

DEVELOPMENT OF AN INDIRECT ELISA TO DETECT ANTIBODIES AGAINST *Actinobacillus suis* USING BOILED WHOLE CELL ANTIGEN



A Thesis Submitted in Partial Fulfillment of the Requirements  
for the Degree of Master of Science in Veterinary Biosciences

Department of Veterinary Anatomy

Faculty of Veterinary Science

Chulalongkorn University

Academic Year 2019

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การพัฒนาวีธีอินไดเร็ค อีไลซ่า เพื่อตรวจหาแอนติบอดีต่อเชื้อ แอคติโนบาซิลลัส ซูอิส โดยใช้ไฮลเชลล์  
แอนติเจน



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต  
สาขาวิชาชีวศาสตร์ทางสัตวแพทย์ ภาควิชากายวิภาคศาสตร์  
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ศิริลักษณ์ จันทอุตสาห : การพัฒนาวิธีอินไคเร็ค อีไลซ่า เพื่อตรวจหาแอนติบอดีต่อเชื้อ  
 แอคติโนบาซิลลัส ซูอิส โดยใช้โฮลเซลล์แอนติเจน. ( DEVELOPMENT OF AN  
 INDIRECT ELISA TO DETECT ANTIBODIES AGAINST *Actinobacillus suis* USING  
 BOILED WHOLE CELL ANTIGEN ) อ.ที่ปรึกษาหลัก : รศ. สพ.ญ.ดร.ศยามณ ศรีสุ  
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 รุ่งสิทธิชัย

เชื้อแอคติโนบาซิลลัส ซูอิส จัดเป็นเชื้อฉวยโอกาสในสุกร ซึ่งสามารถก่อโรคได้ในสุกรทุกช่วงอายุ โดยเฉพาะอย่างยิ่งในฝูงที่มีการติดเชื้อที่สำคัญเป็นหลัก วัตถุประสงค์ของการศึกษานี้เพื่อพัฒนาวิธีอินไคเร็ค อีไลซ่า โดยใช้การต้มโฮลเซลล์แอนติเจน เพื่อตรวจหาแอนติบอดีต่อเชื้อแอคติโนบาซิลลัส ซูอิส โดยศึกษาในฝูงที่มีอาการทางคลินิกเกี่ยวข้องกับการติดเชื้อแอคติโนบาซิลลัส ซูอิส และมีประวัติไม่ได้รับวัคซีนต่อเชื้อแอคติโนบาซิลลัส พลูโรโรโมนิอี รวมทั้งไม่ได้ถูกวินิจฉัยว่ามีการติดเชื้อแอคติโนบาซิลลัส พลูโรโรโมนิอีภายในฝูง เชื้อแอคติโนบาซิลลัส ซูอิส ในการศึกษาแยกได้จากปอดของสุกรที่ติดเชื้อ และนำเชื้อไปเตรียมแอนติเจน โดยใช้วิธีต้มโฮลเซลล์แอนติเจน เพื่อนำแอนติเจนไปเคลือบบนผิวของอีไลซ่าไมโครเพลท นอกจากนี้ มีการเก็บเลือดจากฝูงสุกรเดียวกัน ที่ช่วงอายุ 3, 5, 7, 9, 11, 12, 13, 14, 15, 17, 18, 20, 21-25 และ 25 สัปดาห์ และสุกรแม่พันธุ์ท้องที่ 0 (อายุ 34-36 สัปดาห์), ท้องที่ 1-2, ท้องที่ 3-4 และท้องที่ 5 เพื่อตรวจหาแอนติบอดีต่อเชื้อแอคติโนบาซิลลัส ซูอิส โดยวิธีอินไคเร็ค อีไลซ่า และมีขั้นตอนการหาความเข้มข้นที่เหมาะสมโดยการใช้วิธีเซ็กเกอร์บอร์ดไตเตรชัน ผลการศึกษาพบว่า ที่ความยาวคลื่นแสง 450 นาโนเมตร ค่าการดูดกลืนแสงที่ 0.36 ที่ใช้เป็นจุดแบ่งแยกตัวอย่างให้ผลบวกกับผลลบ นอกจากนี้ยังพบค่าการดูดกลืนแสงที่เพิ่มสูงขึ้นในกลุ่มสุกรที่อายุมากกว่า 20 สัปดาห์ ( $p < 0.05$ ) และมีค่าการดูดกลืนแสงระดับสูงสุดในกลุ่มสุกรแม่พันธุ์ ท้องที่ 1-4 ซึ่งพบว่าระดับแอนติบอดีแตกต่างจากกลุ่มสุกรสาวที่อายุน้อยกว่า 25 สัปดาห์ ( $p < 0.01$ ) โดยสรุป การพัฒนาวิธีอินไคเร็ค อีไลซ่าอาจจะไม่ได้เป็นวิธีตรวจมาตรฐาน และยังเป็นวิธีที่ใช้ในการวินิจฉัยที่ไม่สมบูรณ์ เนื่องจากมีความยากในการตรวจหาตำแหน่งที่จำเพาะของแอนติเจนต่อเชื้อแอคติโนบาซิลลัส ซูอิส แต่วิธีอินไคเร็ค อีไลซ่าก็เป็นอีกเครื่องมือที่ใช้ศึกษาซีโรโลยีของสุกรที่สงสัยการติดเชื้อแอคติโนบาซิลลัส ซูอิสภายในฝูง และจากข้อมูลซีโรโลยีสามารถพัฒนาโปรแกรมวัคซีนที่มีต่อเชื้อโรคนั้น เพื่อการจัดการสุขภาพที่ดีภายในฝูง

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# # 5875331231 : MAJOR VETERINARY BIOSCIENCES

KEYWORD: Actinobacillus suis, boiled whole cell antigen, indirect ELISA, pigs

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*Actinobacillus suis* is considered an opportunistic pathogen in swine that can cause disease in animals of all ages, especially in high health status herds. The objective of this study was to develop an indirect enzyme-linked immunosorbent assay (ELISA) using boiled whole cell antigen for the detection of antibody against *A. suis*. The selected herd has clinical problems associated with *A. suis* infection. In addition, this herd was not received any *Actinobacillus pleuropneumoniae* (APP) vaccine nor diagnosed with APP infection before the study. The isolation of *A. suis* was recovered from lung of the infected pig in the selected herd. Moreover, the antigen of *A. suis* was prepared in form of boiled whole cell antigen for coated ELISA plate. Blood samples were collected from pigs in the same herd at 3, 5, 7, 9, 11, 12, 13, 14, 15, 17, 18, 20, 21-25 and 25 weeks of age. Moreover, blood collection was collected from Parity 0 (34-36 weeks of age), P1-P2, P3-P4, and P5 of sows for detection of antibodies against *A. suis* in indirect ELISA test. The ELISA was optimized by using checkerboard titration. As a result, the cut-off value was set at 0.36 (OD<sub>450</sub>). The higher level of optical density (OD) was observed when the pigs more than 20 weeks of age ( $p < 0.05$ ). The highest level of OD related *A. suis* antibody was observed in the sows at the Parity 1-4 in which the level of the *A. suis* antibody was different to gilt aged less than 25 weeks ( $p < 0.01$ ). In conclusion, the development of indirect ELISA technique may not be a gold standard or completely used as diagnostic tool due to the difficulty to detect the conserved antigenicity of *A. suis* antigen. It could be used as an alternative tool for study the dynamic serological profile of the suspected pigs infected with *A. suis* in the herd and these data could be applied to develop the appropriate vaccination protocol against these pathogens for herd health management.

