การพิสูจน์เชื้อ *มัยโคพลาสมา ฮีโมซูอิส* ณ ฟาร์มสุกรในประเทศไทยด้วยวิธีทั่วไป และวิธีทางอณูชีวโมเลกุล

นางสาวเรืองอุไร กิจโชดก

จุฬาลงกรณ์มหาวิทยาลัย
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THE IDENTIFICATION OF *MYCOPLASMA HAEMOSUIS* IN THAI SWINE FARMS

WITH CONVENTIONAL AND MOLECULAR TECHNIQUES

Miss Ruangurai Kitchodok

A Thesis Submitted in Partial Fulfillment of the Requirements
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Department of Veterinary Pathology
Faculty of Veterinary Science
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เรืองอุไร กิจโชดก : การพิสูจน์เชื้อ *มัยโคพลาสมา ฮีโมซูอิส* ณ ฟาร์มสุกรในประเทศไทยด้วยวิธีทั่วไปและวิธีทางอณูชีวโมเลกุล. (THE IDENTIFICATION OF *MYCOPLASMA HAEMOSUIS* IN THAI SWINE FARMS WITH CONVENTIONAL AND MOLECULAR TECHNIQUES) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. น.สพ. ดร. ปิยนันท์ ทวีถาวรสวัสดิ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. น.สพ. ดร. รุ่งโรจน์ ธนาวงษ์นุเวช, นาง สุดจิตต์ จุ่งพิวัฒน์, 100 หน้า.

โรคฮีโมพลาสมาสในสุกร มีสาเหตุมาจากเชื้อ *มัยโคพลาสมา ฮีโมซูอิส* (*M. haemosuis*) หรือ *มัยโคพลาสมา ซูอิส* (*M. suis*) หรือในอดีตทราบกันในชื่อ *อีเพอริโทรซูน ซูอิส* (*E. suis*) เป็นโรคติดเชื้อโดยมีแมลงดูดเลือดเป็นตัวนำโรค ซึ่งสภาวการณ์เกิดโรคและความชุกของโรคชนิดนี้ในประเทศไทยยังไม่มีข้อมูลชัดเจน ดังนั้นจุดประสงค์ของการศึกษาในครั้งนี้ เพื่อตรวจวินิจฉัยเชื้อ *M. suis* ในฝูงสุกรในประเทศไทยด้วยวิธีทั่วไปและเทคนิคทางอณูชีวโมเลกุล เก็บตัวอย่างเลือดสุกรสงสัยที่มีแนวโน้มการติดเชื้อ *M. suis* จำนวน 300 ตัวอย่างจากสุกรฟาร์มเปิดใน 6 จังหวัดของประเทศไทย ได้แก่ จังหวัดนครปฐม ฉะเชิงเทรา ปราจีนบุรี ราชบุรี ศรีษะเกษ และเชียงใหม่ นำมาตรวจโดยทำฟิมล์เลือดบางย้อมด้วยสี 10% ยิมซา อะไครดีน ออเรนจ์ (AO) และวิธีพีซีอาร์ทั่วไปโดยใช้เลือดที่ให้ผลบวกต่อ 10% ยิมซาและ AO จำนวน 10 ตัวอย่าง และวิธีเนสเตด พีซีอาร์ โดยเลือกยีนอนุรักษ์ (16srRNA) จำนวน 27 เสตรนของเชื้อจากสุกรเลี้ยงและสุกรป่าที่มีใน GenBank ออกแบบไพรเมอร์คู่นอกด้วยสายตาจากโปรแกรม Bioedit V7.2.5 และ Multalin และไพรเมอร์คู่ในได้จากสายลำดับเบสของรีคอมบิแนนท์ดีเอ็นเอ โดยใช้โปรแกรม Primer3 ซึ่งเป็นตัวควบคุมบวกที่ได้รับจากประเทศญี่ปุ่น ไพรเมอร์ที่ได้รับและไพรเมอร์จากการออกแบบในครั้งนี้ถูกทดสอบความไวและความจำเพาะด้วยเชื้อ *มัยโคพลาสมา* (*Mycoplasma*) ชนิดอื่นที่พบได้จากในแมวและสุกร ร่วมกับการทดสอบเชื้อ *M. suis* ในเลือดสุกรจากฟาร์มที่ตรวจพบเชื้อ *M. suis* ด้วยวิธีย้อมสี 10% ยิมซา และ AO จำนวน 10 ตัวอย่าง ผลการทดสอบไพรเมอร์ พบว่าไพรเมอร์ที่ออกแบบมีความจำเพาะสูงต่อเชื้อ *M. suis* ที่ตำแหน่ง 603 bp (วงนอก) และ 222 bp (วงใน) หรือเฉพาะ 222 bp (วงใน) และมีความไวสูงสามารถตรวจปริมาณดีเอ็นเอได้อย่างน้อย 2.351×10^{-6} copies/µl จากตัวอย่างเลือดสุกร 300 ตัวอย่างด้วยการย้อมสี 10% ยิมซาพบเชื้อมีลักษณะจุดกลม ติดสีม่วงอมชมพูเรียงเป็นแถวหรือเดี่ยว จำนวน 23 ตัวอย่าง (7.67%) การย้อมด้วย AO พบเชื้อ 45 ตัวอย่าง (15%) และตรวจด้วยวิธีเนสเตด พีซีอาร์ พบเชื้อ 70 ตัวอย่าง (23.33%) ดังนั้นการศึกษาในครั้งนี้เป็นครั้งแรกในการตรวจหาเชื้อ *M. suis* ภายในฟาร์มสุกรในประเทศไทยด้วยวิธีอณูชีวโมเลกุล (พีซีอาร์ และเนสเตด พีซีอาร์) ซึ่งผลการทดลองในครั้งนี้สามารถนำไปใช้เป็นทางเลือกสำหรับตรวจวินิจฉัยโรคโดยเฉพาะในสุกรพาหะของโรคเพื่อการควบคุมการระบาดและวางแผนการรักษาต่อไป

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ภาควิชา พยาธิวิทยา ลายมือชื่อนิสิต ....

สาขาวิชา พยาธิชีววิทยาทางสัตวแพทย์ ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก ....

ปีการศึกษา 2556 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม ....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม ....

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RUANGURAI KITCHODOK: THE IDENTIFICATION OF *MYCOPLASMA HAEMOSUIS* IN THAI SWINE FARMS WITH CONVENTIONAL AND MOLECULAR TECHNIQUES. ADVISOR: ASST. PROF. DR. PIYANAN TAWEETHAVONSAWAT, CO-ADVISOR: PROF. DR. ROONGROJE THANAWONGNUWECH, MRS. SUDCHIT CHUNGPIVAT, 100 pp.

Swine hemoplasmas caused by *Mycoplasma haemosuis* (*M. haemosuis*) or *Mycoplasma suis* (*M. suis*) or previously known as *Eperythrozoon suis* (*E. suis*) is the infectious disease via insect vectors that the information of its pathogenesis and prevalence had been still unclear. Therefore, this study aimed to diagnose *M. suis* in swine herds in Thailand by using conventional and molecular techniques. Three hundred suspected pigs tending to *M. suis* infection were collected for blood samples from open-air swine farms in six provinces of Thailand such as Nakhon Pathom, Chachoengsao, Prachinburi, Ratchaburi, Sisaket, and Chiang Mai. Blood were detected by thin blood smear staining with 10% Giemsa, acridine orange (AO) and conventional PCR using 10 samples that were positive to *M. suis* by 10% Giemsa and AO and nested PCR based on conserve gene (16srRNA) of 27 strains of *M. suis* from domestic pigs and wild pigs in GenBank. Outer primers were designed using Bioedit V7.2.5 and Multalin programs and inner primers were designed based on DNA sequence of derived recombinant DNA as positive control from Japan using Primer3 program. Those primers in this study were examined sensitivity and specificity tests with other *Mycoplasma* in feline and swine together with 10 blood samples that were positive to *M. suis* based on Giemsa and acridine orange. The result of primers investigation found that designed primers were highly specificity to *M. suis* at position 603 bp (outer) and 222 bp (inner) or only 222 bp (inner) and highly sensitivity that could scrutinize a quantity of DNA at least 2.351×10^{-6} copies/µl. Based on 300 blood samples, twenty-three samples (7.67%) showed basophilic discoid shape using 10% Giemsa. Forty-five blood samples (15%) were found using acridine orange and 70 samples (23.33%) using nested PCR were positive. Consequently, this study is the first to investigate *M. suis* within swine farms in Thailand using molecular techniques (PCR and nested PCR). The results of this study could be used as alternative diagnostic assays especially carrier pigs for continuing control of outbreak and treatment planning.

