่สัมพันธ์กับภาวะภูมิแพ้ไรฝุ่นในสุนัขอาโทปีสิบชนิด โดยโปรตีน Der f 28 เป็นโปรตีนที่น่าสนใจและควรได้นำไปพัฒนาเพื่อการวินิจฉัยแบบ component-resolved diagnostics (CRD) และพบว่าเอ็นไซม์แอลฟาอีโนเลส เซอรีนโปรติเอส และอาร์จินีนไคเนสเป็นโปรตีนสำคัญที่ทำให้เกิดการ cross reaction จากผลการตอบสนองทางภูมิคุ้มกันต่อจุลีพบนผิวหนังพบว่า สุนัขอาโทปีมีระดับอิมมูโนโกลบูลินอีที่เฉพาะต่อจุลชีพบนผิวหนังทั้งสองชนิดในระดับที่สูงกว่าสุนัขปกติ ชี้ให้เห็นว่าจุลชีพบนผิวหนังสามารถทำหน้าที่เป็นสารก่อภูมิแพ้ได้ในสุนัขอาโทปี ระดับอิมมูโนโกลบูลินทั้งจีวันและจีทูซับคลาสที่เฉพาะกับจุลชีพทั้งสองสูงขึ้นเช่นกัน แต่อย่างไรก็ตามไม่พบว่ามีระดับของอิมมูโนโกลบูลินชนิดใดที่สัมพันธ์กับคะแนนที่สะท้อนภาวะความรุนแรงของรอยโรคบนผิวหนังในสุนัขอาโทปีที่ประเมิณความรุนแรงโดยใช้ The Canine Atopic Dermatitis Lesion Index (CADLI) กล่าวได้ว่าระดับอิมมูโนโกลบูลินที่เฉพาะกับจุลชีพบนผิวหนังไม่สัมพันธ์กับความรุนแรงของรอยโรคบนผิวหนัง

สาขาวิชา	พยาธิชีววิทยาทางสัตวแพทย์	ลายมือชื่อนิสิต .....
ปีการศึกษา	2562	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
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Nathrada Khantavee : CORRELATION OF ALLERGEN SPECIFIC IMMUNOGLOBULIN TO HOUSE DUST MITES AND COMMENSAL MICROBES IN ATOPIC DOGS AND IDENTIFICATION OF MAJOR ALLERGEN IN DERMATOPHAGOIDES FARINAE. Advisor: Assoc. Prof. Dr. NUVEE PRAPASARAKUL Co-advisor: Prof. Dr. SANIPA SURADHAT,Assoc. Prof. Dr. Nitat Sookrung

To confirm a causative allergen in atopic dogs could test through intradermal skin testing (IDT) or allergen-specific IgE serology testing (ASIS). ASIS is more practical, convenient and safer. However, the results of ASIS could be affected by source and type of allergen. Crude allergen extracts were still used in commercial testing and protein antigens of skin microbes were not included in the panel of testing. In this study, our group developed serology test for confirming house dust mite allergy called *Dermatophagoides farinae* and *D. pteronyssinus*, and measuring specific immunoglobulin to skin microbes on dogs like Malssezia *pachydermatis* yeast and *Staphylococcus pseudintermedius* bacteria. The results was found that high IgE levels to house dust mites could be used to differentiate atopic dog with allergy to house dust mites and atopic dogs without allergy to house dust mites. Moreover, IgG1 levels could also use to differentiate house dust mite allergy and non-house dust mite allergy by using lower sample volumes than IgE about 100 times. In the other hand, total IgG and IgG2 levels could not be used to differentiate house dust mite allergy and non-house dust mite allergy in atopic dogs. Both of IgE and IgG1 levels showed the same degrees of agreement to IDT. By investigation through two-dimensional IgE blotting, ten proteins of house dust mites that related to house dust mite allergy in atopic dogs were found, Der f 28 protein is interesting and should be selected to apply for a component-resolved diagnostics. Alpha-enolase, serine protease, and arginine kinase are the critical protein to be a cause of cross reaction. The results of specific immune response to skin microbes were found that atopic dogs had higher specific IgE levels to both skin microbes than healthy dogs. This indicated that skin microbes could act as allergen in atopic dogs. Other specific IgG1 and IgG2 to these skin microbes also high. However, none of any types specific immunoglobulin to skin microbes related to skin severity scores in atopic dogs determining through the Canine Atopic Dermatitis Lesion Index. We concluded that levels of specific immunoglobulin to skin microbes did not relate to skin severity.

Field of Study:	Veterinary Pathobiology	Student's Signature .....
Academic Year:	2019	Advisor's Signature .....
		Co-advisor's Signature .....
		Co-advisor's Signature .....

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Nathrada Khantavee

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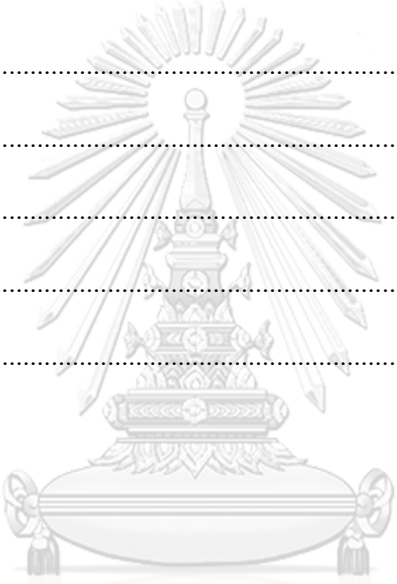

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CHAPTER I

Importance and rationale

Atopic dermatitis (AD) is one of the important allergic skin diseases in both humans and dogs (Halliwell, 2006; Bieber, 2010). AD is a complex disease involving skin barrier defects, immune dysregulation, allergic sensitization, colonization of skin microbes and, environmental factors (Nuttall, 2013). The pathogenesis of AD in dogs is still unclear. About 10-15% of dogs suffering from AD present severe pruritus with chronic dermatitis associated with allergies (Chamberlain, 1974; Scott, 2001). Previously, incidences of AD have believed to associate with genetic predisposition and dog breed heritability. Some certain dog breeds indicated to closely relate with AD such as West Highland white terriers, Boxer, Golden retriever, Labrador retriever, German shepherd, Shar-pei, Dalmatian, French bulldog and Jack Russell terrier (Hillier and Griffin, 2001; Sousa and Marsella, 2001; Nuttall, 2013; Hensel et al., 2015). Regarding to genetic factors, the genes associated with immune deviation response and impaired skin barrier such as increased transepidermal water loss (TEWL), decreased ceramide levels and filaggrin mutations are assumed as the relative factors to increase of allergen-specific immunoglobulin E (IgE) (Hill et al., 2001; Osawa et al., 2011; Salzmann et al., 2011; Nuttall, 2013). To date, the guideline for diagnosis of canine atopic dermatitis focuses on three steps including; the ruling out of other skin problems with clinical signs overlapping, matching historical and clinical feature of patients by Favrot's criteria, and allergic determination by intradermal test or serum specific IgE (Favrot et al., 2010; Hensel et al., 2015). The results from allergy testing are necessary information for further avoidance and immunotherapy. Nowadays, allergen sensitization can also determine by both intradermal skin testing (IDT; detection allergen-specific IgE via skin reaction) and allergen-specific IgE serology testing (ASIS; *in vitro* measuring allergen-specific IgE in circulation) (DeBoer and Hillier, 2001; Griffin and Hillier, 2001; Hillier and DeBoer, 2001; Hensel et al., 2015). Even though IDT considered as the gold standard test amongst veterinary dermatologists, ASIS is more practical and convenient in veterinary practitioners. Furthermore, ASIS is not required the withdrawal time for antihistamines, non-steroidal anti-inflammatory drugs, or short-acting glucocorticoids before testing. Moreover, this testing is less

invasive, as this is no need for sedation (Hensel et al., 2015). For ASIS, the level of allergen-specific IgE is detectable, which this level reflects allergic reactive markers triggering by immediate hypersensitivity (e.g., Sensitest and ALLERCEPT®) (Plant et al., 2014). Unfortunately, commercial ASIS is expensive and still not widely available in Thailand; therefore, the development of appropriate ASIS required. Even though increasing allergen-specific IgE usually found in canine AD, a high level of IgE did not guarantee as an actual causative allergen in all cases of dog (Foster et al., 2003; Lauber et al., 2012). Potential pitfalls have effected the reliability of ASIS include dog's background, nonspecific-IgE antibody binding, allergenic extract variability, nonspecific signal molecule activation, and establishing clinically relevant cut-off values (Plant et al., 2014). Moreover, subisotypes and half-life of serum IgE may lead to complicated serological diagnosis of AD (Peng et al., 1997). IgE has suggested as a major effector in antigen capture that lead to an allergic reaction. Interestingly, high IgG response often coincides with high IgE response in canine AD (Halliwell and DeBoer, 2001). Therefore, the monitoring of the allergen-specific IgG levels may also apply as an alternative marker for canine AD, as previously proposed in human AD (Boluda et al., 1997). Nevertheless, the roles of the specific immune response via allergen specific-IgG or their IgG subclasses are still unclear and limited in atopic dogs. Allergy testing contains several seasonal and non-seasonal allergens, but some allergens are not relevant in a tropical country like Thailand (Hensel, 2012). The source of used allergen is the influential key to validate the allergic sensitization test regarding the variable concentration of allergenic components in each source (Nuttall et al., 2001). Hence, identification of relevant allergens or common allergens in each country should be concerned about the type and source of allergen extracts. House dust mites (HDM) are predominantly allergens in canine AD, especially *Dermatophagoides farinae* and *D. pteronyssinus* (Halbert et al., 1995; Hill and DeBoer, 2001). In Thailand, over 70% and 50 % of canine AD dogs were positive to *D. farinae* and *D. pteronyssinus* by IDT, respectively (Chanthick et al., 2008). In advance medicine, the component-resolved diagnosis (CRD) and component-resolved immunotherapy (CRT) are well-described in human with allergy, their techniques have applied in patients according to high accuracy of diagnosis and increasing

success rate of treatment (Kraft et al., 1999; Valenta et al., 1999; Treudler and Simon, 2013). In contrast, CRD and CRT have not been applied and evaluated in dogs. A study about allergenic components of HDM still lacks in canine AD. Not only HDM, *Staphylococcus pseudintermedius,* and *Malassezia pachydermatis* are members of skin commensal in dogs and can act as opportunistic pathogens causing secondary skin infection and enhance the severity of skin inflammation in canine AD (DeBoer and Marsella, 2001). By IDT and ASIS determination, *S. pseudintermedius* and *M. pachydermatis,* probably assigned as self-microbial allergen, (Morales et al., 1994; Morris et al., 1998; Nuttall and Halliwell, 2004; Bexley et al., 2013), but they have not included in a panel of commercial allergy testing. The immune response of canine AD to the skin microbes has not elucidated when compared to mite allergens. The study of allergen-specific immune response to these microbes in canine AD probably links to immune dysregulation. The database of allergen and a system for nomenclature of allergens are not established in dogs. For comparative study to allergic patients, the study of allergen characterization, structure, function, molecular biology, and bioinformatics are required in canine AD. Therefore, the study of canine AD should focus on mapping allergenome or identified specific protein in each important allergens that relate to allergic condition in dogs.

Objectives of the study

1. To develop an in-house ELISA and determine the level of allergen-specific IgE, IgG and its subclasses (IgG1&IgG2) in healthy and atopic dogs
2. To investigate the level of allergen-specific IgE and IgG subclasses (IgG1&IgG2) responding in healthy and atopic dogs against four major allergens by using in-house ELISA
3. To identify major allergens of *D. farinae* recognized by atopic dogs in Thailand

Hypotheses

1. Developed-in house ELISA can detect the level of allergen-specific IgE, IgG, and its subclasses, and can use to can confirm a causative allergen in dog
2. Level of allergen-specific IgE or IgG subclass (IgG1 and IgG2) show the agreement to IDT or related to skin severity score.
3. Reactive proteins belonging to *D. farinae* can be represented in local *D. farinae* and can be used to differentiate between healthy and atopic dogs.

Advantages of study

1. This finding leads to further development of available local allergen-specific serology testing specific to Thailand
2. The patterns of allergen-specific IgG subclasses (IgG1 and IgG2) might be used as a prognosis marker of treatment
3. Identified reactive protein in *D. farinae* related to allergic condition can be used for further development of CRD and CRT

Keywords (Thai):
อาโทปี, สุนัข, อิมมูโนโกลบูลินอี, อิมมูโนโกลบูลินจีวัน, อิมมูโนโกลบูลินจีทู, จุลชีพบนผิวหนัง
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Literature review

1. Canine atopic dermatitis and allergen-specific IgE and IgG subclasses

AD is associated with the multiplication of IgE antibodies to environmental allergens. The basic pathogenesis of AD started from allergen exposure and followed by allergen processing. In dog, routes of allergen exposure could pass through skin regard to skin barrier defects in predisposing dogs or through the respiratory system as the initial concept following human allergy. However, both route of allergen exposure in atopic dogs is still confusing regard to uncompleted investigation in many previous literatures mentioned in reviewed literature by the American College of Veterinary Dermatology (ACVD) that appointed the Task Force on Canine Atopic Dermatitis. This task force was composed of eight Board-certified dermatologists selected because of their clinical and research interest in canine AD (Olivry and Hill, 2001). Next, the bias of T-helper 2 cells under allergic condition in AD dogs, could manipulate B-cells to produce allergen-specific IgE. High allergen-specific IgE could circulated as a free form IgE and bonding form to a mast cell. So, secondary exposure to a causative allergen and that allergen was captured by specific IgE bonding mast cell, its consequence is a histamine-releasing and following by allergic reaction and inflammation of the skin (Vercelli, 2008). So specific IgE is used as a maker of allergic reaction in AD (Halliwell and DeBoer, 2001). Following the updated guideline of canine AD diagnosis (Hensel et al., 2015), start with history taking, ruling out other pruritic skin diseases (parasite infestation, flea bite allergy, and food allergy) and confirming the clinical diagnosis of canine AD with allergy testing. The common allergy testing in dogs is IDT and ASIS. IDT is the in vivo test to detect allergen-specific IgE bonding mast cells, while ASIS is the in vitro test to detect free form of circulated allergen-specific IgE. So, most dermatologists seem to prefer IDT rather than ASIS. Unfortunately, IDT is not a practical test in all cases of dogs, this test requires a professional to perform and drug-withdrawal time of anti-inflammatory drugs is need before testing. ASIS is more practical in most case of AD,

This test is used to detect a high amount specific IgE response against a panel of allergens (e.g., HDM, pollen, mold, and epidermal allergens) (DeBoer and Hillier, 2001;

Olivry et al., 2001) because in allergic condition IgE production was induced. Then, the outcome of ASIS could use to guide veterinarians and owners for allergen avoidance or formulate allergen-specific immunotherapy to the AD dogs as IDT (Griffin and Hillier, 2001). Detection specific IgE with monoclonal antibodies anti-dog IgE or high affinity-Fc-epsilon receptor alpha chain protein (FcεRIα; Mast cell-bound IgE) seemed to show a higher specificity than using polyclonal antibodies or non-specific IgE receptor (DeBoer and Hillier, 2001; Foster et al., 2003; Saevik et al., 2003). For the big point of considering in ASIS, allergen-specific IgE levels are affected by many factors such as allergen sources, allergen types, breeds, geographical localization of dogs, season of sampling, sex and age of dogs, therefore IgE analysis could not be a maker for canine AD in all aspects (Lauber et al., 2012; Nuttall, 2013; Bjelland et al., 2014). The agreement between IDT and ASIS is not good (Foster et al., 2003). Moreover, IgE takes less than one percent of total Ig in the serum, so this might be a cause of low sensitivity (Ucan et al., 2003). In an allergic condition, not only IgE production, high allergen-specific IgG production could found in atopic dogs (Hill et al., 1995; Kang et al., 2014). In human, IgG concentrations have elevated in various allergic conditions (Shakib et al., 1977; Gondo et al., 1987) and its linkage to specific allergens uncovered by immunoglobulin isotypes and IgG subclasses (IgG1, IgG2, IgG3, and IgG4) (Schur, 1988). Non-IgE anaphylactic antibodies have identified as IgG4 in human and guinea pigs, IgG1 in mice, IgG2a in rats and IgG2 in sheep (Nussenzweig and Benacerraf, 1964; Reid et al., 1966; Bach et al., 1971; Margni and Hajos, 1973; Esteves et al., 1974). Restricted IgG isotype response in humans involved with specific classes of antigen, for example, protein antigens and membrane protein preferred IgG1 response while carbohydrate antigens activated IgG2 response (Sigal, 2012). Moreover, IgG4 antibody responses in long-term antigen exposure, such as allergen-specific immunotherapy (ASIT) (Vidarsson et al., 2014). However, the response of IgG and their subclasses have less understood in canine AD and other diseases of dog. In the 1990s, Massa and colleagues found that dog sera with immune-mediated or inflammatory diseases exhibited a high level of IgG2 markedly, while IgG1 level was low. This finding was the first connection between IgG subclass response and dog diseases. Moreover, it suggested that the composition of IgG

subclasses had changed toward the specific patterns in a variety of clinical diseases, and confirmed that a degree of IgG restriction might be dependent upon the nature of the allergen, especially IgG1 and IgG4 (Day et al., 1996). Many researchers have been focused on the level of total IgG, total IgG1 and total IgG4 in canine AD, but not focus on the level of allergen-specific IgG subclasses to relevant allergen (Fraser et al., 2004; Hou et al., 2006; Lauber et al., 2012). Parasitism, atopic dermatitis, and ASIT induced a high concentration of IgG1 in dog sera (Fraser et al., 2004). The types and levels of allergen-specific IgG subclasses in AD have been less defined, especially on the proportion of IgG subclasses amongst AD dogs. The evidences of allergen-specific IgG subclass by type, level, and their proportions in AD dogs compared to healthy can contribute the knowledge of immune dysregulation by allergens and being detective markers in atopic dogs and convalescence.

2. Major Allergens: House dust mites

In both humans and dogs, HDMs *(e.g., D. farinae and D. pteronyssinus)* are the most intimate allergens. Positive IDT and increasing allergen-specific IgE levels in ASIS to house dust mites dramatically found in 40-80% and 60-90% in allergic patients and AD dogs, respectively (DeBoer, 1989; Ricci et al., 1999; Zur et al., 2002). *Dermatophagoides* genus are closely similar in species level but have differences in some physical characteristics (Thomas et al., 2004). To date, ALLERGEN NOMENCLATURE, this website is the official site for the systematic allergen nomenclature that is approved by the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee, has reported over 30 different allergen groups of *Dermatophagoides spp.*, relating HDM allergy in allergic human patients. The group 1 allergens (e.g., Der f 1 and Der p 1) are low molecular weight cysteine protease and the most abundant allergens to human, having about 70-80% seropositive in atopic patient sera (Le Mao et al., 1998; Thomas et al., 1998). However, in contrast, sera of atopic dogs often recognized the high molecular weight allergen proteins of house dust mites such as Der f 15 and Der f 18, defined as chitinase (Nuttall et al., 2001; Moya et al., 2016). Excluding of Der f/p 1, 2, and 15/18, other allergens of *Dermatophagoides spp.* have been less or no

reported in atopic dogs. These observations indicated that dogs and humans have different major allergen recognition of HDM crude extracts. In diagnosis and immunotherapy, group 1 allergens have been used as a control for allergic activity and potency of HDM in both humans and dogs, even though allergen group 1 is not a major recognized allergen in most of atopic dogs. The major allergen recognized by atopic dogs also depends on the geographic region of dogs. Indeed, standardized allergens for diagnosis and treatment of canine AD should be considered based on its major allergen type and frequency in the dog's habitat. Both concepts of "component-resolved diagnosis (CRD)" and "component-resolved immunotherapy (CRIT)" are introduced in human AD to improve the determination of IgE sensitivity to individual allergen molecules and increase the successful outcome of allergen-specific immunotherapy (Valenta et al., 1999; Treudler and Simon, 2013). However, many factors were affecting the result of allergen-specific IgE to house dust mites such as allergen source, culture media, and investigation methods (Nuttall et al., 2001; Avula-Poola et al., 2012; Yella et al., 2013). The frequency binding and affinity to the full range of *Dermatophagoides* allergens have clearly defined in human AD but still lack in canine AD. Overall, allergens belonging to *D. farinae* and *D. pteronyssinus* are closely related with 80-90% homology and shown cross-reaction activity in immunoblotting study in atopic dog (Mueller et al., 1998). However, the reactive proteins of these two HDM were not characterized (Nuttall et al., 2001; Nuttall et al., 2006).