Field of Study: Veterinary Biosciences

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## ACKNOWLEDGEMENTS

Firstly, I would like to express my sincere gratitude to my advisor Assoc. Prof. Dr. Sayamon Srisuwatanasagul for obtaining from her lot of helps and giving me an opportunity for my study and research. Her guidance helped me in all the time of research and writing of this thesis. Besides my advisor, I would like to thank the rest of my thesis co-advisors: Assoc. Prof. Dr. Sukanya Manee-In and Asst. Prof. Dr. Atthaporn Roongsitthichai for their suggestion and comments in my study life. My sincere thanks to Dr. Pornchalit Assavacheep who is numerous helpful suggestions and supports the sample used in this study. Likewise, I would like to thank Asst. Prof. Dr. Saritvich Panyaboriban who supports statistic in this research.

Moreover, I would also like to thank Mrs. Pussadee Makum and Mrs. Sukuma Samngamnim who support all in the laboratory. In addition, I would like to thank all of the instructors and staffs at biochemistry unit, department of physiology, department of anatomy, Faculty of Veterinary Science, Chulalongkorn University, for all kind supports. I would like to thank Faculty of Veterinary Sciences, Maharakham University and Faculty of Veterinary Science, Chulalongkorn University for supporting the grants in education fee and my research.

Last but not least, I would like to thank my family for supporting me throughout my study. Finally, my friends are willing to help me.

Siriluck Juntautsa

## TABLE OF CONTENTS

	Page
.....	iii
ABSTRACT (THAI).....	iii
.....	iv
ABSTRACT (ENGLISH).....	iv
ACKNOWLEDGEMENTS.....	v
TABLE OF CONTENTS.....	vi
LIST OF TABLES.....	viii
LIST OF FIGURES.....	ix
CHAPTER I INTRODUCTION.....	2
CHAPTER II LITERATURE REVIEW.....	4
2.1 <i>Actinobacillus suis</i> .....	4
2.2 <i>A. suis</i> identification.....	6
2.3 ELISA.....	7
2.4 Using boiled whole cell as the antigen in ELISA.....	7
CHAPTER III MATERIALS AND METHODS.....	9
3.1 Animals.....	9
3.2 Identification of <i>A. suis</i> .....	9
3.3 Preparation of <i>A. suis</i> antigens.....	10
3.4 Positive control serum.....	12
3.5 Sample collection.....	12
3.6 Development of ELISA.....	12

3.6.1 Indirect ELISA Procedure .....	12
3.6.2 Checkerboard Titration .....	13
3.6.2.1 Antigen Titration.....	13
3.6.2.2 Serum and Conjugate .....	14
3.6.3 Detection of antibodies to <i>A. suis</i> .....	15
3.7 Statistical Analysis .....	18
CHAPTER IV RESULTS .....	19
4.1 Quantification of <i>A. suis</i> antigen.....	19
4.2 Optimization of ELISA.....	19
4.3 Results of Indirect ELISA .....	21
CHAPTER V DISCUSSION .....	24
CHAPTER VI FUTURE PERSPECTIVE .....	28
REFERENCES .....	29
VITA.....	34



## LIST OF TABLES

	Page
Table 1 The optical density values of serum samples measured by ELISA reader at 450 nm.....	18
Table 2 OD value of antigen titration of <i>A. suis</i> positive serum.....	20
Table 3 OD value of sera and conjugate titration of <i>A. suis</i> positive serum .....	21



## LIST OF FIGURES

	Page
Figure 1 Pure colony of <i>A. suis</i> for preparation of antigen (a) <i>A. suis</i> colonies on the chocolate agar (b) Colonies of <i>A. suis</i> are small, shiny and sticky.....	10
Figure 2 Preparation of boiled whole cell antigen of <i>A. suis</i> (a) the bacteria are harvested and washed with PBS (b) centrifugation at 4°C (c) the bacterial suspension is boiled at 100°C for 20 minutes (d) the liquid phase is collected and filtered through a filter with 0.22 µm pore size (e) after the last centrifugation, the liquid phase is collected and the pellet is discarded.....	11
Figure 3 Dilution of coating antigen and sera. Single dilution of conjugate sets as 1: 1,000.....	14
Figure 4 Dilution of sera and conjugate. Single dilution of antigen is derived from antigen titration step.....	15
Figure 5 ELISA procedure of indirect boiled whole cell of <i>A. suis</i> based ELISA.....	16
Figure 6 ELISA reader (EL808, BioTek Instruments, Inc., Vermont, USA).....	17
Figure 7 96-well microtiter plate is added with substrate (blue) and stop solution (yellow).....	17
Figure 8 Mean and standard deviation of optical density in the pigs sera is collected from <i>A. suis</i> suspected farm.....	22
Figure 9 Mean and standard deviation of optical density when categorized pigs into 4 different groups: A= aged <20 weeks, B= aged 20-25 weeks, C= Parity 0-2 and D= Parity 3-5. Different lowercase letters are represented significant different ( $p < 0.01$ )..	23

## LIST OF ABBREVIATIONS

<i>A. suis</i>	<i>Actinobacillus suis</i>
APP	<i>Actinobacillus pleuropneumoniae</i>
CFU	Colony forming unit
CPS	Capsular polysaccharide
ELISA	Enzyme-linked immunosorbent assay
H <sub>2</sub> SO <sub>4</sub>	Sulfuric acid
IgG	Immunoglobulin G
LPS	Lipopolysaccharide
M	Molar
ml	Milliliter
mm	Millimeter
nm	Nanometer
OD	Optical density
P	Parity
PBS	Phosphate buffered saline
SD	Standard deviation
TBS	Tris buffered saline
TBS-T	Tris buffered saline with 0.1% Tween 20
TMB	3,3',5,5'-Tetramethylbenzidine
µg	Microgram
µl	Microliter
µm	Micrometer
°C	Degree Celsius

## CHAPTER I

### INTRODUCTION

Nowadays, swine production has become more intensive than it was in the past. Most of the piggeries focus on maximum benefit resulting from all pigs in the farms. Nevertheless, pig removal always occurs in every herd, especially commercial herds. The previous study indicated that the removal of the female pigs from the herds was performed on account of two major patterns: planned (e.g., old, high parity number) and unplanned removal (e.g., lameness, abnormal vaginal discharge, udder problems) (Engblom et al., 2007). In addition, the most common reasons to remove the pigs from the herd is reproductive reasons which account for one-third of culling reasons (Tummaruk et al., 2009). This considerably affects economic status of the herd.

One of the targets of most swine industries is piglet production; a number of quality piglet produced imply an economic prosperity of the herds. Unfortunately, all pregnant sows cannot carry the fetuses through the parturition. Undoubtedly, fetal loss or abortion can take place at any time of pregnancy period with infectious and non-infectious reasons. Focusing on infectious agents, a number of pathogens leads to swine abortion, such as porcine Parvovirus, porcine respiratory and reproductive syndrome, porcine Circovirus, and so on. Previously, a study reported that *Actinobacillus suis* contributed to high rate abortion in Croatia. Furthermore, the abortion from *A. suis* infection takes place more frequently in herds with minimal diseases and high replacement rate (Mauch and Bilkei, 2004). Since the early 1990s, *A. suis* has been reported in an increasing number of outbreaks of severe clinical signs in swine of varying ages, particularly in North America, Canada, the United States, and more recently Australia where it is considered an emerging disease

(MacInnes and Desrosiers, 1999; Wilson and McOrist, 2000). In recent data, MacInnes et al. (2008) showed that as many as 94% of the tested herds were suspected to be infected by *A. suis*, although no clinical signs were observed. The laboratory of Minnesota has shown an increased *A. suis* isolation of 0.4% swine during in 2005 compared with 2002 (Oliveira, 2006). Although originally reported as causing septicemia and death in only suckling and recently weaned pigs, disease can be observed in suckling, weaning, fattening pigs, and even adult animals, especially in high health status herds (Yaeger, 1995; Yaeger, 1996). Prevalence of disease may be greater in relatively new herds, before animals develop immunity (Wilson and McOrist, 2000). Therefore, *A. suis* has emerged as a new threat to swine production, particularly in high health status herds; and the occurrence of the disease in this group of pigs has been attributed to lack of herd immunity in these closed herds, but there is minimal information about the immunogenicity of *A. suis* (Yaeger, 1996; MacInnes and Desrosiers, 1999; Wilson and McOrist, 2000; Lapointe et al., 2001; Oliveira, 2007).