Department:	Veterinary Pathology	Student's Signature	....
Field of Study:	Veterinary Pathobiology	Advisor's Signature	....
Academic Year:	2013	Co-Advisor's Signature	....
		Co-Advisor's Signature	....

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Figure 19 Virulence score (0-5) of Giemsa-stained blood smears. (**C**) only one *M. suis*-like on more than one infected RBCs was seen as score 2 (black arrow) (bar = 10 μm) and (**D**) more than one *M. suis*-like harboring on infected erythrocytes was observed as score 3 (black arrow) (bar = 10 μm). 63

Figure 20 Virulence score (0-5) from Giemsa-stained blood smears. (E) more than one or one suspected *M. suis* (black arrow) on almost infected RBCs was found as score 4 (bar = 10 μm)..64

Figure 21 Bar graph of virulence score (0-5) in seventy positive blood samples based on nested PCR. Score 0 could be observed most up to 52 samples. Score 1 was seen in 6 samples. Score 2 was seen in 10 samples. Score 3 was observed in 1 sample. Score 4 was seen in 1sample. Score 5 was not found. 65

LIST OF ABBREVIATION

ELISA	Enzyme-linked immunosorbent assay
GADPHs	Glyceraldehydes-3-phosphate dehydrogenase
HsP1	Heat shock protein
HIV	Human immunodeficiency virus
IHA	Immunohaemagglutination test
PCR	Polymerase chain reaction
PE	Porcine Eperythrozoonosis
PP	Pentose phosphate pathways
Ppa	Inorganic pyrophosphate gene
PRRS	Porcine reproductive and respiratory syndrome
RBCs	Red blood cells
rnpB	RNase P RNA
Sec-tRNA	Selenocysteine tRNA
WBCs	White blood cells

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CHAPTER I
INTRODUCTION

Important and rationale

Mycoplasma haemosuis (*M. haemosuis*) or *Mycoplasma suis* (*M. suis*) or previously so-called *Eperythrozoon suis* (*E. suis*) is uncultivable hemotrophic agent in all age domestic pigs (*Sus scrofa domestica*) and wild pigs (*Sus scrofa*) (Splitter, 1950; Baker et al., 1971; Hoelzle et al., 2011), which presently is belonging to genus *Mycoplasma* depended on closely phylogenetic typically relationship in 16S rRNA gene (Neimark et al., 2001; Neimark et al., 2002; Messick, 2004; Neimark et al., 2005; Hoelzle, 2008; Hoelzle et al., 2011).

This agent generally affected causing anemic sign in all aged pigs, reproductive disorders in sows and gilts, icteroanemia and growth retardation in fattening pigs (Messick, 2004; Givens and Marley, 2008; Hoelzle, 2008). What's more, this agent mostly can be found in nursery pigs which compatible with upper and lower respiratory and also gastro-enteric disorders (Hoelzle, 2008).

Surprisingly, potentially zoonosis between swine and immunocompromised human beings in particular pregnant women, human immunodeficiency virus (HIV) infection and neonatal birth are noted particularly large-scale batches in China. Based on many publications in recent, *M. suis* is a wide-range host including swine, human and laboratory mice (Yang et al., 2000; Wu et al., 2006; Tasker et al., 2010; Huang et al., 2012). Until today, *M. suis* has been still a novel agent in many countries because of uncultured in *vitro* (Messick, 2004; Hoelzle, 2008), little report, difficultly investigation and isolation during very low parasitemia.

In addition, *M. suis* affirmation within swine herds with only routine techniques is recorded in some countries including Thailand. Transmission of this disease is two main factors are insect vectors including mostly blood-sucking insects, contaminated semen, cannibalism and mechanics such as contaminated surgical instruments and recycle needles in sensitivity farms (Hoelzle, 2008). Currently, well-known pathogenesis concept was broaden explained relied on parasitic characteristics, abnormally red blood cells (RBCs) life spanning and host immune modulating that directly associated with clinical signs and criteria of hematologic values especially low blood glucose and low erythrocytes (Prullage et al., 1993; Messick, 2004; Hoelzle, 2008; Guimaraes et al., 2011; Oehlerking et al., 2011).

Mortality and morbidity rates in various infected herds approximately 10%-60% (Yang et al., 2000; Watanabe et al., 2012) are acclaimed in each farm based on host immunity, farm biosecurity policy, vector control, drug treatment and virulent factors particularly strain of *M. suis* (KI3806) (Messick, 2004; Hoelzle, 2008). Recently, two genomes of *M. suis* strains namely Illinois isolated from North America (Non-invasive) and KI3806 isolated from Europe (Invasive) (Groebel et al., 2009) had been entire sequenced in 2011 (Guimaraes et al., 2011; Oehlerking et al., 2011). Those results displayed the difference of those strains in genome size and the numbers of hypothetical proteins (Guimaraes et al., 2011; Oehlerking et al., 2011).

In the past, *M. suis* diagnosis predominantly as following by three core methods, For instance, clinical signs and pathological lesions criteria of inoculated splenectomized pigs, conventional techniques (Romanowsky staining and acridine orange) (Messick, 2004; Hoelzle, 2008) and serological diagnostic assay (Indirect Immunohaemagglutination test; IHA and Enzyme-linked immunosorbent assay; ELISA) which those techniques has been established until present for monitoring herd health status or confirmation of this disease in farms (Hoelzle et al., 2007; Liu et al., 2012).

However, the laboratory methods mentioned above are limited to scrutinize during very low parasitemia, host humoral immunity (immunoglobulin M (IgM) or Immunoglobulin G (IgG)) from different ages of pigs and duration of infection (Hoelzle, 2008) except highly sensitivity and specificity techniques as subsequently PCR and real time-PCR. As a consequent, PCR has been continuously developed and widely used to corroborate this agent in swine herds worldwide from 1990s until it becomes gold standard to date (Gwaltney et al., 1993; Messick et al., 1999; Hoelzle et al., 2003).

M. suis treatment had been still essential to ease clinical signs, pathological lesions, virulence and economic losses in farms serving as cyclin and arsenic antibiotics. Even through, those drugs can not enough to successfully eradicate this agent in circulatory system and finally bring about carrier pigs in farms continuously (Messick, 2004; Hoelzle, 2008). However, these drugs are one of a good prevention paths in both small and large-scales herds by virtue of unavailable commercial vaccine protecting *M. suis* infection.

In Thailand epidemic circumstance in pronto, *M. suis* had ever been little reported in some documents including Chulalongkorn University publications during 1980s. Both conventional techniques and *M. suis*-inoculated splenectomized pigs mainly were used for *M. suis* verification due to easy (Hoelzle, 2008). However, those techniques including Giemsa displayed ambiguous morphologic characteristics and mostly showed mislead results due to color background (Luengyosluechakul and Nithiuthai, 1989).

Thereby, this agent in swine herd in Thailand has been taken for granted until present according to difficultly investigation by conventional techniques and non-typically clinical signs. Little is known about exactly existence of *M. suis* in swine farms in Thailand because of lacking of its confirmation by molecular identification and DNA sequencing.

Therefore, the aim of this present study is to identify existence of *M. suis* in swine herds in Thailand by conventional and molecular techniques and how to detect this agent accurately using more sensitive tool in Thailand in the near future.

Objectives of study

To identify *M. suis* existence in swine herds in Thailand with conventional and molecular techniques

Hypothesis

Existence of *M. suis* in swine farms in Thailand can be identified with both conventional and molecular techniques

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CHAPTER II
LITERITURE REVIEWS

Background and rationale

Porcine hemoplasmas, nowadays caused by *Mycoplasma suis* (*M. suis*) or *Mycoplasma haemosuis* (*M. haemosuis*) or formerly known as *Eperythrozoon suis* (*E. suis*), is the infectious vector-borne disease which triggers icteroanemia and growth retardation in all age pigs in America, Europe and Asia (Splitter, 1950; Messick, 2004; Hoelzle, 2008). This agent led to almost 100% morbidity and up to 60% mortality relied on host immune status, naïve herds, biosecurity policy and vector control in swine farms (Messick, 2004; Hoelzle, 2008). *M. suis* or *E. suis* was firstly reported in 1930s in United states (Kinsley, 1932) and called infectious icteroanemia disease, Anaplasmosis-like and yellow belly. Later, this agent was name *E. suis* for the reasons of cell target and spherical characteristics (Splitter, 1950).