3. Bacterial allergens of *S. pseudintermedius* in atopic dermatitis

Staphylococcal infection is a cause of secondary infection, and lead to be a major complication in canine and human atopic dermatitis (DeBoer and Marsella, 2001; Hensel et al., 2015). During AD episode, the risen staphylococcal colonization and adherence were allowed on the skin lesion and non-lesion, but this could not observe in healthy (Harvey and Noble, 1998). Staphylococcal protein antigens can penetrate via impaired skin epithelium following erythematous and severe pruritus caused by mast cell degranulation, which leads to the activation of immune response. Up to 25% of *S. pseudintermedius* in dogs can produce exotoxins,

especially superantigens (Hendricks et al., 2002). Superantigens are the active proteins induced Staphylococcus-specific IgE, which can bind receptors on mast cells or Langerhans cells (Mason et al., 1996; Hendricks et al., 2002; Lloyd et al., 2007). In atopic humans, the levels of *Staphylococcus*-specific IgE was claimed as a key role in disease pathogenesis (Ong, 2006; Ong and Leung, 2006). *S. aureus* commonly presented on the skin of healthy and atopic humans, but progressively worsening dermatitis related to amount and action of enterotoxins. The increasing level of *S. aureus* enterotoxin B (SEB) specific-IgE, IgG1 and IgG4 were found in atopic humans, while the level of IgG2 and IgG3 were not different (Orfali et al., 2015). Their finding indicated that IgE, IgG1, and IgG4 has associated with disease detection. While the levels of IgG1 and IgG 3 seem to correlate with the severity of the disease. In the veterinary field, the elevated IgE to *S. pseudintermedius* have also detected in canine AD, but their specific antigens and superantigens are less defined (Morales et al., 1994; Bexley et al., 2013). This finding suggested an immunopathogenic role for anti-staphylococcal IgE as a human. However, the level of *S. pseudintermedius* specific total IgG in AD and non-AD with pyoderma was not different (Bexley et al., 2013). Until now, a lot of inquiries on the role of bacterial infection in atopic dogs have been still unclear, such as a correlation between exotoxin and clinical symptoms (Burkett and Frank, 1998), identification of allergenic proteins and different reaction between total IgG and their subclasses.

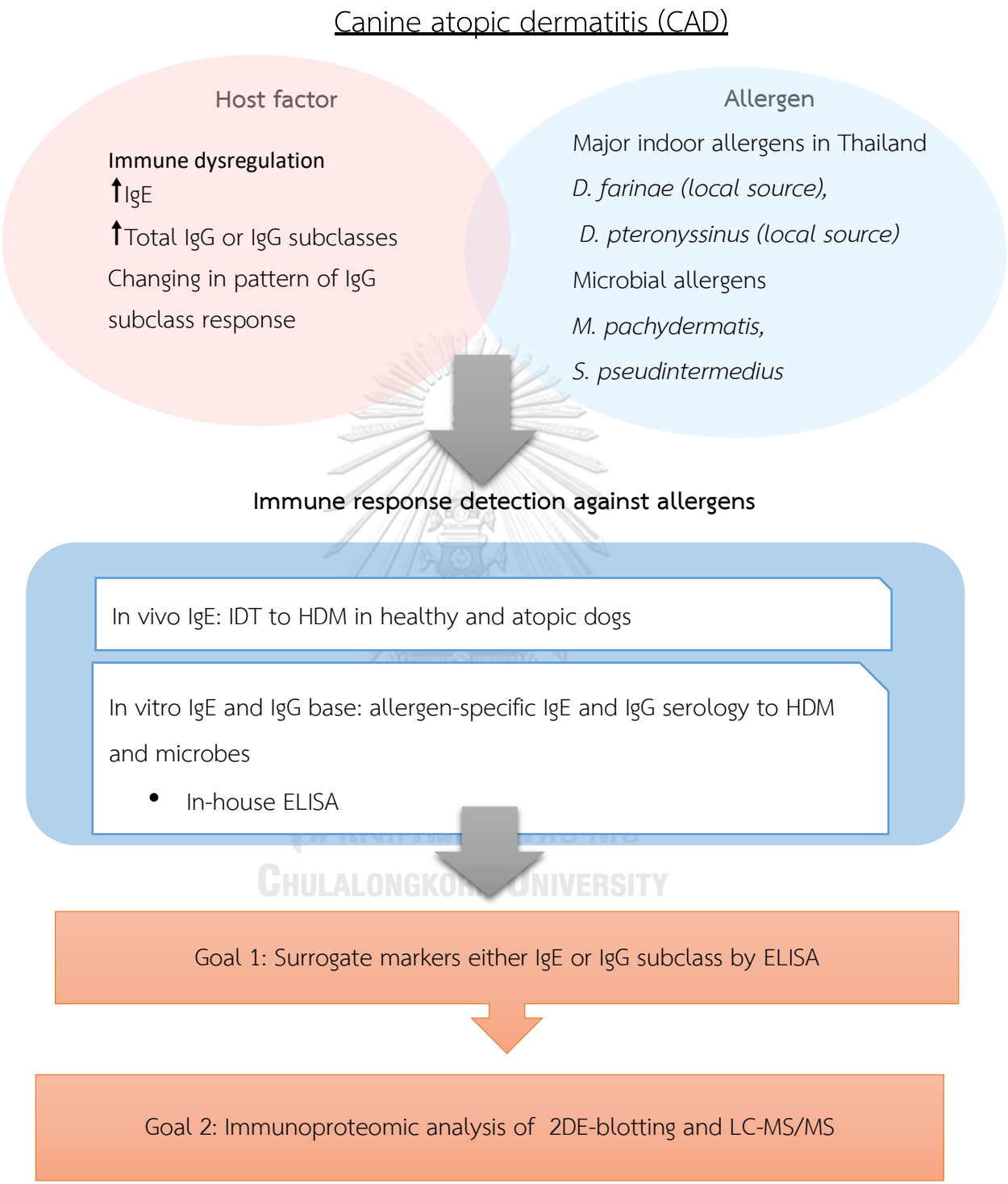
4. Fungal allergen of *M. pachydermatis* in atopic dermatitis

Recurrent colonization and infection by *Malassezia spp*. are commonly observed in both humans and dogs with atopic disease. *Malassezia spp*., itself, can induce interleukin-1β, interleukin-6, interleukin-8, and tumor necrosis factor-α production in normal human keratinocytes (Watanabe et al., 2001). *M. furfur* acted as allergenic in humans, and nine allergens (Mal f1- Mal f 9) defined by immunoblotting technique (Zargari et al., 1994; Lintu et al., 1997; Rasool et al., 2000). Besides, a positive relation between *Malassezia*-specific IgE levels and clinical severity of “head and neck dermatitis” are were reported (Bayrou et al., 2005). In atopic dogs, observation of greater wheal-and-flare reactions after intradermal

injection by *M. pachydermatis* extract revealed that *M. pachydermatis* was also capable of promoting immediate hypersensitivity reactions in canine atopic dermatitis phenotype (Morris et al., 1998).

Moreover, multiple major and minor antigens recognized by sera total IgG and IgE from atopic dogs (Chen et al., 2002). However, the levels of *Malassezia*-specific IgE were indifferent between dogs with recurrent *Malassezia* otitis externa and healthy; thus, serum IgE reactivity for *M. pachydermatis* could not use to differentiate between diseased and healthy patients (Layne and DeBoer, 2016). To date, the role of *Malassezia* antigens in atopic dogs is still inconclusive, identification and characterization of *M. pachydermatis* antigens reacted to IgG subclass, and IgE simultaneously can be completed this aspect.

Conceptual Framework



CHAPTER II

Immunoglobulin G1 subclass responses can use to detect specific allergy to the house dust mites *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* in atopic dogs

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Abstract

Background: In dogs with atopic dermatitis, intradermal testing (IDT), or allergen-specific IgE serological testing (ASIS) routinely employed to identify causative allergens. These allergens can use for allergen-specific immunotherapy (ASIT) and allergy management. The clinical relevance of this testing is affected by the source of allergen, and other biomarkers that are more related to specific allergens still need to be identified. This study aimed to investigate levels of specific IgE, total IgG, and IgG1 and IgG2 subclasses against the local house dust mites (HDM) *Dermatophagoides farinae* (DF) and *D. pteronyssinus* (DP) as biomarkers by using in-house ELISAs in healthy (n =33) and atopic dogs (AD) (n = 44) that were either positive or negative by IDT to HDM.

Results: Being over three years of age was a risk factor for AD (OR= 4.10, 95% CI 1.57 - 10.75, p =0.0049), but there was no relation to IDT outcomes (OR= 0.9091, 95% CI 0.22 -3.74, p =1.00) (table 2). High levels of all antibody isotypes (IgE, IgG, IgG1, and IgG2) against HDM found in aged healthy dogs (> three years old). In AD, HDM-IgE and IgG1 levels were higher in dogs that were IDT positive to HDM than in IDT negative animals. Levels of IgE and IgG1 could use to distinguish the specific allergens, whereas total IgG and IgG2 levels were not different between IDT-positive and IDT-negative AD. Similar to IgE, serum IgG1 concentration was also relevant to IDT outcomes.

Conclusions: Our in-house ELISAs coated with local HDM were useful for evaluating antibody levels, and we propose the use of the HDM-specific IgG1 subclass as a biomarker to detect HDM specific allergens in AD, potentially together with an IgE based platform.

Keywords: Antibody, Biomarker, Canine atopic dermatitis, House dust mites, Immunoglobulin G1 subclass

Background

Canine atopic dermatitis (CAD) is a major chronic immune-mediated inflammatory and pruritic skin disease with a genetic predisposition (Halliwell and DeBoer, 2001). Underlying allergic reactions can direct at otherwise harmless substances such as grass, mold spores, house dust mites, and other environmental allergens. The diagnosis of CAD based on patient history, clinical signs, and elimination of other forms of allergic skin disease. Intradermal testing (IDT) or allergen-specific IgE serological testing (ASIS) are needed to indicate relevant allergens in atopic dogs. This information can then use to avoid allergen exposure and to formulate allergen-specific immunotherapy (ASIT) (Hensel et al., 2015). IDT and ASIS still have some problems, as their outcomes can confound by factors such as allergen type, allergen source, dog breed, dog age, presence of ectoparasites, and the laboratory techniques used. Different sources of house dust mite extract affect the level of IgE recognition from atopic dog sera (Nuttall et al., 2001), and a high background of specific IgE levels can found in some predisposed dog breeds without clinical signs of AD (Lauber et al., 2012). For practical use by veterinary dermatologists, serological detection is reliable, minimally invasive, and convenient to undertake. In serological detection in atopic dogs using allergen-specific IgE and IgGd, compared to IgE, a high IgGd response had a low specificity and was irrelevant to IDT results, implying its uncertain role in atopic dogs (Lian and Halliwell, 1998). In later studies, the role of allergen-specific IgG in the pathogenesis of CAD was not well defined. IgG divided into four subclasses (IgG1-4), and certain of these have an interesting relationship with allergic dermatitis (Mazza et al., 1994; Fraser et al., 2004). In a previous study, levels of total non-allergen specific IgG1 subclass in sera were affected by parasitic-infestation, atopic dermatitis (AD), and ASIT (Fraser et al., 2004). In human allergic patients, besides IgE, increases in IgG1 and IgG4 levels were associated with some specific allergens, and also were inducible after ASIT, especially with high IgG4 levels corresponding to the relief of symptoms (Einarsson et al., 1992). On the other hand, IgE, IgG1 and IgG4 levels were used as an indicator set to differentiate between non-atopic and atopic dogs, but neither levels of specific immunoglobulins could differentiate both groups of dogs (Lauber et al., 2012). The

model of antigen specific-IgG subclass responses also has been explored in canine leishmaniasis. Concerning different outcomes of Leishmania infection, IgG1 levels were noted as a biomarker during the active stage, while IgG2 levels were associated with subclinical infection or disease resistance (Lima et al., 2017). However, the role of IgG subclasses has not been fully elucidated in CAD. The aim of the study was to identify an improved biomarker for use in CAD. This involved investigating levels of allergen-specific IgE, total IgG, IgG1 and IgG2 antibody against the local house dust mites (HDM) *Dermatophagoides farinae* (DF) and *D. pteronyssinus* (DP) extracts in healthy dogs and in atopic dogs which had positive or negative IDT reactions to the HDM.

Methods

Serum samples

A total of 33 healthy dogs were enrolled with the following inclusion criteria: normal at physical examination, not suffering from any diseases, no previous history of skin problems, and normal skin appearance. Serum samples were collected and stored at -20 ^{o}C until used.

CAD was defined in 44 dogs by a combination of their clinical histories, clinical signs, match to Favrot's criteria (Favrot et al., 2010), ruling out other pruritic skin disease, and little improvement of skin condition after eight weeks of dietary restriction using a hypoallergenic food or a novel protein (Hensel et al., 2015). AD subjects were not previously prescribed with any steroid regimen. IDT was undertaken in all AD cases after a withdrawal period of at least two weeks for anti-inflammatory and anti-pruritic drugs. IDT was performed according to the standard protocol recommended by the manufacturer, using 45 allergen extracts from ALK Abello (ALK Abello), excluding DF and DP. Commercial HDM extracts for IDT obtained from Siriraj House Dust Mite Center (SDMC) (Mahidol University, Thailand). Allergen extracts were diluted as recommendations by the manufacturer. After interpretation with the same criteria as in a previous report (Chanthick et al., 2008), AD cases were divided into IDT negative and IDT positive to HDM (for DF and DP). Serum samples were collected on the same day as IDT testing and were stored at -20 ^{o}C until used.

Preparation of HDM extract

Purified DF (batch no. DF-SDMC 080158) and DP (batch no. DPT-BKK 060158), both with >99% purity, were obtained from SDMC. Two grams of each mite had been individually resuspended in 8 mL of 0.01 M phosphate-buffered saline (PBS), pH 7.4, and then were homogenized using an Omni Sonic Ruptor 4000 (Omni International) at 35% amplitude with 0.5 cycles (15 min, on ice). The supernatant was collected after centrifugation at 12,000 x *g*, 4 °C for 5 min (Sorvall Legend X1R, Thermo Scientific). Composition analysis of extracts was performed for quality control. All the processes of validation and production of DF and DP extracts were executed following good manufacturing practice (GMP) regulations, under the supervision of an SDMC specialist. Protein content was measured by Bradford's assay (Protein Assay Kit II, Bio-Rad), and the supernatant was kept in aliquots at -20 °C until used. Antigenic bands of HDM extracts were checked by SDS-PAGE, as previously reported (Choopong et al., 2016). HDM extracts were examined for adequate content of the major allergens Der f 1 and Der p 1 using commercial ELISAs (Der f 1 ELISA kit (6A8/4C1) and Der p 1 ELISA kit (5H8/4C1), Indoor biotechnologies).

In-house DF/DP-specific IgE ELISAs

Pooled serum samples from five AD dogs and five young, healthy dogs were used in a checkerboard titration. AD dogs were selected for their strongly positive IDT (+4) to DF/DP and also their positive results to both DF and DP in a commercial ASIS (Avacta Animal Health). Sera from young, healthy dogs (six to eight months old) negative by commercial ASIS were used as negative controls. DF and DP extracts were separately prepared in 0.2 M sodium carbonate-bicarbonate buffer, pH 9.4 (BupH™ Carbonate-Bicarbonate Buffer Packs, Thermo Fisher Scientific). One hundred microliters of HDM solution was added into the wells of 96-well plates (Costar 3590 EIA, Corning) and incubated overnight at 37 °C. After washing with washing buffer consisting of PBS containing 0.05% Tween 20 (Affymetrix, Fisher Scientific) (PBST), 200 µl of blocking buffer (PBST containing 1% Bovine serum albumin (BSA)) was added and incubated at 37 °C for 1 h. Plates were rinsed before adding 100 µl of diluted pooled serum samples in duplicate, consisting of two-fold serial dilutions in blocking

buffer starting at 1/5. Plates were incubated at 37 °C for 2 h before washing. Monoclonal anti-dog IgE (clone E6-2A1, Bio-Rad) diluted in blocking buffer (1/2,000) were added and incubated at 37 °C for 1 h. After washing, 100 µl of a phosphatase conjugate (1/2,000 in blocking buffer) (goat anti-mouse IgG & Human ads-alkaline phosphatase, Southern Biotech) was added, incubated at 37 °C for 1 h, and washed as before. Alkaline phosphatase substrate (Sensitest Canine IgE Substrate, Avacta Animal Health Limited) was added at 25 °C for 20 min, and plates were immediately read at an optical density (OD) of 450 nm using an ELx808 ultra microplate reader and KC4 3.3 Rev 10 software (Bio-Tek Instruments Inc.). Results were subtracted with the OD of the blank control before generating titration curves. The middle point of the near-linear part was selected as an optimal point. The optimal HDM concentration for coating was 20 µg/mL with a serum dilution of 1/5. The reaction was quantified in ELISA units to act as a reference, as previously described (Iniesta et al., 2005). Results from a pooled serum sample from five AD dogs were used for calibration and arbitrarily set at 100 units (U). A cut-off value was established by using the mean +4 standard deviations of the negative control samples. For intra- and inter-assay consistency, results of detection were acceptable with a coefficient of variation (CV) not exceeding 10%.

In-house DF/DP specific-IgG, IgG1, and IgG2 ELISAs

Checker-board titrations for the other antibody classes were performed as described for the IgE ELISA, with some modifications. Three different peroxidase conjugates were used to detect allergen-specific IgG, IgG1 and IgG2, included polyclonal anti-dog IgG (IgG antibody (AAI32P), Bio-Rad.), polyclonal anti-dog IgG1 (IgG1 antibody (AHP947P), Bio-Rad), and polyclonal anti-dog IgG2 (IgG2 antibody (AHP948P), Bio-Rad), respectively. These conjugates were individually diluted in blocking buffer at 1/4,000 before use. Peroxidase substrate (ABTS® peroxidase substrate, KPL Inc.) was added for color development. The optimal serum dilution was 1/500.

Data and statistical analyses

GraphPad Prism (GraphPad Software Incorporated) was used for statistical analyses. Risk factors were analyzed in the sample population. Levels of DF- and DP-specific immunoglobulin between healthy and AD dogs were determined for normality by the D'Agostino & Pearson omnibus, and medians were compared by the Kruskal-Wallis test. Differences were considered significant if *p* values were less than 0.05. The sensitivity and specificity of each assay was calculated in AD by using the IDT results as a gold standard.

Results

Demography of dogs supplying the serum samples

All serum samples were categorized by the source and the dog's clinical signs. In the AD group, 68% (30/44) of the dogs were positive to HDM (DF and DP) by IDT (table 1). The number of female and male dogs were similar, and the neuter status was not recorded. Amongst the healthy dogs, those from internal medicine were younger than dogs from the blood bank. In the AD, both sub-groups (+ and – IDT to HDM) were of near median age and were older than the healthy dogs. Breeds varied in each sub-group, including animals with and without known breed predisposition. In contrast to other sources, Boxer and Rottweiler breeds over three-year-old were the main donors in the blood bank. In AD that were either +IDT or – IDT to HDM, food allergy was found at a rate of about 10-14%. Both sex and age were included in the risk analysis between healthy dogs and AD, but dog breed was not included due to the large number of breeds represented amongst the relatively small number of samples. Sex was not a confounding factor between healthy dogs and AD (OR= 0.58, 95% CI 0.22 -1.52, p = 0.3359) (Table 2). Being over three years of age was a risk factor for AD (OR= 4.10, 95% CI 1.57 -10.75, p =0.0049), but there was no relation to IDT outcomes (OR= 0.9091, 95% CI 0.22 -3.74, p =1.00) (table 2).

Table 1 Demographic data for the serum samples

Data	Healthy		AD	
Source/ IDT results	Internal medicine	Blood bank	+ IDT to HDM	- IDT to HDM
Number	20	13	30	14
Sex				
-female	11	6	16	5
-male	9	7	14	9
Age range in years (median)	0.17-3.00 (1.00)	2.00-5.00 (3.00)	1.30-15.75 (6.17)	1.16-13.33 (7.08)
Breed	Chihuahua (6), Crossbreed (6), Labrador Retriever (3), Siberian Husky (2), Beagle (1), French bulldog (1), Bangkraw (1)	Boxer (7), Rottweiler (5), Golden Retriever (1)	Poodle (7), Shitzu (5), French bulldog (4), Beagle (3), Cross breed (2), Golden Retriever (2), Shiba inu, Labrador retriever, Westy white terrier, Chihuahua, Jack Russel, American Pitbull, Bangkraw (1)	Shitzu (4), Cross breed (4), Pomeranian (3), Bulldog, Golden Retriever, Westy white terrier (1)
Food allergy (%)			10.00	14.28

Table 2 Risk factors in healthy dogs and AD from different sources and with different IDT outcomes

Risk factor	Between groups of dogs		
	Healthy (H)	Atopic Dog (AD)	
	Internal medicine and Blood bank	+ IDT and - IDT to HDM	H and AD
Sex			
OR[a]	1.83	2.06	0.58
(95% CI)[b]	(0.47- 7.13)	(0.56-7.61)	(0.22 -1.52)
p[c]	0.4998	0.3419	0.3359
Age >3 yr			
OR[a]	1025	0.9091	4.103
(95%CI) [b]	(19.09 – 55048)	(0.22 - 3.74)	(1.57 -10.75)
p[c]	< 0.0001	1.00	0.0049

[a] Odds ratio; [b] 95% Confidence interval; [c] P-value; significantly if $p < 0.05$

Quality of local HDM extracts for coating ELISA plates

HDM extracts containing at least 32 mg of crude protein per gram of purify lived HDM. The concentration of group 1 allergens in DF and DP extracts were 40 and 36.75 µg/ml, respectively. As shown in Figure 1, protein bands of Der f 15, Der f 18, and Der f/ Der p 1 were present at 97-109 (a), 60 (b), and 25 (c) kDa respectively in SDS PAGE.

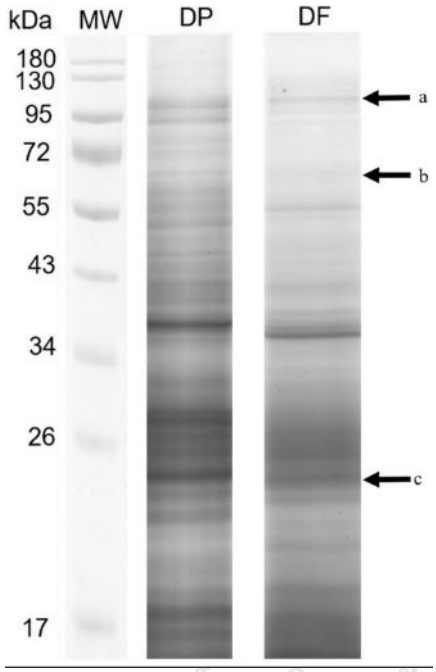

Figure 1 Analysis of protein components in crude extracts of DP and DF.