To determine the manifestation of *A. suis*, bacterial culture and biochemical tests can be a confirmative approach (Yaeger, 1996). In suspected pigs, lung, liver, spleen, lymph nodes, joints, and exudates are organs to collect to culture (Mauch and Bilkei, 2004). Nevertheless, sample collection and bacterial culture might be considered a complicated and time-consuming method. Moreover, the information pertaining to serological diagnosis for *A. suis* has been limited and in dispute. The present study, accordingly, aims to develop the diagnostic method for detecting antibody against *A. suis* by an indirect ELISA using boiled whole cell antigen in order to be one of the diagnostic tools for *A. suis* infection.

## CHAPTER II

### LITERATURE REVIEW

#### 2.1 *Actinobacillus suis*

In pigs, *A. suis* is considered an opportunistic pathogen (Sanford et al., 1990) which has been first described by van Dorssen and Jaartsveld (1962). It is categorized as a member of Family *Pasteurellaceae*, genus *Actinobacillus*. In microbiological aspect, it is a gram-negative, non-motile, facultative anaerobic, coccobacillus bacteria (MacInnes and Lally, 2006) growing well on blood and MacConkey agars with round, 1–2 mm smooth translucent colonies within 12–24 hours after inoculation. In addition,  $\beta$  hemolysis was found on calf and sheep blood agars. Although the survival ability of *A. suis* in the environment has not been fully reported, the evidence of persistence showed that it existed in the affected herd for at least 18 months (Miniats et al., 1989). Nevertheless, the destruction of *A. suis* can be taken place within 15 minutes at 60°C. In addition, it is sensitive to most kinds of disinfectants, and will be disappeared within a few days in clinical specimens (Oliveira, 2007). Although it is generally considered a pathogen in swine, *A. suis* or *A. suis*-like bacteria have been isolated sporadically from other species, including a Canadian goose (Maddux et al., 1987), an alpaca (Hill and Johnstone, 1992), a cat (Daignault et al., 1999), calves (DeBey et al., 1996), and horses (Kim et al., 1976). In humans, *A. suis* has been reported to initiate severe infection after getting bitten by a pig (Escande et al., 1996). Therefore, it is important that the infected animals in the herd should be identified and treated.

*A. suis* has emerged as a new threat to swine production, particularly in high health status herds of all ages since these pigs lack specific and cross-reactive immunity (MacInnes and Desrosiers, 1999). The infection of *A. suis* is able to be

happened via aerosol route or by close contact with the infected animals. Generally, tonsils, nostrils, and upper respiratory tract are the organs where *A. suis* was found. Besides, it often resides asymptotically within nasopharynx and palatine tonsils of the pigs (Sanford et al., 1990; MacInnes et al., 2008). Moreover, it can be isolated as a commensal organism from the alimentary and genital tracts in the healthy sows (MacInnes and Bosse, 2004).

Clinical signs manifest septicemia and sudden death in suckling and weaned piglets, and is associated with various clinical conditions in older animals, including pneumonia, meningitis, arthritis, pericarditis, endocarditis, enteritis, mastitis, metritis, erysipelas-like cutaneous lesions, and abortion. In addition to septicemia, sudden death could be found, but mortality is usually low (Miniats et al., 1989; Sanford et al., 1990; Yaeger, 1996; MacInnes and Desrosiers, 1999). Differential diagnoses should be focused on since its lesions and clinical signs are similar to other diseases. The outbreak of diseases caused by *A. suis* resembling erysipelas has been reported in sows in Canada (Miniats et al., 1989). Apart from that, rhomboid erythematous skin lesions produced by *Erysipelothrix rhusiopathiae* and *A. suis* are very similar to each other and cannot be distinguished clinically (Sanford and Miniats, 1988; Yaeger, 1995). In particular, septicemic form of *A. suis* in neonates can easily be confused with septicemic lesions from *Escherichia coli* (Mauch and Bilkei, 2004). In addition, pneumonic lesions from *A. suis* and *A. pleuropneumoniae* are highly comparable (Yaeger, 1995). Furthermore, fibrinous pleuritis and pericarditis lesions produced by *Haemophilus parasuis* resemble those caused by *A. suis*.

Presently, the pathogenicity resulted from *A. suis* is not clearly understood. Previous reports demonstrated that potential virulence factors of this microorganism include the production of toxins, capsule, and urease, including the resistance to complement-mediated destruction. Genetically *A. suis* come with genes encoding

pore-forming protein toxins belonging to the RTX (repeats in the structural toxin) family and are very similar to *ApxI* and *ApxII* of *A. pleuropneumoniae* (Kamp et al., 1994; Frey and Kuhnert, 2002), which results in the development of similar lesions caused by these two bacteria (Jeannotte et al., 2002). Considering virulence of *A. suis*, it includes two RTX toxins (*ApxI* and *ApxII*), capsular polysaccharides (CPSs) and lipopolysaccharides (LPSs), iron-regulated outer-membrane proteins, and resistance to complement-mediated killing (Bahrami and Niven, 2005; Ojha et al., 2007). Despite many similarities between *A. pleuropneumoniae* and *A. suis* was reported, host of *A. suis* was broader than that of *A. pleuropneumoniae* (Jeannotte et al., 2002).

Commercial vaccination and serodiagnostic tests for *A. suis* are complicated due to multiple serotypes and cross-reaction with other microorganisms (MacInnes and Desrosiers, 1999; Lapointe et al., 2001). In addition, some farmers administer autogenous vaccines against *A. suis* to the pigs in the herd in order to enhance specific humoral immunity in the replacement gilts (Lapointe et al., 2001), but the vaccine efficacy has not been critically assessed. To determine the duration of passive immunity, serological profile could be applied in a specific herd to help implement the strategy of the best vaccination program.

## 2.2 *A. suis* identification

The identification of *A. suis* can be done by biochemical test as well as genetically test. Additionally, the sensitivity test to antimicrobial drugs is used for the classification of *A. suis*. The bacteriological analysis can be taken from different tissues such as liver, lung, kidney of infected animals and can be inoculated on blood agar (Jeannotte et al., 2002). Gram stain of *A. suis* showed gram-negative pleomorphic rods. In addition, this bacterium showed catalase positive and fermented lactose, hydrolyzed esculin but not produced acid from mannitol. The



biochemical characteristic of *A. suis* isolation has been concluded by Rullo et al (2006).

### **2.3 ELISA**

The principle of an ELISA is the binding ability of antigen and antibody that can be used to detect either antigen or antibody in the unknown samples which depend on the design of the test procedure (Hongbao et al., 2006). The main four steps of ELISA, consist of coating, blocking, reacting between antigen and antibody, and developing color.

