การเกิดขึ้นของ exfoliative toxin genes และความไวรับต่อยาต้านจุลชีพของเชื้อ
Staphylococcus hyicus ที่แยกได้จากฟาร์มสุกรในภาคกลางของประเทศไทย

นางเงียน ทิฮาย

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OCCURRENCE OF EXFOLIATIVE TOXIN GENES AND ANTIMICROBIAL

SUSCEPTIBILITY OF *STAPHYLOCOCCUS HYICUS* ISOLATED

FROM PIG FARMS IN CENTRAL THAILAND

Mrs. Nguyen Thi Hai

A Thesis Submitted in Partial Fulfillment of the Requirements

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Department of Veterinary Medicine

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เงี่ยน ทิฮาย: การเกิดขึ้นของ exfoliative toxin genes และความไวรับต่อยาต้านจุลชีพของ เชื้อ *Staphylococcus hyicus* ที่แยกได้จากฟาร์มสุกรในภาคกลางของประเทศไทย (OCCURRENCE OF EXFOLIATIVE TOXIN GENES AND ANTIMICROBIAL SUSCEPTIBILITY OF *STAPHYLOCOCCUS HYICUS* ISOLATED FROM PIG FARMS IN CENTRAL THAILAND).

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การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาการเกิดขึ้นของยีนที่สร้างสารพิษ exfoliative และ ความไวต่อยาต้านจุลชีพของเชื้อ *Staphylococcus hyicus* (*S. hyicus*) ในสุกรที่มีและไม่มีรอย โรค exudative epidermitis (EE) จากฟาร์มสุกรจำนวน 12 แห่ง ในภาคกลางของประเทศไทย เชื้อ *S. hyicus* ที่แยกได้จากตำแหน่งต่าง ๆ ของตัวสุกรที่มีและไม่มี EE และถูกทดสอบด้วยวิธีการ ทางชีวเคมีและวิธีปฏิกิริยาลูกโซ่โพลีเมอเรส และตรวจหา ยีนชีวพิษทั้ง 4 ชนิด (*exhA, exhB, exhD* และ *exhA* และ *exhD*) ด้วยวิธีปฏิกิริยาลูกโซ่โพลีเมอเรสแบบมัลติเพล็กซ์ ทดสอบความไว ของเชื้อสายพันธุ์ที่มียีนชีวพิษและไม่มียีนชีวพิษอย่างละ 40 ตัวอย่างต่อยาต้านจุลชีพ 7 ชนิด ด้วย วิธี broth microdilution ผลการศึกษาพบเชื้อจำนวน 248 ตัวอย่างให้ผลบวกต่อการตรวจยืนยันว่า เป็นเชื้อ *S. hyicus* โดยพบยีนชีวพิษ (*exhA, exhB, exhD, and exhA and exhD*) จาก 63 ตัวอย่างเชื้อ (25.40%) มีอัตราการตรวจพบยีน *exhA* สูงที่สุดจากเชื้อจำนวน 41 ตัวอย่าง (65.07%), ตามมาด้วยยีน *exhD* จำนวน 13 ตัวอย่าง (20.63%), และมี 8 ตัวอย่าง (12.69%) ที่มี การตรวจพบทั้งยีน *exhA* และ *exhD* และมีเพียงตัวอย่างเดียวที่ให้ผลบวกกับ *exhB* อัตราความไว ของเชื้อสูงที่สุดต่อยา Trimethoprim-sulfamethoxazole (81.25%) ตามด้วย methicillin (80%) อัตราการดื้อยาสูงสุดคือ penicillin (75%) ตามด้วย cephalexin (27.5%) โดยสรุปพบการเกิดขึ้น ของยีนชีวพิษ exfoliative สูงในสุกรที่มี EE กว่าสุกรที่ไม่เป็น EE โดยพบยีน *exhA* มากที่สุด เชื้อ *S. hyicus* มีความไวต่อยาต้านจุลชีพที่ไม่แตกต่างกันอย่างมีนัยสำคัญระหว่างสายพันธุ์ที่มีและไม่ มียีนชีวพิษ.

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NGUYEN THI HAI: OCCURRENCE OF EXFOLIATIVE TOXIN GENES AND ANTIMICROBIAL SUSCEPTIBILITY OF *STAPHYLOCOCCUS HYICUS* ISOLATED FROM PIG FARMS IN CENTRAL THAILAND. ADVISOR: ASSOC.PROF. SUPOL LEUNGYOSLUECHAKUL, Ph.D., CO-ADVISOR: PORNCHALIT ASSAVACHEEP, Ph.D., SUPOT WATTANAPHANSAK, Ph.D.,64 pp.

This study was conducted to investigate the occurrence of exfoliative toxin genes and antimicrobial susceptibility of *Staphylococcus hyicus (S. hyicus)* in pigs with and without exudative epidermitis (EE) in the central Thailand. Twelve pig farms were chosen in this study. The *S. hyicus* was isolated from different parts of the pigs with and without EE and was identified by biochemical test, PCR test. Four exfoliative toxin genes *(exhA, exhB, exhD, and exhA and exhD)* were detected by multiplex PCR test. Forty toxin producing strains and forty non-toxin producing strains from isolated strains were selected for their susceptibility test to 7 antimicrobial agents by using the broth microdilution method.

Two hundred and forty eight isolates from collected samples were positive with *S. hyicus.* Exfoliative toxin genes (*exhA, exhB, exhD, and exhA and exhD)* were detected in 63 (25.40%) isolates. The *exhA* gene was the highest prevalent in these toxin genes with 41 isolates (65.07%)*,* following by *exhD* gene with 13 isolates (20.63%), eight (12.69%) isolates were positive for the combination of *exhA* and *exhD* genes. Only one isolate was positive with *exhB*. The highest susceptibility rate was trimethoprim-sulfamethoxazole (81.25%), followed by methicillin (80%). The highest resistant rate was penicillin (75%), followed by cephalexin (27.5%). In conclusion, the prevalence of exfoliative toxin genes was higher in pigs with EE than that of the pigs without EE. The *exh*A was the most popular in the pigs in the central Thailand. Antimicrobial susceptibility was not significantly different between toxigenic strains and non-toxigenic strains.

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

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

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LIST OF ABBREVIATION

µl	=	Microliter
^{0}C	=	Degree Celsius
bp	=	Base pair
Cef	=	Cefotaxime
Cep	=	Cephalexin
CFU	=	Colony - forming unit
CLSI	=	Clinical and Laboratory Standards Institute
DNA	=	Deoxyribonucleic acid
EE	=	Exudative epidermitis
ELISA	=	Enzyme-linked immunosorbent assay
ETA	=	Exfoliative toxin A
ETB	=	Exfoliative toxin B
ETC	=	Exfoliative toxin C
Exh	=	Exfoliative toxin gene
ExhA	=	Exfoliative toxin of *Staphylococcus hyicus* A
ExhB	=	Exfoliative toxin of *Staphylococcus hyicus* B
ExhC	=	Exfoliative toxin of *Staphylococcus hyicus* C
ExhD	=	Exfoliative toxin of *Staphylococcus hyicus* D
Gen	=	Gentamicin
HCl	=	Hydrochloric acid
Met	=	Methicillin
MHB	=	Mueller Hinton broth
MIC	=	Minimum inhibitory concentration
Ntx	=	Non-toxin
PBS	=	Phosphate buffer solution
PCR	=	Polymerase chain reaction
Pen	=	Penicillin

pH	=	The negative logarithm of hydrogen ion concentration
S. hyicus	=	*Staphylococcus hyicus*
SHETA	=	*Staphylococcus hyicus* exfoliative toxin A
SHETB	=	*Staphylococcus hyicus* exfoliative toxin B
SodA	=	Superoxide dismutase A
TAE	=	Tris-Acetate-EDTA
TE	=	Tris-EDTA
Tri-Sul	=	Trimethoprim-sulfamethoxazole
TSA	=	Tryptic soy agar
Tx	=	Toxin
UV	=	Ultraviolet
Van	=	Vancomycin
cm^2	=	Square centimeter
O-F	=	Oxidation fermentative test
Rpm	=	Round per minute

CHAPTER I
INTRODUCTION

Exudative epidermitis (EE) is an infection disease caused by *Staphylococcus hyicus (S. hyicus)*. The disease has been known for over 160 years, which has various names such as greasy pig disease, greasy skin, and marmite disease. The disease mostly occurs in suckling and recently weaned piglets in individual herds, especially in newly established or repopulated herds.

Acute or subacute infection can be found in the conventional pig farms. The acute infected of piglets frequently die because of dehydration or septicemia with 90% mortality rate (Jones, 1956; Wegener and Skov-Jensen, 2006). The disease has been reported in several pig producing countries including Denmark, Russia, Germany, Japan (Kanbar et al., 2006; Futagawa-Saito et al., 2007; Kanbar et al., 2008). The infected piglets can recover after treatment in the early stage of disease with minor clinical signs. In contrast, the recovery time will be longer when piglets are in the late stage of disease with skin lesions covered large parts or all over the piglet body.

The cause of EE is *S. hyicus*, which is a small, round, gram positive bacterium. The colony diameter is about 3-4 mm, white and non-hemolytic on sheep blood agar after 24 hours incubation. The bacterium is coagulase negative by testing on slide, but it may be, sometime, coagulase positive. Fermentation with mannitol and acetoin is negative, and other biochemical characteristics such as heat-stable DNase, lipase, and hyaluronidase are positive. These biochemical characteristics are the significant features to distinguish *S. hyicus* from other staphylococci which found in pigs by conventional methods (L'Ecuyer, 1967; Sato et al., 1991).

Exfoliative toxin products are the main factors causing this disease. These toxins induce EE with special clinical signs; therefore, the virulent strains are named by toxin producing or toxigenic strains. The virulent strains of *S. hyicus* produce several

different exfoliative toxins; these toxins are distinguished by amino acid structures. However, they all seem to have the same functions in causing the disease with particular skin lesions (Andresen et al., 1997). Either crude or purified exfoliative toxins from culture supernates of *S. hyicus* also caused the disease when piglets were injected subcutaneously in the pig skin (Sato et al., 1991; Andresen et al., 1993a). The exfoliative toxins of the virulent *S. hyicus* have been isolated from the affected piglets in Japan and Denmark, and these exfoliative toxins were named as SHETA and SHETB in Japan (Sato et al., 2000) and ExhA, ExhB, ExhC, and ExhD in Denmark (Andresen and Ahrens, 2004).

In order to prevent EE in effective ways, the first thing needs to do is offering pregnant sows a high standard of sanitation, especially housing, and brushing for the pregnant sows also valuable. This is important because *S. hyicus* can be passed from the vagina of the farrowing sows to the piglets at birth. Besides doing these methods, autogenous vaccine of *S. hyicus* has been proposed as a replacement method in the prevention (Wegener et al., 1994). In case of the vaccinated sows with autogenous vaccine, the piglets would be protected from EE by maternal antibody derived from colostrum in the early stage of their lives (Wegener and Skov-Jensen, 1992). Moreover, the autogenous vaccine was made from the virulent strains would be more effective in producing antibody againts *S. hyicus*.

Control of EE may depend mainly on preventing pain and improving the environment with better air ventilation, cleaner and drier farrowing pens. The humidity control also is a useful factor in the prevention program. The last one is stocking density. The crowed herds can cause the piglets fighting and creating the skin injury from on the piglet body; *S. hyicus* will be, therefore, infected to the lesions.

Similar to other pig producing countries, the pig industry in Thailand also has a problem in economic loss because of the EE. Base on pathognomonic lesions on skin of

affected piglets, the disease is easily recognized and continuously identified for several decades in pig farms. One observed the veterinarians and pig producers normally use antimicrobial agents for treatment EE in Thai pig farms. Antimicrobial resistance also occurred in Thai pig farms because of the antibiotic use as a growth promotor in feed and for control and prevention measures. Moreover, autogenous vaccine for this disease was not widely applicable. Therefore, the result of the treatment was not high, and the economic losses still continuously occur in Thai pig producing industry. The important aspects for the control and prevention of this disease in Thailand are that which toxin genes are more prevalent and the parttern of antimicrobial susceptibilities of *S. hyicus* in Thai pig farms.

Therefore, determination of the encoding gene of exfoliation toxins of *S. hyicus* from infected pigs might be benefit in prevention this disease in the future. Autogenous vaccine will be made from toxin producing strains of *S. hyicus* in order to inject to sows prior to farrowing. On the other hand, determination of the antimicrobial susceptibilities from the isolated pathogens will be useful for chosing right antibiotics in treatment EE. Regarding the above benefits, the current research was conducted to get more information not only the disease but also the disease prevention method in futrure. This could be a valuable guideline for a designation of autogenous vaccine, and perhaps a further application for antimicrobial selection regarding to the toxin producing isolates.

CHAPTER II

LITERATURE REVIEW

2.1 Etiology

S. hyicus is generally considered as the main cause of EE, particularly toxic strains. *S. hyicus* contains two strains: virulent and avirulent strains. This classification depends on the capability of producing exfoliative toxins (Andresen et al., 1993; Wegener et al., 1993). Though only the virulent strains can cause the EE, but both virulent and avirulent strains of *S. hyicus* can be isolated from the skin of pigs with and without EE (Park and Kang, 1986). The same ability of producing the exfoliative toxins, some other staphylococci bacteria such as *S. aureus* (van Duijkeren et al., 2007), *S. chromogenes* (Andresen et al., 2005), and *S. sciuri* (Chen et al., 2007), have been identified and isolated from pigs and other animal, although rarely, from cases of EE.

Under a microscope, *S. hyicus* appears small, gram positive cocci, and often stand together in two, short chains of 4 or 5 cocci but sometime in unusual packets. The cells' diameter is 0.6-1.2 µm. In terms of morphology, the bacteria cannot be differentiated between the virulent and avirulent strains. The bacteria grow in nutrient agar after 18-24 hour incubation. The differentiation between *S. hyicus* and other staphylococci based on the biochemical characteristics by conventional methods (L'Ecuyer, 1967; Devriese, 1977; Sato et al., 1991; Sampimon et al., 2009). *S. hyicus* has a strong ability to resist to adverse conditions, and endure in the environment for a long time. In addition, the bacterium can survive several weeks on equipments, floor, and surfaces. Moreover, they can spread in the air and transfer from pens to others (Wegener, 1992). Other animals such as horses, dogs, cattle, goats, and chickens may be considered as carriers (Wegener and Skov-Jensen, 2006).