Protein bands of Der f 15, Der f 18, and Der f/ Der p 1 are located at 97-109 (a), 60 (b) and 25 (c) kDa respectively.

Reproducibility of ELISA

The IgE ELISA for HDM presented an acceptable reproducibility value, and %CV of intra-and inter-assay were 5.4 % and 7.1%, respectively. For HDM specific IgG and its subclasses, the % CV of intra-and inter-assay was about 3.0% and 5.8%, respectively.

DF-specific IgG1 reflects DF allergy in AD

Aged healthy dogs had significantly higher DF specific-IgE levels than both younger healthy dogs and AD (Figure 2a). Considering the groups by IDT outcomes, high levels of DF specific-IgE measured by ELISA were consistent with DF allergy detected by IDT. Nevertheless, elevated DF-specific IgE levels were also found in healthy dogs. The aged healthy dogs had much higher levels of DF-specific total IgG than they did to the other antibodies (Figure 2b), which were not different between healthy and AD or HDM allergen types.

For DF-specific IgG1 levels, the aged healthy dogs showed the highest median levels compared to the other groups (Figure 2c). In the AD group, DF-IgG1 levels were significantly higher in AD that were positive to DF by IDT (+IDT to DF) compared to those that were negative (-IDT to DF). Interestingly, the patterns of DF-IgE (Figure 2a) and DF-IgG1 (Figure 2c) closely corresponded. In contrast, levels of the DF-specific IgG2 subclass were not different between AD, whether they were positive or negative to DF, and healthy dogs still showed a high median level (Figure 2d).

Similarly, the pattern of antibody levels specific to the DP antigen (Figure 2e-h) reassembled the response to DF as well as to the pattern of DF specific detection (Figure 2a-d). The aged healthy dogs showed the highest levels for all detected markers. According to the cut-off values, IgE and IgG1specific to DP could distinguish between healthy dogs and AD that had positive results to DP by IDT, and between AD (+IDT to DP) and AD (-IDT to DP) (Figure 2e and 2g).

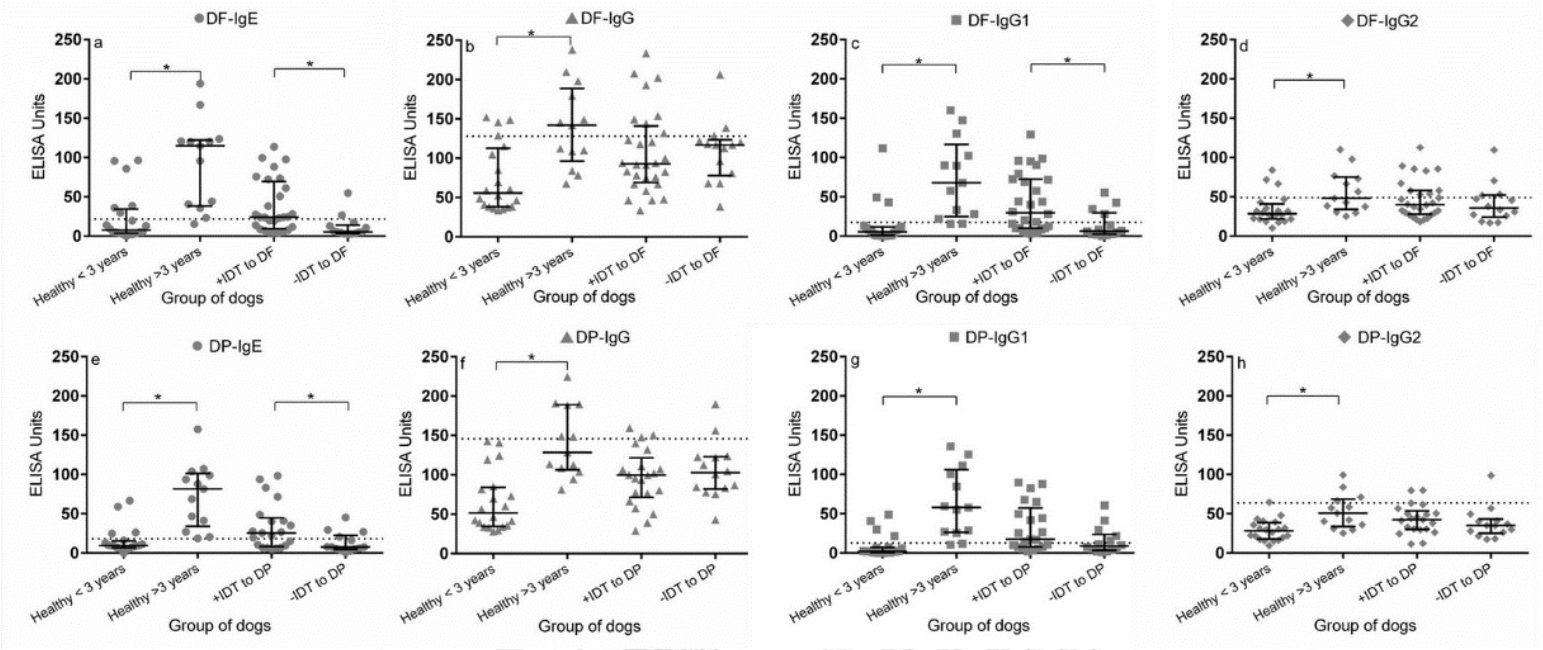

Figure 2 Median and interquartile range of DF- and DP-specific IgE, IgG antibodies and IgG subclasses levels

Aged healthy dogs (> 3 years) had high levels of all antibody types to both species of HDM. In cases of CAD (+IDT or -IDT to DF/DP), high IgE and IgG1 levels were in accordance with IDT results. Dotted line; cut-off levels for each ELISA.

Ratio of DF- or DP-IgG1/IgG enhances the validity of allergic detection in AD

The ratios of DF-IgG1/IgG and IgG2/IgG calculated to reduce the individual confounder of their total specific IgG difference. A low DF-IgG1/IgG ratio and high value for the DF-IgG2/IgG ratio were observed in the young, healthy dogs, whereas the patterns were switched in the aged healthy dogs (Figure 3a). The ratio of DF-IgG1/IgG significantly increased in AD dogs that were DF positive by IDT (AD+IDT to DF), but this not seen for DF-IgG2/IgG. The DP-IgG1/IgG ratio level was higher in the healthy aged group than in the young group (Figure 3b), as with the results from the DF panel. These ratios also showed a significantly higher level in AD (+IDT to DP) than in AD (-IDT to DP), while in certain samples, detection just with DP-IgG1 levels could not separate between AD (+IDT and -IDT to DP) (Figure 3g).

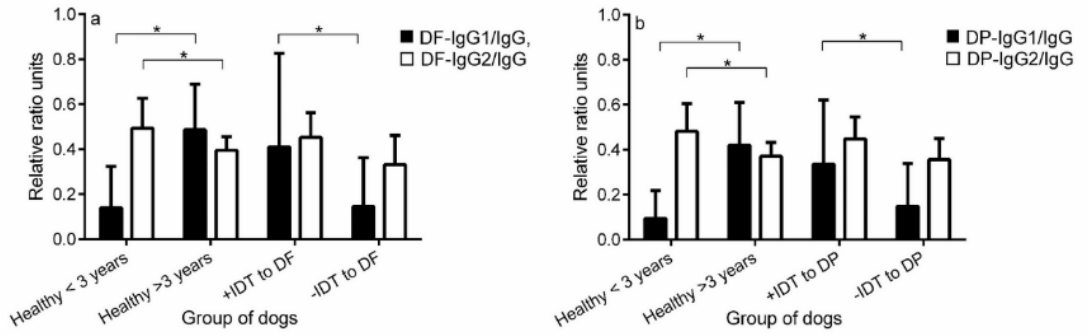

Figure 3 The pattern of specific IgG1 and IgG2 in total IgG against DF and DP in healthy and AD dogs.

A low IgG1/total IgG ratio and high value for the IgG2/total IgG ratio were observed in the young, healthy dogs (< 3years) and in AD with negative IDT to DF/DP, whereas the patterns were switched in the aged healthy dogs (> 3years). AD that were positive in IDT to DF/DP showed a high ratio of both IgG1/total IgG and IgG2/total IgG.

HDM-specific IgG1 has similar sensitivity as HDM-specific IgE

By using our in-house ELISAs, the sensitivity and specificity values achieved for the different antibody classes and antigens varied considerably (Table 3). For confirming DF allergy, either IgE or IgG1 showed better sensitivity than IgG and IgG2, even though IgG1 had lower specificity than IgE (by about 14%). For DP allergy, using DP-specific IgE or IgG1 levels gave better sensitivity than IgG and IgG2. By assessment agreement between IDT and ELISA, both IgE and IgG1 against both HDM antigens showed a similar value of agreement (Table 4), whereas the agreements for IgG or IgG2 against IDT were poor (k = 0.057 and 0.078, respectively).

Table 3 Sensitivity and specificity (%) of in-house ELISAs through different type of specific immunoglobulins

Allergen	Number IDT+	Number IDT -	Sensitivity (%)				Specificity (%)			
			IgE	IgG	IgG1	IgG2	IgE	IgG	IgG1	IgG2
DF	28	14	60.7	28.6	60.7	35.7	85.7	85.7	71.4	71.4
DP	21	14	61.9	14.3	61.9	9.52	71.4	92.9	64.3	92.9

Table 4 Agreement analysis between IDT and in-house ELISA with different type of specific immunoglobulins

Allergen	Agreement between IDT and ELISA (k[a]/strength of agreement[b])			
	IgE	IgG	IgG1	IgG2
DF	0.400/fair	0.108/poor	0.286/fair	0.057/poor
DP	0.318/fair	0.086/poor	0.253/fair	0.078/poor

[a] kappa value; [b] strength of agreement, the interpretation if kappa value =0.00-0.20 (poor); 0.21-0.40 (fair); = 0.41-0.60 (moderate); = 0.61-0.80 (substantial); = 0.81-0.99 (almost perfect)

Discussion

In this study, we explored levels of specific IgE, IgG, IgG1, and IgG2 antibody against local sources of DF and DP extracts in healthy dogs and AD dogs by using in-house ELISAs. IgE levels measured by ELISA were not satisfactory as a screening test without clinical diagnosis because healthy dogs still presented high levels of DF- and DP- IgE (Figure 2a, e). In general, age, sex, and breed have an impact on allergen-specific IgE production (Bjelland et al., 2014). In our study, healthy dogs over three years old had a greater likelihood of presenting high levels of DF- and DP-IgE than did younger dogs, in agreement with findings in previous studies (Lauber et al., 2012; Bjelland et al., 2014). These high levels might be associated with exposure to natural ectoparasites, and thus the age of the patient should be considered as part of the interpretation of results (Racine et al., 1999). In this study, the breed was not included in the risk analysis because of the small numbers of animals available in each breed type. However, Boxers and Rottweilers over 3-year-old were the major population, and these showed quite high levels of intrinsic background IgG and IgE. A previous study was reporting that Boxers and Rottweilers have higher serum IgE levels against HDM than other breeds (Bjelland et al., 2014). This observation confirmed that false-positive results could be found in aged-dogs or some predisposed breeds. Moreover, some AD dogs that were positive to HDM had IgE levels lower than the cut-off. However, the specific IgE related to HDM type was detectable in dogs with atopic dermatitis (Fig 2 a and e), and this response was in accordance with their IDT outcomes.

In this study, both HDM extracts were prepared from the Siriraj Dust Mite Center for Services and Research (SDMC). The HDM extracts especially standard groups 1 allergen extracts (Der f 1 and Der p 1) (Larsen and Dreborg, 2008) are routinely used for diagnosis in patients with allergic rhinitis and allergic field research. The concentration of group 1 allergens in DF and DP extracts was adequate as specified for FDA reference preparations (Filep et al., 2012), and the other major allergenic bands Der f 15 (98-105 kDa) and Der f 18 (60 kDa) were confirmed in our extracts(Thomas et al., 2004). Regarding the crude protein extracts, the cross-reactivity between HDMs and other invertebrates, and the variety of allergenic

components could affect immunoglobulin E levels (Nuttall et al., 2001; Minami et al., 2015; Waldron et al., 2019). The proper protein components of DF and DP extracts related to allergic conditions in dogs should further investigate to improve sensitivity and specificity.

In addition to the IgE level, the finding of high levels of HDM–specific IgG, IgG1 and IgG2 among the healthy aged group confirmed the need for caution about background antibody levels: although the antibodies were specific to HDM the latter still consist of some proteins (Hou et al., 2006). Conversely, the levels of DF- or DP-IgG1 could be used to identify the type of HDM allergen in AD. The sensitivity results for the HDM-IgG1 ELISA were the same as the sensitivity with the IgE ELISA (Table 3). Moreover, the IgG1 ELISA showed the same strength of agreement to IDT, as did IgE (Table 4). These results suggested that the specific IgG1 concentration could identify HDM allergens while using about 100 times less serum than needed for IgE. The use of low volumes of blood is beneficial in terms of animal welfare, and the test can duplicate. Moreover, specific IgG1 seemed to play a role in an immunopathogenesis of atopic dermatitis.

Regarding the IgG subclass volume in dogs, the proportions of IgG1 and IgG2 are approximately equal and deviate during episodes of inflammation; for instance, high IgG2 levels occur in furunculosis, otitis externa and autoimmune hemolytic anemia (Mazza et al., 1994). Varying background amounts of immunoglobulins in each dog could be a major drawback leading to misinterpretation in serological detection. To circumvent this problem, measuring the ratio of IgG1/ total IgG and IgG2/ total IgG should reduce any individual bias. The results of this study confirmed the validity of this approach, with the proportions of both *D. farinae*- and *D. pteronyssinus*-specific IgG1/total IgG reflecting true positives. In contrast, the IgG2/ total IgG levels clearly distinguished the healthy and HDM-IDT negative group from the HDM-IDT positive group, indicating an increase in specificity. A bias towards IgG1 is believed to occur as a response in TH2-mediated disease in mice and dogs, especially in the case of canine leishmaniasis (Jankovic et al., 2006; Lima et al., 2017). On the other hand, a predominance of Th1 cytokines and *Leishmania chagasi*-specific IgG2 was present in vaccinated dogs or asymptomatic dogs exposed to this

protozoan (Barbieri, 2006). Our findings imply that an IgG1 response may reflect a type 1 hypersensitivity triggered by specific HDM allergens, whereas IgG2 levels may represent a predominance of Th1 cytokines. Moreover, the bias towards allergen-specific IgG1/IgG2 could enhance diagnosis for AD and could help to predict the outcome of ASIT.

In this study, IDT was used as the gold standard, but associated technical problems were not fully evaluated in clinical samples. Regarding the agreement between IDT and ELISA, serological detection might serve as a tool for specific allergen identification as confirmed by using HDM allergenic model, but this was not recommended for investigation in healthy dogs. In Thailand, IDTwidely used because the commercial ELISA is costly and time-consuming. Identification of DF allergy through our IgE ELISA had a similar sensitivity to a commercial IgE ELISA (Foster et al., 2003). Interestingly, the DP-IgE ELISA in this study showed better sensitivity than a commercial test. Different sources of DP might influence the results of sensitivity (Nuttall et al., 2001), and locally prepared DP extracts are more likely to be recognized by AD dogs in the same area. The agreement between the results of IDT and IgE ELISA in this study was only fair, even though the same source of HDM used. This phenomenon might be affected by different forms of IgE: IgE on skin has a longer life-span than that in the circulation (Lawrence et al., 2017), so this may explain why some AD (+IDT) had lower levels of HDM-specific IgE and a fair agreement was found between IDT and IgE ELISA results. There is still little evidence in support of an immunopathological role in respect of the immunoglobulin isotype.

There has been a lack of information on the role of IgG1, which may act as another allergic marker, or possibly function as a blocking antibody after an IgE response. In humans with allergic rhinitis, IgG1 production depends on the frequency of protein exposure, and its response becomes dominant after ASIT (Einarsson et al., 1992). The lack of difference in DF-IgG1 levels found between AD and healthy dogs in this study was consistent with the results of a previous study (Lauber et al., 2012), but a relationship between aging and IgE and IgG1 titers was found. Moreover, if the positive or negative results from IDT against DF or DP were considered in the inclusion criterion, this could well enhance the specificity of the results.

Nevertheless, we suggest that levels of IgG1 and IgG2 could be used for longitudinal monitoring of responses in different stages of AD or HDM specific immunotherapy.

Conclusions

Levels of IgG1 and IgE had similar patterns during episodes of HDM allergy in CAD. An in-house ELISA using IgG1 could be used to differentiate between DF or DP allergy, and to monitor the immune response in clinical cases. Unfortunately, this serological approach gave less consensual results in dogs over three years old. The detection platform for HDM allergy identification using IgG1 and ratio of IgG1/IgG was affordable, valid, and only required a very small serum sample.

CHAPTER III

Antibody levels to *Malassezia pachydermatis* and *Staphylococcus pseudintermedius* in atopic dogs and their relation to lesion scores

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Abstract

Background – Elevated IgE levels to *Malassezia* or *Staphylococcus* species in human atopic dermatitis are related to the skin severity index; a similar association has not been reported in atopic dogs.

Objectives – To investigate serum levels of allergen-specific IgE, total specific IgG, and IgG subclasses (IgG1 and IgG2) for *M. pachydermatis* and *S. pseudintermedius* and to correlate them with the severity of dermatitis in dogs.

Animals – Serum samples were collected from dogs categorized by age and disease status. Groups 1 and 2: < three-year-old healthy (n=9) and atopic dogs (n=9), respectively; and Groups 3 and 4: ≥ three-year-old healthy (n=11) and atopic dogs (n=14),respectively.

Methods and materials – Antibody levels were measured by ELISA. The Canine Atopic Dermatitis Lesion Index (CADLI) was analyzed in relation to antibody levels.

Results – Specific IgE and total IgG against *M. pachydermatis* and *S. pseudintermedius* were significantly increased in atopic dogs of all ages. While differences between atopic and healthy dogs, about specific IgG1 and IgG2 levels to each microbe, varied insignificance within age groups. No significant relationships were found between the CADLI and any specific immunoglobulin levels for both microbe types.

Conclusions and clinical importance – In dogs, skin microbes may act as allergens triggering inflammatory responses via IgE- and IgG-depended pathway(s). The affinity of the IgG subclass produced may vary according to antigen type. Specific IgE levels may be related to clinical disease in dogs and not to skin lesion severity.

Introduction

Canine atopic dermatitis (CAD) is an inflammatory skin disease that is associated with allergen-related IgE antibodies (Hensel et al., 2015). Concurrent carriage of both *Malassezia pachydermatis* and *Staphylococcus pseudintermedius* in atopic dogs has been related to specific IgE production (Bexley et al., 2013; Oldenhoff et al., 2014). In human AD, specific IgE levels to *Malassezia* species or *Staphylococcus* species are reported to be related to the skin severity index (Motala et al., 1986; Glatz et al., 2015); such an association has not been reported for cAD. Four IgG subclasses have been described in humans and each subclass is associated with different types of antigens and has different effector functions (Vidarsson et al., 2014). In atopic humans, IgG1 and IgG4 levels are related to allergen sensitization with high specific IgE levels, simultaneously (Einarsson et al., 1992; Hales et al., 2006), while the IgG2 response is restricted to polysaccharide antigens (Mikolajczyk et al., 2004). In dogs, four subclasses of IgG have been reported. One study reported elevated IgG2 levels with decreasing IgG1 levels in sera from dogs with a range of immune-mediated or inflammatory diseases (Mazza et al., 1994). Another study reported on allergen-specific IgG subclass and suggested that the response depended on the nature of the allergen and that *Dermatophagoides farinae*-IgG1 levels were rarely detected in atopic dogs (Day et al., 1996). A further study reported a high level of IgG1 against *D. farinae* in atopic dogs and that it increased after immunotherapy (Lauber et al., 2012). Due to a lack of availability of commercial anti-dog IgG subclass antibodies only IgG1 and IgG2 have been studied (Bird et al., 2011; Agallou et al., 2016). The aim of this study was to investigate levels of allergen-specific IgE, IgG1, and IgG2 directed against *M. pachydermatis* and *S. pseudintermedius*, with total IgG levels, then correlate them with lesion severity in cAD as defined by the Canine Atopic Dermatitis Lesion Index (CADLI) (Plant et al., 2012).

Material and methods

Study population

The animal use protocol was approved under the Institutional Animal Care and Use Committee of Chulalongkorn University Animal Care and Use Protocol (CU-ACUP); Animal Use Protocol No. 1731046. Four groups of dogs were enrolled; healthy dogs were categorized into Groups 1 (< three years; n=9) and 3 (≥ three years; n=11), respectively. Atopic dogs were categorized into Groups 2 (< three years; n=9) and 4 (≥ three years; n=14), respectively (Table S1). Atopic dogs were defined according to published guidelines (Hensel et al., 2015). All atopic dogs had suffered from recurrent infections with *M. pachydermatis* and/or *S. pseudintermedius*, based on cytological findings (Mendelsohn et al., 2006; Hillier et al., 2014). Cutaneous adverse reaction to food was ruled out by elimination diet for a minimum of eight weeks. After successful treatment with antimicrobial therapy and two weeks withdrawal of anti-inflammatory, anti-pruritic and antimicrobial drugs (none of the dogs had treated with steroids) (Olivry et al., 2013), intradermal testing (IDT) with environmental allergens was performed according to previous reports (Chanthick et al., 2008). All atopic dogs had at least one positive intradermal reaction to an allergen. The severity of the skin lesions was determined based on the CADLI (Plant et al., 2014). The same investigator evaluated all subjects.