#### **Indirect ELISA**

The indirect ELISA system has the advantage that several antisera can be examined by using a single antispecies conjugate. The indirect ELISA has been widely used in diagnostic applications, especially when examining large numbers of samples. In general, the antigen is coated onto the solid phase by passive adsorption. Then, the unknown samples are incubated with the solid phase and attach to the antigen if there is any specific antibody to that attached antigen. An antiglobulin enzyme conjugate is added and incubated, followed with the addition of enzyme substrate in order to develop color. In the final step, the color will be measured by the amount of the conjugate, which can be calculated for the proportion of the antibody level in the tested sample (Voller et al., 1978).

#### **2.4 Using boiled whole cell as the antigen in ELISA**

There are several advantages of using boiled whole cell antigen for indirect ELISA. By coating the ELISA plate with this antigen, the level of sera antibody could be detected. The preparation of the whole cell antigen is simple and need no laborious laboratory work. In addition, this technique provides the antigenicity to the

antibody of interested which allowed to use in the test that need high sensitivity and, in the case, that other technique is not available for the antigen of interest (Elder et al., 1982). However, the whole cell antigen ELISA presented the low specificity and may give high false positive of the test. As it has been demonstrated by the detection of *A. pleuropneumoniae* using whole cell ELISA that false positive results could be observed. Thus, the specificity of the whole cell ELISA assay could be improved to 88% by using mean OD plus 4 times SD as the cut-off value (Gottschalk et al., 1994). In addition, to avoid this problem and to increase the specificity of the test, the whole cell antigen may need the formaldehyde pre-treatment in order to eliminate cell growth if the antigen was coated on ELISA plate overnight. Though formaldehyde can slightly modify the structure on the protein, it can also stabilize protein and membrane so that the surface antigen still be able to be recognized by the antibody.

Regarding the use of saline extract boiled formalinized whole cell ELISA in the *Actinobacillus* assay, the cross reactions were also observed between sera against *A. suis* and *A. pleuropneumoniae* serotype 1. However, the specificity was increase by using the mean OD of negative sample plus 4 SD as mentioned above or using the long chain LPS as the antigen for *A. pleuropneumoniae* instead (Gottschalk et al., 1994). In general, the boiled whole cell antigen of *A. pleuropneumoniae* mainly composed of LPS, proteins and minimal amount of CPS which seem to be responsible for cross-reactions (Radacovici et al., 1992). However, until present, there still be no biochemical characterization of the antigenic *A. suis* whole cell.

## CHAPTER III

### MATERIALS AND METHODS

#### 3.1 Animals

The present study was conducted in commercial farrowing-to-fattening swine herd in an intensive pig producing area of Thailand. The number of sows-on-production and fattening pigs have 3,800 sows and 20,000 pigs, respectively. Moreover, the pigs were accommodated in the open houses through the study period. The selected herd had a history of porcine reproductive and respiratory syndrome (PRRS) infection before the experiment that caused contributed to *A. suis* bacterial infection. In addition, the selected swine herd was not received any *A. pleuropneumoniae* (APP) vaccine and not detected APP in both bacterial culture and ELISA ApxIV.

#### 3.2 Identification of *A. suis*

The isolation of *A. suis* was recovered from lung of infected pig submitted for necropsy with suspected clinical signs of *A. suis* infection including septicemia, purple discoloration of the ears and swollen joints.

Briefly, the lung of necropsy pig was swabbed by a sterile cotton that was streak on 5% sheep blood and MacConkey agar. Then, the inoculated plates were incubated at 37°C with 5% CO<sub>2</sub> for 24-72 hours. Thereafter, pink colonies can grow on MacConkey agar and another sheep blood agar plate had  $\beta$  hemolysis and small colonies that was picked up for test positive catalase (Quinn et al., 1994).

Next, the colony on sheep blood agar plate was picked up to streak on blood agar for subculture to examine biochemical test of *A. suis* including urease, esculin,

lactose, maltose, sucrose positive and mannitol negative. Finally, the identified isolation of *A. suis* was stored at  $-20^{\circ}\text{C}$  in skimmed milk until preparation of antigen.

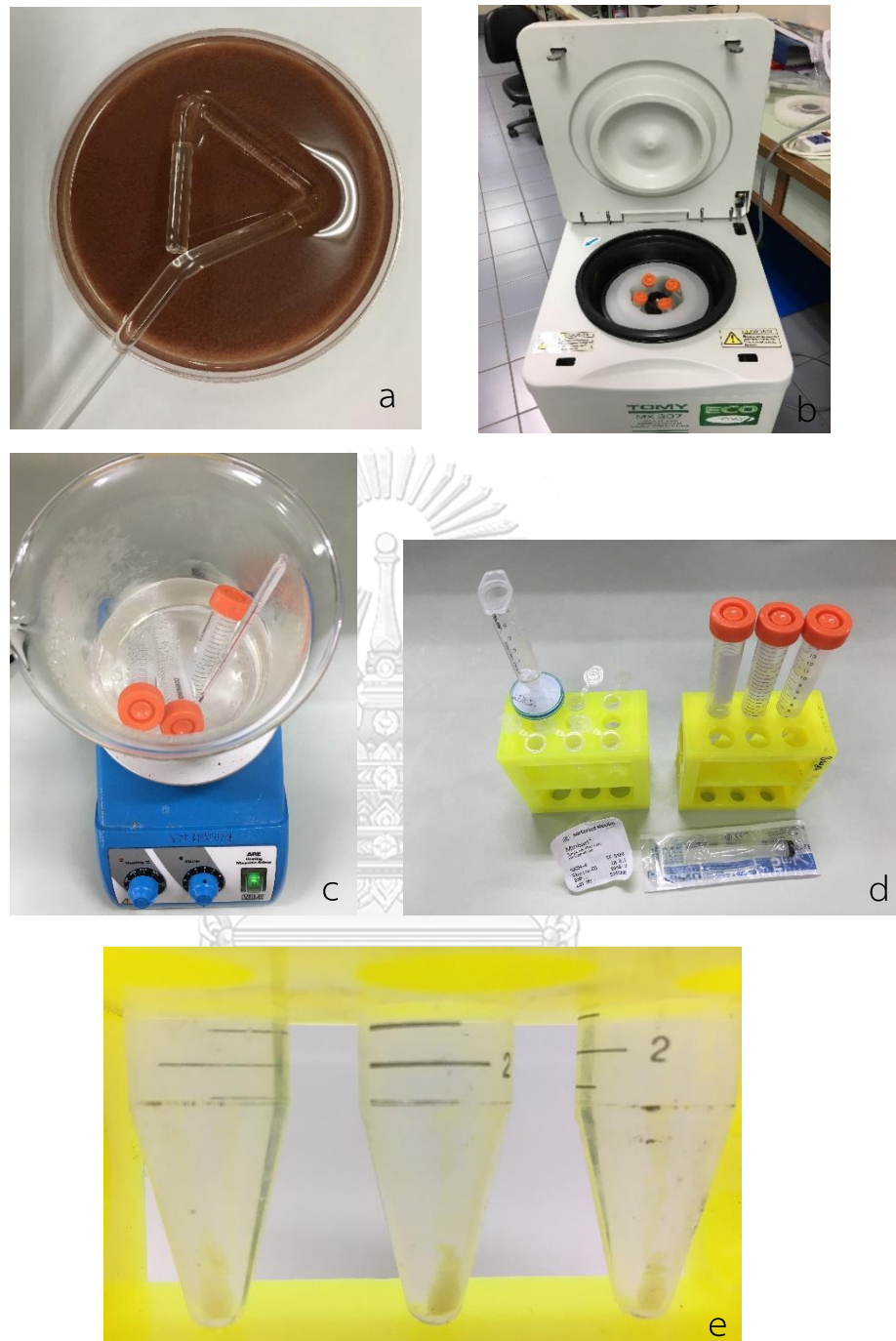
### 3.3 Preparation of *A. suis* antigens