2.2 Exfoliative toxins

The *S. hyicus* exfoliative toxins are considered as the main factors causing EE with the specific skin lesions (Sato et al., 1991; Wegener et al., 1993b; Andresen et al., 1993; Andresen et al., 1997). However, the role of other factors may also be necessary, but not the main factors to induce EE in piglets.

Recently, the mechanism of action of staphylococcal exfoliative toxins was found and explained as "molecular scissors". *S. hyicus* exfoliative toxins and *S. aureus* exfoliative toxins were showed the same in mechanism activity. The keratinocyte cell-cell bond in the superficial epidermis was cut by the staphylococcal exfoliative toxins (Nishifuji et al., 2008). Three isoforms of *S. aureus* exfoliative toxins, namely as ETA, ETB, and ETD, were glutamate-specific serine proteases. These exfoliative toxins specially and proficiently cut a single peptide bond in the extracellular region of human and mouse desmoglein 1, which was a desmosomal intercellular adhesion molecule. In addition, the same results were also found that 4 isoforms of *S. hyicus* exfoliative toxins, namely as ExhA, ExhB, ExhC, and ExhD, cut the swine desmoglein 1, resulting in the skin exfoliation similar to that with the observed pigs with EE (Fudaba et al., 2005).

Skin exfoliation stimulates sebaceous gland to secrete excessively and serous exudates, resulting in the characteristic "greasy" exterior of the lesions. At these lesions, the skin integrity is damaged and dehydration might occur from losing of fluids and septicemia is possible because the defending wall provided by the skin is lost.

The exfoliative toxins were determined as 27 and 30 kDa (Tanabe et al., 1993; Andresen et al., 1997). The antigenic diversity of *S. hyicus* among exfoliative toxins has been reported. Moreover, at least 6 exfoliative toxins of the virulent *S. hyicus* have been described. In spite of the difference in terms of amino acid sequence, these exfoliative toxins seemed to have the same role of activity in pig skin (Andresen et al., 1997) and

their existence was species-dependent (Takeuchi et al., 2000). The exfoliative toxins of virulent *S. hyicus* have been determined from the affected piglets in some countries such as Japan and Denmark, and were named as SHETA and SHETB in Japan (Sato et al., 2000) and ExhA, ExhB, ExhC and ExhD in Denmark (Andresen, 1998; Andresen and Ahrens, 2004).

The exfoliative toxins designing as SHETA and SHETB in Japan are heat-labile. Targets of SHETs are also epidermal cells in the granular layer of pig skin (Sato et al., 1991; Sato et al., 2000). The difference between two types of exfoliative toxin has been distinguished by immunodiffusion using antisera against exfoliative toxin which is produced by the *S. hyicus* strains. The toxigenic strains can be isolated from both diseased piglets and healthy piglets. However, one strain can produce more than one toxin (Tanabe et al., 1996). The virulent strains may carry a 42 kb plasmid, which carries a gene (*shetB*) encoding for SHETB, whereas the *shetA* gene is located in chromosomal DNA (Sato et al., 1999). A recent research has, however, suggested that the SHETA-encoding gene (*sheta*) is a subtype of *exhB* (Onuma et al., 2011).

The antigenic variation of *S. hyicus* exfoliative toxins was also reported in Denmark. In one study about purification and demonstration of the diversity among the exfoliative toxins, the result showed that polyclonal and monoclonal antibodies against the exfoliative toxins only responded with two of nine well characterized virulent *S. hyicus* strains in both immunoblot analysis and indirect ELISA (Andresen et al., 1997). This result showed different antigenic variants of the toxins (Andresen et al., 1997). Of the cases without EE, toxigenic strains also could be found (Andresen, 1998, 1999a). By in-house indirect ELISA method, researchers have distinguished three different exfoliative toxins that were then designated the three proteins as exfoliative toxins ExhA, ExhB and ExhC, respectively (Andresen, 1999b). It was also showed that virulent *S. hyicus* could produce one of at least three antigenically distinct exfoliative toxins. Although, both ELISA and immunoblot methods could not detect all kinds of exfoliative

toxin. The recent studies of the DNA sequences of the different exfoliative toxins from *S. hyicus* offered a chance to develop a PCR method for detection of the toxin genes. A multiplex PCR method was developed to detect the toxin encoding genes. This method could find four toxin genes that encoded toxin ExhA, ExhB, ExhC, and ExhD of virulent *S. hyicus*. The development multiplex PCR method to detect the toxins provided a more comprehensive method to investigate the prevalence of *S. hyicus* exfoliative toxins in pig herds (Andresen and Ahrens, 2004).

These toxins which were produced by virulent *S. hyicus* were distinctive in the virulent strains compared to avirulent strains (Andresen et al., 1993). Crude or purified exfoliative toxins, which probably appeared in culture supernates of the bacteria, could cause the skin changes when injected subcutaneously into piglet skin (Sato et al., 1991; Andresen et al., 1993; Wegener et al., 1993). Though, a range of other factors of *S. hyicus* may also be necessary, but not much important as the exfoliative toxins to reproduce EE in piglets.

The exfoliative toxin genes such as *exh*A*, exh*B*, exh*C, and *exh*D were detected in Denmark. These toxin genes were also isolated in Japan by a study on the molecular surveillance of *S. hyicus* exfoliative toxin genes in pigs through the country. Actually, the most prevalent of exfoliative toxin genes was *exh*A (Futagawa-Saito et al., 2007). The rate of toxin producing strains that was isolated from the diseased pigs was higher than that from the pigs without EE. Nevertheless, the prevalence of various toxin genes was also different from countries to countries (Andresen, 2005; Futagawa-Saito et al., 2007).

In order to investigate the characteristics of exfoliative toxins of staphylococcus, some studies confirmed a number of phage types could isolated concurrently from the skin of the diseased pig (Wegener et al., 1993; Wegener, 1993a, 1993b). Moreover, the results were also revealed that only one of several clones of *S. hyicus* from diseased pigs could induce EE (Wegener et al., 1993).

2.3 Pathogenesis

It is generally agreed that along with the presence of the virulent strains of *S. hyicus*, there is a requirement for skin wounds which allow the bacteria to invade the epidermis. In addition, the environment and host are also factors that influence on causing the skin lesions.

In order to confirm about the causative agent of EE, purified cultures of virulent *S. hyicus* was scarified skin of a non immunized pigs (Underdahl et al., 1965; L'Ecuyer and Jericho, 1966) or inject subcutanously to specific pathogen free piglets (Underdahl et al., 1965; Wegener et al., 1993) to see clinical of EE. Both of these methods can cause the EE with specific clinical skin lesions, but conventional pigs may be opposed to such methods. This leads to the suggestion the presence of protective immunity in conventional herd. Studies indicated that other elements of the skin bacteria, especially other staphylococci, may contribute to this resistance to colonization (Allaker et al., 1988).

The lesions, that come from fighting, biting, scratching due to rough floor, or pen walls, can allow *S. hyicus* pass through pig skin easily (Wegener and Skov-Jensen, 2006). However, *S. hyicus* may get through the epidermis directly. After the bacteria pass through the epidermis, the earliest change of pig skin is reddish, together with the multiplication of the bacteria on the skin surface. Bacteria grow between the corneocytes of the epidermis. However, inflammation process causes hyperplasia of the stratum corneum, and neutrophils invade to this layer. These lead the epidermis thickenning, continued by erosion. The stratum germinativum becomes disorganized and penetrates deeply into the dermis (Wegener and Skov-Jensen, 2006). Clinical signs develop in gnotobiotic piglets when the number of organisms on the skin exceeds $10^5/cm^2$ (Allaker et al., 1988). *S. hyicus* may adhere to fibronectin in the dermis by fibronectin-binding proteins on the bacterial surface (Lämmler et al., 1985)

In order to prevent the expansion of the bacteria in pig body, the phagocyte-opsonin organization, the first defense action, motivated to fight against the infection; therefore, *S. hyicus* needs to prevent themselves from host defense mechanisms including phagocytosis. There are some factors that *S. hyicus* escapes from phagocytosis. The protein A, for example, is present in the cell wall of most porcine *S. hyicus* strains, potentially reduces opsonization by binding with immunoglobulins at the Fc terminal (Takeuchi et al., 1990). Moreover, capsule of all virulent strains, but not avirulent strains of *S. hyicus* inhibits phagocytosis by neutrophils and macrophages (Wegener, 1990). All porcine *S. hyicus* strains coagulate pig plasma, suggestion a potential for forming combination which may raise protection of the bacterium against phagocytosis. In addition, the production of catalase may protect the bacterium from being killed by the phagocytic cells. All of these properties may support to overcoming the first immune reaction of piglet (Wegener and Skov-Jensen, 2006).

2.4 Clinical signs

The clinical signs of EE are a consequence from the action of exfoliative toxins of virulent *S. hyicus*: the clinical signs firstly appear with redness, swelling at the site of infection in the pig skin. The clinical signs become more severe with series of features such as exfoliative, thick, odorous, and brownish in the axillae and groin. These signs spread to parts or throughout body skin within 3-5 days and after that the lesions quickly turned into dark color and greasy in pig body surface (L'Ecuyer, 1966). Lesions regularly initiate around the face and expand to other parts of the pig body. The skin is covered by dirt and feces. Ulcers may appear in the mouth, and separation of the horn may happen in the hooves. There is no pruritis, and fever is not common.

The disease severity depends on pig age, density, and housing (Wegener and Skov-Jensen, 2006). Some diseased pigs may die from the severity of clinical signs and

dehydration or septicemia, but some individuals can tolerate from chronic disease. In the chronic cases, the lesions remain in some areas of the pig body. Mildly infected piglets may have a yellowish skin, apparently hairy, and have only a few flakes of exudation in the axilla, groin, face, damage on the knees, or adjacent area to badly clipped teeth (L'Ecuyer, 1966).

2.5 Epidemiology

EE was a significant disease in young pigs worldwide. This disease also appear in all major pig-producing countries with the increasing incidence in some regions (Wegener and Skov-Jensen, 1992). This increase may be due to changes in the pig industry. The highest prevalence and the most severe special skin lesions of the disease are normally reported in suckling pigs within the first few days of life.

The factor that affected to the prevalence of this disease was the fighting among the piglets. The fighting occurred in the first 48 hours as the piglets establishing teat. This leads to piglets bite the faces of each others. To reduce the injury of the face among piglets, the cutting of "needle teeth" at birth was a common practice in many pig farms. Another point of time occurred when pigs became at risk of developing EE was the first week after weaning. Piglets from different litters are usually mixed together at weaning and fighting which occurred in order to set up a social order and this frequently results in facial abrasions, which may allow for infection to occur (Wegener and Skov-Jensen, 2006).

Morbidity and mortality rate vary from 20% - 80%. Mortality also depends on the secondary infecting agents present, so mortality may increase up to 90% (Wegener and Skov-Jensen, 2006). After an outbreak, the growth rate of the recovered pigs could be decreased. This leads to a lower productivity of herds down to 35% during an outbreak and to 9% in the year, following infection (L'Ecuyer and Jericho, 1966). The disease

could happen infrequently and with low morbidity among litters in some pig herds. In other herds, however, the disease occurs with high morbidity and infected all litters. This suggests that role of immunity is an important part in terms of disease prevention in individual animal as well as in the herd level. Nevertheless, the significance of immunity in relation to EE has not been comprehensively investigated.

The disease occurs almost in the herds that disease carrier gilts are introduced into a nonimmune herd. Therefore, the offsprings from the nonimmune sows may be potentially infected. All litters in an infected herd may be affected, and the mortality can be up to 70% of affected piglets. The duration of the disease normally lasts for 2–3 months. The disease may vane itself but can be more severe or reoccurred if nonimmune sows are brought into infected buildings or exposed to infected pigs.

The disease can be found at early age as one week of age or among the weaning piglets, possibly as a result of mixing nonimmune litters and litters of immune carriers, and then spread to the farrowing area of the herd. *S. hyicus* can frequently be recovered from the nasal mucosa of healthy pigs, from the conjunctiva, the skin of the snout or ear, and from the vagina in gilts and sows (Wegener and Skov-Jensen, 1992). *S. hyicus* strains *that* present in the vaginal of sows have been recovered from the skin of their offspring. This means that the bacteria can infect the piglets during birth (Wegener and Skov-Jensen, 1992).

2.6 Treatment and prevention

The disease could be treated by antibiotics, and prevented by vaccinating of pregnant sows with autogenous vaccine in order to reduce mortality rate and economic losses (Wegener and Skov-Jensen, 2006). The diseased pigs should be treated with antibiotics at an early stage of disease. Because, treatment is only effective when used at early stage of the disease, thus harshly infected pigs were not often recovered from

the disease. Moreover, the treatment was more responding when combining some certain drugs together such as combination of the trimethoprim and sulfonamides or lincomycin and spectinomycin (Wegener et al., 1994). Antibiotics make a decrease of the severity of the skin lesions, so the lesions only developed on the skin surface. The period of treatment must last for at least 5 days, and clinically affected piglets may slowly recover or remain stunt.

In order to prevent the disease, the sows should be vaccinated by autogenous vaccine. The immunity from the vaccinated sows will confer a protection for piglets for the first period in their life. Antibodies can protect the piglets effectively by neutralizing the effect of the exfoliative toxins in the skin. The autogenous vaccine can be made by using bacterial cell of the strains isolated from infected pigs, or by the exfoliative toxins that extracted from the virulent strain. The occurrence of EE with the specific clinical signs in pigs is remaining a problem for farmers in Thailand. In order to prevent and to treat effectively this disease, the autogenous vaccine should be used in the pregnant sows.

2.7 Antimicrobial susceptibilities

S. hyicus is frequently resistant to antimicrobial agents, but the resistance is different from herd to herd. This resistance has been shown to be predominantly mediated by plasmids (Wegener and Schwarz, 1993). Because of the antimicrobial resistance, the antimicrobial susceptibility test should be done before applying in the treatment.

The resistant rate of *S. hyicus* to some antibiotics in Germany was reported as 66% of isolates resistant to tetracyclines, 100% to sulphonamides, 43% to streptomycin, 25% to penicillin and 3% to erythromycin (Schwarz and Blobel, 1989). In Belgium, the resistant rate of *S. hyicus* to tetracyclines, penicillin, erythromycin was 60%, 60%, 74% respectively, (Devriese, 1977).