Preparation of microbe extracts

Malassezia pachydermatis strain 4180 and *S. pseudintermedius* strain AK30 were used as representative strains (Yurayart et al., 2011; Perreten et al., 2013). AK30 lacked the *spa* gene, so protein A could not be produced and interfere with the IgG-binding assessments. The identities of both microbes were confirmed based on morphological and physiological characteristics according to routine laboratory methods (Yurayart et al., 2011; Perreten et al., 2013). Colonies of *M. pachydermatis* were sub-cultured onto Sabouraud's dextrose agar and incubated at 35 ^{o}C to obtain enough organisms representing all stages of the growth phase (Kim et al., 2010). In parallel, colonies of *S. pseudintermedius* were subcultured onto Trypticase Soy Agar containing 5% sheep blood and incubated at 37^{o} C. The colonies of *M.*

pachydermatis, and *S. pseudintermedius* were individually harvested at 72 h and 24 h post-incubation, respectively, and were suspended in phosphate-buffered saline (PBS; pH 7.4) at 4° C. After washing three times with PBS, the pellets were re-suspended with an equal volume of PBS, followed by adding an equal volume of SiLibeads Type S (SiLibeads; Warmensteinach, Germany). For extraction, the microbes were subjected to 10 cycles of vigorous shaking for 1 min using a Vortex Genie® 2 with replacement tube inserts (Scientific Industries; New York, NY, USA) and kept on ice for 2 min per cycles. After elution and centrifugation at 6000 x ***g*** for 5 min, the supernatant was filtered with Minisart filters of pore size 0.45 µm (Sartorius Stedim Biotech GmbH; Goettingen, Germany) and the protein concentration measured using a Protein Assay Kit II (Bio-Rad; Irvine, CA, USA). The filtrate was kept in aliquots at -20 °C until used.

Specific IgE ELISA

Pooled serum samples from five atopic dogs and a pool from five young healthy dogs were used in checker-board titrations. Costar 3590 EIA plates (Corning; New York, NY, USA) were coated with microbial extracts diluted in 0.2 M BupH™ Carbonate-Bicarbonate Buffer Packs, pH 9.4 (Thermo Fisher Scientific; Rockford, IL, USA) with two micrograms of total protein used per well. After washing with PBS containing 0.05% tween 20 (PBST) plates were blocked with blocking buffer (PBST containing 1% bovine serum albumin BSA). All serum samples, antisera, and conjugate were diluted in blocking buffer before being added into the wells. First, diluted serum samples (1:5) were added into wells. Next, diluted monoclonal mouse anti-dog IgE (1:2,000; clone E6-2A1, AbD Serotec; Kidlington, Oxford, England) was added, followed by diluted phosphatase conjugate (1:2,000; goat anti-mouse IgG & Human ads-AP, Southern Biotech; Birmingham, AL, USA). Finally, the Sensitest Canine IgE substrate (Avacta Animal Health; Wetherby, UK) was used for color development. The optical density (OD) at 450 nm was measured using an ELx808 ultra reader (Bio-Tek Instruments Inc., Winooski, VT, USA). After subtracting the OD of the blank control, the OD values were quantified in ELISA units. The pooled serum samples

from atopic dogs were used for calibration and were arbitrarily set at 100 units (U) as previously described (Iniesta et al., 2005). All samples were run in duplicate.

Total specific IgG and IgG subclasses ELISA

The IgG and IgG subclass assays were developed as described for the IgE ELISA, with some modifications. The optimal serum dilution for IgG and IgG subclass assays was 1:2,000. Diluted polyclonal sheep anti-dog IgG conjugated-Horseradish Peroxidase (HRP) (AAI32P, AbD serotec Ltd); polyclonal goat anti-dog IgG1-HRP (AHP947P, AbD serotec Ltd) and polyclonal sheep anti-dog IgG2-HRP (AHP948P, AbD serotec Ltd) in blocking buffer (1:4,000) were applied to detect allergen-specific total IgG, IgG1 and IgG2, respectively. Regarding the commercial anti-dog IgG1 and anti-dog IgG2 used for antibody detection, the manufacturer claimed there was no cross-reactivity to other IgG subclasses and these antibodies were selected for use in determining the bias of IgG subclasses in previous studies (Bird et al., 2011; Agallou et al., 2016). Peroxidase substrate (ABTS® peroxidase substrate, KPL Inc.; Gaithersburg, MD, USA) was added for color development. The OD of the blank control was subtracted from the OD and quantified in ELISA units.

Data and statistical analyses

GraphPad Prism6 (GraphPad Software Incorporated; San Diego, CA, USA) was used for analysis of results. Age distributions between healthy and atopic dogs in the same category were compared by the Mann-Whitney U test. Median levels of antibody were compared by the Kruskal-Wallis ANOVA Ranks with a Dunn's post hoc test. Pearson correlation (r) was used to assess relationships between CADLI scores and antibody levels in atopic dogs.

Results

Characteristics of dog samples

There were various breeds of dogs in each group (Table 5). The median ages of dogs in Groups 1 and 2 were 1.67 and 1.58 years, respectively. In Groups 3 and 4,

the median ages were 3.85 and 4.71 years, respectively; a significant difference in age between the younger and older groups was shown ($P < 0.05$; Mann-Whitney U test).

Reproducibility

For all *M. pachydermatis* and *S. pseudintermedius* ELISAs, the mean intra- and inter-assay variations did not exceed 4.88% and 10.15%, respectively.

Antibody levels to skin microbial extracts

Immunoglobulin levels for the yeast and bacteria extracts are reported in Figure 4. Atopic dogs (Groups 2 and 4) showed significantly higher antibody levels to microbial extracts compared with healthy dogs (Groups 1 and 3). Apart from IgG1 levels to *M. pachydermatis* in older dogs and IgG2 levels to *S. pseudintermedius* in younger dogs, all differences between healthy and atopic dogs were significant within the same age category ($P < 0.05$; ANOVA, Figure 4). There were no statistical differences within either the healthy or atopic dogs according to age (i.e., Group 1 versus 3 and Group 2 versus 4; Figure 4).

CADLI scores (see Figure 5)

The median of CALDI scores in young atopic dogs was nine (range 0 to 40) while the median scores in aged atopic dogs was 18 (range four to 30)($P = 0.049$; Mann-Whitney U test). Two young dogs were classified atopic and had CADLI scores of "0" on the day of sampling when the only residual clinical sign was pruritus. The CADLI scores in atopic dogs were not correlated to any of the types of specific antibodies for either *M. pachydermatis* (r = -0.053 to 0.283, $P > 0.05$) or *S. pseudintermedius* (r = -0.385 to -0.035, $P > 0.05$). Overall, the IgE level to *S. pseudintermedius* had the most negative correlation to CADLI scores (r = -0.35, Figure 5E) even though no significant difference was found.

Table 5 Demographic details of dogs in each group

Group	Number of dogs	Sex (Male: Female)	Median age and range (Years)	Breed
1	9	5:4	1.67 (1.0-2.5)	Chihuahua (1); Labrador retriever (2); Shiba inu (1); Crossbreed (5)
2	9	4:5	1.58 (1.17-2.58)	American pitbull (1); Beagle (2); Bulldog (1); French bulldog (1); Labrador retriever (1); Pomeranian (1); West highland (1); Crossbreed (1)
3	11	7:4	3.85 (3.5-4.2)	Boxer (6); Rottweiler (3); Labrador retriever (1); Crossbreed (1)
4	14	10:4	4.71 (3.5-6.0)	Bangkraw (1); Beagle (1); Chihuahua (1); French bulldog (2); Golden retriever (2); Pug (1); Pomeranian (2); Poodle (1); Shitzu (1); Shiba inu (1); Crossbreed (1)

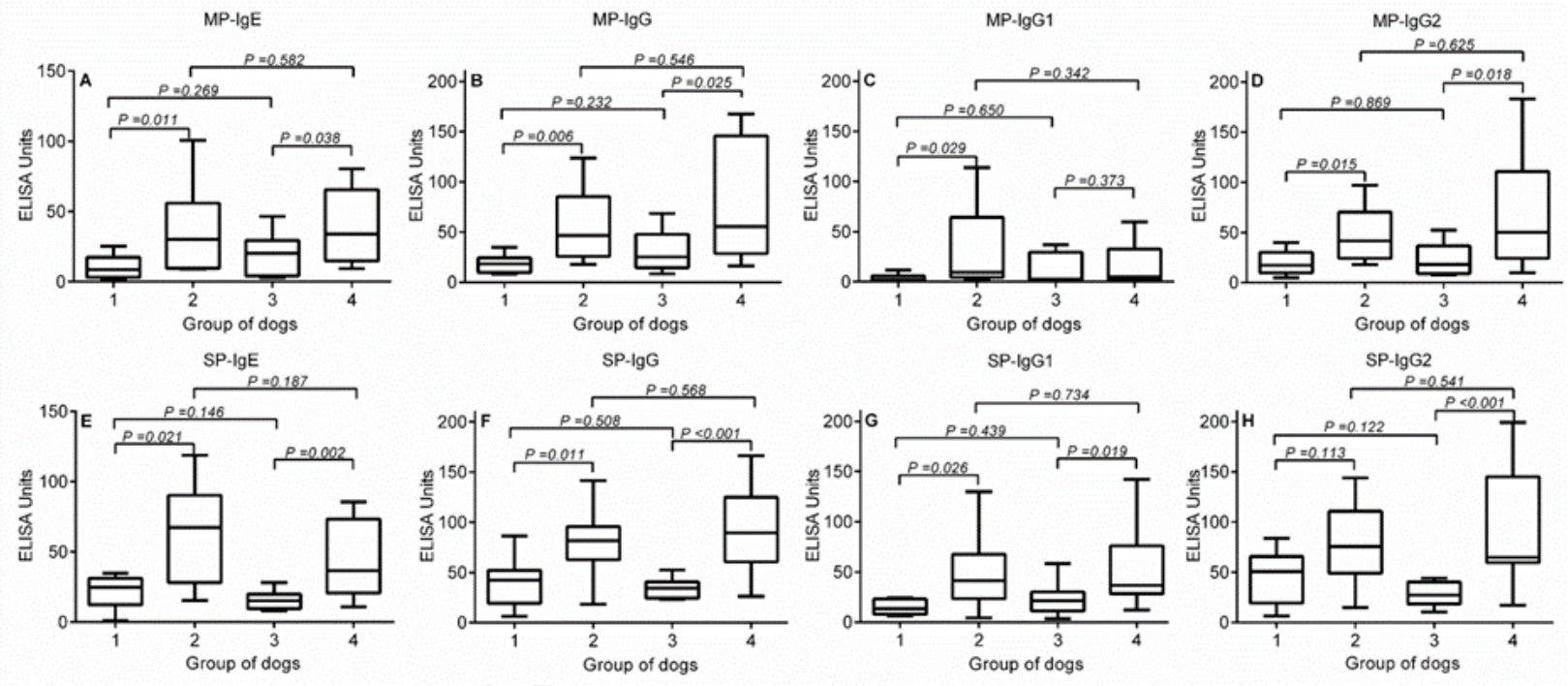

Figure 4 Median levels of specific antibody to *Malassezia pachydermatis* (MP) and *Staphylococcus pseudintermedius* (SP) in each group of dogs.

Group 1 healthy dogs (< three years old), group 2 atopic dogs (< three years old), group 3 healthy dogs (≥ three years old), and group 4 atopic dog (≥ three years old). The median levels were compared by Kruskal-Wallis ANOVA Ranks, with significance accepted if $P < 0.05$.

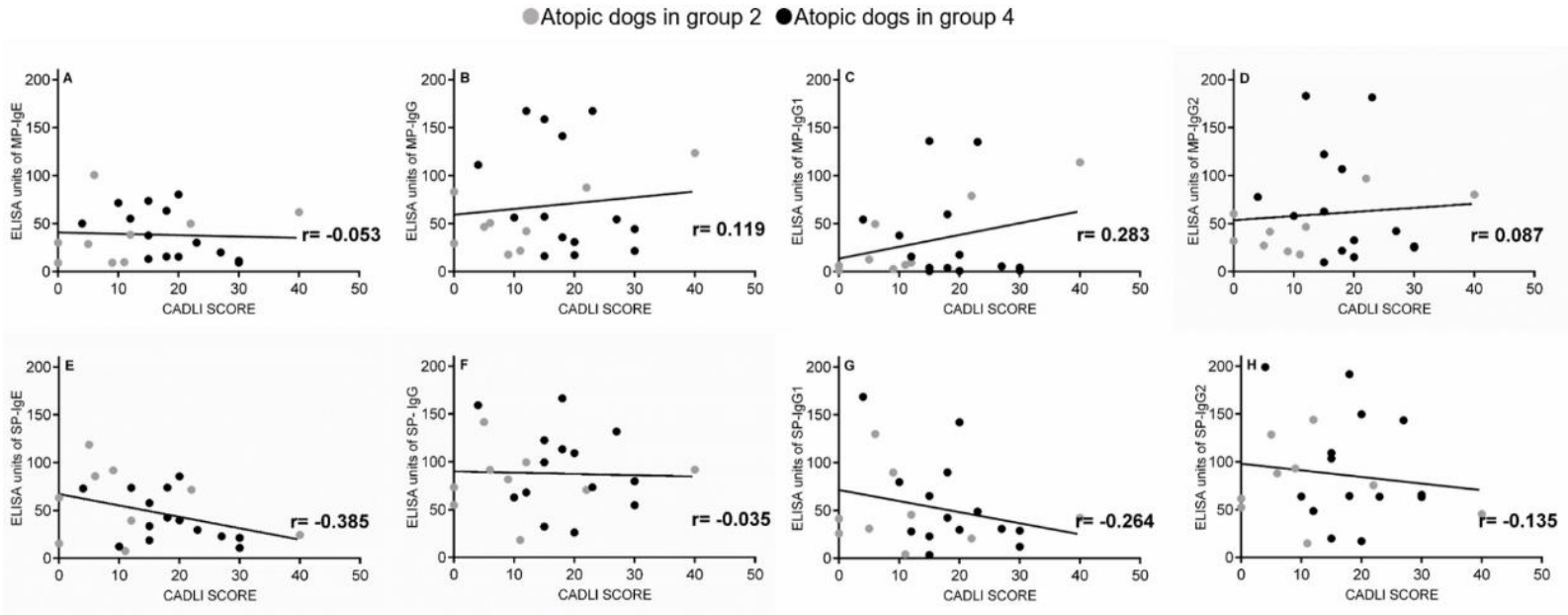

Figure 5 The Pearson correlation (r) of CADLI scores and antibody levels to *Malassezia pachydermatis* (MP) and *Staphylococcus pseudintermedius* (SP) in atopic dogs (groups 2 and 4). *Significantly different with $P < 0.05$.

Discussion

This study found that both *M. pachydermatis* and *S. pseudintermedius* are capable of eliciting humoral immune responses (IgE, IgG, IgG1, and IgG2) in atopic dogs. This humoral response may be associated with the pathogenesis of CAD (Bexley et al., 2013; Oldenhoff et al., 2014). The age of the dog was included as a factor for the evaluation of antibody levels because a previous study showed that allergen-specific IgE levels increased with age in dogs with CAD (Bjelland et al., 2014). However, in our study, age was not associated with a difference in specific IgE or IgG antibody levels to either microorganism. Overall, the increased antibody levels in atopic dogs may be a consequence of dermatitis in atopic dogs. However, the lack of standard recommendations for preparing skin microbial extracts is a limitation to confirming hypersensitivity reactions that arise from these microbes. Following a previous study (Kim et al., 2010), we harvested microbial colonies from subcultured plates for preparing microbial extracts. We assumed that this would include all proteins from any growth phases, even though further quantitative immunoelectrophoretic techniques are required to find standardized allergens.

In humans and a murine model, the IgG subclass response depends both on the antigen components and on immune deviation leading to pathogen clearance through T-cell responses (Ashbee et al., 1994; Ashbee et al., 1997; Jung et al., 2012; Selle et al., 2016). *Malassezia pachydermatis* organisms contain large polysaccharides, and these may drive a strong IgG2 response (Ashbee et al., 1994). This is consistent with our results for anti-*M. pachydermatis* IgG2, which was significantly higher in atopic dogs than in healthy controls for both age groups. However, we found that anti-*M. pachydermatis* IgG1 was not increased in older atopic dogs compared to healthy controls. This is potentially consistent with the report that human patients with pityriasis Versicolor or seborrhoeic dermatitis have an IgG1 response to *M. furfur* serovar A that is similar to healthy controls (Ashbee et al., 1994). Differences in IgG subclass responses to *Malassezia spp*. probably are dependent on their serovars and *Malassezia*-associated dermatoses (Ashbee et al., 1994).

In the case of *Staphylococcus* species, whole cells or their components, including peptidoglycan and teichoic acid, elicit both IgG1 and IgG2 responses (Jung et al., 2012; Selle et al., 2016). In human patients with acne vulgaris, specific IgG1, and IgG2 to *S. epidermidis* were higher than in controls (Ashbee et al., 1997). This is in concordance with our findings for IgG subclasses specific to *S. pseudintermedius*. So, the types of antigens involved may influence IgG subclass production in dogs, as previously suggested (Day et al., 1996). In vaccination models or when classifying the severity of disease, up-regulation of IgG1 or IgG2 often is influenced by Th2 or Th1 responses, respectively (Bird et al., 2011; Agallou et al., 2016; Lima et al., 2017). However, IgG subclass responses with T-helper cell regulation and the role of each IgG subclass remain ambiguous in dogs. In a study of canine IgG subclass function the synthesis of IgG A subclass, that was specific to commercial anti-dog IgG1 antibody, was found to have a similar function as human IgG2, while the synthesis of canine IgG B subclass, specific to commercial anti-dog IgG2, had a similar function to human IgG1 (Bergeron et al., 2014). These findings lead to still unanswered questions about the specificity of the commercial antibodies used in canine IgG subclass detection and differences in translation of IgG subclass responses between humans and dogs. The claimed specificities of the commercial antibodies used in the current study were based on the results of previous studies (Bird et al., 2011; Agallou et al., 2016). T cell regulation of immunoglobulin class expression is known to differ between species of mammals. In a murine model, IgG1 production dominated following Th2 regulation (Mosmann and Coffman, 1989), while in humans, the IgG1 response was associated with Th1-mediated cytokine production (Kawasaki et al., 2004). The pathogenesis of CAD has shown to be associated with Th2 responses, although Th1 regulation was involved in chronic lesions (Nuttall et al., 2002). The study reported here has shown high IgG2 levels to antigens from both microbes in atopic dogs, even though an elevated IgG2 level is more consistent with non-atopic inflammatory diseases (Mazza et al., 1994). This result implies the involvement of a Th1 response to commensal microbes in cAD, but rigorous investigation of cytokines still needed to help confirm this. In humans, immunity driven by T-helper 1 cytokines required for protection from *Staphylococcus* spp. infection (Brown et al., 2015), while these

cytokines promote inflammation in *Malassezia* spp. infections (Jacob et al., 2003). Given the complexity of immune responses shaped by different microbes, clarification is still needed regarding the bias of IgG subclass-associated T helper cell polarization in dogs.

In our study, the older dogs with cAD (Group 4) had a higher median CADLI score than the younger atopic dogs (Group 2). However, no correlation found between skin lesion severity and any of the specific antibodies to commensal microbes; this may reflect the complexity of the immune network in cAD, which involves many other factors such as keratinocyte-derived mediators, neuroimmune mediators, and inflammatory cytokines which contribute to the itch-scratch cycle and promote skin inflammation. Based on our results, the detection of specific IgE to skin microbes should not be recommended as a biomarker for CAD or a gauge of its severity.

Conclusion

The increased levels of IgE specific to *M. pachydermatis* and *S. pseudintermedius* in dogs with cAD suggest a role for these organisms as allergens but do not correlate with skin lesion severity as assessed by CADLI. These skin microbes also elicited different patterns of specific IgG subclass responses. The bias in IgG subclass relating to T helper cell polarization requires further investigation in dogs.

CHAPTER IV

Allergen components of *Dermatophagoides farinae* that are related to atopic dermatitis in dogs

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Abstract

Background: *Dermatophagoides farinae* (DF) is a common causative allergen of atopic dermatitis (AD) in dogs. However, data on the relevant components of DF are scarce and require exploration.

Objective: We aimed to provide data on the relevant allergen and cross-reactive components of DF for further application in component resolve diagnosis and immunotherapy for atopic dogs.

Methods: We explored the allergen component profiles of DF through two-dimensional gel electrophoresis and IgE immunoblotting in five healthy dogs, nine DF-allergic atopic dogs, and seven non-DF-allergic atopic dogs. The relevant allergen components and cross-reactive components in both groups of atopic dogs were analyzed by liquid chromatography coupled with tandem mass spectrometry.

Results: Majority of DF-allergic atopic dogs were sensitized to the Alt a 10 allergen, elongation factor 1-alpha (EF1-**α**), gelsolin-like allergen Der f 16, Der f 28, and an allergen similar to mite group 2, whereas Der f 3, Der f 10, Der f 20, and Der f 32 were recognized as minor allergens. Alpha-enolase, serine protease, arginine kinase, and a few hypothetical proteins were found as dominant cross-reactive components between both groups of atopic dogs. Surprisingly, Der f 15 (chitinase) was not a major component in atopic dogs but it was exposed as a minor cross-reactive component.