M. suis was found on red blood cells (RBCs) surfaces in infected pigs which was abruptly 0.2-2 μm in size, pleomorphic configurations including discoid, oval or rod (Messick, 2004; Hoelzle, 2008) based on using different laboratory equipment (Pospischil and Hoffmann, 1982). Depended on Giemsa microscopy, this bacterial performs basophilic pleomorphic shapes arranging in chain-like or single harboring on RBCs structures and illustrates bright orange in acridine orange (Heinritzi, 1990; Messick, 2004; Hoelzle, 2008; Hoelzle et al., 2010a). According to unlike bacterial characteristics and vector relevant, this agent initially was categorized in order Rickettsiales, family Anaplasmataceae and in genus *Eperythrozoon* before reclassification in 2002.

The novel clustering of this disease was recorded as followed by prokaryotic committees' agreement because of phylogenetic typically in 16S rRNA gene as a conserve gene that closely regarding genera *Mycoplasma* in pneumonia bacterial group (Neimark et al., 2001; Neimark et al., 2002; Neimark et al., 2005). Additionally, sensitivity to beta-lactam, cell wall disappearance, uncultivable in *vitro*, vector relationship and minute genome size are also involved in genus *Mycoplasma* (Messick, 2004; Hoelzle, 2008).

In the past, four hemoplasma prokaryotes isolated from domestic animals and livestock were reclassified and declared in 2002 namely *Candidatus* Mycoplasma suis (former; *Eperythrozoon suis*, present; *Mycoplasma suis*) in swine, *Candidatus* Mycoplasma haemofelis (former; *Haemobartonella felis*, present; *Mycoplasma haemofelis*) in feline, *Candidatus* Mycoplasma wenyonii (former; *Eperythrozoon wenyonii*, present; *Mycoplasma wenyonii*) in large ruminants and *Candidatus* Mycoplasma haemomuris (former; *Haemobartonella muris*, present; *Mycoplasma haemomuris*) in rodents.

Until present, *M. suis* taxonomic nomenclature has been belonging to kingdom Bacteria, phylum Firmicutes, class Mollicutes, order Mycoplasmatales, family Mycoplasmataceae and genus *Mycoplasma* as phylogenetic status consideration that showed in below. (Neimark et al., 2002; Hoelzle, 2008).

Kingdom Bacteria

Phylum Firmicutes

Class Mollicutes

Order Mycoplasmatales

Family Mycoplasmataceae

Genus *Mycoplasma*

Species *haemosuis* or *suis*

Class Mollicutes, the word Mollicutes was derived from Latin language called mollis meaning soft or fragile and cutes meaning skin that classified in most cell wall less bacteria. Scientists suggested that *M. suis* related to evolution of gram positive bacteria lists without cell wall depending on branching of coherent phylogenetic analysis among bacteria and similarity of housekeeping genes (Dybvig and Voelker, 1996). Basically, bacteria in class Mollicutes mostly showed uniquely very small genome size around less than 600 Kb (Kilo base pair), low G+C contents, universal genetic code disappearance, numerous A-T, and encoding tryptophan from stop codon (TGA) that differs from most other organisms (Dybvig and Voelker, 1996). Likewise, minute size and losing capacity encoding key enzymes are also observed in genome that affect to its survival and replication in eukaryotic hosts (Dybvig and Voelker, 1996; Guimaraes et al., 2011; Oehlerking et al., 2011).

Class Mollicutes in update is distinguished into five orders namely Acholeplasmatales, Anaeroplasmatales, Entomoplasmatales, Haloplasmatales and Mycoplasmatales and six genera including *Acholeplasma*, *Anaeroplasma*, *Asteroleplasma*, *Mycoplasma*, *Spiroplasma* and *Ureaplasma* which each order manifests in different typical characteristics, target organ and host specific (Dybvig and Voelker, 1996). However, hemoplasmas and *Mycoplasma* isolated from swine are also different size, target organs and pathogenesis pathways (Ross and Duncan, 1970; Barden and Decker, 1971; Messick, 2004; Hoelzle, 2008).

Examples of *Mycoplasma* in respiratory tract in swine are *M. hyopneumoniae*, *M. hyorhinis* (Ross and Duncan, 1970; Barden and Decker, 1971; Kobisch and Friis, 1996; Maes et al., 1996; Maes et al., 2008b). *M. hyosynoviae* and two of them (*M. hyorhinis* and *M. synoviae*) can cause joint disease (Ross and Duncan, 1970; Barden and Decker, 1971). Besides, stomach cancer had ever been reported in *M. hyorhinis*-infected pigs (Gong et al., 2008).

At present, those agents can be cultured in *vitro* by selective media or be strenuously cultured in monolayers (Zielinski et al., 1990). However, time consuming and expert labors are also limited even if this technique is a gold standard to confirm those agents (Thacker, 2004). Several hemotrophic agents in creatures were publicly documented in many countries in recent year including Asia (Splitter, 1950; Messick, 2004; Neimark et al., 2005; Hoelzle, 2008). However, those agents were heeded in circulatory system and finally caused anemia during stress (Messick, 2004; Hoelzle, 2008). Examples of hemoplasmas isolated from swine and others had ever been noted in many publications as shown in Table 1.

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Table 1 The taxonomic nomenclature of bacterial agents in wildlife, domestic animals and livestock animals

Host	Name	Rename (Mycoplasma)	References
Swine	*E. suis*	*M. haemosuis*	(Splitter, 1950; Hoelzle, 2008)
	E. parvum	*M. parvum*	(Splitter, 1953; do Nascimento et al., 2013)
Cattle	*E. wenyonii*	*M. wenyonii*	(Neimark and Kocan, 1997; Neimark et al., 2002)
Feline, lion	*H. felis*	*M. haemofelis*	(Neimark et al., 2002; Kewish et al., 2004)
	H. haemominutum	*M. haemominutum*	(Foley and Pedersen, 2001; Kewish et al., 2004)
	Candidatus M. turicensis	-	(Novacco et al., 2011; Willi et al., 2011)
Canine	*H. canis*	*M. haemocanis*	(Compton et al., 2012; do Nascimento et al., 2012)
	Candidatus M. haematoparvum	-	(Sykes et al., 2005; Compton et al., 2012)

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Host	Name	Rename (Mycoplasma)	References
Mice	*E. coccoides*	*M. coccoides*	(Neimark et al., 2002; Neimark et al., 2005)
Sheep, deer	*E. ovis*	*M. ovis*	(Neimark et al., 2004)
Monkey	*Candidatus* M. haemomacaque	-	(Maggi et al., 2013)
Sheep, goat	*Candidatus* M. haemoovis	-	(Hornok et al., 2012)
Llamas, alpacas	*Candidatus* M. haemolamae	-	(Almy et al., 2006; Tornquist et al., 2011; Crosse et al., 2012)
California sea lion	*Candidatus* M. haemozalophi sp. nov.	-	(Volokhov et al., 2011)
Rat	*H. muris*	*M. haemomuris*	(Neimark et al., 2001; Harasawa et al., 2002; Sashida et al., 2013)
Opossum	*Candidatus* M. haemodidelphis	-	(Hoelzle, 2008)
Squirrel, Monkey	*Candidatus* *M. kahanei*	-	(Hoelzle, 2008)

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Interestingly, medicine and public health fields industriously are interested in novel hemotrophic agent particularly *M. suis* isolated from human blood which was confirmed by polymerase chain reaction (PCR) based on 16S rRNA gene (conserve gene) and RNase P RNA gene (*rnpB*). Besides, the questions of potentially zoonosis of other hemotrophic agents including *M. ovis* (sheep), *M. haemominutum* (feline) and *M. haemofelis* (feline) are clear based on nested PCR (Yang et al., 2000; Wu et al., 2006; dos Santos et al., 2008; Yuan et al., 2009; Sykes et al., 2010; Tasker et al., 2010; Steer et al., 2011; Huang et al., 2012). In addition, *M. ovis* has been reported that it occasionally always infects together with *Bartonella hensalae* (*B. hensalae*) in immunocompromised humans (Yang et al., 2000; Wu et al., 2006; dos Santos et al., 2008; Yuan et al., 2009; Sykes et al., 2010; Tasker et al., 2010; Steer et al., 2011; Huang et al., 2012).