A research in Japan found 22% of isolates resistant to tetracyclines, 4% resistant to penicillin, 2% resistant to streptomycin and 40% resistant to erythromycin (Teranishi et al., 1987). On the other hand, the activities of thirteen antimicrobial agents have been tested against 100 *S. hyicus* strains isolated from pigs with EE (Wegener et al., 1994). The result showed that novobiocin was the most active antimicrobial agents against *S. hyicus.* Ceftiofur and enrofloxacin were the second antibiotics against *S. hyicus,* and these antibiotics suggested being use in the treatment of EE in pigs. Furthermore, lincomycin and spectinomycin in combination gave a more activity than the tested individually against the strains (Wegener et al., 1994).

Another study of antimicrobial sensitivity test confirmed the increasing antibiotics resistant ability of *S. hyicus* (Aarestrup and Jensen, 2002). According to this study, penicillin and ampicillin were the most resistant to *S. hyicus,* followed by erythromycin, trimethoprim-sulfamethoxazole, chloramphenicol, kanamycin; however, doxycycline was the most sensitive to *S. hyicus.*

Another study investigated on isolates from 28 healthy and 72 diseased pigs on 100 Danish herds in terms of antimicrobial resistance. Differences in antimicrobial resistance between *S. hyicus* from healthy pigs and pigs affected with EE have been observed. The rates of resistance to penicillin, tetracycline, and streptomycin among the diseased pig isolates were higher than the isolates from healthy pigs (Wegener and Schwarz, 1993).

On the other hand, in Japan, there was no difference in terms of resistance to macrolides and incosamides among strains from both pigs with and without EE (Teranishi et al., 1987). This study used isolates collected from 74 healthy and 50 diseased pigs between 1979 and 1984. The result showed that resistance to at least one antimicrobial was seen in 74% of all strains, in 89% of isolates from healthy

pigs and in 52% of isolates from diseased pigs. The resistant rates of *S. hyicus* to ampicillin, tetracycline, and streptomycin were much higher in strains from healthy pigs than in strains from the diseased pigs. However, the resistant rates of erythromycin were approximately the same in strains isolated from both healthy and diseased pigs.

A possible association between toxin gene carrying strains and resistance gene carrying strains were also investigated (Futagawa-Saito et al., 2009). They used 207 *S. hyicus* strains in the study to test for nine groups of common antimicrobials. These strains included 150 toxigenic and 57 non-toxigenic strains from healthy and diseased pigs. The samples were collected between 1994 and 2002. The result showed that resistant rates in toxigenic strains were higher than in non-toxigenic strains to chloramphenicol (33% and 0%) and trimethoprim-sulphamethoxazole. In contrast, the resistant rates were higher in non-toxigenic strains than toxigenic strains to kanamycin (47% and 9%) and erythromycin. However, there was no difference between the two groups with using penicillin/ampicillin.

The results from studies showed that the correlation could not be found between toxin gene carrying strains and resistance gene carrying strains. However, higher rates of multiple resistances were seen in the toxigenic strains. This was also showed in Wegener and Schwarz's study (1993). Their isolates were not identified as toxigenic or non-toxigenic but multiple resistances were more prevalent in isolates from pigs with EE than from healthy pigs. Nevertheless, there was only one study showed opposite results (Teranishi et al., 1987).

The differences regarding resistance patterns in multiple resistant strains also observed in Japan (Futagawa-Saito et al., 2009). The result shows that resistance to two or more antimicrobials were observed in 85.5% of total strains, with a significantly higher occurrence in toxigenic strains in 89.3% comparing with 75.4% in non-toxigenic strains. This finding is supported by Teranishi et al. (1987). The dominant patterns in strains from

diseased and healthy pigs included two and five antimicrobials, respectively. In a Danish study the dominating pattern with four antimicrobials was seen in 39% of strains from pigs with EE but just 7% of strains from healthy pigs (Wegener and Schwarz., 1993).

The cause of resistance in *S. hyicus* is the antimicrobial use in pig farms. Antimicrobials have long been used in feed as growth-promotion factor in pig farms. The use of antibiotics causes the development of resistance in *S. hyicus.* (Aarestrup and Jensen, 2002; Futagawa-Saito et al., 2009). Tylosin, a macrolide normally added in pig feed in Denmark, was banned for use as a growth-promoter in 1999. Because of reducing amount of tylosin, the percentage of resistant *S. hyicus* decreased from 62 to 26%. After the ban, the resistance rate stayed around 15%. In Japan, chloramphenicol also was added in feed as tylosin in Denmark. Resistance to chloramphenicol was 82% in 1998, and banned as a growth-promoter.

CHAPTER III

MATERIALS AND METHODS

The study consisted of two main parts. The first part consisted of a longitudinal study, conducted in farm A and farm B in Ratchaburi province for one year from June 2010 to May 2011 and a cross sectional study that conducted in 10 different farms in central provinces of Thailand. The second part was the antimicrobial susceptibility testing of the collected *S. hyicus* isolates from those 12 farms.

The research was conducted as the following conceptual framework.
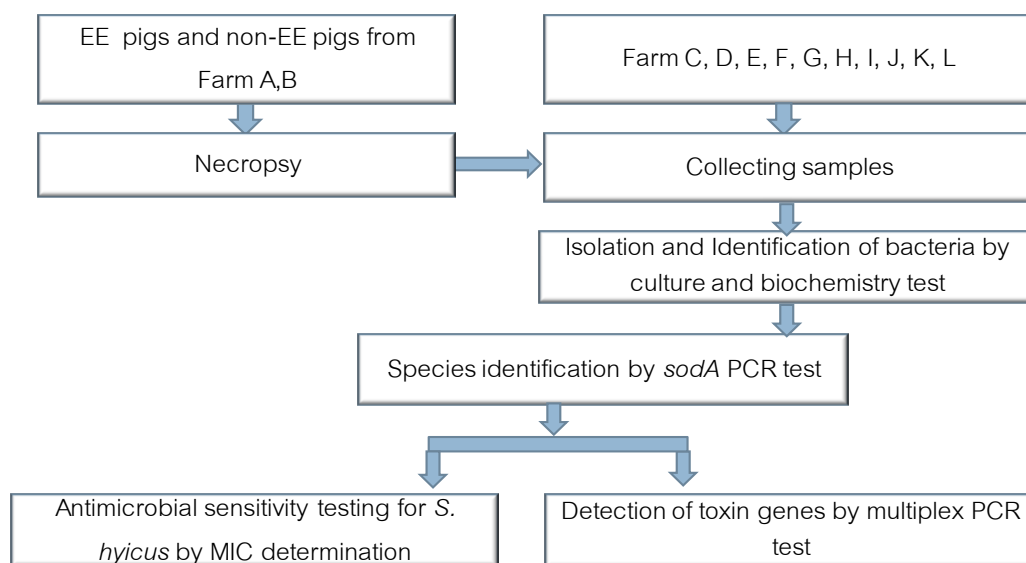
Figure 3.1 The conceptual frame work in this study

3.1 Sample collection

3.1.1 Sample collection for the longitudinal study

Description of pig herds

Two commercial pig farms with around 2000 sows on reproduction were chosen for this investigation. The occurrence of *exh* genes of *S. hyicus* was followed in piglets in two pig farms. The first one, farm A had prehistory of EE and presented EE in piglets

during the time of sampling collection. Farm B had no prehistory and without the EE lesion for at least one year. Piglets were randomly selected from the litters of lactation sows. Four pens per farms and one piglet per litter were chosen. These two farms were selected in order to know the different of the *exh* genes occurrence in pigs with and without EE lesion.

In this study, the skin lesion of EE piglets was classified into three levels, depending on the skin lesions' severity. Level 1 was mild of the skin lesions and lesion cover to only less than one third of the body. The severity of skin lesions was moderate. Level 2 was equivalent to the skin lesions that appeared less than two third of pig body surface. Level 3 was the most extreme; the skin lesions covered most or entire of the body surface covered with a thick, brown, greasy, and odorous layer. Normal skin was classified by without any skin lesion.

Figure 3.2 Skin lesions in piglets affected with EE. A: skin lesions at level 3; B: skin lesions at level 2; C: skin lesions at level 1; D: normal skin

Four to six piglets per farm were delivered monthly to the hospital in the period of one year from June 2010 to May 2011. All of the delivered piglets were necropsied at the Livestock Animal Hospital in Chulalongkorn University in Nakhon Pathom province. The piglets were injected intravenously 2-5 ml of Nembutal 5% (pentobarbitone sodium) to anesthesia the piglets, and then the piglets were euthanized by injecting 2-5 ml of saturated magnesium sulfate solution. The bacterial samples were collected from skin and joint fluid by using cotton-tipped while lymph nodes were applied directly onto sheep blood agar immediately.
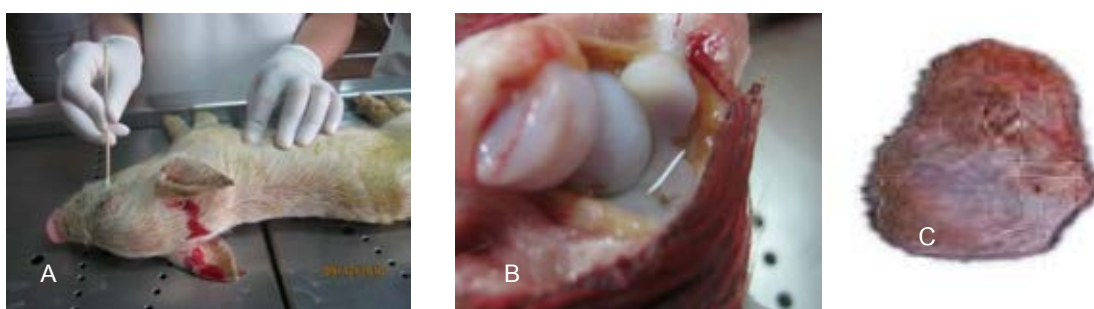
Figure 3.3 Sample collection from various organs: A: from skin; B: from joint; C: from lymph node

3.1.2 Sample collection for the cross sectional study with other ten farms: C-L

Ten pig farms in Nakhon Pathom, Saraburi, Ratchaburi, Ayuthaya, were purposively selected for the study. Sow on production varied from 300 to 500 sows per each farm. Thirty samples per farm were randomly chosen (10 suckling pigs, 10 weaning pigs, and 10 sows).
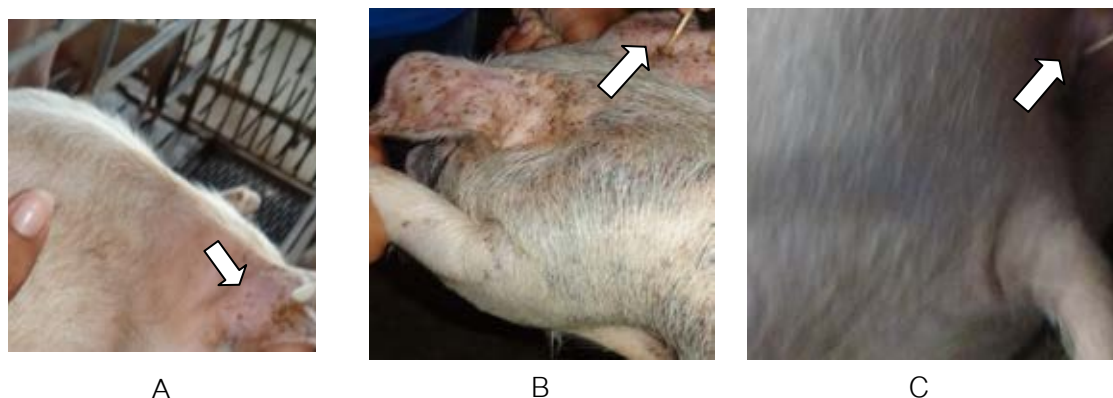
Figure 3.4 Collecting samples from various stages of pigs: A: suckling pigs; B: nursing pig; C: lactating sow.

In the suckling and nursery pigs, the sterile cotton swabs were dipped in 0.9% sterile sodium chloride and applied to approximately 10 cm^2 area behind the right ear of the EE pigs or non EE piglets. Then, the swab samples were placed in liquid Stuarts medium. In sows, vaginal swabs were collected by rigorous rubbing with a sterile cotton swab inserted approximately 10 cm into sow vagina. The swab tip was then put into a plastic tube containing 5 ml Stuart's medium. Then, the samples were submitted to the bacterial laboratory, Livestock Animal Hospital in Chulalongkorn University, Nakhon Pathom campus.

3.2 Bacterial isolation and identification by biochemical and PCR test

3.2.1 Bacterial isolation and identification by biochemical characteristics

S. hyicus was isolated and identified following the conceptual frame work in Figure 3.4. All of the collected samples were inoculated directly onto 5% sheep blood agar (Oxoid Ltd, Basingstoke, Hampshire, England) and McConkey agar (Merck KGaA, 64271 Darmstadt, Germany) and then incubated at 37^{0}C for 18h-24h. If bacterial culture heavily contaminated with other species, the colonies should be subcultured on mannitol-salt agar (Oxoid Ltd, Basingstoke, Hampshire, England). After incubation, three single colonies with size of about 3–4 mm, slightly opaque white, and non-hemolytic were selected for gram staining. Continuously, the gram staining was positive; the

colonies were subcultured and tested for further other biochemistry tests such as catalase, oxidase, and oxidative fermentation test.