Conclusions & Clinical Relevance: New major allergen components of DF related to AD in dogs were demonstrated, including Alt a 10, EF1-**α**, gelsolin-like Der f 16, Der f 28, and allergen with similar to mite group 2. These components had the potential to be a set of candidate proteins to confirm a DF allergy. While some of the cross-reactive components, like alpha-enolase, serine protease, and arginine kinase, could the benefit for immunotherapy and further study about pathogenesis in atopic dogs.

Keywords: allergen components, atopic dermatitis, dog, house dust mite

Introduction

Atopic dermatitis (AD) is a genetically predisposed inflammatory and pruritic skin disease in dogs, which is associated with multiple factors, such as skin barrier disruption, imbalance of immune response, imbalance of microbial colonization, and IgE production to environmental allergens (Hensel et al., 2015). From the prevalence of allergy testing in different regions, including Thailand, house dust mite (HDM) acts as a dominant causative allergen in dogs with AD, especially the HDM *Dermatophogoides farinae* (DF) (Masuda et al., 2000; Foster et al., 2003; Chanthick et al., 2008). Up to now, allergy testing or allergen-specific immunotherapy (ASIT) in dogs still uses crude extracts, which contain a varied concentration of allergen and non-allergenic components (Hillier and DeBoer, 2001). Similar to a commercial preparation for DF extracts in humans, the Der f 1 allergen has been used as a standardized allergen in dogs (Larsen and Dreborg, 2008). However, the Der f 1 allergen is not a major allergen in atopic dogs, so this allergen is not suitable for allergen standardization in batch-to-batch control (Noli et al., 1996; Nuttall et al., 2006).

Accordingly, using crude extracts could affect the accuracy of allergy testing and probably reduce the success rate of ASIT (Nuttall et al., 2001). Based on using a recombinant allergen in a component-resolved diagnostics and immunotherapy (CRD and CRIT) in humans, these approaches could improve the accuracy of diagnosis and ability to achieve ASIT (Pittner et al., 2004; Nicolaou et al., 2011). From the database in Allergen Nomenclature, thirty-five allergen components of DF-related allergic diseases in humans have been defined. Of these, Der f 1, Der f 2, Der f 10, Der f 11, Der f 14, Der f 15, Der f 16, Der f 18, Der f 25, Der f 29, and Der f 30 have been reported as the major allergens in allergic patients. Knowledge of the types and prevalence of allergens can enhance the knowledge about the pathogenesis-related to the biological activity of the allergen, which can then apply for designing therapeutic agents, such as specific drugs or monoclonal antibodies (mAbs). In contrast to humans, the prevalence of the relevant components of DF is still poorly defined and classified in atopic dogs. Only a few of the allergen components of DF

have been mentioned in atopic dogs. Previous studies found that more than 50% of atopic dogs strongly recognized high molecular weight proteins, like chitinase (Der f 15) and chitin-binding protein (Der f 18) (McCall et al., 2001; Weber et al., 2003; Moya et al., 2016). While low molecular weight proteins, such as Der f 1, were less important in dogs, and the prevalence of IgE reactivity to Der f 2 depended on the geographic region (Nuttall et al., 2001; Yamashita et al., 2002; Moya et al., 2016). Unfortunately, other allergen components of DF have not been thoroughly explored and unidentified in dogs.

Thus, the aim of this study was to explore and identify allergen components of DF by using two-dimensional electrophoresis (2DE) and doing proteomic analysis of the IgE-reactive components of DF related to allergic conditions in atopic dogs.

Materials and methods

Serum samples

This study protocol was approved by the Institution Animal Care and Use Committee (IACUC) of Chulalongkorn University Animal Care and Use Protocol (CU-ACUP); Animal Use Protocol No. 1731046. Serum samples were collected from five healthy dogs and 16 dogs with AD from the Veterinary Teaching Hospital of Kasetsart University and Small Animal Teaching Hospital of Chulalongkorn University. All the healthy dogs, which had a different age range, were enrolled with the inclusion criteria determined by a normal physical examination, of not suffering from any diseases, and no previous history or current clinical signs of skin problems. The atopic dogs were recruited following the guideline of clinical diagnosis of AD, and an intradermal skin test (IDT) with 47 allergen extracts was performed as previously reported (Chanthick et al., 2008; Hensel et al., 2015). Anti-inflammatory and anti-pruritic drugs were withdrawn at least two weeks before the IDT. According to the IDT protocol, local sources of DF extracts and *D. pteronyssinus* were obtained from Siriraj House Dust Mite Center (SDMC) at Mahidol University (Choopong et al., 2016). Allergen extracts were diluted as recommendations by the manufacturer's

instructions and a previous report (Chanthick et al., 2008). All serum samples were collected and kept at -20 °C until used.

Preparation of *DF* extracts

Two grams of purified DF (batch no. DF-SDMC 080158) with > 99% purity from SDMC were washed three times and resuspended in 8 mL of 0.01 M phosphate-buffered saline pH 7.4 (PBS). Then the suspension was homogenized in an Omni Sonic Ruptor 4000 (Omni International, Kennesaw, GA, USA) at 35% amplitude with 0.5 cycles (15 min, on ice) and the supernatant was collected after centrifugation at 12,000 x *g*, 4 °C, for 5 min (Sorvall Legend X1R, Thermo Scientific, Waltham, MA, USA). The protein concentration in the supernatant was measured using the Bradford assay and was kept in aliquots at -20 °C until used.

Antigen analysis by 2DE and mass spectrometry

The DF extract was cleaned to eliminate non-protein contaminants using a 2D-clean up kit (GE Healthcare, Pittsburgh, PA, USA), and then the protein concentration was determined using a 2D-Quant kit (GE Healthcare, Pittsburgh, PA, USA). Then 100 µg of DF extract was added to the DeStreak Rehydration Solution containing 0.5% (w/v) IPG buffer pH 3–11 nonlinear (NL; GE Healthcare, Pittsburgh, PA, USA) and transferred to a strip holder. An IPG strip (Immobilien DryStrip pH 3–10NL, 7 cm; GE Healthcare, Pittsburgh, PA, USA) was placed into the strip holder, with the gel side down and then covered in the rehydration solution cover fluid at 25 °C overnight before running the 2DE. The running protocol for the first-dimensional isoelectric focusing electrophoresis was an initial 0.2 kV/h for 30 min, then a gradient step at 0.3 kV/h for 30 min, 4.5 kV/h for 90 min, and held for 30 min. The focused IPG strip was then transferred to reduction buffer [50 mM Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) bromophenol blue, and 1% (w/v) dithiothreitol (DTT)] at 25 °C for 15 min and then placed into alkylation buffer [50 mM Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) bromophenol blue, and 2.5% (w/v) iodoacetamide] for 15 min. The second

dimension was then performed by 12% (w/v) polyacrylamide gel electrophoresis, and the resolved proteins spots were revealed by silver staining.

IgE immunoblotting

The 2DE separated DF extract was blotted onto a nitrocellulose membrane (0.45 µm pore, Thermo Scientific, Waltham, MA, USA), and the pattern of protein spots was stained by Ponceau S staining. The membrane was washed with PBST washing buffer [PBS containing 0.05% (v/v) tween 20) and then soaked in blocking buffer [5% (w/v) skimmed milk in PBST] for 1 h. After washing, the membrane was incubated with individual serum samples from atopic dogs (diluted 1:5 in blocking buffer) at 4 °C for overnight. In the case of healthy dogs, five sera samples were pooled before use. The membrane was washed again and incubated for 2 h with diluted 1:2,000 mAb mouse anti-dog IgE (clone E6-2A1; Bio-rad, Watford, Hertfordshire, UK) in blocking buffer. The membrane was washed and incubated with goat anti-mouse IgG conjugated to horseradish peroxidase (HRP) diluted 1:4,000 in blocking buffer (Southern Biotech, Birmingham, AL, USA) for 1 h. After the final wash, the membrane was reacted with enhanced chemiluminescent HRP substrate (SuperSignal West Pico, Thermo Scientific, Rockford, USA) and visualized using the gel doc system (G: BOX Chemi, Syngene, Cambridge, UK).

Protein identification by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS)

For protein spot selection, the common spots were culled out and the unique spots, which were visualized among the atopic dog's membranes, were selected for identification using LC-MS/MS. The silver-stained protein spots, corresponding to those visualized spots on the membranes, were excised from the gel and destained by incubating for 30 min in 30 mM potassium ferricyanide [$K_3Fe(CN)_6$; Merck, USA] and 100 mM sodium thiosulfate (Merck USA) solution. For digestion, gel pieces were reduced with 4 mM dithiothreitol (DTT) at 60 °C for 15 min and were then alkylated by 250 mM iodoacetamide at room temperature for 30 min. The gel pieces were then dehydrated with 100% (v/v) acetonitrile (ACN; Thermo

Scientific, Waltham, MA, USA) and digested by trypsin (T6567; Sigma-Aldrich, New Road Gillingham, Dorset, UK) overnight at 37 °C. The peptides were extracted by adding 100% (v/v) ACN and incubated for 20 min. The samples were stored at −20 °C until analyzed. All protein samples were analyzed using an Ultimate® 3000 Nano-LC system (Thermo Scientific, Waltham, MA, USA) coupled with a microTOF-Q II (Bruker Daltonics, Bremen, Germany), both controlled by HyStar™ version 3.2 (Bruker, Bremen, Germany) (Sookrung et al., 2014). For selection, MS and MS/MS spectra covered a mass range of m/z 400–2000 and m/z 50–1500, respectively. For identification, the raw data format (.d) files were processed and converted to mascot generic files (.mgf) using the Compass DataAnalysis™ software version 3.4 (Bruker, Bremen, Germany) and screened for matched against the *Dermatophagoides* database and insect database using the Mascot Daemon software (Matrix Science, Boston, MA, USA). Only proteins identified with the highest scores were reported.

Results

Characteristics of the study samples

Regarding the dog's history and IDT results, all 16 dogs were classified into nine atopic dogs with positive IDT to DF (AD +IDT to DF) and other atopic dogs with negative IDT to DF (AD -IDT to DF). Breed, sex, and age were varied in each group of dogs, as shown in Table 6.

Table 6 Characteristics of the serum samples from dogs used in this study

Subject	Age (y)	Sex	Breed	Clinical diagnosis	IDT to DF	Positive IDT to other allergens
H1	2	F	Rottweiler	Non-AD	N/A	N/A
H2	4	F	Siberian	Non-AD	N/A	N/A
H3	5	M	Beagle	Non-AD	N/A	N/A
H4	3	F	Rottweiler	Non-AD	N/A	N/A
H5	3	F	Rottweiler	Non-AD	N/A	N/A
AD1	13	M	Poodle	AD	+	-
AD2	11	F	Cross breed	AD	+	*D. pteronyssinus*
AD3	1	M	West highland white terrier	AD	+	*D. pteronyssinus*
AD4	1	F	French bulldog	AD	+	*D. pteronyssinus*
AD5	15	M	Shitzu	AD	+	*D. pteronyssinus*
AD6	4	M	Beagle	AD	+	*Periplaneta americana*
AD7	3	M	Beagle	AD	+	*D. pteronyssinus*
AD8	3	F	Beagle	AD	+	*D. pteronyssinus*
AD9	8	F	Jack russel	AD	+	*D. pteronyssinus*
AD10	15	M	Cross breed	AD	-	*Aspergillus fumigatus*
AD11	10	M	Labrador Retriever	AD	-	*Periplaneta Americana*

Subject	Age (y)	Sex	Breed	Clinical diagnosis	IDT to DF	Positive IDT to other allergens
AD12	1	M	Thai Ridgeback	AD	-	*Culex pipiens*
AD13	6	F	Crossbreed	AD	-	*Periplaneta americana*
AD14	2	M	West highland white terrier	AD	-	*Mixed ants*
AD15	7	F	Poodle	AD	-	*Culex pipiens*
AD16	5	M	Pug	AD	-	*Periplaneta americana*

AD, Atopic dermatitis; IDT, intradermal skin test; DF; *Dermatophagoides farinae*

The 2DE pattern of the DF extracts and DF-specific IgE spots in dogs

A representative 2DE pattern of the DF extracts shown in Figure 6 after silver staining. From the possible spots on the 2DE pattern of DF extracts, only 43 were determined to be DF-specific IgE spots from the blotting results. In each group of dogs and individual dogs, the results of the DF-specific IgE spots were different in terms of the number and pattern of recognition, as shown in Figure 7. However, nine common DF-specific IgE spots were found among all groups of dogs, and the sizes of these spots were above 30 kDa. In total, 34 DF-specific IgE spots recognized by serum samples from both groups of atopic dogs were selected for LC-MS/MS analysis. After identification, 14 specific IgE spots to DF were found to be cross-reactive components between the AD +IDT to DF and AD -IDT to DF groups, as shown in Figure 8. For the unique reactive spots in each group of atopic dogs, 10 specific IgE components to DF were identified in the AD +IDT to DF group, while only one specific component found in the AD -IDT to DF group.

The type of allergen/proteins, frequency, and percentage of allergenicity are presented in Table 7, while the accession number of the sequence and biological function of each respective protein spot is summarized in Table 8. At least half of the dogs in each group of atopic dogs reacted to alpha-enolase, serine protease, arginine kinase, and two different types of hypothetical proteins (spots no.6 and 22). In the AD +IDT to DF group, the Alt a 10 allergen, elongation factor 1-alpha (EFI-**α**), gelsolin-like allergen Der f 16, Der f 28, and an allergen similar to mite group 2, were the dominant components for only this group. Whereas, Der f 28 was the major component reacting to serum samples from only the AD +IDT to DF group only. Whereas, other components, such as Der p II, Der f 3, Der f 10, Der f 20, Der f 32, three hypothetical proteins (spot no.11, 21, and 24), and an unnamed protein product, were the minor proteins that recognized in only the AD +IDT to DF group. In the AD -IDT to DF group, all reactive components of this group were also found in the AD +IDT to DF group, except for the hypothetical protein spot no. 30, glyceraldehyde 3-phosphate dehydrogenase (G3PDH), which was a major reactive protein in serum samples from this group only.

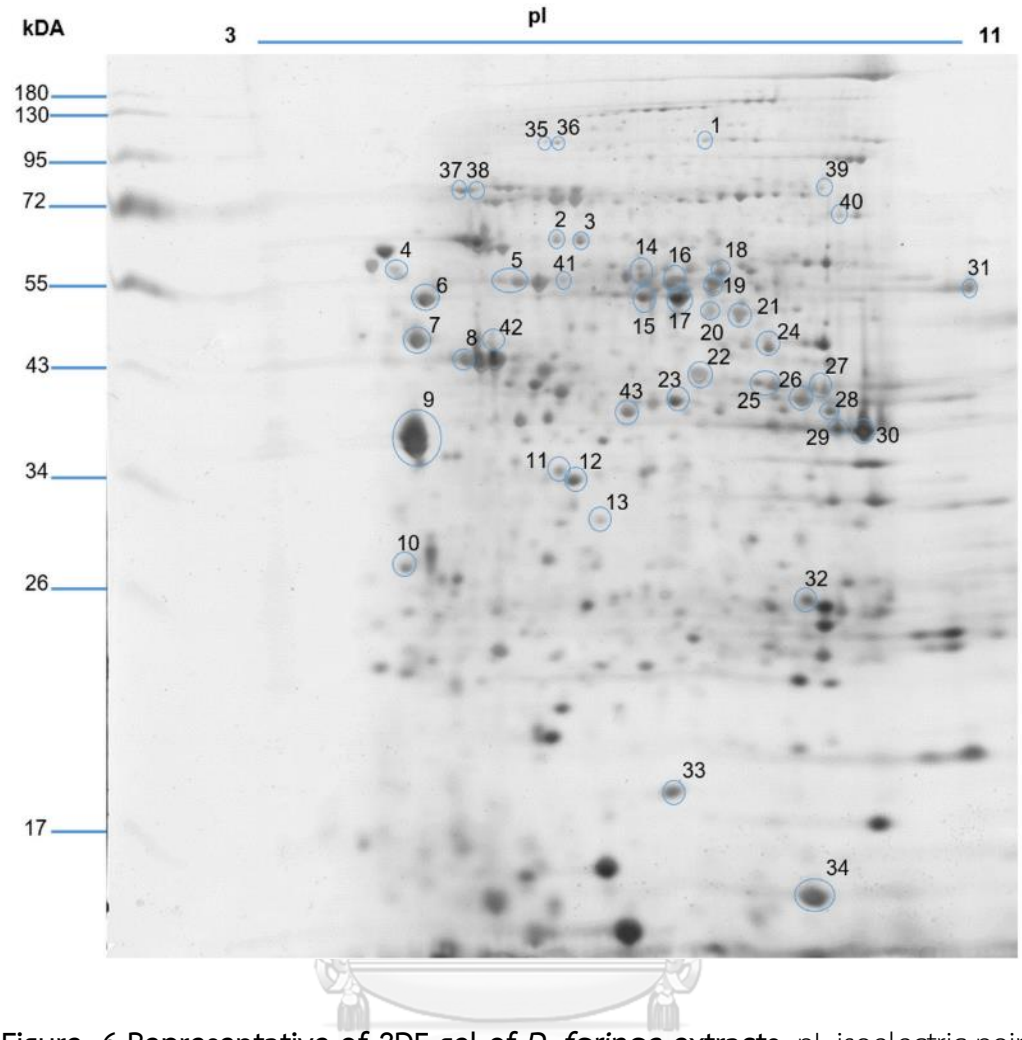

Figure 6 Representative of 2DE-gel of *D. farinae* extracts. pI, isoelectric point; ○; IgE-reactive spots that were found among the study population in this study

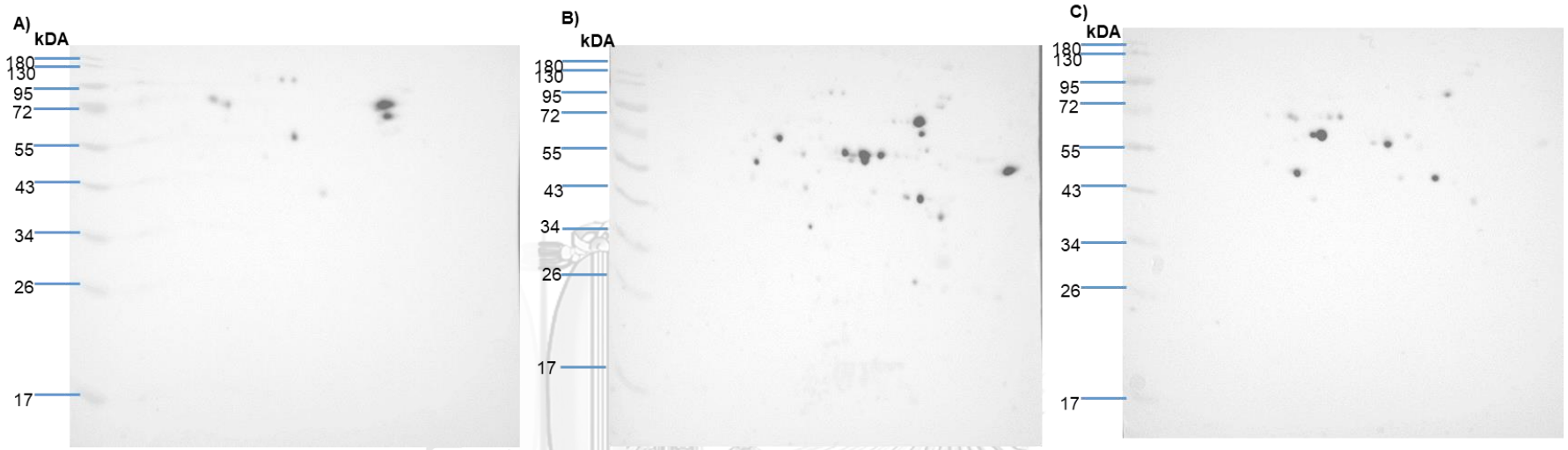

Fig 7. Representative results of 2DE-IgE immunoblotting in healthy and atopic dogs.