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Unfortunately, little is elucidated clearly about *M. suis* pathogenesis and pathogenicity by reasons of uncultured in *vitro* (Messick, 2004; Hoelzle, 2008) and difficultly investigation during very low parasitemia (Hoelzle, 2008). However, some details of mechanisms from their entire genomes of two strains including Europe (KI3806) and America (Illinois strain) are observed from gene encoding proteins and existing key metabolism pathways (Guimaraes et al., 2011; Oehlerking et al., 2011).

Two years later, whole genome of *M. parvum* was isolated in swine farms in America (Indiana strain) (do Nascimento et al., 2013). Its genome was entirely sequenced (do Nascimento et al., 2013). Those results were directly deal with previously study on survey and existence of this agent in Japan based on 16S rRNA and RNase P RNA genes (Watanabe et al., 2011; Watanabe et al., 2012). In entire genome, it can insist that *M. parvum* is not different development stages of *M. suis* but it is the smallest bacterial hemotrophic agent in pig circulatory system (Splitter, 1953; do Nascimento et al., 2013).

M. *parvum* or formerly so-called *E. parvum* is the smallest hemotrophic agent in pig blood causing anemia signs. This agent firstly found in swine farms in 1950's by Splitter (Splitter, 1953) manifesting discoid characteristics harboring on erythrocytes as well as *M. suis* (Splitter, 1950). The differentiation between *M. parvum* and *M. suis* was size measurement and clinical signs observation. Even through, some documents reported that *M. parvum* could contribute to severe symptoms in splenectomized or immunosuppressive pigs as well as *M. suis* (Barnett, 1963).

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M. suis epidemiology

M. suis firstly reported in swine herds in America in 1930s by Kinsley (Kinsley, 1932) and recorded *Anaplasma* – like characteristics by Splitter in 1950 (Splitter, 1950). Moreover, a smaller agent called *E. parvum* was documented in 3 years later (Splitter, 1953). Until present, *M. suis* has been still reported worldwide over 70 years (Splitter, 1950; Heinritzi, 1990; Gwaltney et al., 1993; Messick et al., 1999; Yang et al., 2000; Wu et al., 2006; Ritzmann et al., 2009; Yuan et al., 2009; Hoelzle et al., 2010a; Huang et al., 2012; Watanabe et al., 2012). The prevalence of this disease in several countries was noted approximately 1-15% (Ritzmann et al., 2009; Watanabe et al., 2012).

In Asian epidemic outbreaks, *M. suis*-infected humans had ever been recorded in China during 1994-2007 based on molecular detection (Hu et al., 2009). More than 1,000 infected pigs were investigated from 1997 to 2007. *M. suis* could be detected around 600,000 during 2003 that impacted on economic losses in many regions of China (Hu et al., 2009). The prevalence of this bacterial agent in China up to 80% (Yuan et al., 2009) but *M. suis* prevalence in swine herds in Japan showed less than those (5%) (Watanabe et al., 2012). In addition, mixing between *M. parvum* and *M. suis* was documented in swine farms in Japan around 15% using real-time PCR (Watanabe et al., 2012). In Thailand epidemic circumstance, actually prevalence of this agent is remaining unknown because of source-limited molecular methods for confirmation. Conventional techniques including Giemsa have been remained used in laboratory in Thailand since 1980s (Luengyosluechakul and Nithiuthai, 1989).

M. suis morphology and genome characteristics

M. suis had minute genome size and pleomorphic configuration including discoid, ring and rod (Pospischil and Hoffmann, 1982; Messick, 2004; Hoelzle, 2008). It showed basophilic *Anaplasma*-like arranging in single or chain-like harboring on RBCs by using Romanowsky staining (Hoelzle, 2008). Besides, bright orange generally can be seen using acridine orange which easily differentiate between dark green RBCs and bacteria (Messick, 2004; Neimark et al., 2004; Hoelzle, 2008).

Analysis of core phylogenetic clade relationship among hemoplasma based on almost entire of 16S rRNA gene can be divided into two groups namely hemoplasma and pneumonia (Zhou et al., 2009). Subdivision of hemoplasma species in current are recognized as hemominutum and hemofelis groups based on 16S rRNA (conserve gene) relevant. *M. suis* was categorized in hemominutum group as well as hemotrophic agent derived from cattle (*M. wenyonii*), sheep (*M. ovis*) and feline (*Candidatus* M. haemominutum). *M. haemofelis* and *M. haemocanis* were belonging to hemofelis group. The remaining group was bacteria isolated from humans including *M. hominis* and *M. pneumonia*. Interestingly, all bacterial hemotrophic agents were noted in single clade and closely associated with *M. pneumonia* group (Zhou et al., 2009).

Two years later, whole genomes of two strains of *M. suis* namely KI3806 and Illinois are entirely sequenced (Guimaraes et al., 2011; Oehlerking et al., 2011). Relied on whole genomes of those strains, pathogenicity, metabolism pathways, parasitic characteristics, highly adaptation and immune evasion are summarized which insist that this agent is Mollicutes bacterial group from molecular mimicry such as stop codon and ori*C* in prokaryotes (Guimaraes et al., 2011).

Its pathogenicity including causing RBCs fragile during acute phase based on metabolisms process, some enzyme orthologs and host immunomodulatory status are noted. Paralogous genes are better understanding during chronic phase. Furthermore, some details of lacking nutrients in those studies can be used for development of suitable selective media for culture of this agent in *vitro* in the future (Guimaraes et al., 2011; Oehlerking et al., 2011).

Based on whole genome of Illinois strain (Accession number: CP002525), it has a single circular chromosome approximately more than 740,000 base pairs (bp) in size and was composes of single copies of 3 rRNA and 32 tRNA. This agent is similarity to Mollicutes from using UTA for tryptophan codon and selenocysteine tRNA (Sec-tRNA) for stop codon. CDSs can be found around almost 90% and in length 783 bp that is similar to *Rickettsia* group. Moreover, CDSs proteins are observed up to 30% and the remaining is hypothetical protein (more than 60%) and conserved hypothetical protein (4%).

In *M. suis* strain KI3806 (Accession number: FQ790233), it has a circular chromosome and was comprised of the main composition including 32 tRNAs and a single-copy 16S rRNA as a Illinois strain from America. However, the differences between those are genome size and the numbers of hypothetical proteins (Guimaraes et al., 2011; Oehlerking et al., 2011). KI3806 has a smaller size (709,270 base pairs) with 31% GC content, and 70% hypothetical proteins. In addition, this research notes that *M. suis* is lacking necessary metabolic pathways for its survival and replication by itself (Oehlerking et al., 2011).

Some enzymes, glycolysis process and transporters of *M. suis* are encoded for nutrient uptake (Oehlerking et al., 2011). Glycolysis metabolism is noted in its genome but other ATP synthesis pathway including arginine hydrolysis and dephosphorylation of dihydroxyacetone phosphate are unapparent.

Based on glycolysis mechanism, complete process are composed of the preparatory and pay-off phases (Romano and Conway, 1996). Derived sugar from conversion by glucose 6-phosphate isomerase is taken by *M. suis* that caused hypoglycemia pigs from its nutrients requirement (Hoelzle, 2008; Guimaraes et al., 2011; Oehlerking et al., 2011). NAD+, NADPH and Coenzyme A also are needed to its survival in host but enzymes ortholog and pentose phosphate pathways (PP) are not functional. This factor is the best choice to develop selective media for its replication in *vitro* (Guimaraes et al., 2011). According to lacking some pathways, *M. suis* tended to use exogenous fatty acids to create plasma membrane by phosphatidylglycerol and cardiolipin for transferring vitamins, sugar and amino acid via ABC transporters.

Generally, several biomolecules such as glucose, inosine, hypoxanthine, amino acids, NADH/NADPH and ribose are released from infected RBCs before their devastation. Consequently, the reasons mentioned above can insist that *M. suis* is a blood parasite harboring on erythrocytes structures due to its behaviors and genome characteristics (Guimaraes et al., 2011; Oehlerking et al., 2011). However, DNA repair and some transporters revealved transportation of several molecules via plasma membrane can be apparent. Virulence of this agent is still obscured but some proteins are mentioned (Guimaraes et al., 2011).

Interestingly, O-sialoglycoprotein endopeptidase ortholog is noticed in its genome that is concerned with erythrocyte lysis. Some heat shock proteins are documented including DnaJ, DnaK (ortholog for chaperonin GroEL) and GrpE but chaperonin GroEL does not existed (Guimaraes et al., 2011). Additionally, paralogous genes multi-families revealed antigenic diversity and virulence also are found that can answer in case of unapparent clinical signs during chronic stage (Guimaraes et al., 2011).