Colonies like *S. hyicus*
Gram staining
Catalase reaction
Oxidase reaction
Oxidation fermentative reaction
Coagulase reaction
sodA PCR test
API staph strip test
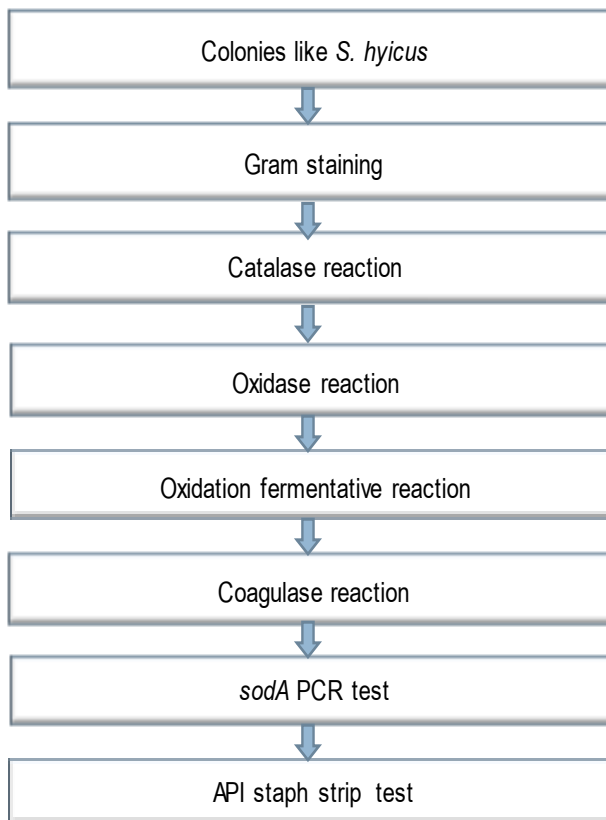
Figure 3.5 The conceptual frame work in identification *S. hyicus* by colony morphology and biochemical characteristics
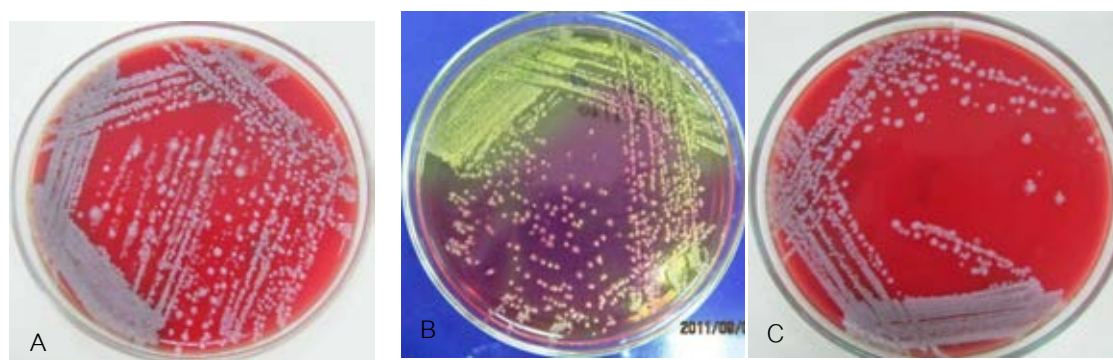
Figure 3.6 Colonies growth on media: A: sheep blood agar; B: mannitol agar; C: sheep blood agar

Gram staining
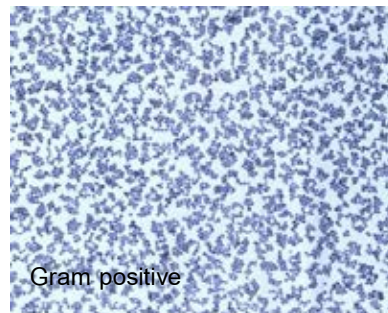
Figure 3.7 Gram staining

The colonies with size of about 3–4 mm, white, nonhemolytic were picked up and smeared on a glass slide. The smear was fixed by quickly passing the slide through the flame and then the smear was treated with crystal violet in one minute. After treated one minute, the slide was washed in water. The smear was added few drop of Gram's Iodine and lasted for one minute. As a result, iodine interacted with crystal violet and formed a large complex of crystal violet and iodine. Iodine absorbed within the inner and outer layers of the cell wall, which made cell wall became purple. The slide was again washed in water and then decolorized in absolute ethyl alcohol. The process of decolorization was fairly quick in 15 seconds. After decolorization, the smear was washed in water immediately. The smear was finally added few drops of counter stain - dilute carbon fuchsine, and washed in water. The excess water was removed using a blotting paper, dried in the air and heat fixed before observing under microscope. If bacteria appeared as single or couple, coccus, and gram positive were selected for further chemical test.

Catalase test
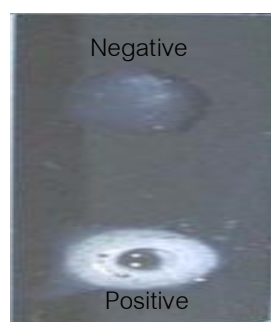
Figure 3.8 Catalase reaction

This method detected the enzyme catalase of *S. hyicus*. This enzyme converted hydrogen peroxide into water and gaseous oxygen. One colony on 5% sheep blood agar after 24 hours incubation was picked up and placed onto the glass slide by a sterile loop. One drop of 3% H_2O_2 was added onto the colony. The result was observed within 10 seconds. The reaction was positive if vigorous bubbling appeared. The reaction was negative if bubbling did not appear.

Oxidase test

Figure 3.9 Oxidase reaction

This method detects cytochrome C oxidase in a bacterial cell. A piece of filter paper was moistened in a Petri dish with 1% aqueous solution of tetramethyl-p-phenylenediamine dihydrochloride. The test bacterium was streaked firmly across filter paper with a glass rod. The result was observed within 10 seconds. The reaction was positive if the filter paper turned into dark purple color. If filter paper did not change color, the reaction was negative.

Oxidation fermentative test (O-F)
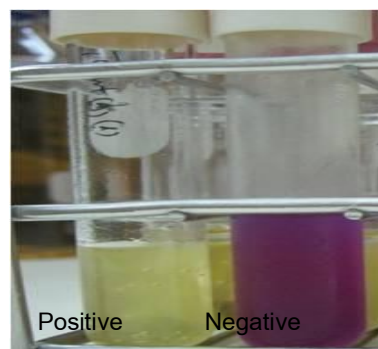
Figure 3.10 Oxidation fermentative reaction

This method was used to determine the oxidative or the metabolism of carbohydrate fermentation by *S. hyicus*. The components of this medium culture were tryptone 10 g, yeast extract 1 g, agar 2 g, bromocresol purple 0.001 g, and water 1000 ml. Boil two tubes of medium in 10 minutes to remove all of the oxygen, then cooled and inoculated by inserting a straight, vertical wire with *S. hyicus*. One tube was incubated aerobically and the second tube that was sealed the surface with a layer of sterile liquid paraffin oil to create anaerobic conditions. Both tubes were incubated with bacteria at 35°C-37°C for 18-24 hours and then observed tubes for color change. Fermentation was positive if color in both tubes changed to yellow color. The result was negative, if neither fermentation was no acid production (purple color in both tubes) nor oxidation.

Coagulase test
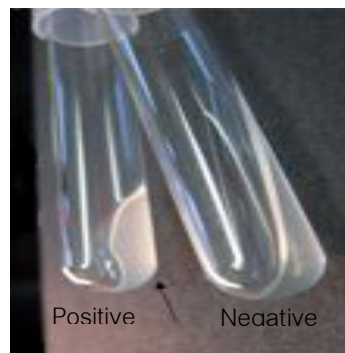
Figure 3.11 Coagulase reaction

Coagulase was a protein produced by *S. hyicus* that enabled the conversion of fibrinogen to fibrin. The coagulase reacted with prothrombin in plasma to form a complex which was *staphylothrombin*. This *staphylothrombin* which enabled the enzyme protease converted fibrinogen to fibrin. This resulted in clotting of plasma.

Three test tubes were taken and labeled "test", "negative control" and "positive control". Each tube was filled with 0.5ml of 1 coagulase plasma. 0.1ml of overnight broth culture of *S. hyicus* was added to the tube labeled test. 0.1ml of overnight broth culture of known *S. aureus* was added to the tube labeled positive control. 0.1ml of sterile broth was added to the tube labeled negative control. All the tubes were incubated at 37^{0}C

and observed up to four hours. Positive result was indicated by gelling of the plasma, which remained in place even after inverting the tube.

API Staph Strip

Figure 3.12 API Staph Strip test. A: negative with *S. hyicus*, B: Positive with *S. hyicus*

The isolates, which were positive catalase, negative oxidase, positive in oxidation fermentative, were further confirmed by the API Staph system (Bio-Merieux SA, France).

The API Staph Strip consisted of 20 microtubes containing dehydrated substrates. These microtubes were inoculated with a *S. hyicus* suspension. During incubation, metabolism created color changes that were either spontaneous or detected by the addition of reagents. The reactions were read according to the reading table and the identification was obtained by using the identification software.

According to the manufacturer's recommendations, inoculum for the API Staph Strip gallery was a *S. hyicus* suspension adjusted to be equal to 0.5 McFarland scale. The API Staph Strip included the following tests: fermentation of glucose, fructose,

mannose, maltose, lactose, trehalose, mannitol, xylitol, melibiose, raffinose, xylose, saccharose, alpha-methylglucoside, and N-acetylglucosamine; reduction of nitrate to nitrite; and production of acetyl methylcarbinol, alkaline phosphatase, arginine dehydrolase, and urease. The strips were developed after 24 hours of incubation at 37°C. Positive reactions were converted to a profile of seven digits. These profiles were then checked by the identification software (https://apiweb.biomerieux.com).

3.2.2 Identification by single *sodA* PCR test

The identification of *S. hyicus* follows the method polymerase chain reaction mediated amplification of species specific sequences of superoxide dismutase A encoding gene (*sodA*) (Voytenko et al., 2006). The targets of the oligonucleotide primer sequences and the thermal cycler program were summarized in Table 3.1.

Table 3.1 Species and genus specific oligonucleotide primer and PCR programs used in the present investigation

Target	Oligonucleotide primer	Oligonucleotide sequences	Size of the PCR products (bp)
sodA *S.hyicus*	STAH-SodI STAH-SodII	GCTTATCGCGAATGTTGACCAAT TCGTGCTGCTGCTTTATCTGAG	205

PCR-program:

1 = 1x (94^{0}C 180 s), 31x (94^{0}C 30 s, 60^{0}C 30 s, 72^{0}C 50 s), 1x (72^{0}C 300s).

Positive control strain was *S. hyicus* strain of NCTC 10350 (kindly provided by Lars Ole Andresen, Danish Institute for Food and Veterinary Research, Copenhagen, Denmark)

DNA preparation

The bacteria genomic DNA were extracted by boiling method. Briefly, three colonies of freshly subcultured strains to be investigated were suspended in 400 µl PBS, pH 7.4. The bacteria suspension was boiled at 100^{0}C for 10 minutes. After centrifugation

at 13, 000 rpm for 3 minutes, the supernatant was carefully collected in a new tube and keep in -20^{0}C until used for PCR.

The PCR reaction mixture (25 µl) contains 1µl of each primer (10 pmol/µl), 12.5 µl of 2 x promega PCR master mix, and 9.5 µl of H_2O. Finally 1µl DNA preparation was added to each reaction tube. The tubes were then subjected to thermal cycler (Gene Amp PCR System 2400, Perkin-Elmer, Rodgau Juegesheim, Germany or T3, Biometra, Goettingen, Germany) as reported in Table 3.1. The presence of PCR products were determined by electrophoresis of 5 µl of reaction product and 1µl loading dye in an 1.0% agarose gel (Gibco BRL, Karlsruhe, Germany) with Tris–acetate electrophoresis buffer (TAE, 4.0 mmol/l Tris, 1 mmol/l EDTA, pH 8.0).

3.3 Detection of *exh* genes by multiplex PCR test

The *S. hyicus* strain of NCTC 10350, 1289D-88, 842A-88, A2869C (kindly provided by Lars Ole Andresen, Danish Institute for Food and Veterinary Research, Copenhagen, Denmark) that produce toxin ExhA, ExhB, ExhC, ExhD, respectively, were used as positive control isolates, negative control was water.

The detection various the *S. hyicus* exfoliative toxins follows by a multiplex PCR test (Andresen and Ahrens, 2004). Samples of template DNA for the PCR from pure cultures were prepared by boiling method as descried previously.

The multiplex PCR reactions were performed by using 25 µl 2 x promega PCR master mixes (Applied Biosystems/Roche, Branchburg, NJ, USA) per sample in a total volume of 50 µl containing 2.0 µl of template DNA preparation, 1 µl of each primer, and 15 µl of distill water. An automatic thermal cycler (T3 Thermocycler; Biometra, Goettingen, Germany) was used during the PCR reaction. DNAs were amplified by an initial denaturation at 94^{0}C for 3 minute, followed by 30 cycles of 1 minute at 94^{0}C, 1 minute at 56^{0}C, and 1 minute at 72^{0}C. The PCR reaction was completed by 10 minute incubation at 72^{0}C in order to ensure full extension of the PCR products. PCR products

were analyzed by gel electrophoresis of samples of 5µl and 1µl of loading dye in 1.0% agarose gels including 2 µl of ethidium bromide. Finally, the PCR products were visualized on an UV transilluminator. Samples from toxigenic *S. hyicus* strains representing each of the four types of exfoliative toxins were run on each gel as positive controls. A 100 bp ladder was used as molecular size marker.

Table 3.2 Oligonucleotide primers used in the multiplex PCR.

Gene amplified	Primer name	Sequence (5'–3')	PCR product size (bp)
*exh*A	MU4FA	GCTACTGGTTTTGTAGTTTCAC	316
	MU3RA	GTAACCTACAACTCTTAGAACC	
*exh*B	F2EB	AACACGCCAATAGAGAATGTATCAC	717
	MU3RB	TATCAAATCTTATACCAGTTAGAATATCTCC	
*exh*C	MU3FC	GAATAAATATTATGGAGTCTCTCCTGATC	525
	MU4RC	CCATAGTATTTCAATCCAAAATCAGTAC	
*exh*D	F2ED	GAACAAATATAATGGAAGAAACCCAC	588
	MU3RD	GATTTCCCTACGTGAATACCTACAATAC	

3.4 Antimicrobial susceptibility test

Minimal inhibitory concentration (MIC) determination was done by broth microdilution method using the 96 well microtiter plates following the Clinical and Laboratory Standards Institute (CLSI) guidelines

After identified by conventional methods and PCR method, all bacteria that positive with *S. hyicus* were stored in -70 ^{0}C freezer. Only 80 of the *S. hyicus* isolates including 40 of toxin producing strains and 40 of non-toxin producing strains were selected to use for antimicrobial susceptibility tests.