The crude extracts of *D. farinae* (DF) were subjected to 2DE and probed with serum of samples. A, probing with the pooled sample from healthy dogs; B, probing with the sample from DF allergy-atopic dog no. AD8; C, probing with the sample from non-DF allergy-atopic dog no. AD13

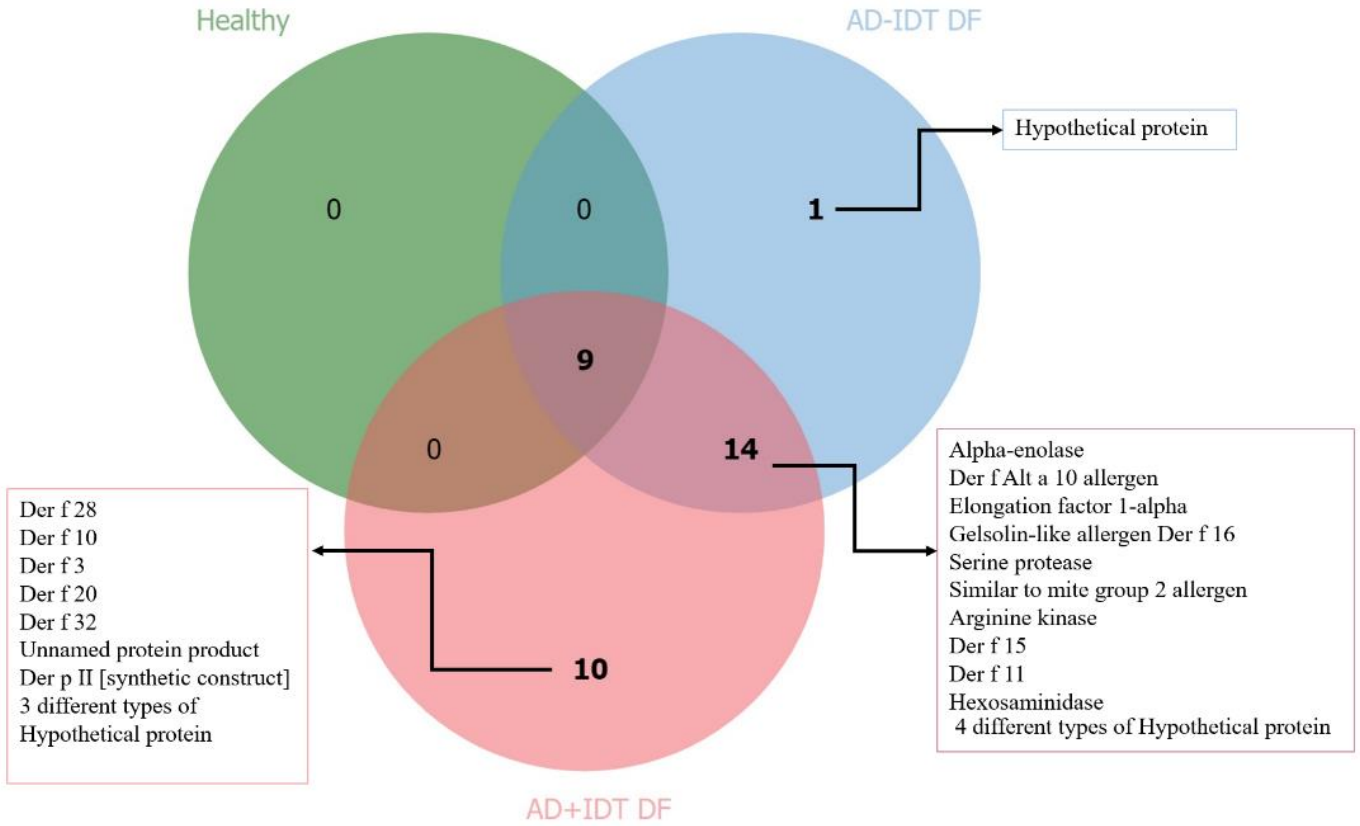

Figure 8 Results of number and types of IgE-reactive spots specific *to D. farinae* extracts in each group of dogs.

AD, atopic dogs; DF, *D. farinae*, +/-IDT, positive or negative skin reaction

Table 7 IgE reactivity to DF components in the AD +IDT to DF and AD -IDT to DF groups

Spot no.	Allergen/ protein name	AD +IDT to DF no.									Frequency	% Allergenicity	AD -IDT to DF no.							Frequency	% Allergenicity
		1	2	3	4	5	6	7	8	9			10	11	12	13	14	15	16		
15,17,19,20	Alpha-enolase	+	+	+	+	+	+	+	+	+	9	100	+	+	-	+	+	+	+	6	85.7
5	Der f Alt a 10 allergen	+	+	+	+	-	+	+	+	+	8	88.9	-	+	+	+	-	-	-	3	42.9
7,31	Elongation factor 1-alpha	+	+	+	+	+	+	+	+	-	8	88.9	+	-	-	-	-	-	+	2	28.6
29, 32	Der f 28	+	+	+	+	+	+	+	+	-	8	88.9	-	-	-	-	-	-	-	0	0
14,16	Gelsolin-like allergen Der f 16	+	-	-	+	+	+	+	+	+	7	77.8	-	-	-	+	-	-	-	1	14.3
18,33	Serine protease	-	-	+	+	+	+	+	+	+	7	77.8	+	+	-	+	+	-	-	4	57.1
34	Similar to mite group 2 allergen	+	+	-	+	+	+	+	+	-	7	77.8	-	-	-	-	-	+	-	1	14.3
22	Hypothetical protein	-	-	-	+	+	+	+	+	+	6	66.7	+	+	-	+	+	+	+	6	85.7
6	Hypothetical protein	+	-	+	+	+	-	-	+	-	5	55.6	+	+	-	-	+	-	+	4	57.1
25,27	Arginine kinase	+	+	-	-	-	+	+	+	-	5	55.6	+	-	+	+	-	-	+	4	57.1
9,10	Der f 10	-	-	+	+	+	+	-	-	-	4	44.4	-	-	-	-	-	-	-	0	0
13	Der f 3	-	-	-	+	-	+	+	+	-	4	44.4	-	-	-	-	-	-	-	0	0

21	Hypothetical protein	-	+	-	-	+	+	+	-	-	4	44.4	-	-	-	-	-	-	-	0	0
23	Hypothetical protein	-	-	-	+	-	-	+	+	+	4	44.4	-	+	-	-	+	-	-	2	28.6
26	Der f 20	-	-	-	-	+	+	+	+	-	4	44.4	-	-	-	-	-	-	-	0	0
2	Der f 15	-	-	-	+	-	+	-	+	-	3	33.3	-	+	+	+	-	-	-	3	42.9
3	Hypothetical protein	-	-	-	+	-	+	-	+	-	3	33.3	-	+	+	+	-	-	-	3	42.9
4	Der f 11	+	+	+	-	-	-	-	-	-	3	33.3	-	+	-	-	-	-	-	1	14.3
8	Hexosaminidase	-	+	-	+	-	+	-	-	-	3	33.3	-	-	+	+	-	-	-	2	28.6
12	Der f 32	-	-	-	+	-	-	+	+	-	3	33.3	-	-	-	-	-	-	-	0	0
28	Unnamed protein product	-	+	-	-	-	+	-	+	-	3	33.3	-	-	-	-	-	-	-	0	0
11	Hypothetical protein	-	-	-	+	-	-	+	-	-	2	22.2	-	-	-	-	-	-	-	0	0
1	Der p II [synthetic construct]	-	-	+	-	-	-	-	-	-	1	11.1	-	-	-	-	-	-	-	0	0
24	Hypothetical protein	-	-	-	+	-	-	-	-	-	1	11.1	-	-	-	-	-	-	-	0	0
30	Hypothetical protein	-	-	-	-	-	-	-	-	-	0	0	+	+	-	+	+	+	-	5	71.4

AD +IDT, Atopic dogs that had positive skin reaction; AD -IDT DF, Atopic dogs that had negative skin reaction; DF, *D. farinae*

Table 8 Accession no. and protein function of each IgE-reactive spot specific to DF components

Spot no.	GenBank No.	Allergen/protein name	Biological function/ identity/conserved domain
15, 17, 19, 20	AHV90299	Alpha-enolase	Enolase
5	AAP35081	Der f Alt a 10 allergen	Aldehyde dehydrogenase
7, 31	ABW99067	Elongation factor 1-α	Elongation factor 1- alpha; Provisional
29, 32	AIO08848	Der f 28	Heat shock 70 kDa protein; Provisional
14, 16	AAM64112	Gelsolin-like allergen Der f 16	Gelsolin sub-domain 1and 3-like domain
18, 33	AAN02510	Serine protease	Trypsin-like serine protease
13	AAA99805	Der f 3	Trypsin-like serine protease
34	ABG35122	Group 2 allergen	NPC2 family
1	CAA00161	Der p II [synthetic construct]	NPC2 family
22	EFY86341	Hypothetical protein	Microcephalin and similar proteins
11	XP_001868513	Hypothetical protein	Microneme/rhoptry antigen; Provisional
6	KJZ80397	Hypothetical protein	Cytochrome c oxidase subunit VIb
25,27	AAP57094	Arginine kinase	Arginine kinase
26	AIO08850	Der f 20	Arginine kinase
9, 10	BAA04557	Der f 10	Tropomyosin

2	AAD52672	Der f 15	Chitinases
28	CAD35266	Unnamed protein product	Chitinases
4	EFP06016	Der f 11	Paramyosin
8	AIO08858	Hexosaminidase	Beta-N-acetylhexosaminidases
12	AIO08849	Der f 32	Inorganic pyrophosphatase
3	EZA54080	Hypothetical protein	Unknown
21	EGX95844	Hypothetical protein	Unknown
23	XP_009014800	Hypothetical protein	Isocitrate/isopropylmalate dehydrogenase
24	EPY82997	Hypothetical protein	Intermediate filament protein
30	ACG63665	Hypothetical protein	Glyceraldehyde-3-phosphate dehydrogenase

Discussion

This study investigated the allergen components of DF extracts that were associated with AD in dogs using 2DE immunoblotting and then identifying reactive components using LC-MS/MS. The DF extracts included a mixture of allergic and non-allergenic components, and so this variable component composition could affect the accuracy of the allergy screening. In accordance with several previous reports (Lauber et al., 2012; Bjelland et al., 2014), we confirmed that some of the components in the DF extracts could be recognized by specific antibodies found in healthy/non-atopic dogs. Moreover, both the AD +IDT to DF and AD -IDT to DF groups also recognized those same components of DF as found in the non-atopic dogs. All components among the three groups of dogs were high molecular weight proteins. Concerning those antigens not associated with the allergic condition, the components common to both the atopic and non-atopic groups were accordingly not selected for analysis.

We suggest that some components of the IgE response to DF may act as a common antigen in dogs from exposure to other natural antigens.

Based on the allergenic profile in atopic dogs, 25 allergen components were identified. Unfortunately, eight hypothetical proteins were found with doubtful characterizations due to the limits of the currently available orthologous proteins in the database. Similar to human AD, atopic dogs showed polysensitization to a broader range of the allergen components of DF, especially in the AD +IDT to the DF group (Park et al., 2018). As shown in Figure 8, 14 intersected allergen components were found between both groups of atopic dogs. However, only four allergen components (Der f Alt a 10, EFI-α, gelsolin-like allergen Der f 16, and similar to mite group 2 allergen) showed an outstanding percentage of allergenicity (> 50%) in the AD +IDT to DF group compared to the AD -IDT to DF group. In humans, Der f Alt a 10 and gelsolin-like allergen Der f 16 have been reported as a new class of allergens in AD (Kawamoto et al., 2002; Park et al., 2018).

Fairly recently, EFI-α was predicted to be a potential allergen in the American cockroach (*Periplaneta americana*) by Algpred (webserver for prediction of allergenic proteins) (Ahmed et al., 2010), but their allergenicity in allergic disease has not been defined. To our knowledge, this is the first study that mentioned four of these allergenic components of DF in atopic dogs. For the allergen related to the group 2 allergen of DF, this allergen is proposed to be Der f 2, which is known as a major allergen component of DF in human allergic patients (Yasueda et al., 1986; Park et al., 2018), but the consensus of this allergen in atopic dogs has been argued due to the different geographical regions of the study dogs (Yamashita et al., 2002; Olivry et al., 2017). In this study, the group 2 allergen acted as the major allergen in Thai atopic dogs.

Interestingly, alpha-enolase, serine protease, arginine kinase, and two different types of hypothetical proteins (cytochrome c oxidase subunit VIb and microcephalin) were found to be major cross-reactive components in atopic dogs in this study. This finding supported that all of these proteins could be sensitized in most atopic dogs

without specificity to DF allergy, and so crude extracts containing these proteins could be a possible cause of false positives in allergen-specific IgE serology screening. In allergic patients, arginine kinase has been reported as a cross-reactive invertebrate pan-allergen (Binder et al., 2001), which might be a reason that our non-DF allergy atopic dogs strongly reacted to this component of DF. Based on previous research, arginine kinase induces Th2 cell differentiation and histamine release (Renand et al., 2014; Xing et al., 2015), whereas alpha-enolase and serine protease jeopardized the epithelial barrier function (Wan et al., 2001; Kato et al., 2009; Tohgasaki et al., 2018).

We agree that arginine kinase, alpha-enolase, and serine protease actually play an essential role in the pathogenesis of canine AD, as also found in allergic humans. Therefore, the idea of further applying these proteins should focus on immunotherapy rather than confirming DF allergy in atopic dogs. For the other two hypothetical proteins, they need to be explored more, due to the lack of previous evidence to support their role in AD. After removing intersected allergen components between both groups of atopic dogs, 10 allergen components related to DF sensitization were revealed, including one dominant protein (Der f 28) and nine minor proteins, as shown in Table 7. By previous findings in asthmatic patients (An et al., 2013; Lin et al., 2015), this study supported that Der f 28 (heat shock protein 70 kDa) was a new allergen component related to DF sensitization in 88.9% (8/9) of atopic dogs. At this point, Der f 28 seems to be an interesting component of DF to apply for CRD and CRIT in atopic dogs.

According to our results, new minor relevant allergen components of DF have disclosed, including Der f 3, Der f 10, Der f 20, and Der f 32. Indeed, Der f 3 (trypsin-like serine protease) and Der f 20 (arginine kinase) may not restricted to DF allergy only but might relate to the pathogenesis of AD in dogs that had hypersensitivities to other relevant allergens, including proteins with serine protease or arginine kinase-like domains (antigens) in their composition. In patients with respiratory allergic diseases, Der f 10 (tropomyosin) has been suspected as a critical cross-reactive component that linked allergic reactions to crustacean shellfish, cockroach, and HDMs, due to their high sequence homology of around 80% (Santos et al., 1999). In

contrast, Der f 10 was suspected as a minor component (44% allergenicity; 4/9) in the AD +IDT to DF group without showing cross-reactivity to the AD -IDT to DF group. However, this recent study is one of only a few studies that elucidated more information on Der f 10 in atopic dogs. Similar to human AD, Der f 32 was one of the minor components of DF that was sensitized in the AD +IDT to DF group (Park et al., 2018).

Surprisingly, in this present study, Der f 15 and an unnamed protein product (spot no. 28), here called chitinase and were identified as a minor allergen in the AD + IDT to DF group. Moreover, some of the AD -IDT to DF group reacted to Der f 15. Hence, Der f 15 could be a cross-reactive component in atopic dogs, which is in contrast to previous studies that reported that Der f 15 and chitinase/ chitin-binding protein (Der f 18) are major relevant allergens of DF in atopic dogs (McCall et al., 2001; Moya et al., 2016). Our source of DF was confirmed to contain Der f 15 (Choopong et al., 2016), but its degradation may compromise the low percentage of allergenicity to this allergen in atopic dogs due to the prolonged process of 2DE-blotting or the different characterization of study samples. Previous studies have often recruited atopic dogs that are positive in IDT or IgE-serology testing for DF while being positive to additional allergens was not evaluated in their studies. In addition, atopic dogs with negative allergy testing to DF have also been excluded. Based on this current finding, it is suggested that Der f 15 is not a suitable candidate allergen component to classify DF allergy in atopic dogs.

Interestingly, samples from the AD -IDT to DF group were strongly associated with the hypothetical protein (spot no. 30), G3PDH, at up to 71.4%. The association of G3PDH with allergy is not well defined and has received less attention. It has been identified as an allergen component in rambutan (Nephelium lappaceum) inducing anaphylaxis (Jirapongsananuruk et al., 2011), while G3PDH or Per a 13 was reported as an allergen component of the American cockroach in the website of Allergen Nomenclature. Hence, further studies on this allergen is required to elucidate its role in the pathogenesis of AD in dogs.

This study still has many limitations that need to be further elucidated. Extending the sample size and sub-classifying according to clinical severity, and single/multiple sensitivity to other relevant allergens, are needed to validate the allergenicity and cross-reactivity to DF components among all the groups of dogs. The identification of standard components should be performed to inform and raise awareness about contaminant components, which affect the accuracy of serology testing in atopic dogs. Moreover, demonstrating IgE-binding to components of DF using purified natural or recombinant allergens is required to confirm a set of major/minor components related to DF allergy. Far from human allergy, a set of allergen components for diagnosis and treatment is required to advance CRD and CRIT.

Conclusions

Atopic AD dogs were shown to have polysensitization to a broader range of allergen components of DF, including high and low molecular weight proteins, through 2DE-IgE immunoblotting. Most components of DF associated with DF allergy were proposed for the first time in the veterinary field for major (Alt a 10 allergen, EF1-α, gelsolin-like allergen Der f 16, Der f 28, and allergen with similar to mite group 2) and minor (Der f 3, Der f 10, Der f 20, and Der f 32) components. Some components were found to be strongly cross-reactive proteins that could also be sensitized in non-DF allergy atopic dogs, including alpha-enolase, serine protease, and arginine kinase. This may be useful to select/manage a set of DF components for diagnostic or therapeutic purposes.

CHAPTER V

General discussion, conclusion, and further recommendations

In this study, we succeeded in developing an in-house ELISA for confirming HDM allergy in atopic dogs, our platform could be used to confirm *D. farinae* and *D. pteronyssinus* allergy in atopic dogs via detection specific IgE or IgG1 levels in serum samples. In our IgE ELISA, this platform is an indirect ELISA, two types of antibodies are required, including with anti-dog IgE (monoclonal Ab) and anti-mouse IgG tagged with alkaline phosphatase enzymes (conjugated Ab). While the direct ELISA using the IgG1 platform required only anti-dog IgG1 tagged with horse reddish peroxidase (polyclonal Ab). Then, the benefits of detection IgG1 are required lower samples volumes than IgE detection about 100 times and used only one type of conjugated Ab for detection. Then, detection IgG1 is cheaper and faster. Both platforms had the same sensitivity, but IgE based assays showed the better specificity that IgG1 assay about 8-15%.

Moreover, both IgE and IgG1 assay showed the same degree of agreement to IDT; this means either IgE or IgG1 could be used to confirm HDM allergy in atopic dogs. This finding support that high IgG1 production to a causative allergen could detect in atopic dogs as IgE, so IgG1 seems to have a role in the pathogenesis of AD in dogs like finding in human (Einarsson et al., 1992). Unfortunately, our study could not clarify the role of IgG1, which might act as a protective antibody after allergen exposure or probably act as another antibody triggering inflammatory process or act as a bystander antibody regard to Th2 regulation. In human allergy, the role of IgG1 is inconsistent and unclear. By using mouse model, IgG1 could contribute airway inflammation. The complex antigen/antibody of allergen and IgG1 could promote allergic responses signal through activation FcγRs on hematopoietic cells (Williams et al., 2012), while another study found that increasing IgG1 could be found after immunotherapy (Einarsson et al., 1992). In an atopic dog, only one study in Labrador- and Golden retrievers found that *D. farinae* specific IgG1 levels were not different between atopic dogs and non-atopic dogs, but increasing could found in atopic dogs after immunotherapy (Lauber et al., 2012).

In contrast to our study that high IgG1 to *D. farinae* found in atopic dogs. In our observation, we found a strong correlation between *D. farinae*-IgE and *D. farinae*-IgG1 in atopic dogs. Then the longitudinal study or monitoring the changing of specific immunoglobulin levels to HDM should be further investigated in clinical atopic dogs, and the correlation between IgE and IgG subclass levels after immunotherapy should be elucidated. If high IgG1 relates to low IgE levels or relates to improved clinical signs after immunotherapy in atopic dogs, consequently, IgG1 might be used as a marker of successful treatment. On the other hand, detection of the IgG1 production after challenging plasma cells from atopic dogs with their causative allergen might support the role of IgG1 in the pathogenesis of CAD. The detection of IgG1 might be useful to identify atopic-like dermatitis dogs who diagnosed with the same criteria as atopic dogs, but they could not detect allergen-specific IgE or not react to any allergen via IDT. Many aspects of IgG1 need further investigation in atopic dogs before widely applying.

In the veterinary field, we still far from precision medicine in the allergy field. In humans, CRD and immunotherapy have proposed to improve the accuracy of diagnosis and raise the promising of successful immunotherapy in allergic patients. Regard to over 3o components of HDM that have reported in human allergy; they could design a set of major allergenic components for immunotherapy that is specific and suitable for individual patients. Our study is the first to demonstrate the components of *D. farinae* that relate to *D. farinae* allergy in Thai atopic dogs. Most of IgE reactive components that recognized in *D. farinae* allergy-atopic dog are high molecular weight protein above than 34 kDa. This finding is following previous study that most of atopic dogs often recognized molecular weight protein (Nuttall et al., 2001), even though we used the different sources of *D. farinae* and used the 2DE-IgE immunoblotting and mass spectrometry. Our result is an excellent fundamental finding to apply CRD in atopic dogs. In addition, this finding supported that standardized allergen of HDM for diagnosis should be different between humans and dogs. Regard to the small groups of atopic dogs in the study, a selection of suitable standardized allergen needs to explore more in Thai atopic dogs. However, some groups of allergenic components have suggested. Der f Alt a 10 allergen, Elongation

factor 1-α, Gelsolin-like Der f 16, allergen group 2 and Der f 28 should be designed for a set of major allergenic components in Thai atopic dogs, whereas Der f 3, Der f 10, Der f 15, Der f 20, Der f 32 and hexosaminidase are a set of minor allergenic components. Development of a serology testing by using a set of major and minor recombinant/ purify allergenic components of *D. farinae* suggested as the secondary test to perform after confirming *D. farinae* allergy by common IDT or ASIS because the results of high IgE level to each major and minor allergenic components of *D. farinae* could help to design a suitable desensitized vaccine for individual *D. farinae* allergy- atopic dog to enhance immunotherapy and might compensate for the variability of natural HDM extracts as current doing in allergic human patients (Calderon et al., 2015). In addition, we suggested raising awareness of cross-reactivity to other allergens in atopic dogs according to high recognized alpha-enolase, serine protease, and arginine kinase. This is the next challenging and critical point of developing a CRD in the veterinary field because atopic dogs also have polysensitization to other allergens.