Entire genome of *M. parvum*, Indiana strain (Accession number: CP006771), was completely sequenced in 2013 (do Nascimento et al., 2013). A genome characteristic is the smallest circular chromosome (564,395 bp) in Mollicutes composing of 27% GC contents. Single copies of arrangement of 16S, 23S and 5S rRNA genes are separated in each operon that can be found in other hemoplasmas in several animals (Messick et al., 2011). Besides, tryptophan synthesis from stop codon, 70% hypothetical protein and others can be found in this genome as well as *M. suis* (do Nascimento et al., 2013).

Some documents were mentioned about antibiotics resistance in invasive strain in Europe known as KI3806 in 2009 (Groebel et al., 2009). Observations of some genes are noted including cell adhesion, cell invasive and antibiotic resistance in entire genomes of two strains (KI3806 and Illinois). However, the difference of interested gene leading to antibiotics resistance between those is not appeared (Guimaraes et al., 2011; Oehlerking et al., 2011). As a result, the study about genes encoding virulent protein and drug resistance must be continued to study for better elucidating in other mechanisms in the future.

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***M. suis* transmission**

Transmission of *M. suis* is insect vectors and mechanics (Messick, 2004; Hoelzle, 2008). *M. suis* can distribute to many farms via blood sucking insects including mosquitoes (*Aedes* spp.), stable fly (*Stomoxys calcitrans*; *S. calcitrans*), house fly (*Musca domestica*; *M. domestica*) and hog louse (*Haematopinus suis*; *H. suis*) (Prullage et al., 1993; Messick, 2004; Hoelzle, 2008). Route of infection of this agent is several ways recognized as oral, intramuscular and intravenous inoculations. Based on previously laboratory experiment, pigs can infect this agent via *M. suis* carrying mosquitoes and they rapidly showed clinical signs around one week (Prullage et al., 1993). Besides, cannibalism behavior and carrier pigs is the main source for its transmission between pigs during environmental stress (Hoelzle, 2008).

Blood sucking and non-blood sucking insects together with swine behaviors could also transmitted between infected pigs and sensitivity splenectomized pigs in the same laboratory room (Prullage et al., 1993). Moreover, those insects were noted it was potentially mechanical transmission because this agent could not replicate in their bodies and their lifetimes were so short (Prullage et al., 1993). Mechanics transmission is a crucial factor as well as vector transmission.

Several publications indicated that recycle needles, contaminated surgical and restraining instruments also contain and distribute this agent between herds (Messick, 2004; Hoelzle, 2008). Interestingly, vertical transmission via genital organs and contaminated blood in semen had ever been reported in recent years (Maes et al., 2008a). However, those routes are also concerned in many farms because those factors enable transmitting this bacterial agent from carrier sow to neonatal pigs. On the other hands, those factors have been still unclear until present particularly *M. suis* attacking fetus and neonatal birth during pregnancy and parturition periods (Messick, 2004; Hoelzle, 2008).

***M. suis* pathogenesis**

Owning to limitation of study on pathogenesis, this bacterial agent is not ability to grow in *vitro* by itself due to lacking mechanisms of nutrients synthesis. That reason has led to affecting on pathogenesis process until present (Messick, 2004; Hoelzle, 2008). Despite, entire genomes of two strains were completed. (Guimaraes et al., 2011; Oehlerking et al., 2011). In the past, several concepts are documented serving as using glucose and amino acids for its survival in circulatory system. Moreover, host immune modulating and its behavior characteristics were concluded (Messick, 2004; Hoelzle, 2008; Guimaraes et al., 2011; Oehlerking et al., 2011).

Depended on fundamental pathogenesis, fibril-like characteristic on *M. suis* surfaces was used for taking biomolecules and caused erythrocytes fragile. Infected RBCs rapidly were removed into spleen for destruction that directly associated with icteroanemia in all age pigs (Messick, 2004; Hoelzle, 2008). Additionally, immunoglobulin M attacking infected erythrocytes induces phagocytosis process for their destroying that also result in hemolytic anemia (Hoelzle, 2008).

Pathogenesis concepts mentioned above were related to the results of hematologic parameters, macroscopic lesions, clinical signs and autoimmune response inducing porcine Eperythrozoonosis (PE) during early infection (Messick, 2004; Hoelzle, 2008). In addition, cold agglutinin inducing autoimmune hemolytic anemia is noted as well as other *Mycoplasma* from humans and animals (Lambert and Nydegger, 2010). Cold agglutinin, as firstly described in 1957, is one of the types of autoimmune response performing by a high concentration of IgM binding with polysaccharide regions of glycoproteins on infected RBCs surfaces under low body temperature at least 28°C (Dacie et al., 1957; Lambert and Nydegger, 2010).

In general, IgM binding antigens known as I, i and Pr can induce classical complement pathway affecting RBCs damages and leading to intravascular or extravascular hemolytic anemia based on membrane attack complex (Dacie et al., 1957; Lambert and Nydegger, 2010; Swiecicki et al., 2013). However, *Mycoplasma* isolated from several creatures can induce both cold agglutinin and warm agglutinin types of autoimmune hemolytic anemia (Lambert and Nydegger, 2010).

Warm agglutinin mechanism is similarity to cold agglutinin but the difference of those are binding IgM or IgG on RBCs surface protein, needing optimal temperature at 37°C, devastating erythrocytes by macrophage and spleen (Lambert and Nydegger, 2010). Hallmark of RBCs characteristics during autoimmune hemolytic anemia is spherocytes and elevating aggregation. Additionally, those blood samples mostly showed positive to Coombs test (Lambert and Nydegger, 2010). Recently, adhesion protein on *M. suis* surface so-called *Mycoplasma suis* glycoprotein or MSG1 (p40) plays a critical role in cell attachment and inducing cold agglutinin during high performing of IgM against infected erythrocytes. Besides, a characteristic of MSG1 is similarity to glyceraldehydes-3-phosphate dehydrogenase (GADPHs) on erythrocytes surfaces (Hoelzle et al., 2007).

Furthermore, several immunogenic proteins had ever been recorded such as two heat shock proteins known as Dnak or GroEL. Dnak (DnaK-like protein) is a heat shock protein A1 (HspA1) that is found in cytoplasm and surface regions of RBCs. RNA helicase is a core enzyme for RNA alignment. Those also are deal with host immune modulating against this bacterial agent (Hoelzle, 2008). In addition, two enzymes for carbohydrate metabolism namely enolase and pyruvate dehydrogenase are immunogenic enzymes to activate host immunity as well as those heat shock proteins mentioned above.

Relied on active immunogenic proteins, establishment of in-house ELISA coating with immunogenic proteins including MSG1 was developed for monitoring host immunity against *M. suis* in porcine sera (Hoelzle et al., 2007). Later, Felder and colleagues reported that hemoplasmas can be involved in warm agglutinin from actin targeting IgG antibodies are up-regulated to induce erythrocytes opsonization in hematopoietic organs or via phagocytosis process (Felder et al., 2012). Furthermore, *M. suis* is ability to attack both epithelial cells and lymphocytic cells during harm infection. Interestingly, detectors found sharing epitope of erythrocytes (α-actin) and MSG1 (*M. suis* surface protein) around 55% thus suggested that cross-reaction between host and bacterial agent accidentally may be occurred (Hoelzle et al., 2007). However, only actin on infected RBCs is a target cells for autoreactive antibodies because alterations of its membrane and compositions and involvement of Ca^+, Na^+ ion channels are contribute to parasitic invasion and host cell death (Hoelzle et al., 2007).

Currently, the novel concept of its pathogenesis is program cell death of infected RBCs is firstly declared in 2012 according to eryptosis characteristic. Several factors revealed eryptosis are documented including chemical residues, malaria, hormone, inflammatory response and free radical substance that are affected to alteration of RBCs structures (Felder et al., 2012). Based on those concepts, infected erythrocytes may suicide by themselves via some signal pathways like other blood parasites including Malaria. However, this concept had not been still clear because the majority of signals inducing program cell death are not discovered (Felder et al., 2012). However, several factors including cell division (metalloproteases CAAX amino terminal protease and ATP-dependent protease FtsH), RBC lysis (O-sialoglycoprotein endopeptidase ortholog) and virulence (Paralogous genes variation) also affect to life spanning of erythrocytes (Guimaraes et al., 2011).