In the present study, antigen of *A. suis* was prepared in form of boiled whole cell antigens which was produced from pure culture of freshly made overnight culture of *A. suis*. Briefly, *A. suis* was cultivated on TYE/ NAD agar and incubated overnight at  $37^{\circ}\text{C}$  with 5-10%  $\text{CO}_2$ . Thereafter, the bacteria were harvested, washed three times with phosphate buffered saline (PBS), and centrifuged at  $4^{\circ}\text{C}$  for 10 minutes at 12,000 g. Afterwards, the bacteria were re-suspended in 20 times of their own volumes of PBS. The bacterial suspension, thereupon, were stored in sterile test tubes, left boiled at  $100^{\circ}\text{C}$  for 20 minutes prior to being stored at room temperature for 1 hour. Thereafter, the solutions were centrifuged at  $4^{\circ}\text{C}$  for 20 minutes at 13,000 g. After the centrifugation, the liquid phase were collected, filtered through a filter with  $0.22\ \mu\text{m}$  pore size, and stored at  $-20^{\circ}\text{C}$  until used.

After preparation, *A. suis* antigen was measured by spectrophotometer at 280 nm in order to measure the level of antigen protein used in the present study.



**Figure 1** Pure colony of *A. suis* for preparation of antigen (a) *A. suis* colonies on the chocolate agar (b) Colonies of *A. suis* are small, shiny and sticky.



**Figure 2** Preparation of boiled whole cell antigen of *A. suis* (a) the bacteria are harvested and washed with PBS (b) centrifugation at 4°C (c) the bacterial suspension is boiled at 100°C for 20 minutes (d) the liquid phase is collected and filtered through a filter with 0.22  $\mu\text{m}$  pore size (e) after the last centrifugation, the liquid phase is collected and the pellet is discarded

### 3.4 Positive control serum

The positive sera to *A. suis* were obtained from four 5-week-old piglets which were inoculated by intramuscular injection of  $1 \times 10^6$  CFU/ml of *A. suis* for three times at two-week interval (Slavic et al., 2000). Two weeks after the last injection, the blood samples were collected from these pigs; thereafter, they were centrifuged and stored at  $-20^\circ\text{C}$  until assay. These sera were used as pooled positive control in all plates of ELISA examined.

### 3.5 Sample collection

Jugular venipuncture was conducted in order to acquire whole blood from pigs aged 3 weeks (n=10), 5 weeks (n=9), 7 weeks (n=10), 9 weeks (n=10), 11 weeks (n=5), 12 weeks (n=5), 13 weeks (n=5), 14 weeks (n=5), 15 weeks (n=5), 17 weeks (n=5), 18 weeks (n=5), 20 weeks (n=29), 21-25 weeks (n=10), 25 weeks (n=5). Moreover, blood collection was collected from P0 (34-36 weeks) (n=9), P1-P2 (n=10), P3-P4 (n=10), and P5 (n=10) sows. The sows in this study had parity 0 to 5. The pigs at each different age in the same herd were randomly selected to the study. The blood samples were centrifuged and the serum was collected and stored at  $-20^\circ\text{C}$  for detection of antibodies against *A. suis* in ELISA test. In addition, before centrifuged whole blood were kept for other further investigations.

### 3.6 Development of ELISA

#### 3.6.1 Indirect ELISA Procedure

Enzyme-linked immunosorbent assays operated on principles very similar to other immunoassay technologies. In an ELISA, an antigen must be immobilized to a solid surface and then complexed with an antibody that was linked to an enzyme. Detection was accomplished by assessing the conjugated enzyme activity via incubation with a substrate to produce a measureable product.

Antibody can be detected or quantitatively determined by indirect ELISA. In this technique, antigen was coated on the microtiter plate. Serum sample containing primary antibody was added to the microtiter plate and allowed to react with the coated antigen. Any free primary antibody was washed away and the bound antibody to the antigen was detected by adding an enzyme conjugated secondary antibody that bound to the primary antibody. Unbound secondary antibody was then washed away and a specific substrate for the enzyme is added. Enzyme hydrolyzed the substrate to form colored products. The amount of colored end product was measured by spectrophotometric plate readers that can measure the absorbance of all the wells of 96-well plate.

### 3.6.2 Checkerboard Titration

The checkerboard titration was used to get the optimal conditions of ELISA results. In this study, three variables had been focused including concentration of coating antigen, primary antibodies (serum) and conjugate. The titration could be performed only two variables in one assay by using the ELISA procedure. Checkerboard titration was done as follows.

**3.6.2.1 Antigen Titration** The antigens were initially diluted from 1: 10 - 1: 100 in column 1 to 10 and the other columns were added with diluent only. Then, the sera were diluted from 1: 10 - 1: 70 in row A to G and row H received only diluent. Finally, constant dilution of conjugate was used at 1: 1,000 to each well.

Antigen		1/10	1/20	1/30	1/40	1/50	1/60	1/70	1/80	1/90	1/100		
Serum		1	2	3	4	5	6	7	8	9	10	11	12
1/10	A	●	●	●	●	●	●	●	●	●	●	○	○
1/20	B	●	●	●	●	●	●	●	●	●	●	○	○
1/30	C	●	●	●	●	●	●	●	●	●	●	○	○
1/40	D	●	●	●	●	●	●	●	●	●	●	○	○
1/50	E	●	●	●	●	●	●	●	●	●	●	○	○
1/60	F	●	●	●	●	●	●	●	●	●	●	○	○
1/70	G	●	●	●	●	●	●	●	●	●	●	○	○
	H	○	○	○	○	○	○	○	○	○	○	○	○

**Figure 3** Dilution of coating antigen and sera. Single dilution of conjugate sets as 1: 1,000.

**3.6.2.2 Serum and Conjugate Titration** From the step of antigen titration, the dilution of conjugate was fixed from the recommended level of manufacturer protocol. Thus, dilution of serum was set and performed from 1: 10 - 1: 70 in columns 1 to 7. The conjugate dilution was set and performed dilution from 1: 1,000 - 1: 10,000 in rows A to G. Columns 8 to 12 and row H received only diluent. These dilutions represent in Figure 4.