In our finding, atopic dogs had high levels of IgE/ IgG/ IgG1 and IgG2 to both skin microbes like *M. pachydermatis* and *S. pseudintermedius*. It supported that high burden/exposure to these skin microbes induce high production of specific immunoglobulin to them. Notably, high specific IgE indicates that these skin microbes could induce allergic reactions in atopic dogs, even though both of these microbes act as commensal microbes in dogs. So, controlling the overgrowth and treatment of secondary infection from skin microbes should be concerned as a long term of treatment regarding to high exposure to these microbes leads to IgE production; consequently, a cycle of allergic reaction might be kicked-off. We suspected that these skin microbes could also act as primary allergen in our atopic dogs, but we cannot confirm this suspicion according to no recommendation of standardized dilution/concentration for performing IDT to these skin microbes at the time we started this study. However, a recent study by Oldenhoff and colleagues supported our suspicion that *M. pachydermatis* can be a cause of *Malassezia* hypersensitivity in atopic dogs (Oldenhoff et al., 2014). Unfortunately, the evidence of *S.*

pseudintermedius inducing hypersensitivity in atopic dogs has not been reported in our current knowledge. Then, performing IDT by allergen of these skin microbes is our further suggestion to clarify the role of skin microbes in atopic dogs. As in humans, overgrowth of *Malassezia spp*. and *Staphylococcus spp.* could contribute a progressively worse of skin inflammation in AD. In human, specific IgE levels to *M. furfur* and *S. epidermidis* related to severity of clinical signs in patients with pityriasis versicolor and acne vulgaris. The benefits of measuring specific IgE level is using it to classify and prognosis the disease.

In contrast to human patients, a high level of specific IgE or other IgG to *M. pachydermatis/ S. pseudintermedius* could not be used to classify the severity of AD progression in atopic dogs. We suspected that using the canine atopic dermatitis lesion index (CADLI) alone cannot reflect the current clinical signs as we expected. CADLI represents the severity of skin lesions, which are the sum of old and new clinical signs (Plant et al., 2012), many factors affected the skin lesions not only the role of IgE. We suggested that monitoring the severity of AD with pruritus visual analog scale (Hill et al., 2007) associating the real clinical signs in atopic dogs should be combined to classify the severity of AD, and might be related to high IgE levels.

Table 9 The summary of specific immunoglobulin levels to HDM and skin microbes in atopic dogs

Allergen	IgE	Total IgG	IgG1	IgG2
D. farinae (Df)	+ IDT to HDM group ↑	No different	+ IDT to HDM group ↑	No different
D. pteronyssinus (Dp)	+ IDT to HDM group ↑	No different	+ IDT to HDM group ↑	No different
M. pachydermatis (Mp)	+/- IDT to HDM group ↑	+/- IDT to HDM group ↑	+/- IDT to HDM group ↑	+/- IDT to HDM group ↑
S. pseudintermedius (Sp)	+/- IDT to HDM group ↑	+/- IDT to HDM group ↑	+/- IDT to HDM group ↑	+/- IDT to HDM group ↑

In table 9, we can conclude that a high level of specific IgE and IgG1 to HDM related to IDT results in atopic dogs, while IgG2 level not related to HDM allergy. So, we can imply that high levels of both specific IgE and IgG1 related to HDM allergy in atopic dogs. Unfortunately, the consensus about high IgG1 production relating to other causative allergen triggering in atopic could not confirm in our study regard to lacking of IDT results to skin microbes in our atopic dogs. In our suspicion, If Th2 cells are truly dominant in atopic dogs without interfering by other factors, then exposure to any type of antigen/allergen should be found only high specific IgE and IgG1 production. However, our results with high production of IgG1 and IgG2 to skin microbes lead us to imply that the type of allergens also affected the IgG subclass production, not only the under Th2 bias condition.

Overall, AD is a complex disease that involved a lot of intrinsic and extrinsic factors. The understanding of a causative allergen triggering allergic conditions seems to be more valuable for increasing the accuracy and efficiency of specific diagnosis and treatment. HDM and skin microbes are the most common allergen/antigens that could be a cause of allergic reactions in atopic dogs. Exploring the major and minor components of allergen/skin microbes are necessary to develop high accuracy allergy testing and enhance the success of immunotherapy. Moreover, determining the levels of specific IgG/ IgG subclasses to various types of allergen/antigens helps us to understand the immune response in dogs underlying AD. This finding is the fundamental of a comparative study to humans and other animal models. Our finding reveals the major and minor components of *D. farinae* that specific to IgE in atopic dogs. Some limitations about the criteria's clinical cases, available commercial antibodies and variable factors in our finding could affect the results of interpretation, so the idea of further investigating is restricted condition of atopic dogs that already rule out or identify the variable factors including breed, age, sex and types of allergens/antigens depending on their characteristics.

Appendix

Appendix 1

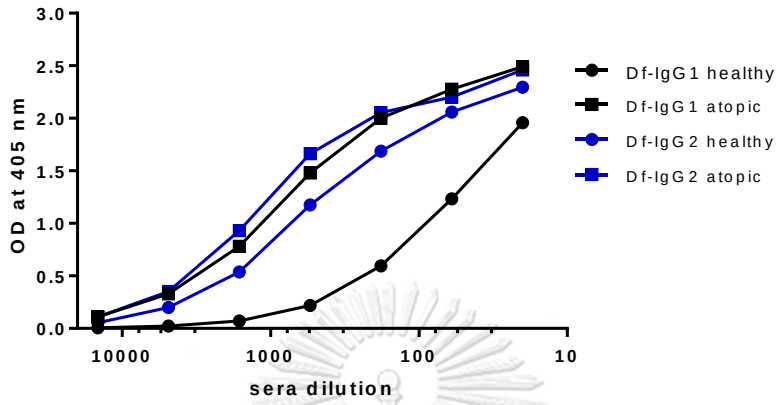

Figure 7 Titration curve of *D. farinae* (DF)-IgG1 and IgG2 for determining serum dilution

Appendix 2

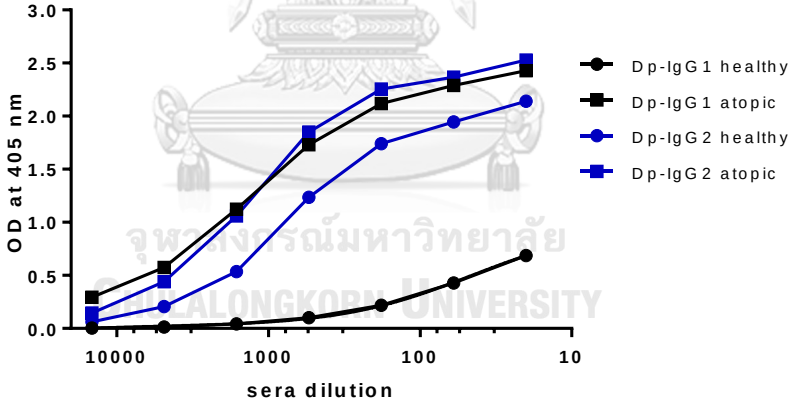

Figure 8 Titration curve of *D. pteronyssinus* (DP)-IgG1 and IgG2 for determining serum dilution

Appendix 3

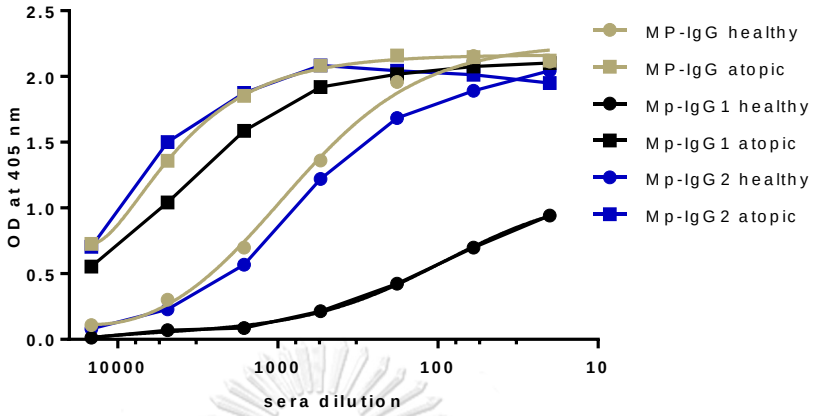

Figure 9 Titration curve of *M. pachydermatis* (MP)- total IgG and IgG subclasses for determining serum dilution

Appendix 4

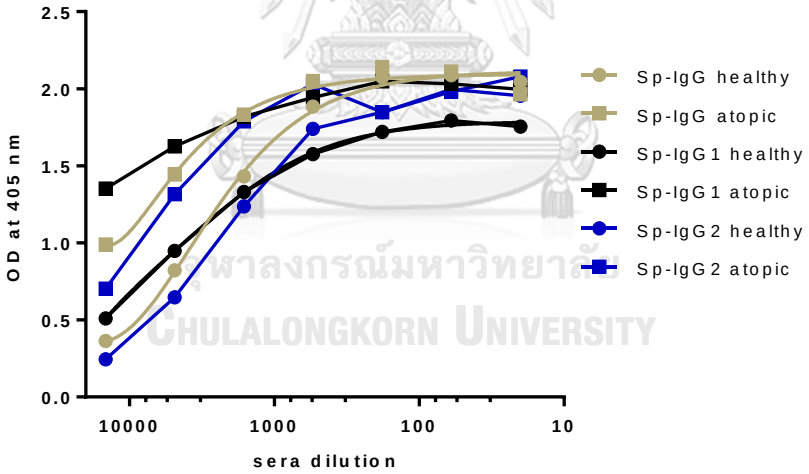

Figure 10 Titration curve of *S. pseudintermedius* (SP)- total IgG and IgG subclasses for determining serum dilution

Appendix 5

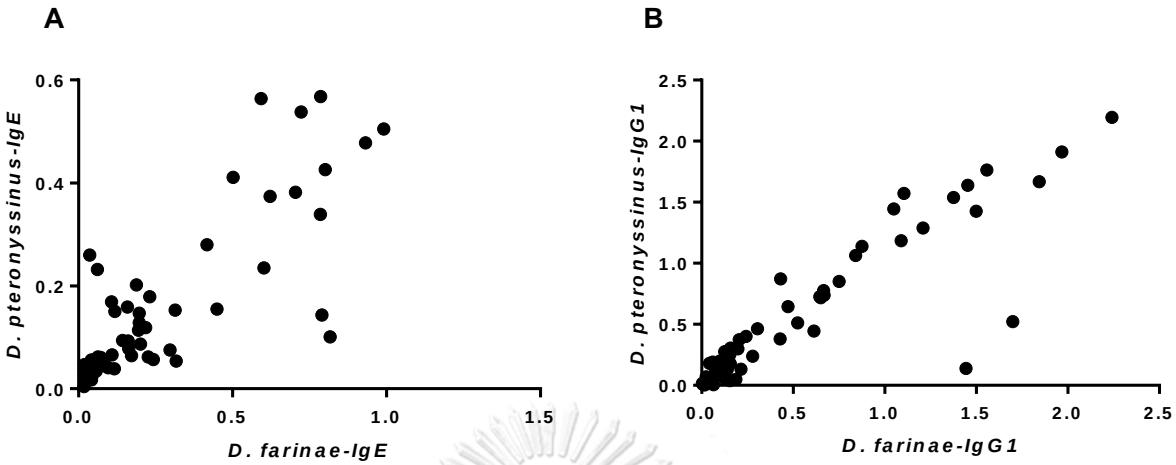

Figure 11 Correlation of specific IgE (A) and IgG 1 (B) levels to different species of HDM

Appendix 6

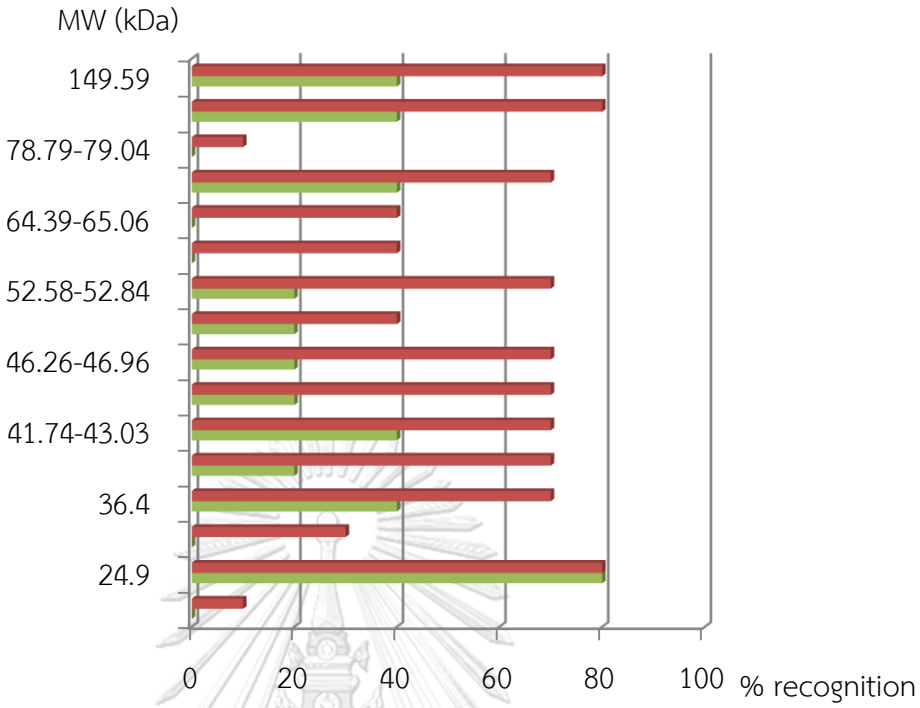

Figure 12 The percentage of IgG2 subclass-specific to *Malassezia pachydermatis* extracts in atopic (orange bar) and non-atopic dogs (gray bar) at titer $1:10^5$

Appendix 7

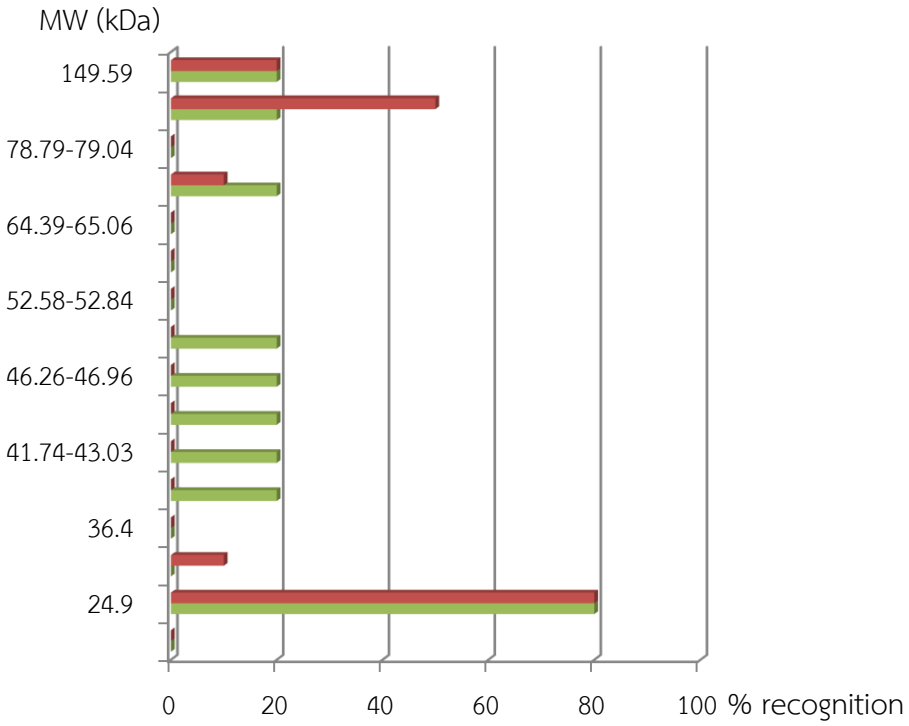

Figure 13 The percentage of IgG1 subclass-specific to *Malassezia pachydermatis* extracts in atopic (orange bar) and non-atopic dogs (gray bar) at titer $1:10^5$

Appendix 8

A **B**

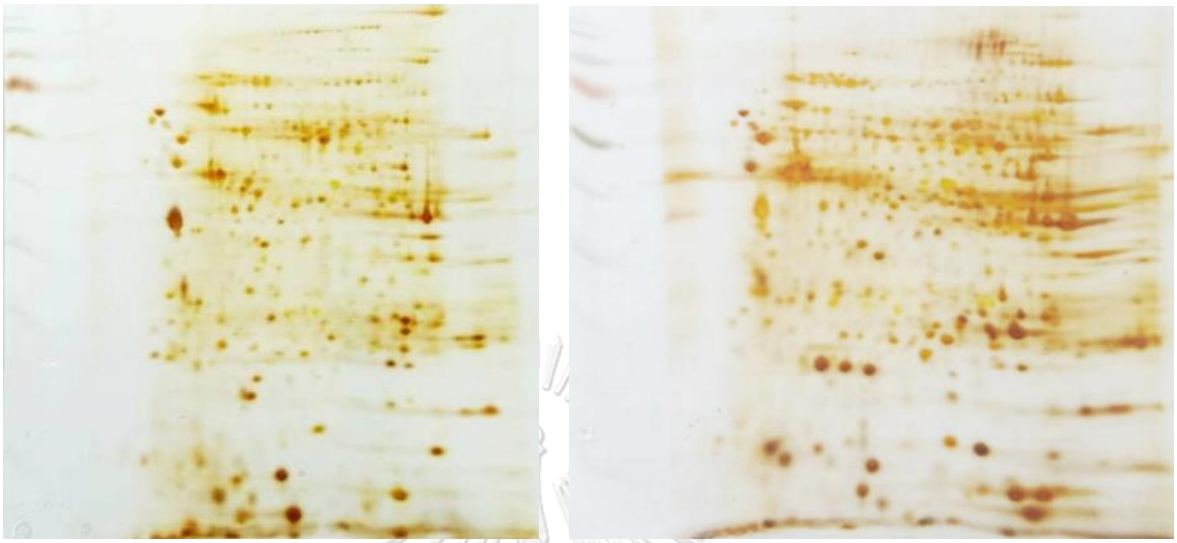

Figure 14 Representative of 2DE-gel of *D. farinae* (A) and *D. pteronyssinus* (B) extracts

REFERENCES

Agallou M, Athanasiou E, Samiotaki M, Panayotou G and Karagouni E 2016. Identification of Immunoreactive *Leishmania infantum* Protein Antigens to Asymptomatic Dog Sera through Combined Immunoproteomics and Bioinformatics Analysis. Plos One. 11(2): 1-22.

Ahmed A, Minhas K, Namood ES, Aftab O and Khan FS 2010. In Silico Identification of Potential American Cockroach (*Periplaneta americana*) Allergens. Iran J Public Health. 39(3): 109-115.

An S, Chen L, Long C, Liu X, Xu X, Lu X, Rong M, Liu Z and Lai R 2013. *Dermatophagoides farinae* allergens diversity identification by proteomics. Mol Cell Proteomics. 12(7): 1818-1828.

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.

Ashbee HR, Muir SR, Cunliffe WJ and Ingham E 1997. IgG subclasses specific to *Staphylococcus epidermidis* and *Propionibacterium acnes* in patients with acne vulgaris. Br J Dermatol. 136(5): 730-733.

Barbieri CL 2006. Immunology of canine leishmaniasis. Parasite Immunol. 28(7): 329-337.

Bergeron LM, McCandless EE, Dunham S, Dunkle B, Zhu Y, Shelly J, Lightle S, Gonzales A and Bainbridge G 2014. Comparative functional characterization of canine IgG subclasses. Vet Immunol Immunopathol. 157(1-2): 31-41.

Bexley J, Nuttall TJ, Hammerberg B, Fitzgerald JR and Halliwell RE 2013. Serum anti-*Staphylococcus pseudintermedius* IgE and IgG antibodies in dogs with atopic dermatitis and nonatopic dogs. Vet Dermatol. 24(1): 19-24.

Bieber T 2010. Atopic dermatitis. Ann Dermatol. 22(2): 125-137.

Binder M, Mahler V, Hayek B, Sperr WR, Scholler M, Prozell S, Wiedermann G, Valent P, Valenta R and Duchene M 2001. Molecular and immunological characterization of arginine kinase from the Indianmeal moth, *Plodia interpunctella*, a novel cross-reactive invertebrate pan-allergen. J Immunol. 167(9): 5470-5477.

Bird RC, DeInnocentes P, Bird AEC, van Ginkel FW, Lindquist J and Smith BF 2011. An autologous dendritic cell canine mammary tumor hybrid-cell fusion vaccine. Cancer Immunol Immunother. 60(1): 87-97.

Bjelland AA, Dolva FL, Nodtvedt A and Saevik BK 2014. Prevalence of and risk factors for increased serum levels of allergen-specific IgE in a population of Norwegian dogs. Acta Vet Scand. 56: 81.

Brown AF, Murphy AG, Lalor SJ, Leech JM, O'Keeffe KM, Mac Aogain M, O'Halloran DP, Lacey KA, Tavakol M, Hearnden CH, Fitzgerald-Hughes D, Humphreys H, Fennell JP, van Wamel WJ, Foster TJ, Geoghegan JA, Lavelle EC, Rogers TR and McLoughlin RM 2015. Memory Th1

Cells Are Protective in Invasive *Staphylococcus aureus* Infection. PLoS Pathog. 11(11): e1005226.

Calderon MA, Kleine-Tebbe J, Linneberg A, De Blay F, Hernandez Fernandez de Rojas D, Virchow JC and Demoly P 2015. House Dust Mite Respiratory Allergy: An Overview of Current Therapeutic Strategies. J Allergy Clin Immunol Pract. 3(6): 843-855.

Chamberlain KW 1974. Atopic (allergic) dermatitis. Vet Clin North Am. 4(1): 29-39.

Chanthick C, Anaman S and Buathet K 2008. The prevalence of positive intradermal allergy tests in 114 dogs with atopic dermatitis in the Bangkok metropolis, Thailand. Vet Immunol Immunopathol. 126(3-4): 256-262.