The following antimicrobials were tested (with tested ranges indicated in parentheses); penicillin (0.06–8 µg/ml), gentamicin (0.5-64 µg/ml), cefotaxime (0.5–64 µg/ml), cephalexin (0.5–64 µg/ml), methicillin (2–32 µg/ml), trimethoprim-

sulfamethoxazole (0.5-8 and 9.5-152 µg/ml, respectively), and vancomycin (0.5–32 µg/ml). *S. aureus* JCM 2874 (ATCC29213) was used as a positive control. A serial two-fold dilution of antimicrobials dissolved in the appropriate solvent was processed according to CLSI 2008.

The MIC testing was performed in microdilution plates. The broth microdilution method was a liquid culture method whereby a standard amount of bacteria were inoculated into the wells of a 96 well microtiter plate that contain different dilutions of antimicrobial drugs. The microdilution was performed in 96-well microtitre plates with U-shaped wells, label with name, date of inoculation, and name of bacterium. Add 100 µl of Mueller Hinton Broth (MHB) in each well in rows A-H from column 1-12. Used the micropipette to dispense 100 µl of test antimicrobial to the first well and mix. This is the first two-fold dilution. Use the micropipette with the same tip to carry out a second two-fold dilution. Continue the series of two-fold dilutions until the well of the column 10 in microwell plate. Discard the quantity in the micropipette from this well. Column 11 was control positive, column 12 was control negative.

The bacteria were cultivated on tryptic soy agar (Oxoid, UK) supplemented with 5% sheep blood. After incubation at 37°C for 18-24 hours, a loopful of bacterial colony was suspended in Mueller Hinton Broth (Difco Lab, USA) and the cell density was adjusted to McFarland standard 0.5, or approximately 10^{8} CFU/ml. The inocula were then diluted ten-fold in sterile normal saline, giving a final cell density of approximately 10^{6}CFU/ml. A serial two-fold dilution of antimicrobial agents in Mueller Hinton Broth (MHB) was inoculated with the standardized inoculums of the *S. hyicus* isolates. After incubation for 18-20 hours at 37^{0}C, the MIC was determined as the lowest concentration of the antimicrobial in the well with no visible growth of bacteria.

Six following single antimicrobial agents were diluted by two-fold dilution: penicillin (0.06–8 µg/ml), gentamicin (0.5-64 µg/ml), cefotaxime (0.5–64 µg/ml), cephalexin (0.5–64 µg/ml), methicillin (2–32 µg/ml, and vancomycin (0.5–32 µg/ml).

Table 3.3 Steps for dilution of trimethoprim-sulfamethoxazole (1:19) in media

Step	Antibiotic solution		Source	Volume	Solvent	Middle concentration		Final concentration Dilution1:10	
	Concentration							in media (µg/ml)	
	(µg/ml)			(ml)	(ml)	(µg/ml)			
	Tri	Sul				Tri	Sul	Tri	Sul
1	1600	-	Stock	1	9	160	-	16	-
2	-	6080	Stock	1	1	-	3040	-	304
3	160	3040	1+2	1+1	-	80	1520	8	152
4	160	3040	3	1	1	40	760	4	76
5	160	3040	3	1	3	20	380	2	38
6	160	3040	3	1	7	10	190	1	19
7	20	380	6	1	1	5	95	0.5	9.5
8	20	380	6	1	3	2.5	47.5	0.25	4.75
9	20	380	6	1	7	1.25	23.75	0.125	2.375
10	2.5	47.5	9	1	1	0.6	11.87	0.06	1.187

Source: CLSI, 2007

Tri: trimethoprim, Sul:sulfamethoxazole

The combination of trimethoprim-sulfamethoxazole with ratio was 1:19. The steps for two fold dilution this combination followed the guideline in CLSI, 2008 and was shown in Table 3.3.

Table 3.4 The breaking point of antimicrobial used in antimicrobial susceptibility test

Antibiotics	Breakingpoit (µg/ml)
Cef	S≤8, R≥64
Cep	S≤ 8, R≥32
Pen	S≤ 0.12, R≥0.25
Met	S≤ 8, R≥16
Gen	S≤ 4, R≥16
Van	S≤ 2, R≥16
Tri-sul	S≤2/38, R≥4/76

S: Sensitive, R: Resistant

Source: CLSI, 2008

3.5 Statistical analysis

All data were reported as number of isolates and percentage of isolates. The data were analyzed using descriptive statistic and Genmod test was applied in analysis of the antimicrobial susceptibility test.

CHAPTER IV
RESULTS

The occurrence of *S. hyicus* and *exh* toxin genes were in section 4.1 in which two studies were separated into a longitudinal study (Farm A and B) and a cross sectional study (Farm C-L). The antimicrobial susceptibility test of Thai *S. hyicus* isolates was included in section 4.2.

4.1. The occurrence of *S. hyicus* and *exh* toxin genes in pig farms with and without EE.

4.1.1 A longitudinal study on the occurrence of *S. hyicus* and *exh* toxin genes in pig farms with and without EE.

The occurrence of *S. hyicus* and *exh* toxin genes was observed in Farm A and B in Ratchaburi province for one year from June 2010 to May 2011. Farm A represented an EE experiencing farm with a history of EE outbreak in suckling and nursery pigs. Farm B had no history of EE at least two years. The EE lesions were particularly found on skin. The lesions began with small, dark, localized areas of infection around the ear, ear base, and face, or on the legs, where the skin had been damaged. The skin along the flanks, the belly, and between the legs became brown and gradually spread over the whole body. The skin began wrinkled around flaking of large areas and moist with when touched it had a greasy feeling. The skin lesion was graded in 3 levels: 1, 2, and 3 according to the severity.

Four pens with 32 litter of each pen were investigated to record the number of piglets affected with EE in monthly interval in period of one year. The number of EE pigs with various skin lesions in farm A in a one year period was shown in Table 4.1. The severity of skin lesions together with number of affected pigs was highest in July (10.30%) and June 2010 (9.38%). The rate of EE pigs with skin lesion decreased in the following months of the studying period. This rate was lowest in February and May 2011 with 0.08% and 0.22%, respectively.

Table 4.1 The occurrence of skin lesion of EE piglets in farm A

Month/year	Number of pigs with various skin lesion severity				
	Level 1 (n)	Level 2 (n)	Level 3 (n)	Total	%
6/2010	56	21	38	115	115/1226 (9.38)
7/2010	45	22	56	123	123/1191 (10.30)
8/2010	2	5	28	35	35/1185 (2.95)
9/2010	5	3	15	23	23/1137 (2.02)
10/2010	11	3	5	19	19/1190 (1.60)
11/2010	7	0	11	18	18/1300 (1.38)
12/2010	6	4	3	13	13/1255 (1.04)
1/2011	12	2	1	15	13/1185 (1.27)
2/2011	0	1	0	1	1/1302 (0.08)
3/2011	19	2	0	21	21/1251 (1.68)
4/2011	31	0	3	34	34/1293 (2.63)
5/2011	1	0	2	3	3/1362 (0.22)

n: number of pigs, Level 1 was mild of the skin lesions, Level 2 was the skin lesions less than two third of pig body surface. Level 3 was the most extreme.

The number of piglets with high severity also decreased from level 3 to level 1 of skin lesion. The percentage of EE piglets with level 3 of skin lesion was higher in first six months of studying period. The number of EE pigs with level 1 of skin lesion was higher late six months. The change rate of EE pigs decreased may be due to the treatment by antibiotics and injection autogenous vaccine.

4.1.1.1 Detection of *S. hyicus* by conventional microbiology and PCR

Detection of *S. hyicus* by conventional microbiology

Three hundred and twenty four samples (177 samples from farm A and 147 samples from farm B) were cultured on sheep blood agar. Following the cultivation, *S.*

hyicus was identified by colony morphology and biochemical characteristics and further and confirmed by a commercial biochemical test (API Staph strip test).

A total of 324 samples, 204 (62.96%) isolates were colonies with no pigment and non-hemolysis. One hundred and ninety-seven isolates (60.08%) were positive with catalase reaction, and 190 (58.64%) were negative with oxidase reaction. One hundred and eighty (55.55%) isolates were positive with O-F test, and 137 (42.28%) isolates was negative with coagulase reaction. The result for identification of *S. hyicus* from skin, lymph node and joint fluid regarding to the conventional biochemical tests was shown in Table 4.2

Table 4.2 The biochemical test results for identification of *S. hyicus* from a total of 324 isolates in a longitudinal study.

Biochemical tests	Number of collected sample from pigs in farm A and B (n,%)						
	Farm A (n=177)			Farm B (n=147)			Total (324)
	Skin (n=59)	LN (n=59)	Joint (n=59)	Skin (n=49)	LN n=(49)	Joint (n=49)	
No pigment	53(89.83)	32(54.23)	28(47.45)	46(93.87)	20(40.81)	25(51.02)	204(62.96)
Non-hemolysis	53(89.83)	32(54.23)	28(47.45)	46(93.87)	20(40.81)	25(51.02)	204(62.96)
Catalase positive	53(89.83)	28(47.45)	27(45.76)	46(93.87)	18(36.73)	25(51.02)	197(60.08)
Oxidase negative	50(84.74)	28(47.45)	27(45.76)	45(91.83)	17(34.69)	23(46.93)	190(58.64)
O-F positive	49(83.05)	28(47.45)	25(42.37)	43(87.75)	15(30.61)	20(40.81)	180(55.55)
Coagulase negative	36(61.01)	17(28.81)	20(33.89)	38(77.55)	11(22.44)	15(30.61)	137(42.28)
Coagulase positive	13(22.03)	11(18.64)	5(8.47)	5(10.20)	4(8.16)	5(10.20)	43(13.27)

n: number of samples; LN: Lymph node

All of the isolates with criteria of *S. hyicus* were collected. These isolates were gram positive, catalse positive, oxidase negative, O-F positive, and coagulase negative or positive. These isolates were collected as *S. hyicus* and were further confirmed by *sodA* PCR test.

The API Staph strip test was further used for identification of randomly selected 30 *S. hyicus* isolates. The number and percentage of *S. hyicus* isolated from skin, lymph node and joint regarding to the API Staph strip test were shown in Table 4.3

Table 4.3 Identification *S. hyicus* by API staph strip test determined by percent of positive results

API Staph strip	Farm A (n=16)			Farm B (n=14)			Total
	Skin (n=10)	Lymph node (n=4)	Joint (n=2)	Skin (n=10)	Lymph node (n=2)	Joint (n=2)	N=30
Glucose	100	100	100	100	100	100	100
Fructose	100	100	100	100	100	100	100
Mannose	100	100	100	100	100	100	100
Maltose	0	0	0	0	0	0	0
Lactose	100	50	50	100	50	50	90
Trehalose	100	100	100	100	100	100	100
Mannitol	0	0	0	0	0	0	0
Xylitol	0	0	0	0	0	0	0
Melibiose	0	0	0	0	0	0	0
NIT (nitrate to nitrite)	30	25	100	20	100	50	26
PAL	100	100	100	100	100	100	100
VP	0	0	0	0	0	0	0
Raffinose	0	0	0	0	0	0	0
Xylose	0	0	0	0	0	0	0
Saccharose	100	100	100	100	100	100	100
MDG	0	0	0	0	0	0	0
NAG	100	100	100	100	100	100	100
ADH	100	100	100	100	100	100	100
Urease	70	50	20	50	100	50	61

Tested isolates were confirmed as *S. hyicus* by Api software. *S. hyicus* was positive in fermentation with glucose, fructose, mannose, lactose, trehalose, NIT (potassium nitrate), PAL (ß-naphthyl phosphate), saccharose, N-acetylglucosamine, and L-arginine. And negative in fermentation with maltose, mannitol, xylitol, melibose, VP (sodium pyruvate), raffinose, xylose, and alpha-methylglucoside.

All tested isolates fermented glucose, fructose, mannose, trehalose, saccharose, and N-acetylglucosamine, whereas maltose, mannitol, xylitol, melibose, raffinose, xylose, and alpha-methylglucoside were not fermented.

The identification of *S. hyicus* was further performed using *sodA* PCR. Totally, 180 *S. hyicus* isolates identified by biochemical identification were further identified by *sodA* PCR technique. Of 180 isolates, only 154 (85.55%) isolates were positive with *S. hyicus*. These isolates were from farm A (88/102, 86.27%) and farm B (66/78, 84.61%). According to the sampling site of pig body, out of 92 isolates from skin, 81 (88.04%) isolates were positive to *sodA* PCR, 36/43 (83.72%) and 37/45 (82.22%) isolates were positive, isolated from lymph nodes and joints, respectively. The *sodA* PCR was shown in Table 4.4 and Figure 4.1.