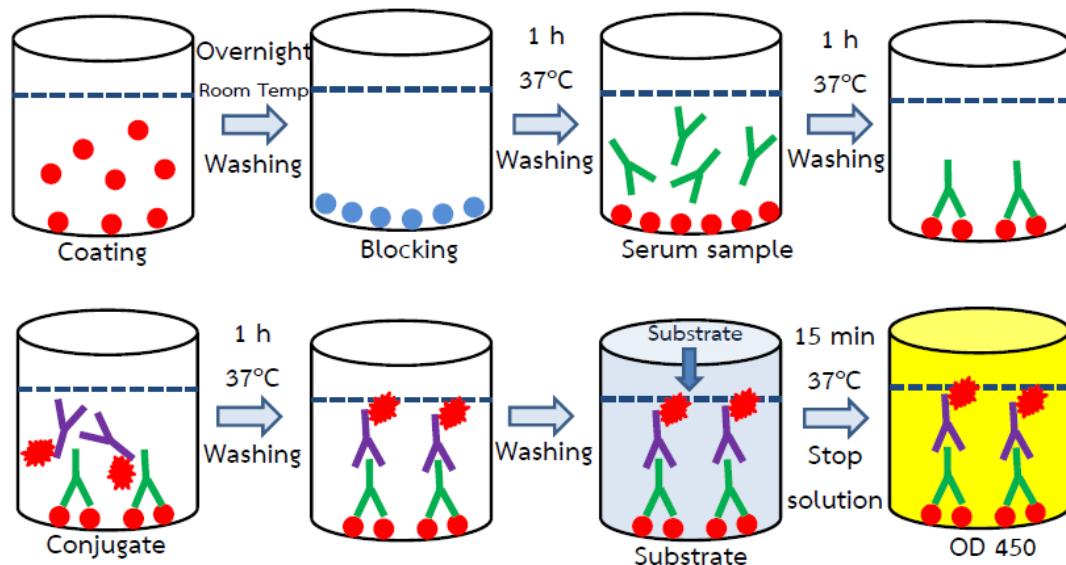
Serum		1/10	1/20	1/30	1/40	1/50	1/60	1/70					
Conjugate		1	2	3	4	5	6	7	8	9	10	11	12
1/1000	A	●	●	●	●	●	●	●	○	○	○	○	○
1/2000	B	●	●	●	●	●	●	●	○	○	○	○	○
1/3000	C	●	●	●	●	●	●	●	○	○	○	○	○
1/4000	D	●	●	●	●	●	●	●	○	○	○	○	○
1/5000	E	●	●	●	●	●	●	●	○	○	○	○	○
1/8000	F	●	●	●	●	●	●	●	○	○	○	○	○
1/10000	G	●	●	●	●	●	●	●	○	○	○	○	○
	H	○	○	○	○	○	○	○	○	○	○	○	○

**Figure 4** Dilution of sera and conjugate. Single dilution of antigen is derived from antigen titration step.

### 3.6.3 Detection of antibodies to *A. suis*

The detection of antibodies to *A. suis* was performed with an indirect ELISA.

The procedure was done as follows (Figure 5).



**Figure 5** ELISA procedure of indirect bovine whole cell of *A. suis* based ELISA

First, each well of 96-well microtiter plate (Thermo Fisher Scientific, Roskilde, Denmark) was coated with 100  $\mu$ l of the diluted *A. suis* antigen 1: 20 in carbonated buffer, pH 9.6 for overnight at room temperature and then 3 times washing with tris buffered saline (TBS) containing 0.1% Tween 20 (TBS-T).

Then, each well of the plate was blocked with 100  $\mu$ l of 4% skimmed milk in TBS-T (blocking buffer) to reduce error from non-specific background and incubated at 37°C for 1 hour following by 3 times washing with TBS-T.

Then, unknown serum samples, negative and positive porcine control diluted serum 1: 20 in blocking buffer were added 100  $\mu$ l into the each well in duplicate and incubated at 37°C for 1 hour following by 3 times washing with TBS-T.

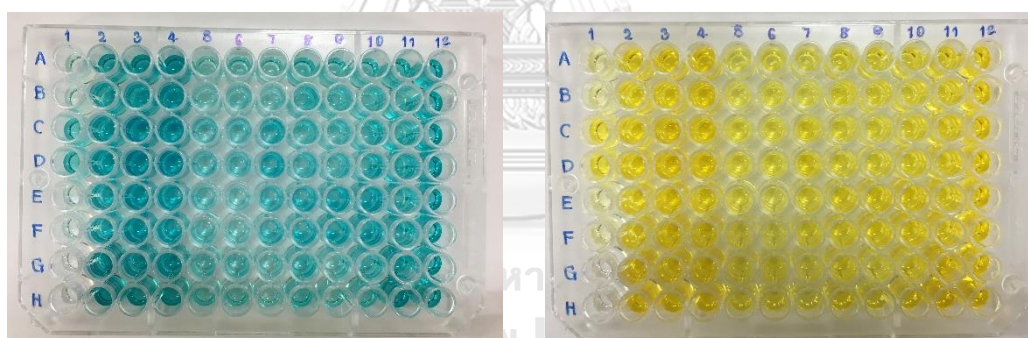
Next, rabbit anti-pig IgG peroxidase antibody (Sigma-Aldrich, St. Louis, Missouri, USA) diluted 1: 2,000 in blocking buffer were added for 100  $\mu$ l into each well and incubated at 37°C for 1 hour following by 3 times washing with TBS-T.

After that, colorimetric reaction was developed by adding 100  $\mu$ l of chromogenic substrate containing tetramethylbenzidine (SeraCare, Milford, USA; TMB = 3,3',5,5'-Tetramethylbenzidine) into each well at incubated 37°C for 15 minutes.

Finally, the reaction was stopped by an addition with 50  $\mu\text{l}$  of 2M  $\text{H}_2\text{SO}_4$  into each well for reading by ELISA reader (EL808, BioTek Instruments, Inc., Vermont, USA) with optical density at the wavelength of 450 nm ( $\text{OD}_{450}$ ).



**Figure 6** ELISA reader (EL808, BioTek Instruments, Inc., Vermont, USA)



**Figure 7** 96-well microtiter plate is added with substrate (blue) and stop solution (yellow).

**Table 1** The optical density values of serum samples measured by ELISA reader at 450 nm

1	2	3	4	5	6	7	8	9	10	11	12
0.280	2.647	3.065	3.238	0.914	1.247	0.954	1.566	1.516	1.470	1.813	2.604
0.278	2.566	3.020	3.403	0.916	1.196	0.957	1.511	1.415	1.577	1.777	2.656
1.465	2.573	3.437	3.268	1.376	1.206	1.885	1.329	1.228	1.752	1.955	2.920
1.489	2.582	4.238	3.366	1.365	1.258	1.895	1.343	1.240	1.698	1.949	2.916
0.173	2.355	3.110	2.230	1.112	0.972	1.518	1.486	1.968	2.470	1.953	2.784
0.160	2.246	3.253	2.211	1.143	0.772	1.456	1.601	2.072	2.471	1.964	2.832
0.045	3.470	2.546	2.680	1.176	1.082	0.772	1.520	1.218	1.616	2.892	2.632
0.045	3.559	2.449	2.726	1.147	1.150	0.814	1.419	1.142	1.631	2.943	2.520

### 3.7 Statistical Analysis

The effect of the age of the pig on the OD results of the serological profile was evaluated with R program. The animals were divided to 17 groups according to ages and parity. Since the data were not normally distributed that calculated by normal quantile plot. The non-parametric method was applied. The effect of age and parity among each group was compared by Kruskal-wallis and Post hoc test. The level of statistical significance was set at  $p < 0.05$ .

## CHAPTER IV

### RESULTS

#### 4.1 Quantification of *A. suis* antigen

The concentration of antigen preparation, boiled whole cell of *A. suis*, was measured by spectrophotometer at absorbance 280 nm. Pooled antigen suspension was added 100  $\mu$ l into cuvette for measuring the protein concentration of *A. suis* antigen. Protein concentration of the pooled antigen was 0.15  $\mu$ g/ml.

#### 4.2 Optimization of ELISA

Checkerboard titration technique was performed with antigen, sera and conjugate titration. To obtain the most controllable results in ELISA, maximum OD values should be about 1.2–1.5 OD units from the plateau maximum color.

Table 2 shows the area in yellow, where there is an optimal amount of antigen coating allowing antibodies to be titrated maximally. Thus, column 2 has enough antigen at dilution 1: 20 for coating microtiter plate with sera and conjugate titration in the new plate of next step.