Choopong J, Reamtone O, Sookrung N, Seesuay W, Indrawattana N, Sakolvaree Y, Chaicumpa W and Tungtrongchitr A 2016. Proteome, Allergenome, and Novel Allergens of House Dust Mite, *Dermatophagoides farinae*. J Proteome Res. 15(2): 422-430.

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.

DeBoer DJ and Hillier A 2001. The ACVD task force on canine atopic dermatitis (XVI): laboratory evaluation of dogs with atopic dermatitis with serum-based "allergy" tests. Vet Immunol Immunopathol. 81(3-4): 277-287.

DeBoer DJ and Marsella R 2001. The ACVD task force on canine atopic dermatitis (XII): the relationship of cutaneous infections to the pathogenesis and clinical course of canine atopic dermatitis. Vet Immunol Immunopathol. 81(3-4): 239-249.

Einarsson R, Dreborg S, Hammarstrom L, Lofkvist T, Smith CI and Svensson G 1992. Monitoring of mite *Dermatophagoides farinae* allergen-specific IgG and IgG subclass distribution in patients on immunotherapy. Allergy. 47(2 Pt 1): 76-82.

Favrot C, Steffan J, Seewald W and Picco F 2010. A prospective study on the clinical features of chronic canine atopic dermatitis and its diagnosis. Vet Dermatol. 21(1): 23-31.

Filep S, Tsay A, Vailes LD, Gadermaier G, Ferreira F, Matsui E, King EM and Chapman MD 2012. Specific allergen concentration of WHO and FDA reference preparations measured using a multiple allergen standard. J Allergy Clin Immunol. 129(5): 1408-1410.

Foster AP, Littlewood JD, Webb P, Wood JL, Rogers K and Shaw SE 2003. Comparison of intradermal and serum testing for allergen-specific IgE using a Fcepsilon RIalpha-based assay in atopic dogs in the UK. Vet Immunol Immunopathol. 93(1-2): 51-60.

Fraser MA, McNeil PE and Gettinby G 2004. Examination of serum total IgG(1) concentration in atopic and non-atopic dogs. J Small Anim Pract. 45(4): 186-190.

Glatz M, Buchner M, Von Bartenwerffer W, Schmid-Grendelmeier P, Worm M, Hedderich J and Folster-Holst R 2015. *Malassezia spp*.-specific Immunoglobulin E Level is a Marker for Severity of Atopic Dermatitis in Adults. Acta Dermato-Venereologica. 95(2): 191-196.

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.

Halbert AR, Weston WL and Morelli JG 1995. Atopic dermatitis: is it an allergic disease? J Am Acad Dermatol. 33(6): 1008-1018.

Hales BJ, Martin AC, Pearce LJ, Laing IA, Hayden CM, Goldblatt J, Le Souef PN and Thomas WR 2006. IgE and IgG anti-house dust mite specificities in allergic disease. J Allergy Clin Immunol. 118(2): 361-367.

Halliwell R 2006. Revised nomenclature for veterinary allergy. Vet Immunol Immunopathol. 114(3-4): 207-208.

Halliwell RE and DeBoer DJ 2001. The ACVD task force on canine atopic dermatitis (III): the role of antibodies in canine atopic dermatitis. Vet Immunol Immunopathol. 81(3-4): 159-167.

Hensel P, Santoro D, Favrot C, Hill P and Griffin C 2015. Canine atopic dermatitis: detailed guidelines for diagnosis and allergen identification. BMC Vet Res. 11: 196.

Hill PB and DeBoer DJ 2001. The ACVD task force on canine atopic dermatitis (IV): environmental allergens. Vet Immunol Immunopathol. 81(3-4): 169-186.

Hill PB, Hillier A and Olivry T 2001. The ACVD task force on canine atopic dermatitis (VI): IgE-induced immediate and late-phase reactions, two inflammatory sequences at sites of intradermal allergen injections. Vet Immunol Immunopathol. 81(3-4): 199-204.

Hill PB, Lau P and Rybnicek J 2007. Development of an owner-assessed scale to measure the severity of pruritus in dogs. Vet Dermatol. 18(5): 301-308.

Hill PB, Moriello KA and DeBoer DJ 1995. Concentrations of total serum IgE, IgA, and IgG in atopic and parasitized dogs. Vet Immunol Immunopathol. 44(2): 105-113.

Hillier A and DeBoer DJ 2001. The ACVD task force on canine atopic dermatitis (XVII): intradermal testing. Vet Immunol Immunopathol. 81(3-4): 289-304.

Hillier A and Griffin CE 2001. The ACVD task force on canine atopic dermatitis (I): incidence and prevalence. Vet Immunol Immunopathol. 81(3-4): 147-151.

Hillier A, Lloyd DH, Weese JS, Blondeau JM, Boothe D, Breitschwerdt E, Guardabassi L, Papich MG, Rankin S, Turnidge JD and Sykes JE 2014. Guidelines for the diagnosis and antimicrobial therapy of canine superficial bacterial folliculitis (Antimicrobial Guidelines Working Group of the International Society for Companion Animal Infectious Diseases). Vet Dermatol. 25(3): 163-+.

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.

Iniesta L, Gallego M and Portus M 2005. Immunoglobulin G and E responses in various stages of canine leishmaniosis. Vet Immunol Immunopathol. 103(1-2): 77-81.

Jacob SE, Nassiri M, Kerdel FA and Vincek V 2003. Simultaneous measurement of multiple Th1 and Th2 serum cytokines in psoriasis and correlation with disease severity. Mediators Inflamm. 12(5): 309-313.

Jankovic D, Steinfelder S, Kullberg MC and Sher A 2006. Mechanisms underlying helminth-induced Th2 polarization: default, negative or positive pathways? Chem Immunol Allergy. 90: 65-81.

Jirapongsananuruk O, Jirarattanasopa N, Pongpruksa S, Vichyanond P and Piboonpocanun S 2011. Glyceraldehyde-3-phosphate dehydrogenase as a major allergen in rambutan-induced anaphylaxis. Ann Allergy Asthma Immunol. 106(6): 545-547.

Jung DJ, An JH, Kurokawa K, Jung YC, Kim MJ, Aoyagi Y, Matsushita M, Takahashi S, Lee HS, Takahashi K and Lee BL 2012. Specific serum Ig recognizing staphylococcal wall teichoic acid induces complement-mediated opsonophagocytosis against *Staphylococcus aureus*. J Immunol. 189(10): 4951-4959.

Kang MH, Kim HJ, Jang HJ and Park HM 2014. Sensitization rates of causative allergens for dogs with atopic dermatitis: detection of canine allergen-specific IgE. J Vet Sci. 15(4): 545-550.

Kato T, Takai T, Fujimura T, Matsuoka H, Ogawa T, Murayama K, Ishii A, Ikeda S, Okumura K and Ogawa H 2009. Mite serine protease activates protease-activated receptor-2 and induces cytokine release in human keratinocytes. Allergy. 64(9): 1366-1374.

Kawamoto S, Suzuki T, Aki T, Katsutani T, Tsuboi S, Shigeta S and Ono K 2002. Der f 16: a novel gelsolin-related molecule identified as an allergen from the house dust mite, *Dermatophagoides farinae*. FEBS Lett. 516(1-3): 234-238.

Kawasaki Y, Suzuki J, Sakai N, Isome M, Nozawa R, Tanji M and Suzuki H 2004. Evaluation of T helper-1/-2 balance on the basis of IgG subclasses and serum cytokines in children with glomerulonephritis. Am J Kidney Dis. 44(1): 42-49.

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.

Kraft D, Ferreira F, Vrtala S, Breiteneder H, Ebner C, Valenta R, Susani M, Breitenbach M and Scheiner O 1999. The importance of recombinant allergens for diagnosis and therapy of IgE-mediated allergies. Int Arch Allergy Immunol. 118(2-4): 171-176.

Larsen JN and Dreborg S 2008. Standardization of allergen extracts. Methods Mol Biol. 138: 133-145.

Lauber B, Molitor V, Meury S, Doherr MG, Favrot C, Tengvall K, Bergvall K, Leeb T, Roosje P and Marti E 2012. Total IgE and allergen-specific IgE and IgG antibody levels in sera of atopic dermatitis affected and non-affected Labrador- and Golden retrievers. Vet Immunol Immunopathol. 149(1-2): 112-118.

Lawrence MG, Woodfolk JA, Schuyler AJ, Stillman LC, Chapman MD and Platts-Mills TAE 2017. Half-life of IgE in serum and skin: Consequences for anti-IgE therapy in patients with allergic disease. J Allergy Clin Immunol. 139(2): 422-428.

Lian TM and Halliwell REW 1998. Allergen-specific IgE and IgGd antibodies in atopic and normal dogs. Vet Immunol Immunopathol. 66(3-4): 203-223.

Lima LVD, Carneiro LA, Campos MB, dos Santos TV, Ramos PK, Laurenti MD, Teixeira CEC and Silveira FT 2017. Further evidence associating IgG1, but not IgG2, with susceptibility to canine visceral leishmaniasis caused by *Leishmania (L.) infantum* chagasi-infection. Parasite. 24.

Lin JL, Wang YY, Xiao XJ, Wu YL, Sun BQ, Gao AJ, Liu ZG, Li J, Yang PC and Liu XY 2015. Characterization of a new subtype of allergen in *Dermatophagoides farinae*-Der f 28. J Thorac Dis. 7(10): 1842-1849.

Masuda K, Sakaguchi M, Fujiwara S, Kurata K, Yamashita K, Odagiri T, Nakao Y, Matsuki N, Ono K, Watari T, Hasegawa A and Tsujimoto H 2000. Positive reactions to common allergens in 42 atopic dogs in Japan. Vet Immunol Immunopathol. 73(2): 193-204.

Mazza G, Whiting AH, Day MJ and Duffus WPH 1994. Development of an Enzyme-Linked-Immunosorbent-Assay for the Detection of IgG Subclasses in the Serum of Normal and Diseased Dogs. Res Vet Sci. 57(2): 133-139.

McCall C, Hunter S, Stedman K, Weber E, Hillier A, Bozic C, Rivoire B and Olivry T 2001. Characterization and cloning of a major high molecular weight house dust mite allergen (Der f 15) for dogs. Vet Immunol Immunopathol. 78(3-4): 231-247.

Mendelsohn C, Rosenkrantz W and Griffin CE 2006. Practical cytology for inflammatory skin diseases. Clin Tech Small Anim Pract. 21(3): 117-127.

Mikolajczyk MG, Concepcion NF, Wang T, Frazier D, Golding B, Frasch CE and Scott DE 2004. Characterization of antibodies to capsular polysaccharide antigens of *Haemophilus influenzae* type b and *Streptococcus pneumoniae* in human immune globulin intravenous preparations. Clin Diagn Lab Immunol. 11(6): 1158-1164.

Minami T, Fukutomi Y, Lidholm J, Yasueda H, Saito A, Sekiya K, Tsuburai T, Maeda Y, Mori A, Taniguchi M, Hasegawa M and Akiyama K 2015. IgE Abs to Der p 1 and Der p 2 as

diagnostic markers of house dust mite allergy as defined by a bronchoprovocation test. Allergol Int. 64(1): 90-95.

Morales CA, Schultz KT and Deboer DJ 1994. Antistaphylococcal Antibodies in Dogs with Recurrent *Staphylococcal* Pyoderma. Vet Immunol Immunopathol. 42(2): 137-147.

Morris DO, Olivier NB and Rosser EJ 1998. Type-1 hypersensitivity reactions to *Malassezia pachydermatis* extracts in atopic dogs. Am J Vet Res. 59(7): 836-841.

Mosmann TR and Coffman RL 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol. 7: 145-173.

Motala C, Potter PC, Weinberg EG, Malherbe D and Hughes J 1986. Anti-Staphylococcus aureus-specific IgE in atopic dermatitis. J Allergy Clin Immunol. 78(4 Pt 1): 583-589.

Moya R, Carnes J, Sinovas N, Ramio L, Brazis P and Puigdernont A 2016. Immunoproteomic characterization of a *Dermatophagoides farinae* extract used in the treatment of canine atopic dermatitis. Vet Immunol Immunopathol. 180: 1-8.

Nicolaou N, Murray C, Belgrave D, Poorafshar M, Simpson A and Custovic A 2011. Quantification of specific IgE to whole peanut extract and peanut components in prediction of peanut allergy. J Allergy Clin Immunol. 127(3): 684-685.

Noli C, Bernadina WE and Willemse T 1996. The significance of reactions to purified fractions of *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* in canine atopic dermatitis. Vet Immunol Immunopathol. 52(3): 147-157.

Nuttall T 2013. The genomics revolution: will canine atopic dermatitis be predictable and preventable? Vet Dermatol. 24(1): 10-18 e13-14.

Nuttall TJ and Halliwell REW 2004. Serum antibodies to *Malassezia* yeasts in canine atopic dermatitis (vol 12, pg 327, 2001). Vet Dermatol. 15(5): 339-339.

Nuttall TJ, Hill PB, Bensignor E, Willemse T and Derm ITFCA 2006. House dust and forage mite allergens and their role in human and canine atopic dermatitis. Vet Dermatol. 17(4): 223-235.

Nuttall TJ, Knight PA, McAleese SM, Lamb JR and Hill PB 2002. T-helper 1, T-helper 2 and immunosuppressive cytokines in canine atopic dermatitis. Vet Immunol Immunopathol. 87(3-4): 379-384.

Nuttall TJ, Lamb JR and Hill PB 2001. Characterisation of major and minor *Dermatophagoides* allergens in canine atopic dermatitis. Res Vet Sci. 71(1): 51-57.

Oldenhoff WE, Frank GR and DeBoer DJ 2014. Comparison of the results of intradermal test reactivity and serum allergen-specific IgE measurement for *Malassezia pachydermatis* in atopic dogs. Vet Dermatol. 25(6): 507-511, e584-505.

Olivry T, Dunston SM, Favrot C, Prelaud P and Tsukui T 2017. The novel high molecular weight *Dermatophagoides farinae* protein Zen-1 is a major allergen in North American and European mite allergic dogs with atopic dermatitis. Vet Dermatol. 28(2): 177-e138.

Olivry T and Hill PB 2001. The ACVD task force on canine atopic dermatitis (IX): the controversy surrounding the route of allergen challenge in canine atopic dermatitis. Vet Immunol Immunopathol. 81(3-4): 219-225.

Olivry T, Saridomichelakis M and Icada 2013. Evidence-based guidelines for anti-allergic drug withdrawal times before allergen-specific intradermal and IgE serological tests in dogs. Vet Dermatol. 24(2).

Osawa R, Akiyama M and Shimizu H 2011. Filaggrin gene defects and the risk of developing allergic disorders. Allergol Int. 60(1): 1-9.

Park KH, Lee J, Lee JY, Lee SC, Sim DW, Shin JU, Park CO, Lee JH, Lee KH, Jeong KY and Park JW 2018. Sensitization to various minor house dust mite allergens is greater in patients with atopic dermatitis than in those with respiratory allergic disease. Clin Exp Allergy. 48(8): 1050-1058.

Perreten V, Chanchaithong P, Prapasarakul N, Rossano A, Blum SE, Elad D and Schwendener S 2013. Novel pseudo-*staphylococcal* cassette chromosome mec element (psiSCCmec57395) in methicillin-resistant *Staphylococcus pseudintermedius* CC45. Antimicrob Agents Chemother. 57(11): 5509-5515.

Pittner G, Vrtala S, Thomas WR, Weghofer M, Kundi M, Horak F, Kraft D and Valenta R 2004. Component-resolved diagnosis of house-dust mite allergy with purified natural and recombinant mite allergens. Clin Exp Allergy. 34(4): 597-603.

Plant JD, Gortel K, Kovalik M, Polissar NL and Neradilek MB 2012. Development and validation of the Canine Atopic Dermatitis Lesion Index, a scale for the rapid scoring of lesion severity in canine atopic dermatitis. Vet Dermatol. 23(6).

Plant JD, Neradelik MB, Polissar NL, Fadok VA and Scott BA 2014. Agreement between allergen-specific IgE assays and ensuing immunotherapy recommendations from four commercial laboratories in the USA. Vet Dermatol. 25(1): 15-+.

Racine BP, Marti E, Busato A, Weilenmann R, Lazary S and Griot-Wenk ME 1999. Influence of sex and age on serum total immunoglobulin E concentration in Beagles. Am J Vet Res. 60(1): 93-97.

Renand A, Newbrough S, Wambre E, DeLong JH, Robinson D and Kwok WW 2014. Arginine kinase Pen m 2 as an important shrimp allergen recognized by TH2 cells. J Allergy Clin Immunol. 134(6): 1456-1459 e1457.

Salzmann CA, Olivry TJ, Nielsen DM, Paps JS, Harris TL and Olby NJ 2011. Genome-wide linkage study of atopic dermatitis in West Highland White Terriers. BMC Genet. 12: 37.

Santos AB, Chapman MD, Aalberse RC, Vailes LD, Ferriani VP, Oliver C, Rizzo MC, Naspitz CK and Arruda LK 1999. Cockroach allergens and asthma in Brazil: identification of tropomyosin as a major allergen with potential cross-reactivity with mite and shrimp allergens. J Allergy Clin Immunol. 104(2 Pt 1): 329-337.

Scott DW, Miller, W.H. 2001. Small Animal Dermatology, 6th Edition. W.B. Saunders, Philadelphia,. pp. 574–601.

Selle M, Hertlein T, Oesterreich B, Klemm T, Kloppot P, Muller E, Ehricht R, Stentzel S, Borker BM, Engelmann S and Ohlsen K 2016. Global antibody response to *Staphylococcus aureus* live-cell vaccination. Scientific Reports. 6.

Sookrung N, Wong-din-Dam S, Tungtrongchitr A, Reamtong O, Indrawattana N, Sakolvaree Y, Visitsunthorn N, Manuyakorn W and Chaicumpa W 2014. Proteome and allergenome of Asian wasp, *Vespa affinis*, venom and IgE reactivity of the venom components. J Proteome Res. 13(3): 1336-1344.

Sousa CA and Marsella R 2001. The ACVD task force on canine atopic dermatitis (II): genetic factors. Vet Immunol Immunopathol. 81(3-4): 153-157.

Thomas WR, Smith WA and Hales BJ 2004. The allergenic specificities of the house dust mite. Chang Gung Med J. 27(8): 563-569.

Tohgasaki T, Ozawa N, Yoshino T, Ishiwatari S, Matsukuma S, Yanagi S and Fukuda H 2018. Enolase-1 expression in the stratum corneum is elevated with parakeratosis of atopic dermatitis and disrupts the cellular tight junction barrier in keratinocytes. Int J Cosmet Sci. 40(2): 178-186.

Treudler R and Simon JC 2013. Overview of Component Resolved Diagnostics. Current Allergy and Asthma Reports. 13(1): 110-117.

Ucan US, Altunok V and Maden M 2003. Total serum IgG, lgM IgA and IgE immunoglobulin concentrations in purebred Anatolian Shepherd Dogs. Revue De Medecine Veterinaire. 154(4): 307-310.

Valenta R, Lidholm J, Niederberger V, Hayek B, Kraft D and Gronlund H 1999. The recombinant allergen-based concept of component-resolved diagnostics and immunotherapy (CRD and CRIT). Clinical and Experimental Allergy. 29(7): 896-904.

Vercelli D 2008. Discovering susceptibility genes for asthma and allergy. Nat Rev Immunol. 8(3): 169-182.

Vidarsson G, Dekkers G and Rispens T 2014. IgG subclasses and allotypes: from structure to effector functions. Front Immunol. 5: 520.

Waldron R, McGowan J, Gordon N, McCarthy C, Mitchell EB and Fitzpatrick DA 2019. Proteome and allergenome of the European house dust mite *Dermatophagoides pteronyssinus*. PLoS One. 14(5): e0216171.

Wan H, Winton HL, Soeller C, Taylor GW, Gruenert DC, Thompson PJ, Cannell MB, Stewart GA, Garrod DR and Robinson C 2001. The transmembrane protein occludin of epithelial tight junctions is a functional target for serine peptidases from faecal pellets of *Dermatophagoides pteronyssinus*. Clin Exp Allergy. 31(2): 279-294.

Weber E, Hunter S, Stedman K, Dreitz S, Olivry T, Hillier A and McCall C 2003. Identification, characterization, and cloning of a complementary DNA encoding a 60-kd house dust mite allergen (Der f 18) for human beings and dogs. J Allergy Clin Immunol. 112(1): 79-86.

Williams JW, Tjota MY and Sperling AI 2012. The contribution of allergen-specific IgG to the development of th2-mediated airway inflammation. J Allergy (Cairo). 2012: 236075.

Xing P, Yu H, Li M, Xiao X, Jiang C, Mo L, Zhang M, Yang P and Liu Z 2015. Characterization of arginine kinase, anovel allergen of *dermatophagoides farinae* (Der f 20). Am J Transl Res. 7(12): 2815-2823.

Yamashita K, Fujiwara C, Azuma R, Sawazaki T, Nakao Y and Hasegawa A 2002. Determination of antigenic proteins of housedust mites in 90 dogs suffering from atopic dermatitis. J Vet Med Sci. 64(8): 673-676.

Yasueda H, Mita H, Yui Y and Shida T 1986. Isolation and characterization of two allergens from D*ermatophagoides farinae*. Int Arch Allergy Appl Immunol. 81(3): 214-223.

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.

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AWARD RECEIVED

1. Excellent Presentation Award 17thCUVC for oral presentation in the title "Diagnostic evaluation of specific-IgG1 to house dust mite allergen in atopic dogs by an-in house ELISA