Table 4.4 *S. hyicus* identification by *sodA* PCR

Sampling Location	Number of isolates positive by *sodA* PCR/total isolates (%)		
	Farm A	Farm B	Total
Skin	41/49 (83.67)	40/43 (93.02)	81/92(88.04)
Lymph node	25/28 (89.28)	11/15 (73.33)	36/43(83.72)
Joint	22/25 (88.00)	15/20 (75.00)	37/45(82.22)
Total	88/102 (86.27)	66/78 (84.61)	154/180(85.55)
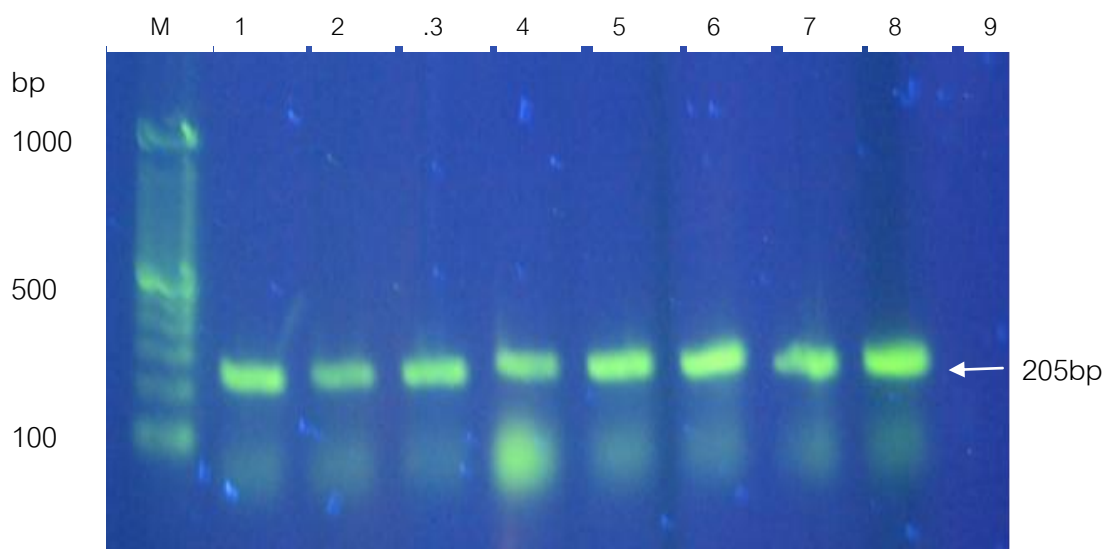
Figure 4.1 Typical agarose gel electrophosis pattern of the *sodA* PCR detecting *S. hyicus*, Lane M: 100 bp plus DNA marker (GeneRuler™, Fermentus, Canada); Lane 1. Pos: Positive control; Lane 2-8: Sample isolates; Lane 9: Negative control

4.1.1.2 Occurrence of three *exh* toxin genes in a longitudinal observation.

The *exh* toxin genes were detected by multiplex PCR from farm A and B. The PCR band was shown in Figure 4.2.
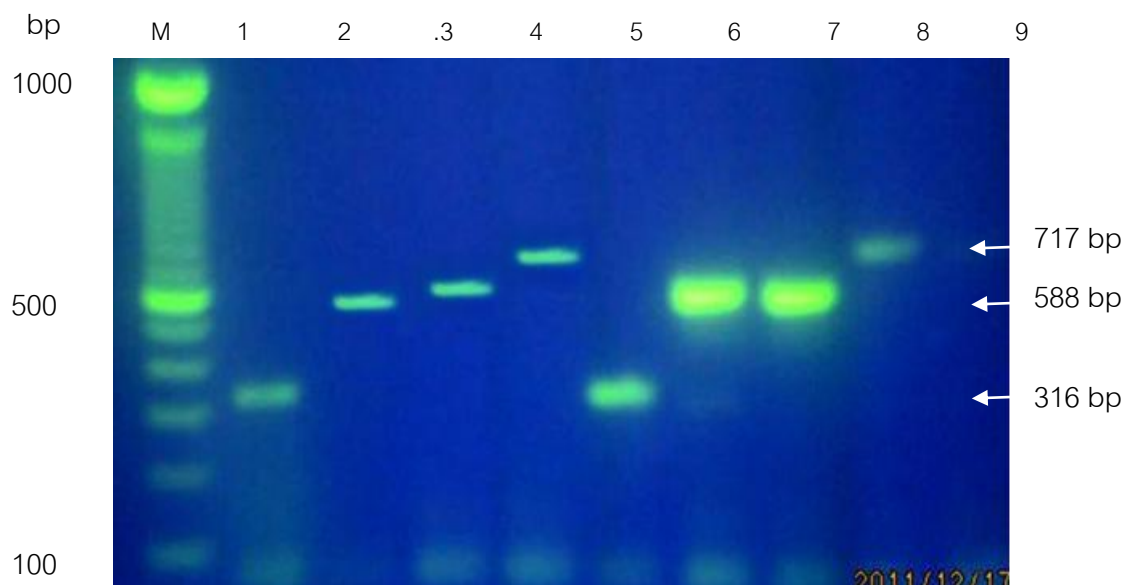
Figure 4.2 Typical amplicons of the multiplex PCR detecting the genes encoding exfoliative toxins from *S. hyicus*. Lane M: 100 bp plus DNA marker (GeneRuler™,

Fermentus, Canada); Lane 1-4: positive control *exhA, exhC, exhD, exhB;* Lane 5-8: samples; Lane 9: Negative control

Occurrence of *exh* toxin genes from various location sites.

The *exh* toxin genes were further determined regarding to sampling locations (Table 4.5). Of 154 S. *hyicus* isolates, 32 (20.78%) isolates harbored *exh* toxin genes, including 24 and 8 isolates from farm A and B, respectively. The percentage of toxin producing strains was highest from skin (14/41, 34.15%), following by joint (6/22, 22.27%) and lymph node (4/25, 16.00%) in EE pigs. In pig without EE, the rate of *exh* genes carrier was lower than this in EE pigs. The *exh* gene carrier from skin was 6/40 (15%), but only 1/11 (9.09%) and 1/15 (6.66%) isolates were toxigenic strain from joint and lymph node, respectively.

Table 4.5 Detection of *exh* toxin genes in isolates of *S. hyicus* from various sampling locations from pigs with and without EE in the farm A and B.

Sampling locations	Number of toxigenic isolates (%)		
	Farm A	Farm B	Total
Skin	14/41 (34.15)	6/40 (15.00)	20/81 (24.69)
Lymph node	4/25 (16.00)	1/11 (9.09)	5/36 (13.88)
Joint	6/22 (22.73)	1/15 (6.66)	7/37 (18.91)
Total	24/88(26.13)	8/66(12.12)	32/154(20.77)

Distribution of *exh* genes from *S. hyicus* isolates in farm A and B was shown in Figure 4.3. The *exhA* gene was predominant among *exh* toxin genes. Out of 32 toxigenic strains, 27 (84.37%) strains harbored *exhA,* whereas 4 (12.5%) and 1 (3.12%) isolates carried *exhD* and *exhB* genes.
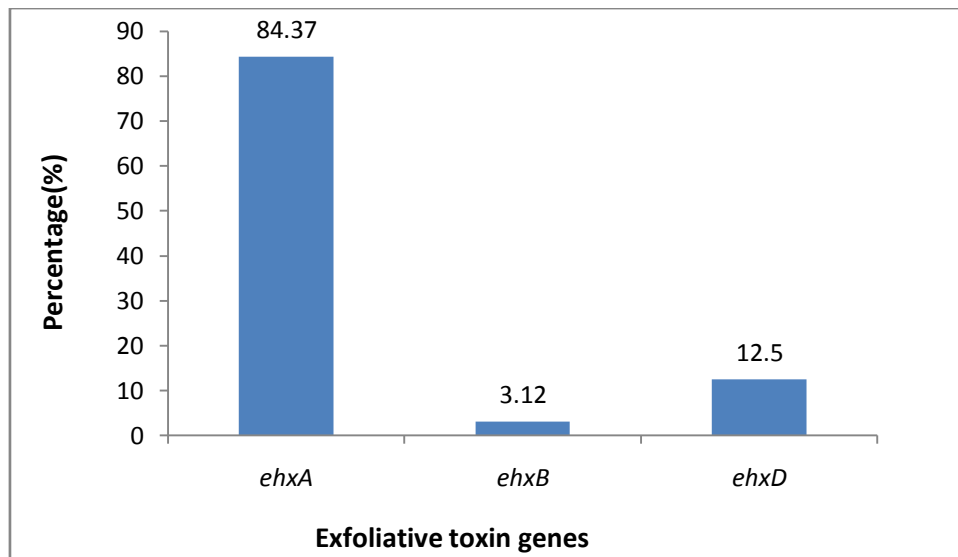
Figure 4.3 The distribution of *exh* genes of *S. hyicus* isolated from farm A and B

Additional analysis was performed on a special interest with respect to the occurrence of *exh* toxin genes in different sampling site of pigs in both farms (Table 4.6).

The *exhA* were found in 21 out of 24 *S. hyicus* isolates (87.50%) in farm A. Among this, 13/24 isolates (54.16%) were isolated from skin, and the equal degree of detection rate was 4/24 isolates (16.66%) from lymph node and joint fluid. In farm B, only 8 isolates were Exh toxin producing strains, 6 (75.00%) isolates carried *exhA* gene, whereas 4/8 (50.00%) isolates were from skin, and 1/8 (12.50%) isolate were from lymph node and joint fluid samples.

Interestingly, the *exhB* gene was found in only 1 out of 24 *exh* gene carrying strains. This isolate was from skin of the EE pig (farm A). The *exhD* gene of *S. hyicus* was found in both EE and non EE pigs. Two *exhD* carrying strains were from joint of EE pigs and another two *exhD* carrying strains were from skin of non EE pigs.

Table 4.6 Distribution of *exh* genes from various sampling locations from pigs in farm A and B

Location of sampling	Farm A (n=24)			Farm B(n=8)	
	exhA	*exhB*	*exhD*	*exhA*	*exhD*
Skin	13/24 (54.16)	1/24 (4.16)	0/24 (0)	4/8 (50)	2/8 (25)
Lymph node	4/24 (16.66)	0/24 (0)	0/24 (0)	1/8 (12.5)	0/8 (0)
Joint	4/24 (16.66)	0/24 (0)	2/24 (8.33)	1/8 (12.5)	0/8 (0)
Total	21/24 (87.50)	1/24 (4.16)	2/24 (8.33)	6/8 (75.00)	2/8 (25.00)

The toxigenic strains were found highest in skin of pigs with level of skin lesion 3 (8/14, 75.14%). For lymph node samples, toxigenic *S. hyicus* only found in the pigs with skin lesion 2 (2/4, 50%) and level of skin lesion 3 (2/4, 50%). Finally, toxigenic strains of *S. hyicus* isolated from joint fluid of pigs with level of skin lesion 3 was highest (4/6, 66.7%) and toxigenic strain of *S. hyicus* from pigs level of skin lesion 1 and 2 was the same (1/6, 16.6%). The results of the correlation between severity of skin lesion with toxin gene isolated strains was shown in table 4.7.

Table 4.7 The correlation between severity of skin lesion with toxin gene isolated strains

Sample isolated from	Level of pig skin lesions in farm A			*Exh* genes (n,%)		
	1 (n,%)	2 (n,%	3 (n,%)	*exhA*	*exhB*	*exhD*
Skin	3 (21.43)	3 (21.43)	8 (57.14)	13 (92.85)	1 (7.15)	0
Lymph node	0	2(50.0)	2(50.0)	4 (100)	0	0
Joint	1(16.7)	1(16.6)	4 (66.7)	4 (66.7)	0	2 (33.3)

4.1.2 A cross-section investigation on occurrence of *S. hyicus* and *exh* toxin genes in pig farms with and without EE.

4.1.2.1 Detection of *S. hyicus*

A total of 300 samples collected from 10 commercial pig farms (farm C-L) were tested for *S. hyicus*. The number and percentage of *S. hyicus* identified by conventional biochemical test was shown in Table 4.8

Table 4.8 The biochemical test results for identification of *S. hyicus* from 300 isolates of *S. hyicus* isolated from 10 pig farms

Conventional test	Number of collected sample from pig in 10 farms (C-L) (n)										
	C	D	E	F	G	H	I	J	K	L	Total (n,%)
No pigment	21	23	18	15	17	11	18	17	20	13	173 (57.66)
Non-hemolysis	21	23	18	15	17	11	18	17	20	13	173 (57.66)
Catalase (+)	20	23	17	15	16	11	17	17	18	13	167 (55.66)
Oxidase (-)	19	21	15	15	15	10	16	15	17	13	156 (52.00)
O-F (+)	18	18	15	12	15	10	14	13	15	12	139 (46.33)
Coagulase (-)	13	14	10	11	11	8	11	10	11	11	110 (36.66)

n: number of samples

Based on criteria to identify *S. hyicus* in longitudinal study, all isolates that grown on sheep blood agar with no pigment, non hemolysis, positive for catalase, O-F, and negative for oxidase reaction were collected.

Out of 300 samples, 173 (57.66%) isolates were colonies with no pigment and non-hemolysis. Similar, but lesser extent, 167 (55.66%) isolates were positive with catalase reaction, and 156 (52.00%) were negative with oxidase reaction. To O-F test 139 (46.33%) isolates were positive, while 110 (36.66%) isolates was negative with coagulase reaction.

The API Staph strip test was also used for identification of randomly selected 30 *S. hyicus* isolates. All *S. hyicus* strains fermented glucose, fructose, mannose, trehalose, saccharose, and N-acetylglucosamine, but maltose, mannitol, xylitol, melibose, raffinose, xylose, and alpha-methylglucoside were not fermented.

Identification of *S. hyicus* was further performed using *sodA* PCR from EE pigs in farm C and D was shown in Table 4.9

Table 4.9 *S. hyicus* identification by *sodA* PCR from EE pigs

Farm	Sample from	Samples (n)	*sodA* PCR positive (%)
C	Suckling	10	8/10 (80)
	Nursing	8	5/8 (62.5)
D	Suckling	10	7/10 (70)
	Nursing	8	4/8 (50)
Total		36	24/36 (66.67)

In two EE farms, 24/36 (66.67%) isolates were positive by *sodA* PCR test. Fifteen isolates were from suckling (8 and 7 isolates from farm C and D, respectively). Nine isolates were from nursing pigs in which 5 and 4 isolates were derived from farm C and D, respectively.

The *sodA* PCR was also performed in eight remaining pig farms which were no history of EE (Table 4.10). Totally, 103 isolates were tested. Seventy isolates (53.84%) were positive with *S. hyicus*. Of 70 positive isolates, 38 and 32 isolates were taken from suckling and nursing pigs, respectively. None of 80 samples from sow vagina was positive with *sodA* PCR.