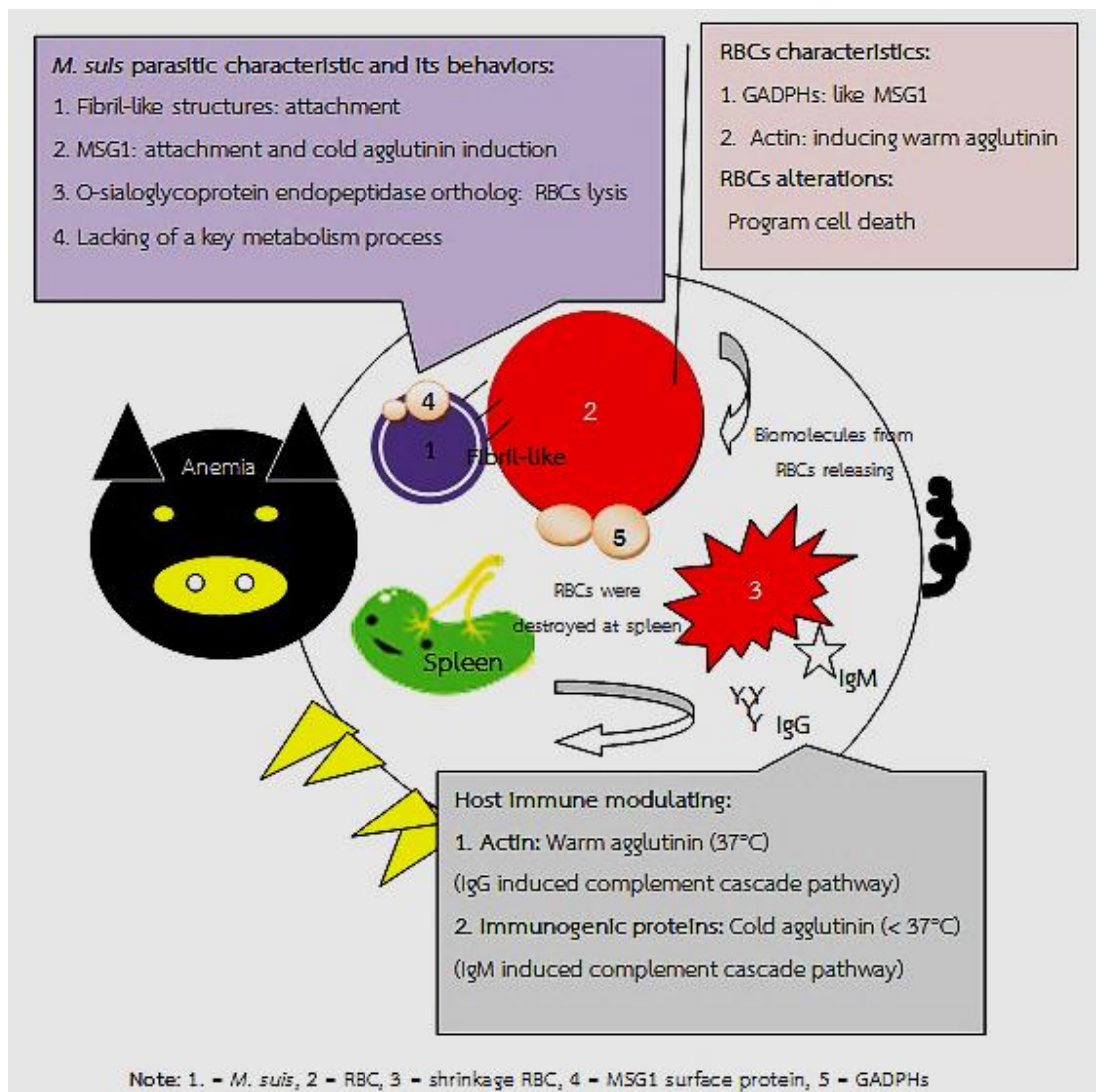

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Figure 1 Conclusion of *M. suis* pathogenesis in several concepts are followed by three main factors including its parasitic behaviors and genomic characteristics, RBCs life spanning and host immunity known as warm agglutinin and cold agglutinin.

***M. suis* clinical signs**

M. suis infected pigs mostly showed mild to severe icteroanemia, pyrexia and low growth performance in all age pigs and manifested reproductive disorders in sows and gilts (Baker et al., 1971; Messick, 2004; Hoelzle, 2008; Dent et al., 2013). Unfortunately, this disease is difficult to differentiate from other swine diseases caused by virus, bacteria and parasites depended on clinical signs observation (Messick, 2004; Hoelzle, 2008). Infected pigs usually show clinical symptoms in acute, subacute and chronic stages based on duration of infection (Messick, 2004; Hoelzle, 2008).

During acute phase, neonatal pigs mostly show pyrexia and severe anemia. Occasionally, pigs died suddenly for 4-5 days after infection in particular naïve farms. Moreover, mild anemia and low growth performance can be observed in subacute and chronic stages (Messick, 2004; Hoelzle, 2008). Those stages are difficult to investigate this agent by Romanowsky-stained blood smears due to misidentification and misclassification. So, established PCR and real-time PCR have used as gold standard for its confirmation since 1990s until present instead of routine techniques (Henderson et al., 1997; Messick, 2004; Hoelzle, 2008).

Post-weaning pigs mostly showed icteroanemia and growth retardation as well as younger pigs in acute phase. In subacute and chronic stages, infected pigs showed mild anemia (Messick, 2004; Hoelzle, 2008). Ear tip necrosis was one of clinical signs of *M. suis*-inoculated pigs in laboratory experiment. Although this sign is not specific to only *M. suis* infection. Examples of other diseases manifesting ear tip necrosis are swine behavior (biting) (Sutton, 1976), porcine circo virus type II (PCV II) (Ichijo et al., 1982), porcine reproductive and respiratory syndrome (PRRS) (Feng and Grizzle, 1992), mycotoxin, (Lesaffre et al., 1993), staphylococcosis (*Staphylococcus hyicus*) (Sutton, 1977) and spirochetosis (Dixon et al., 1993).

Icteroanemia is the main clinical sign of *M. suis*-infected fattening pigs during acute stage. In chronic phase, fattening pigs mainly showed growth retardation and apparent or unapparent anemia. However, this agent can return and replicate in host's circulatory system causing high parasitemia during animal movement and environmental stress (Messick, 2004; Hoelzle, 2008). In sow and gilt, high fever up to 42°C, severe anemia, low growth performance and abnormally maternal behaviors can be observed during acute phase.

Additionally, reproductive disorder in sows and gilts is noted in this disease (Messick, 2004; Hoelzle, 2008). Those pigs mostly showed moderate to severe abortion risks, delay estrous cycle, high stillbirth and embryonic death that impacts on economic losses based on host immune status and biosecurity strategies in each farm (Givens and Marley, 2008). Clinical signs of infected sows and gilts during chronic phase are growth retardation, dysgalactia, mild abortion, long estrous cycle (Strait et al., 2012) and maternal behavior changing in some pigs (Messick, 2004; Hoelzle, 2008). What's more, unapparent clinical signs of sows and gilts after *M. suis* infection are appeared in most cases.

Recent documents reported Infected boars mainly showed growth retardation and anemia during acute, subacute and chronic phases as fattening pigs (Messick, 2004; Hoelzle, 2008). However, abnormally genital organs and genital transmission between boars and sows had never been mentioned except contaminated semen (Hoelzle, 2008). Hence, only observation of clinical sign in all age pigs is not enough to confirm *M. suis* infection and impossible to differentiate this disease from other swine disorders circulating in sensitivity farms.

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M. suis diagnosis

Diagnosis of porcine hemoplasmas in current can be divided into four main concepts including conventional techniques, clinical signs observation of *M. suis* inoculated in splenectomized pigs, serological diagnostic assay and molecular identification (Messick, 2004; Hoelzle, 2008). In the past, conventional techniques namely Romanowsky staining (Giemsa, Wright-Giemsa and Diff Quick) and acridine orange were easily to detect this agent in circulatory system especially during high parasitemia (Hoelzle, 2008). In Romanowsky staining, *M. suis* displays basophilic pleomorphic shapes (discoid, rod, spherical and ring) in size abruptly 1 µm arranging in single or chain-like harboring on erythrocytes after 3-7 days or 2 weeks after infection (Messick, 2004; Ha et al., 2005; Hoelzle, 2008). Generally, this technique is the first step to use for scanning of blood parasites because of convenient, cheap and easy before confirmation with other methods (Messick, 2004; Hoelzle, 2008).