**Table 2** OD value of antigen titration of *A. suis* positive serum

Antigen	10	20	30	40	50	60	70	80	90	100	-	-
Serum												
10	2.28	1.75	1.49	1.51	1.00	1.28	0.84	1.15	0.75	0.87	0.31	0.35
20	1.86	1.26	1.13	1.12	0.74	1.01	0.60	0.79	0.60	0.64	0.25	0.26
30	1.61	1.08	1.01	0.88	0.61	0.80	0.50	0.69	0.54	0.60	0.20	0.22
40	1.52	1.01	0.92	0.85	0.61	0.76	0.50	0.65	0.47	0.52	0.17	0.18
50	1.43	0.94	0.85	0.77	0.53	0.68	0.46	0.59	0.44	0.40	0.15	0.16
60	1.29	0.83	0.76	0.67	0.46	0.62	0.40	0.49	0.38	0.33	0.13	0.14
70	1.24	0.82	0.71	0.65	0.45	0.57	0.38	0.49	0.39	0.30	0.14	0.15
-	0.05	0.05	0.05	0.05	0.05	0.07	0.06	0.06	0.06	0.06	0.07	0.07

The optimal dilution of the conjugate in table 3 is therefore taken from assessing the plateau maximum. In this study, a dilution of conjugate of 1: 2,000 appears optimal with the serum dilutions used. Thus, the optimal concentrations of antigen, serum and conjugate were 1: 20, 1: 20 and 1: 2,000, respectively.

**Table 3** OD value of sera and conjugate titration of *A. suis* positive serum

Serum	10	20	30	40	50	60	70	-	-	-	-	-
Conjugate												
1000	2.22	1.80	1.49	1.33	1.28	1.17	1.14	0.41	0.32	0.11	0.13	0.14
2000	1.99	1.55	1.34	1.18	1.10	1.06	1.01	0.38	0.31	0.13	0.15	0.16
3000	1.86	1.37	1.26	1.05	1.04	0.92	0.86	0.35	0.30	0.13	0.14	0.15
4000	1.76	1.26	1.10	0.94	0.95	0.87	0.84	0.31	0.26	0.10	0.11	0.12
5000	1.62	1.20	1.02	0.96	0.84	0.82	0.77	0.32	0.27	0.13	0.14	0.16
8000	1.28	0.94	0.83	0.75	0.67	0.64	0.60	0.28	0.24	0.14	0.15	0.16
10000	1.21	0.89	0.76	0.68	0.61	0.57	0.55	0.27	0.24	0.15	0.16	0.17
-	0.06	0.06	0.06	0.07	0.07	0.08	0.09	0.10	0.10	0.12	0.13	0.13

### 4.3 Results of Indirect ELISA

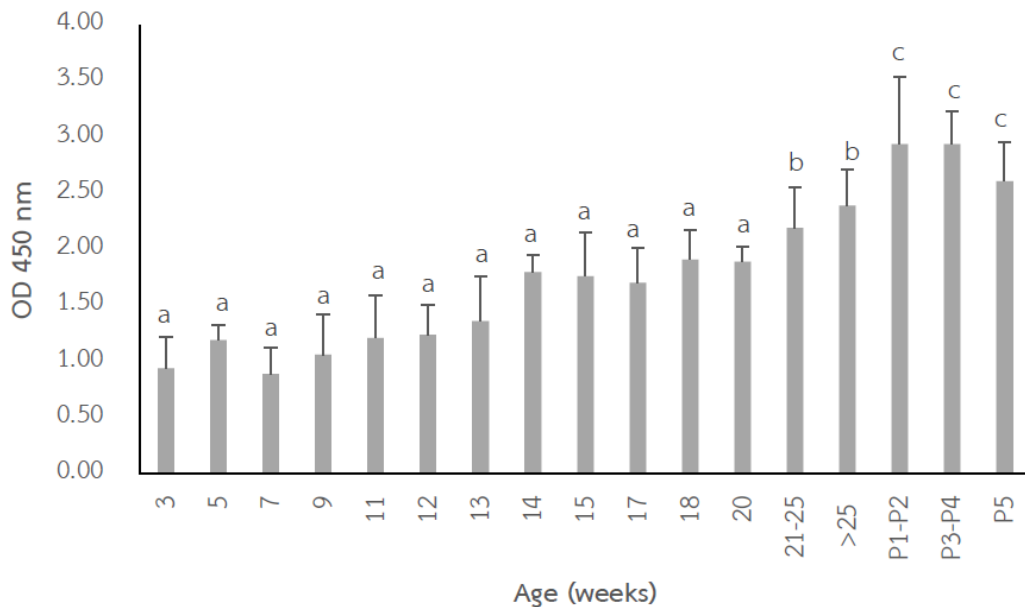
#### The cut-off value

The cut-off value in this study was calculated by the mean OD of the negative serum plus 3 times of the standard deviation. According to the calculation, the cut-off value was 0.36 and therefore, the OD value higher than 0.36 was classified as seropositive against *A. suis* antibodies.

#### ELISA

The results of the mean OD of indirect ELISA using boiled whole cell antigen show in Table 4 according to the different ages of the pig. From the table, it shows that all pigs in the farm have the antibody to *A. suis* by using ELISA method. On the other hand, the OD of the negative control from each plate of ELISA was 0.36 which was used to calculate the cut-off value for this protocol. The positive control sera

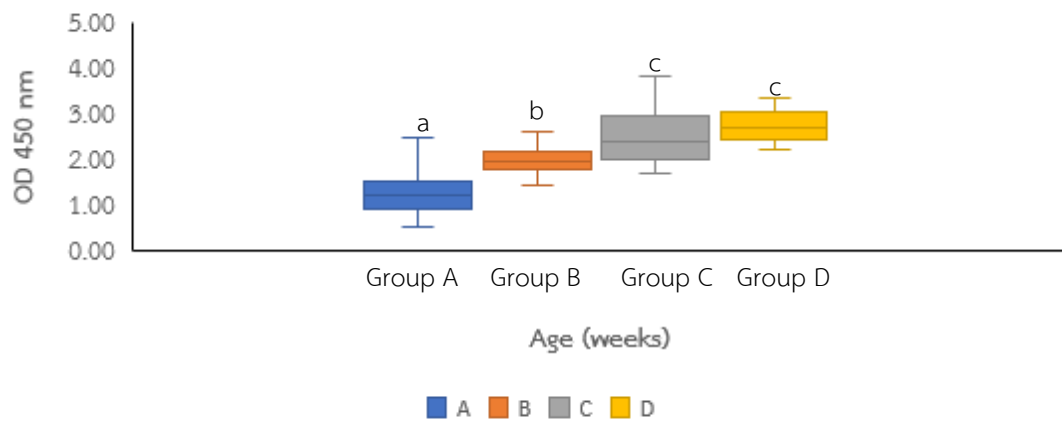
showed high OD for all the plate examined. The mean OD of positive control were 1.20, 1.31, 1.37 and 1.48 (average=1.32).



**Figure 8** Mean and standard deviation of optical density in the pigs sera is collected from *A. suis* suspected farm.

When compared between different ages of the pigs, it showed that higher level of OD was observed when the pigs more than 20 weeks of age ( $p < 0.05$ ). Furthermore, the pig with higher parity showed higher OD related antibody level though it was not statistically significant. The highest level of OD related *A. suis* antibody was observed in the sows at the P1-P4 in which the level of the *A. suis* antibody was different to gilt aged less than 25 weeks ( $p < 0.01$ ).





**Figure 9** Mean and standard deviation of optical density when categorized pigs into 4 different groups: A= aged <20 weeks, B= aged 20-25 weeks, C= Parity 0-2 and D= Parity 3-5. Different lowercase letters are represented significant different ( $p < 0.01$ ).