Table 4.10 *S. hyicus* identification by biochemical test and *sodA* PCR

Farm	Sample collected from	Biochemical test (%)	*sodA* PCR (%)
E	Suckling	9/10 (90)	6/9 (66.67)
	Nursing	6/10 (60)	5/6 (88.33)
F	Suckling	6/10 (60)	4/6 (66.67)
	Nursing	6/10 (60)	4/6 (66.67)
G	Suckling	8/10 (80)	5/8 (62.5)
	Nursing	7/10 (70)	6/7 (85.71)
H	Suckling	7/10 (70)	5/7 (71.42)
	Nursing	3/10 (30)	2/3 (66.67)
I	Suckling	6/10 (60)	4/6 (66.67)
	Nursing	5/10 (50)	4/5 (80)
J	Suckling	8/10 (80)	6/8 (75)
	Nursing	5/10 (50)	3/5 (60)
K	Suckling	9/10 (90)	5/9 (55.55)
	Nursing	6/10 (60)	4/6 (66.67)
L	Suckling	7/10 (70)	3/7 (42.85)
	Nursing	5/10 (50)	4/5 (80)
Total		103/160 (81.25)	70/103 (53.84)

4.1.2.2 Detection of *exh* toxin genes

The occurrence of *exh* genes of *S. hyicus* was higher in EE pigs (farm C and D) than non-EE pigs (farm E-L) (Figure 4.4). A total 31 isolates from 10 farms was toxin producing strains, including 17 isolates from EE pigs and 14 isolates from non EE pigs. Of 17 toxigenic isolates from EE, 9 (52.50%) isolates were positive for *exhD* and 8

(47.05%) isolates were positive for combination of *exhA* and *exhD.* All of 14 (100%) toxigenic strains from non EE pigs were positive for *ExhA*.
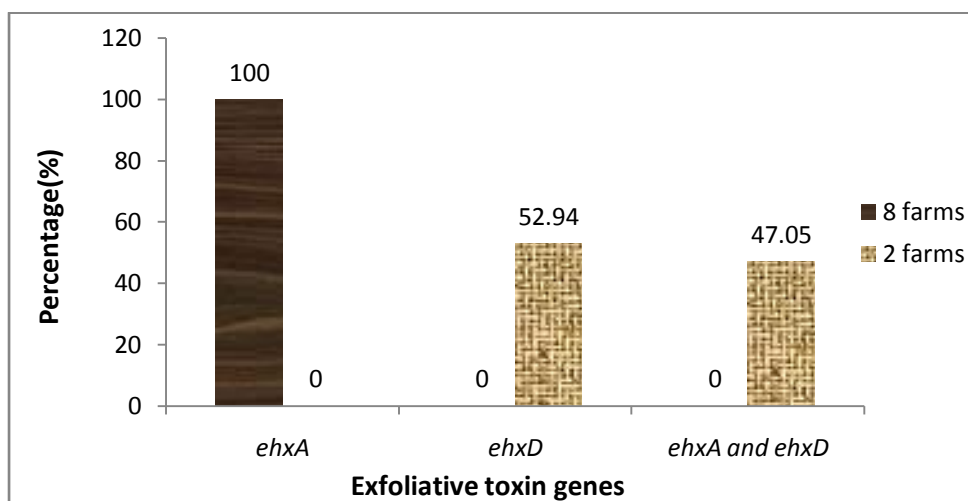
Figure 4.4 Distribution of exfoliative toxin genes in the pigs with and without skin lesion from 10 pig farms.

Detection of *S. hyicus exh* toxin genes from EE pigs in farm C and D was shown in Table 4.11.

Table 4.11 The detection of *exh* genes of *S. hyicus* isolated from EE farm C and D

Farm	*S. hyicus* isolated from	*S. hyicus* (n)	*Exh* genes (%)	
			Exh D	*Exh A* and *Exh D*
C	Suckling	8	2/8 (25.00)	4/8 (75.00)
	Nursing	5	0/5 (0.00)	4/5 (80.00)
D	Suckling	7	4/7 (57.14)	0/7 (0.00)
	Nursing	4	3/4 (75.00)	0/4 (0.00)
Total		24	9/24 (37.50)	8/24 (33.34)

Total 17 out of 24 *S. hyicus* isolates (70.83%) were toxigenic. Ten toxigenic isolates were from suckling pigs (6 from farm C and 4 from farm D). Seven (29.16%) toxigenic isolates were from nursing pigs (4 from farm C and 3 from farm D). Nine

isolates contained *exhD* gene (37.50%), whereas eight isolates carried *exhA* and *exhD* (33.34%).

Detection of *S exh* toxin genes from non-EE pigs in farm E-L was shown in Table 4.12.

Table 4.12 Detection of *exh* genes of *S. hyicus* isolated from non-EE farm E-L

Farm	Sample from	*S. hyicus* (N)	*Exh* genes A (%)
E	Suckling	6	2/6 (33.34)
E	Nursing	0	0
F	Suckling	4	1/4 (25.00)
F	Nursing	4	2/4 (50.00)
G	Suckling	0	0
G	Nursing	6	2/6 (33.33)
H	Suckling	0	0
H	Nursing	0	0
I	Suckling	4	1/4 (25.00)
I	Nursing	4	1/4 (25.00)
J	Suckling	6	2/6 (33.33)
J	Nursing	0	0
K	Suckling	5	2/5 (40.00)
K	Nursing	4	1/4 (25.00)
L	Suckling	0	0
L	Nursing	0	0
Total		70	14/70 (20.00)

In a total 70 *S. hyicus* isolates, 14 (20.00%) isolates were toxigenic. Eight toxigenic isolates were from suckling pigs (farm E, F, I, J, and K). Six (29.16%) toxigenic isolates were from nursing (F, G, I, and K). All of 14 toxigenic strains harbored *exhA* gene. Others *exh* genes were not found in non EE pigs in these eight farms.

4.2 Antimicrobial susceptibility test

Table 4.13 The MIC_{50}, MIC_{90}, and breaking point of antimicrobial agents in this test

Antibiotics	MIC_{50} (µg/ml)	MIC_{90} (µg/ml)	MIC Range (µg/ml)	Breakingpoits (µg/ml)
Cef	16	32	4-32	S≤ 8, R≥64
Cep	4	64	0.5-32	S≤ 8, R≥32
Pen	0.5	8	0.06 - 8	S≤ 0.12, R≥0.25
Met	4	8	0.5-8	S≤ 8, R≥16
Gen	4	8	0.5-64	S≤ 4, R≥16
Van	0.5	8	0.5 - 8	S≤ 2, R≥16
Tri-sul	2/38	8/152	0.5/9.5 - 8/152	S≤ 2/38, R≥4/76

The MIC result of seven antimicrobials against 80 Thai *S. hyicus* isolates were shown in Figure 4.5. Overall, the antimicrobial to which *S. hyicus* became most susceptible was trimethoprim-sulfamethoxazole (81.25%), followed by methicillin (80%) and cephalexin (57.5%), respectively. In contrast, the highest resistance was observed with penicillin (75%), followed by cephalexin (27.5%) and methicillin (20%), respectively. Vancomycin appeared to fall in the MIC range of sensitive and intermediate against all isolates.
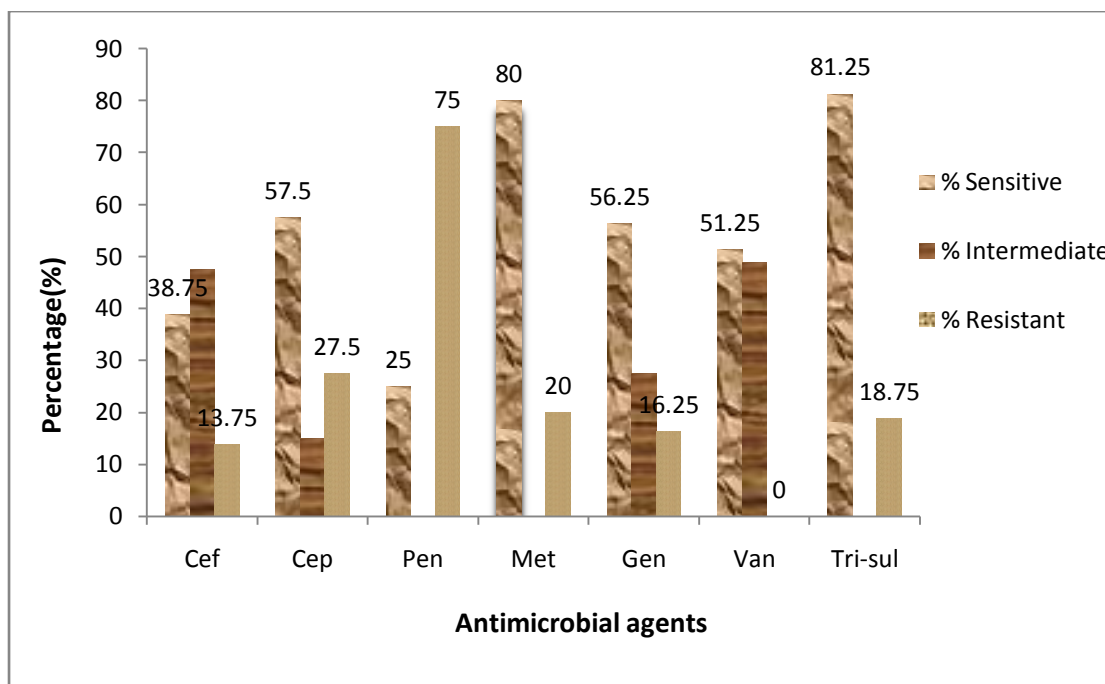
Figure 4.5 Antimicrobial susceptibility of 80 *S. hyicus* isolates

With respect to toxigenic characteristic of *S. hyicus*, the MIC of seven antimicrobials to *S. hyicus* isolates was shown in Table 4.14. The highest sensitive rate of both toxigenic and non-toxigenic isolates showed similarity as trimethoprim-sulfamethoxazole, methicillin, gentamicin, vancomycin, cephalexin, cefotaxime and penicillin, respectively. The percentage of non- toxigenic isolates was higher than those of toxigenic isolates in term of sensitivity to all antimicrobials. The highest frequency of sensitivity to both non-toxigenic and toxigenic isolates was trimethoprim-sulfamethoxazole (87.50% and 75%) follow by methicillin (85.00% and 75.00%), cephalexin (67.50% and 47.50%), gentamicin (60.00% and 52.00%), vancomycin (52.50% and 50%), cefotaxime (45% and 32.5%) and penicillin (12.50% and 10.00%), respectively. The number of isolates sensitive to penicillin was lowest in both non-toxigenic (12.5%) and toxigenic (10%) isolates.

Table 4.14 The percentage of toxin producing and non-toxin producing strains that sensitive, intermediate and resistant to seven antibiotics

Antibiotics	Sensitive		Intermediate		Resistant	
	Tx	Ntx	Tx	Ntx	Tx	Ntx
	n (%)	n (%)	(%)	n (%)	n (%)	(%)
Cef	13 (32.50)	18 (45.00)	21 (52.50)	17 (42.50)	6 (15.00)	5 (12.50)
Cep	19 (47.50)	27 (67.50)	7 (17.50)	5 (12.50)	14 (35.00)	8 (20.0)
Pen	4 (10.00)	5 (12.50)	0 (0)	0 (0)	36 (90.00)	35 (87.50)
Met	30 (75.00)	34 (85.00)	0 (0)	0 (0)	10 (25.00)	6 (15.00)
Gen	21 (52.50)	24 (60.00)	12 (30.00)	10 (25.00)	7 (17.50)	6 (15.00)
Van	20 (50.00)	21 (52.50)	20 (50.00)	19 (47.50)	0 (0)	0 (0)
Tri-sul	30 (75.00)	35 (87.50)	0 (0)	0 (0)	10 (25.00)	5 (12.50)

n= number, Tx = toxigenic *S. hyicus*, Ntx = non-toxigenic *S. hyicus*

Total number of toxigenic *S. hyicus* tested = 40 isolates, Total number of non-toxigenic *S. hyicus* tested = 40 isolates

The highest resistant rate was found with penicillin and cephalexin to both toxin and non-toxin producing strains, but higher in toxin producing than non-toxin producing (90.00% and 35.00% for toxigenic isolates, and 87.50% and 20.00% for non-toxigenic isolates). The lower frequency of resistance was found with trimethoprim-sulfamethoxazole, follow by methicillin, cefotaxime, gentamicin to toxin and non-toxin producing strains, vancomycin was not resistant to both toxin and non-toxin producing strains. But this was not significantly different between toxigenic strains and non-toxigenic strains. The intermediate was found highest with cefotaxime and vancomycin 52.50%, 50.00% to toxigenic strains and 42.50% and 47.50% to non-toxigenic strains, respectively.

Based on antimicrobial resistance patterns, *S. hyicus* isolates could be categorized into 17 distinctive groups (Table 4.15), the single resistance was found

commonly with penicillin to both toxigenic and non-toxigenic strains with a degree of finding of 22.50% and 40.00% respectively, with a total of 33.75%.

Resistance to two antimicrobial agents was revealed with a group of penicillin and others (methicillin, gentamicin, cephalexin, cefotaxime and trimethoprim-sulfamethoxazole) Combination of two antimicrobial resistant was unveiled with a rate of 17.5% and 10% to toxigenic and non-toxigenic strains, respectively). A combination of cephalexin and trimethoprim-sulfamethoxazole was also found. Totally, the frequency of resistance to two antimicrobials was 41.25% to both toxigenic and non-toxingenic strains.

The highest prevalent resistance of toxigenic strains to three antimicrobials was found with penicillin/methicillin/ trimethoprim-sulfamethoxazole (12.50%), but for non-toxigenic strains was not found. *S. hyicus* was also resistant to other groups of three antibiotics, but much lesser extent (2.50-3.75%). Totally, the resistant rate of both toxigenic and non-toxigenic strains to the groups of three antimicrobials was equal to 20%. Multiple resistances to 4 and 5 antimicrobials was shown in a combination of penicillin, cefotaxime, methicillin and gentamicin, and penicillin, cefotaxime, cephalexin, gentamicin, and trimethoprim-sulfamethoxazole, 3.75%, 1.25%, respectively.

Resistance to two or more antimicrobials (multiple antimicrobial resistance) was observed in 53/80 (66.25%) of the total strains. A higher resistant rate was observed in toxigenic strains (34/40, 85.00%) than non-toxigenic strains (19/40, 47.50%) ($p < 0.05$).