Acridine orange, firstly described in 1942 by Hilbrich and Strugger (Mirrett, 1982), is the sensitivity technique (Messick, 2004; Hoelzle, 2008) that binds DNA or RNA and forms complex condition with intercalation and electrostatic attractions. Those showed green fluorescence in DNA and red or orange in RNA in different wave whereas emission which those can be easily differentiate between agents and host cells (Mirrett, 1982). *M. suis* detection using acridine orange illustrated bright orange on dark green RBCs structures surrounding bright yellow leucocytes under fluorescent microscope (Hoelzle, 2008; Groebel et al., 2009). However, those techniques are also limited to investigate during very low parasitemia, early infection and needing expert persons. Moreover, color precipitation and nuclear fragment are the main factors that derogate the result as false positive (Messick, 2004; Hoelzle, 2008).

In the past, *M. suis* inoculated in splenectomized pigs was the famous way to investigate and confirm hemotrophic Mycoplasmosis because this agent is cleared from circulatory system via spleen sequestration (Maede, 1979). Removal spleen drives high parasitemia (bacteremia) after inoculation within 2 days to 2 weeks depended on a quantity of this agent, route of infection and percentages of parasitemia (Messick, 2004; Hoelzle, 2008; Dent et al., 2013). In the previous publications, ≥ 90% parasitemia are mostly used for inoculation in splenectomized pigs via intraperitoneal or intravenous to activate severe symptoms before scanning this agent by routine techniques and observing clinical signs (Guimaraes et al., 2011). Infected pigs showed severe pyrexia up to 42°C, severe anemia or jaundice, low growth performance and ear tip necrosis in some cases (Hoelzle, 2008; Dent et al., 2013).

Macroscopic lesions of *M. suis*-inoculated splenectomized pigs showing fluid in abdomen, thoracic cavity and pericardium, hepatomegaly and hemorrhage at kidney, heart and lung regions were documented (Messick, 2004; Hoelzle, 2008). However, clinical signs and macroscopic lesions of those pigs are not enough to confirm this agent circulating in farms (Messick, 2004). Furthermore, surgical procedures, treatment, time and labor consuming and expensive naïve pigs for study are concerned. Hematologic parameters of *M. suis*- infected pigs illustrated low hematocrit, low blood glucose, reticulocytes less than 2.5% and low red blood cells count that related to causing anemia, hypoglycemia, parasitic characteristics and behaviors for needing blood nutrients (Messick, 2004; Hoelzle, 2008; Guimaraes et al., 2011; Oehlerking et al., 2011). Later, serological techniques were very popular to detect and monitor this agent in large-scale batches based on humoral immunity against *M. suis* including indirect haemagglutination (IHA) and enzyme-linked immunosorbent assay (ELISA) (Messick, 2004; Hoelzle, 2008).

IHA had been used in swine farms in America in 1950s for monitoring that was involved in quantification of both bacteria and virus. The advantages of this method are easy, simple and rapid test depended on surface protein or envelope protein. However, this technique is limited to investigate immunoglobulin G (IgG) or immunoglobulin M (IgM) targeting *M. suis* due to humoral immunity in different ages of pigs and duration of infection (Messick, 2004; Hoelzle, 2008). Humoral immunity of neonatal pigs is slightly higher than others that cause high titer as false positive. Furthermore, fragile RBCs from bacterial infection could induce high immunoglobulin to attack and remove them into spleen for destroying. That has been affected to IgG and IgM decreasing in circulatory system which contributes to vague investigation in particular false negative as well as the result of ELISA (Messick, 2004).

IHA technique for antibody against *M. suis* detecting in splenectomized pigs compared to non-splenectomized pigs during 80-190 days after inoculation was noted in previously study which results were associated with clinical signs appearance. During acute phase (≤two months), antibody titer was increased up to 1:640 and then it rapidly decreased around 2-3 months. Later, antibody increased again and easily for detection of a high titer as the first time of infection (Baljer et al., 1989). Hence, it showed host immunity and clinical signs slightly are fluctuated in different time (Baljer et al., 1989).

ELISA is a rapid test to scrutinized IgG in host' blood circulation with antigen coated solid plate, secondary antibody conjugated and substrate. Substrate digests enzyme containing in secondary antibody that demonstrated color in each dilution of porcine serum (Engvall and Perlmann, 1971; Lequin, 2005). In present, specific ELISA based on several specific genes had been developed and widely used in many countries due to rapid and cheap. However, antigen preparation for coating plates is trouble those affect to available commercial ELISA for investigation of host immunity against *M. suis*.

In 1992, some documents reported that ELISA was more sensitive tool (nearly 90% positive) than IHA (around 80% positive) and immune cross reaction between agents isolated from pigs were not observed (Hsu et al., 1992). To date, several immunogenic genes of *M. suis* are used for antigen coated ELISA plate namely *Mycoplasma suis* glycoprotein 1 (MSG1) (Hoelzle et al., 2007), heat shock protein (HsP1) (Hoelzle et al., 2007) and inorganic pyrophosphate gene (ppa) (Hoelzle et al., 2010b), respectively.

Novel ELISA based on those genes are more specific and sensitive than the former ELISA and IHA. In addition, cross reaction between species of *Mycoplasma* isolated from pigs or others had never been recorded. Currently, novel blocking ELISA based on monoclonal antibodies (Mab) producing from BALB/c mice immunized with recombinant *M. suis* MSG1 protein expressing in *E. coli* showed 100% specificity and more than 90% sensitivity for detection *M. suis*. That technique can be used in serodiagnosis of *M. suis* for monitoring in large scale batches (Dacie et al., 1957).

Polymerase chain reaction (PCR), firstly reported in 1983 by Kary Mullis, is one of the best techniques for confirmation in many interested agents. This technique can amplify many copies of DNA in one reaction with 5 steps known as Initial denaturation, denaturation, annealing, extension and final extension (Bartlett and Stirling, 2003). In the past, 16S rRNA gene of several bacteria was chosen for designed universal primers for examining of *M. suis* in blood samples (Gwaltney et al., 1993; Messick et al., 1999; Guimaraes et al., 2011). Accordingly, designed primers based on 16S rRNA gene with BamHI (Gwaltney et al., 1993; Messick et al., 1999; Guimaraes et al., 2011) and RNase P RNA (*rnpB*) gene can differentiate *M.suis* from other bacteria by manifesting different band (Watanabe et al., 2011).

RNase P RNA and 16S rRNA genes were used together for differentiating between *M. suis* and *M. parvum* existed in swine farms in Japan (Watanabe et al., 2011) (Watanabe et al., 2012). Recent those studies prove that *M. parvum* is not different developmental stages of *M. suis*. Later, entire genome of *M. parvum* was completely sequenced in 2013 (do Nascimento et al., 2013) that insisted this agent is one of the hemoplasmas in circulatory system causing anemia as well as *M. suis* (do Nascimento et al., 2013).

In addition, various rapid and accurate tests were developed and applied for *M. suis* detection such as nested PCR assay. Two pairs of primers (outer and inner primers) are needed to investigate *M. suis* DNA especially derived blood from chronic stage or using Dexamethasone (immunosuppressive drug) due to highly sensitive method (Yuan et al., 2007). At present, conventional PCR is accepted as gold standard for prove and identify exactly existence of *M. suis* in swine farms based on specific genes (Gwaltney et al., 1993; Messick et al., 1999; Ha et al., 2005; Groebel et al., 2009; Zhou et al., 2009; Watanabe et al., 2011; Watanabe et al., 2012).

Recently, developing highly sensitive and specific real-time PCR or known as quantitative PCR (Groebel et al., 2009) can be employed concerning a better understanding of the exactly consequences of infection in infected pigs especially chronic stages and carrier pigs. Recently, designed specific gene based on MSG1 using quantitative real-time PCR was developed suggesting 100% sensitivity and nearly 100% (96.7%) specificity for investigation and study prevalence of this disease (Hoelzle et al., 2007). Moreover, designed primers and probes rely on 16S rRNA gene in real-time PCR were optimized for study prevalence of this agent in Japan (Watanabe et al., 2012).

Besides, a new tool for *M. suis* detection using *In situ* hybridization (ISH) together with nonradioactive digoxigenin-labelled DNA probe at 839 base pairs in various formalin-fixed and paraffin wax-embedded tissues organs in *M. suis*-infected 6 weeks splenectomized pigs. The technique is capability investigates this agent in spleen, kidney and blood using Romanowsky staining under light microscope in three days after infection. Fixed- formalin organ samples of euthanized pigs are used for detecting such as liver, lymph node, tonsil, spleen, heart, lung, kidney and small and intestines. However, blood vessels, hepatic sinusoids and renal glomeruli showed strongly hybridization signals more than other organs. Therefore, this technique insisted that pathogenesis of this agent related to many organs containing infected RBCs (Ha et al., 2005).