In order to better understanding the overall picture of the antibody titer changes in the pigs from suspected farm, the samples were re-organized into 4 groups which were group A, pigs which aged less than 20 weeks; group B, pigs aged 20-25 weeks; group C, pigs with parity 0-2 and group D, pigs with parity 3-5. The results showed the tendency of higher level of *A. suis* antibody titer with older pigs. Comparing to the parity of the pigs, higher parity of pigs showed higher OD level though it was not significant different. However, when compared between each parity, it was shown that pig in the parity 5 the lowest level of antibody titer though it was not significantly different from one another (Figure 8).

## CHAPTER V

### DISCUSSION

At the present, *Actinobacillus suis* was known to be an important bacterial infection in high health-status herd. This gram-negative bacterium has emerged the importance in swine industrial and it can infect pigs of all ages. In suckling and weaned piglets, *A. suis* has been shown to cause septicemia and sudden death (Sanford et al., 1990). *A. suis* could be detected in tonsils and nostrils of healthy pigs and also in the reproductive tract of healthy sows (MacInnes and Bosse, 2004). As an opportunistic pathogen, the outbreak of *A. suis* always occur sporadically and represented various clinical signs (Mair et al., 1974). Members of the genus *Actinobacillus* including *A. suis* are associated with mucous membrane infection of the several systems such as respiratory, alimentary, and genital tracts. Though, *A. suis* is generally considered commensals, it is be able to cause diseases with low mortality rate.

From the present results, the boiled whole cell antigen of *A. suis* can detect the antibody level in pig sera by using indirect ELISA while the negative results show only low unspecific binding or background of the test. Regarding antigenicity, *A. suis* has been shown to express both capsular polysaccharides (CPSs) and lipopolysaccharides (LPSs) on its outer surface (Monteiro et al., 2000) which considered the main cell surface component for antigenic structure. The earlier study about the *A. suis* LPS show the common structural features with *A. pleuropneumoniae* and that accounts to the cross-reactivity between *A. suis* and *A. pleuropneumoniae*. By using immunoblotting, the core region of *A. pleuropneumoniae* was similar to that of *A. suis* except for the serotypes 1, 6, 9 and 11 of *A. pleuropneumoniae* (Ganeshpillai et al., 2011). Therefore, cross-reaction

between these proteins will not be observed. For this reason, by using the core region of *A. suis* as the antigen, the antibody detected might be contributed to the diagnostic test for *A. suis* and also other serotypes of *A. pleuropneumoniae* except for the serotypes mentioned above. Further, in the study using different antigens i.e. saline boiled extract and CPS to detect *A. pleuropneumoniae* in other serotypes besides serotype 1, 6, 9 and 11, false positive was observed in pig infected with *A. suis*. This problem could be overcome by using long chain lipopolysaccharide as an antigen (Gottschalk et al., 1994). In addition to specific antigen used, the higher specificity can be improved by increasing the cut-off value from mean negative OD plus 2 SD to mean negative OD plus 4 SD (Gottschalk et al., 1994). Until present, there is no data available for different antigens to develop serological diagnostic tool for *A. suis*. Regarding the antigenicity of *A. suis* by using boiled whole cell extract, the only study about the antigenicity of *A. suis* showed that the ELISA test in that study seemed to recognize the antibody against the O-chain LPS by using immunoblot assay (Lapointe et al., 2001). Furthermore, the ELISA assay used to detect antibody to *A. suis* in that study could not detect other proteins or antigens which were noticed by immunoblotting when using the whole bacteria. From the results of the present study, the boiled whole cell extract was prepared in the same way as that previous study (Lapointe et al., 2001); therefore, it could be speculated that the indirect ELISA technique used boiled whole cell antigen could recognize the antibody against the O-chain LPS.

When considered the result of aged related antibody level of the pigs from the present study, it was obviously shown that the level of antibody to *A. suis* increase with age and parity of the gilts. The low OD at 3-7 week of ages may suggest the interference from maternal immunity. However, when the time passed, the exposure to the antigen may activate the antibody to *A. suis* in the herd as it was shown by the increase in the level of antibody. The present results were in

accordance with the earlier study that the higher antibody level was observed after 16 weeks of age (Lapointe et al., 2001). However, the sera used in the presented study were collected from pigs with no APP vaccination history nor autogenous vaccine to *A. suis*; thus, the higher level of antibody may account only to the exposure to the natural active immunity presented from the herd. When comparing the present study to the earlier study using the same ELISA technique (Lapointe et al., 2001), differences were observed that there was subpopulation of lower immunity of the pig in each experimental group. That study suggested that these subpopulations with low antibody level can be a host of bacterial transmission in herds with problems. From our results, the levels of antibody in each group of pig were similar with low standard deviation and therefore it suggested that all pig in the groups have the same level of *A. suis* immunity when evaluated. However, our study could not detect any difference among the pig antibody level until 21-25 weeks of age. After that, the level of antibody increased significantly.

Regarding the cross reaction of *A. suis* to the related pathogen *A. pleuropneumoniae*, the data from earlier study revealed that though the antigenic epitopes using lipopolysaccharide core-lipid (LPS core lipid) is similar between these two pathogens, the antigenic epitopes and structures of CPS and LPS of *A. suis* differed from those of *A. pleuropneumoniae* serovar 15. Therefore, while using boiled whole cell extract, the antigenicity of *A. suis* was expected from the O-chain LPS which may retain the good specificity as well as good sensitivity for detection of the antibody to *A. suis* (Ganeshapillai et al., 2011). Further, the (1→6)-β-D-glucan was observed in *A. suis* both LPS O-chains and CPS which are commonly found as well in the cell wall components of yeasts, fungi and lichens; therefore, the cross reactivity may derive from these organism surrounding in the environments as well (Monteiro et al., 2000). Though the cross reactivity may be observed, the protective immunity from (1→6)-β-D-glucan may also account to the *A. suis* infection.

Regarding using autogenous vaccine in the herd suspected to have the problem with *A. suis*, the different protocol should be applied in each herd according to the serological profile of the pigs in the herd. The earlier study revealed that *A. suis* vaccine can activate the specific humoral immunity of gilts, especially when the level of antibodies was low prior to vaccination (Lapointe et al., 2001). Therefore, the serological profile of each herd should be of importance that the autogenous vaccine should be administered when the level of *A. suis* antibody was low.

In conclusion, the development of ELISA technique by using boiled whole cell as the antigen could be applied to detect the antibody to *A. suis* in the pig sera. However, due to the difficulty to detect the conserved antigenicity of *A. suis* antigen, this method may not be a gold standard or completely used as a routine diagnostic tool. Instead, it could be used as an alternative tool for studying the dynamic serological profile of the pigs suspected with *A. suis* infection in the herd and these data could be applied to develop the appropriate vaccination protocol against these pathogens for herd health management.

## CHAPTER VI

### FUTURE PERSPECTIVE

From the present study, the serum samples were divided to several groups of age since the dynamic of antibody level could be observed better than in groups with wide range of ages. When considered the possible cross reaction with other relevant antigen i.e. *A. pleuropneumoniae* or other microorganisms which can detect the antigen to *A. suis*, the specificity could be improved by increasing the cut-off value to mean negative value plus 4 SD as described by the earlier study conducted in *A. pleuropneumoniae*. In addition, the pathogen-free serum should be added in the study in order to test the level of specificity of this ELISA method if it is possible. Alternatively, other validated methods such as polymerase chain reaction, western blot may also be applied to test the antibody levels from the pig of the present study (MacInnes et al., 2008). However, as the difficulty of specific antigenicity determination of *A. suis*, serological method may not be practical for diagnosis of *A. suis* infection.

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