Table 4.15 Antimicrobial resistance patterns among 80 *S. hyicus* isolates

Patterns	Antimicrobial resistance	Number of *S. hyicus* isolates (%)			
		Toxigenic	Non-toxigenic	Total	
1	Pen	9/40 (22.50)	16/40 (40)	25/80 (31.25)	27/80 (33.75)
2	Cep	1/40 (2.50)	0	1/80 (1.25)	
3	Tri/sul	1/40 (2.50)	0	1/80 (1.25)	
4	Pen,Met	5/40 (12..50)	3/40 (7.5)	8/80 (10.00)	
5	Pen,Gen	2/40 (50.00)	2/40 (5.00)	4/80 (5.00)	
6	Pen,Cep	7/40 (17.50)	4/40 (10.00)	11/80 (13.75)	33/80 (41.25)
7	Pen,Cef	2/40 (5.00)	3/40 (7.50)	5/80 (6.25)	
8	Pen, Tri/sul	3/40 (7.50)	1/40 (2.50)	4/80 (5.00)	
9	Cep, Tri/sul	0	1/40 (2.05)	1/80 (1.25)	
10	Pen,Met,Cef	2/40 (5.00)	0	2/80 (2.50)	
11	Pen,Met,Tri/sul	5/40 (12.50)	0	5/80 (6.25)	
12	Pen,Met,Cep	1/40 (2.50)	1/40 (2.5)	2/80 (2.50)	16/80 (20.00)
13	Pen,Gen,Cep	2/40 (5.00)	1/40 (2.5)	3/80 (3.75)	
14	Pen,Gen,Cef	1/40 (2.50)	1/40 (2.5)	2/80 (2.50)	
15	Pen,Cef,Cep	1/40 (2.50)	1/40 (2.5)	2/80 (2.50)	
16	Pen,Cef,Met,Gen	2/40 (5.00)	1/40 (2.50)	3/80 (3.75)	3/80 (3.75)
17	Pen,Cef,Cep, Gen,Tri/sul	1/40 (2.50)	0	1/80 (1.25)	1/80 (1.25)
	Resistance to ≥ 2 antimicrobials	34/40 (85.00)[a]	19/40 (47.50)[b]	53/80 (66.25)	
	Resistance to <2 antimicrobials	11/40 (27.50)	16/40 (40.00)	27/80(33.75)	

[a] and [b]: Difference between column

P<0.05, Genmod test

CHAPTER V
DISCUSSION

EE is a sporadic disease that causes significant health problems and economic losses on individual farms, particularly for newly establishing farms or repopulated gilt herds (Wegener and Skov-Jensen, 2006). Mortality and morbidity in susceptible piglets in non immune herds may be high during an occurrence of EE. However, even minor performance of the disease can negatively affect the cost of feeder pigs, because it is difficult to sell the weanling pigs with the skin lesions of EE. The increase in EE in the pig producing industry might be due to some recent trends and development of farming scale such as increasing breeding sow population density, efforts to maximize number of piglets in litters, and less cutting of needle teeth at birth.

A few reports on prevalence of toxin productions and antimicrobial resistance of *S. hyicus* are available (Björklund, 2011). In addition, those studies are usually conducted in limited countries with advanced technology in pig production. The data about the occurrence of this disease are not fully geographic completed across the world. Moreover, the research outcomes of each country show differences in prevalence of each toxin genes. This variation is affected by difference in number of sample sizes and research methods in each study (Andresen, 2005).

The first report on prevalence of toxin producing *S. hyicus* from healthy pigs and pigs with EE in several countries was conducted by Andresen (2005). Samples for this study were collected from Europe (Belgium, Germany, UK, Croatia, and Slovenia), Asia (Japan, Korea and India) and North America (USA) and in a period of four decades (1960's -1990's). The bacteria were detected for toxin genes *exhA, exhB, exhC* and *exhD* using a multiplex PCR and immunoblotting methods. More than fifty percents of the EE cases were toxigenic.

The previous studies showed that *exhA* was the most prevalent in Japan, followed by *exhB* and *exh*D (Andresen, 2005; Futagawa-Saito et al., 2007). In contrast, *exhD* was the most prevalent in Germany and followed by *exh*B (Andresen, 2005; Kanbar et al., 2006), whereas in Denmark, *exhB* was the most prevalent followed by *exhA, exhC* and *exhD* (Andresen, 1998; Andresen and Ahrens, 2004). The order of toxin prevalence in Japan also changed after some years by another study. The *exhB* became the most prevalent toxin gene (Onuma et al., 2011), which was inconsistent to previous study. The prevalence of each toxin gene profile appears changing from time to time (Onuma et al., 2011) and from country to country (Aarestrup and Jensen, 2002; Andresen, 2005; Futagawa-Saito et al., 2007; Kanbar et al., 2006). In present study, the outcome was in accordance with the result from Begium. The *exhA* was the most prevalent gene, and followed by *exhD*, and both *exhA and exhD, and exhB* genes. The *Exh*C gene was not found. However, this was different from previous studies in some countries. This difference may be involving in the sample sizes and methods used.

Previous studies indicate that the virulent strains can be found in both healthy as well as pigs with EE (Tanabe et al., 1996; Andresen, 2005; Kanbar et al., 2006; Futagawa-Saito et al., 2007; Hassler et al., 2008). The prevalence of toxin producing strains was four times higher in EE pigs than healthy pigs (Futagawa-Saito et al., 2007). In general, explanation of *S. hyicus* originating from healthy pigs is limited (Tanabe et al., 1996; Andresen, 2005; Futagawa-Saito et al, 2007). In the present study, the toxin producing strains were also found in non-EE and EE pigs. The most predominant toxin gene was *exhA* in both non-EE and EE pigs. Up to now, the reasons for why healthy pigs harbored *exh* toxin genes without causing the EE has not been crystal cleared.

Previous research suggested that *S. hyicus* is considered as normal flora of the skin, lymph node, and other organs (Wegener 1992). In this current study, both toxin producing and non-toxin producing strains were isolated from pigs with and without skin lesions of EE. Both virulent and avirulent strains were found in the skin lesions, lymph node, and joint fluid samples. Among these samples, *S. hyicus* could be mostly isolated

from the pig skin, followed by lymph node and joint fluid samples, which are the systemic infection site. Therefore, skin is apparently preferential site for detection of S. hyicus as well as antimicrobial resistance. This is also beneficial and practical for collecting sample from live pigs.

From this investigation, number of toxin producing strains was higher in pigs with EE than pigs without EE. Number of toxin producing strains was also higher in skin samples than number in lymph node, and joint fluid samples of necropsied pigs. This was due to the target organ for toxin producing *S. hyicus*. The target organ of *S. hyicus* exfoliative toxins was the epidermis of the pig skin (Sato et al., 1991). In addition, experimental transmission in an earlier study showed that when pig infected with *S. hyicus* not only causing the lesions on the skin, but also causing lesion in joint, and lymph node. At necropsy, a serious or occasionally purulent polyarthritis in some cases, and enlarged, edematous superficial lymph node were observable (L'Ecuyer and Jericho. 1966). This supported that it is possible to isolate *S. hyicus* from lymph node and joint fluid.

It was surprising that exfoliative toxin gene carrying pathogens can be found in lymph node and joint fluid of the pigs without lesions of EE in the farm B. Only one toxin producing isolate was found in lymph node and another isolate from joint of different pig without EE. The pigs without EE, but poor health condition were delivered to the hospital for necropsy. Some of them affected with other diseases had swollen joint, so that *S. hyicus* may be a secondary invader to cause joint lesions.

The cross-sectional study in ten pig farms was attempted to isolate the agent from piglet skin and vagina of sows. *S. hyicus* could be found only from pig skin, but not from sow vaginal swabs. The swab samples were collected from lactating sows during weeks 2-4 of lactation. These sows were primiparous and mutiparous, and healthy. No EE skin lesion was observed. However, according to previous study which suggested that *S. hyicus* could be found in the genital tract of pigs in an EE outbreak herd. Another study also unveiled that *S. hyicus* was isolated from vagina of 35 out of 38 healthy

prepubertal gilts, indicating that vaginal carriage by purchased gilts may be a potential source of *S. hyicus* infection. The bacteria were also found in vaginal samples of the gilts within 24 hours before and after breeding (Wegener and Skov-Jensen, 1992). These sows were primiparous sows and gilts were imported from other farm, which may be *S. hyicus* original source. The bacteria were found higher at the time when the gilts were just introduced to the farms than at the time before farrowing.

Investigation on antimicrobial susceptibility is useful as an important guideline to choose effective antibiotics for treatment of this bacterial disease. This information delineates the advantage of promoting a careful use of antimicrobials, which potentially lessens the pressure of antimicrobial resistance induction (McEwen and Fedorka-Cray, 2002). Antimicrobial resistance in *S. hyicus* is common in large pig-producing countries. In some countries where EE is endemic, several studies demonstrated that penicillin, tetracycline, streptomycin, macrolides/lincosamides and sulphonamides are relatively highly resistant to *S. hyicus* (Wegener and Schwarz, 1993; Aarestrup and Jensen, 2002; Futagawa-Saito et al., 2009). However, variation in degree of resistance within these antimicrobial agents among countries, and among farms is according to routine antibiotic use (Aarestrup and Jensen, 2002; Futagawa-Saito et al., 2009). The antibiotics are commonly used as growth promoting factors in pig feed, leading to the prevalence of resistance dramatically increasing in some countries such as Japan and Denmark. After the ban of antibiotic used in pig feed, the rate of the resistance dropped significantly (Aarestrup and Jensen, 2002; Futagawa-Saito et al., 2009).

Similar to other pig producing countries, pig owners in Thailand treat the pigs with bacterial diseases using antibiotic as well as add antibiotic in feed for several purposes. This antibiotic usage potentially causes the increasing of the antimicrobial resistance in pig farms. A survey of drug use in Thailand in the pass showed some proof of overuse of antibiotics among Thai pig farming (FAO, 2002). In detail, firstly, more than 81.00% of investigated farms used antibiotic drugs. Secondly, the owners used antibiotic in the farm for a long time and increased the number of antibiotics. Although

most farmers (62.00%) reported that the trend of drug administration was stable, 25% admitted that they used several antibiotics. About 4-15% of pig farms reported some incidence of communicable diseases on their farms. Other than vaccination, the antibiotic was the second popular choice of viral disease solving. Only 17 farms employed the veterinarian to help solving the problems and 10 farms seek help from the Department of Livestock Development.

According to the present study outcome, the highest frequency of antimicrobial sensitivity of *S. hyicus* was trimethoprim-sulfamethoxazole, followed by methicillin, and cephalexin and others. The frequency of sensitivity was also higher in the non-toxin producing strains than the toxin producing strains. Compared to another study, the result showed that vancomycin, gentamicin, ofloxacin, and cefazolin were, in contrast, most commonly sensitive with *S. hyicus (*Futagawa-Saito et al., 2009). This difference may be due to the common antimicrobial agents used for treatment EE and the use of antibiotics as growth promotor.

In the current study, the highest resistance was penicillin. This was in accordance with previous studies in Japan, Denmark (Wegener and Schwarz, 1993; Aarestrup and Jensen, 2002; Futagawa-Saito et al., 2009). This is due to the fact that penicillin resistance usually dominated for a long time in many countries. The present study also found that *S. hyicus* became resistant to cephalexin and methicillin. According to our update knowledge, this was the first study showing *S. hyicus* was resistant to methicilin. The mecA gene was found in *S. hyicus,* but these *S. hyicus* was not found to be resistant to antibiotics (Hassler et al., 2008).

S. hyicus was found sensitive to gentamicin in Japan, but resistant in current study. A combination of trimethoprim-sulfamethoxazole gave decreasing rate of resistant compared with previous studies (DANMAP, 1996-2008). Not any tested isolates of current study found to be resistant to vancomycin. This agreed with the result found in Japan (Futagawa-Saito et al., 2009). As previous mentioned, antimicrobial resistance is various, depending on several factors.

Multiple resistances are common among *S. hyicus* strains*,* and can be found in isolates from both healthy and pigs with EE (Schwarz and Blobel, 1989; Wegener and Schwarz, 1993; Futagawa-Saito et al., 2009). The prevalence of antibiotics resistance was higher among diseased pig isolates (Wegener and Schwarz, 1993; Futagawa-Saito et al., 2009). Futagawa-Saito et al. (2009) observed differences in resistance patterns in multiple resistant strains. Five patterns exhibiting resistance to three or more antimicrobials were found to be predominant pattern. Three out of five patterns were only seen in toxigenic strains. Other two patterns were seen in strains from both sources, but with higher frequencies in healthy pig strains. Similar findings were reported by Teranishi et al. (1987). The dominant patterns among healthy pig isolates and diseased pig isolates are often different, and the dominant pattern in one source can be identical to the others (Teranishi et al., 1987; Futagawa-Saito et al., 2009).

In the current study, the multiple resistances were found in both toxin producing and non-toxin producing strains. The prevalence of multiple resistances was 73.75% of the tested strains. Resistance to the group of two antibiotics was seen the most common resistant, followed by the group of three antibiotics. Resistance to penicillin and cephalexime was the most common among the group of two antibiotics. Resistance to the group of penicillin/methicillin/trimethoprim-sulfamethoxazole was the highest among the group of three antibiotics. The study in Japan found that *S. hyicus* was the most resistant to the group of penicillin/ampicillin/trimethoprim-sulfamethoxazole, following by the group of penicillin/ampicillin/erythromycin/trimethoprim-sulfamethoxazole (Futagawa-Saito et al., 2009).

Differences in resistance between *S. hyicus* from the pigs with and without EE have been observed. In one study, the higher resistant rates to penicillin, tetracycline and streptomycin were among diseased pig isolates (Wegener and Schwarz, 1993). In another study, the higher resistant rate belonged to the *S. hyicus* isolated from healthy pigs (Teranishi et al., 1987). However, both studies found the resistance to macrolides and lincosamides with the same extent in strains derived from both healthy and the

disease pigs. The result from these studies is in accordance with a similar study with known toxigenic and non-toxigenic strains in Japan. The purpose of that study was to demonstrate any association of the antimicrobial resistance between toxin producing and non-toxin producing strains, but no association could be found (Futagawa-Saito et al., 2009).

In the current studies, the resistant rates to antibiotics were higher in toxigenic strains than non-toxigenic strains, but not of significantly different. This finding may be due to the long term use of antibiotics in treatment of diseased pigs.

In conclusion, the exfoliative toxin genes were detected with relative higher prevalent rate in isolates from skin in the pigs with lesions of EE than isolates from skin of healthy pigs. The *exh*A was the most common toxin gene in the pigs in central Thailand. In one isolate of *S. hyicus* can carry two of exfoliative toxin genes (*exhA* and *exhD*).

S. hyicus was the most resistant to penicillin and cephalexin to both toxigenic strain and non-toxigenic strain. Resistance to two antimicrobial agents also found and dominant in this group was combination of penicillin with other antimicrobial. The multiple antimicrobial resistances found higher in toxigenic strain than in non-toxigenic strains.

In order to treat the diseased pigs in an effective way, pork producers and swine veterinarians should conduct the antimicrobial susceptibility tests prior to the treatment of EE for the diseased pigs. Preventive measure needs to be focused which includes: minimizing wounds by minimizing cross-fostering and non-essential mixing of pigs, and possibly by clipping needle teeth at early day after birth, and in addition, exercising good sanitation, lowering humidity, and treating wounds promptly with an antiseptic. The production of autogenous vaccine that injected for the sows and the gilts in the specific farms will certainly beneficial in an effective prevention method.

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