Besides, convenient, rapid, high specificity and high sensitivity namely colloidal gold-based immunochromatographic assay was currently developed to investigate *M. suis* in porcine plasma by only one step per reaction using nanoparticles coated with polyclonal antibody against this agent. The technique takes time only 10 minutes for detection and specific to *M. suis* (McMahon et al., 1994). However, many new tools for *M. suis* detection must be continued to develop for its investigation but molecular techniques namely PCR and real-time PCR as gold standard are the best techniques for identification of this bacterial agent.

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***M. suis* treatment**

M. suis treatment by antibiotics is the best way to reduce severe clinical signs, pathologic lesions and parasitemia. However, several documents reported that this agent can resist to those drugs including beta-Lactam (β-Lactam) due to its cell wall characteristics (Messick, 2004; Hoelzle, 2008). Basically, β-Lactam antibiotics are commonly used for inhibiting bacterial organism that target is cell wall. Nowadays, commercially β-Lactam antibiotics are developed to devastate both gram-negative and gram-positive bacteria. Unfortunately, *M. suis* cannot be successfully eradicated and has been still circulating in swine farms (Hoelzle, 2008).

Subsequently, cyclin and arsenic drugs are globally used for its prevention. Both drugs can ease this agent in circulatory system. However, arsenic compounds namely roxarsone, carbarsone and arsanilic acid were abandoned by FAO committee for treatment in livestock farms including poultry and swine because of its residues known as inorganic arsenic (iAS). However, some publications documented that cyclin antibiotics could not effectively destroy this agent in carrier pigs. Dose of Oxycyclin for treatment in current is orally 25-30 mg/kg together with dextran and iron (200 mg/pigs) during acute and subacute phases for easing clinical symptoms (Messick, 2004; Hoelzle, 2008).

In Thailand epidemic circumstances, document is little reported about existence of *M. suis* in swine farms due to lacking suitable molecular detection for its identification. However, macroscopic lesions and hematologic parameters of infected splenectromized pigs had been documented in 1980s (Luengyosluechakul and Nithiuthai, 1989). Hence, the present study aimed to identify *M. suis* in swine herds in Thailand by conventional methods and molecular investigation for confirmation actually existence of this agent in Thailand or not.

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CHAPTER III
MATERIAL AND METHODS

Research was experimented in this conceptual framework table as displayed in below.

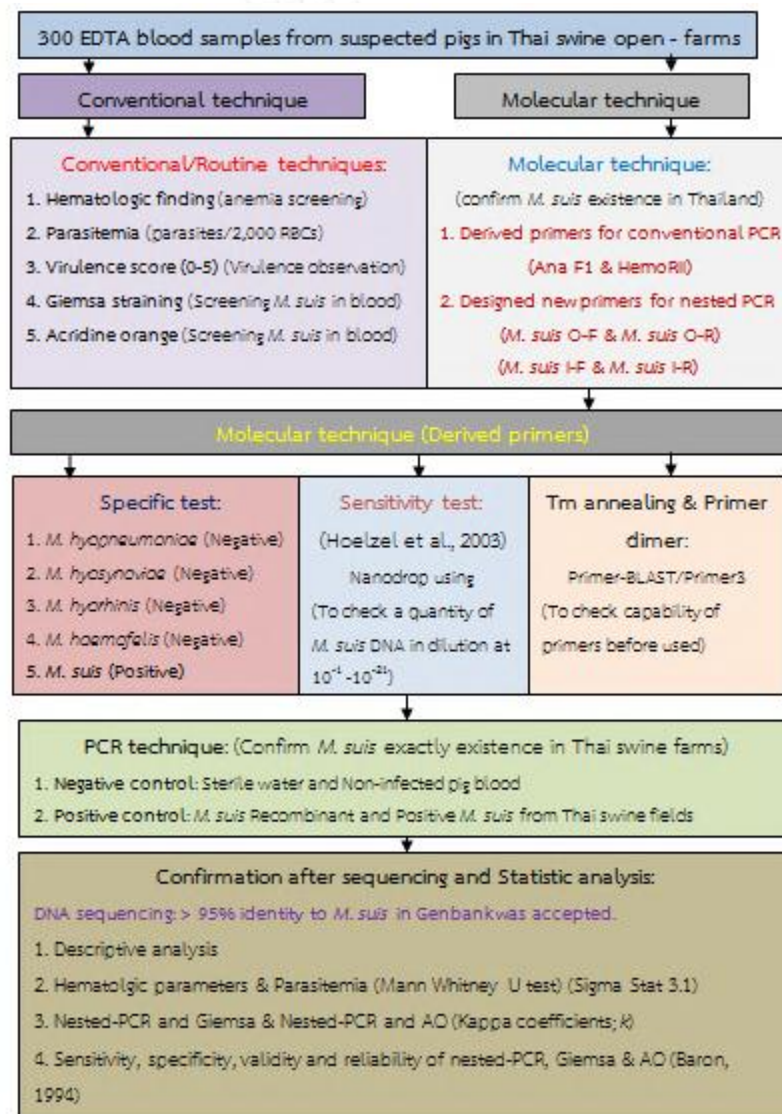

Figure 2 Conceptual framework of this present study on identification *M. suis* in Thailand

Sample size for blood collection

Population sample size in this study was modified from the previous research as this manual solution was illustrated in below.

$$\text{Population sample size (N)} = \frac{P\,(1-P)\,Z^2}{D^2} \quad \text{(Skaik, 2010; Kumar, 2012)}$$

N = Sample size population

P = Population proportion or frequency value expectance (30% or 0.30 proportion is usually used for unknown population)

Z = Area under normal curve according to confidence level or significantly level

D = Maximum difference between population mean and sample mean or accepted error that directly concerned with Z in the level of confidence

Based on the sample size formula as mentioned above, swine blood samples in this study were calculated in 95% confidence level or error appearance at 0.05 (D) and the significant level (Z) at 0.05 (1.96) as demonstrated in below.

$$\text{Population sample size (N)} = \frac{0.30(1-0.30)[(1.96)]^2}{(0.05)^2} \text{ or } 322$$

From the result of sample size manual calculation, it showed 322 blood samples were chosen in swine herds. However, prevalence and epidemiology data of *M. suis* infected pigs in swine farms in Thailand had never been reported.

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In addition, sample size of this study also was modified from other the previous research in the topic on survey this agent in farms in Asia and Europe (Yang et al., 2000; Ritzmann et al., 2009; Yuan et al., 2009; Watanabe et al., 2012). Fifty to one hundred samples of those studies were selected to survey this agent in prevalence around 1-15%. Therefore, 300 blood samples in this study were suitable to investigate and confirm existence of *M. suis* in swine farms in Thailand by the reason of sample size calculation and expectance of *M. suis* prevalence in Thailand approximately 1-30%.

Ethical approval

The procedure of blood collection from suspected pigs in this study was approved by Chulalongkorn University Animal Care and Use Committee (CU-ACUC) in number 12310087.

Criteria of suspected *M. suis* for blood collection

Three-hundred blood samples were collected randomly in eleven open-air swine farms in six provinces of Thailand including Chachoengsao, Chiang Mai, Nakhon Pathom, Prachinburi, Ratchaburi and Sisaket during 2012-2014. Criteria of suspected pigs for blood collection according to the previous study on survey this agent in swine farms (Yang et al., 2000; Watanabe et al., 2012). Those documents reported anemia signs in all age pigs, low growth performance and compatible with severe respiratory and enteric diseases in nursery pigs, reproductive disorders in gilts, icteroanemia signs and low growth performance in fattening pigs (Baker et al., 1971; Messick, 2004; Hoelzle, 2008).

Histories taking from swine farmers including clinical signs, environmental status and animal movement during blood collection were the main factors for collection. Example of clinical signs in suspected fattening pigs in swine farms in china was indicated as manifested in Figure 3.

Figure 3 Infected post-weaning pig showed mild anemia and growth retardation (http://www.carrsconsulting.com/thepig/disorders/diseasebysystem/diseasesgilts.htm).

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Blood preparation

Three-hundred blood samples of suspected pigs that directly consistent with *M. suis* infection were collected 1 ml of blood/1 mg EDTA. 0.5 ml of blood was kept at -20°C for DNA extraction and molecular assays. The remaining was preserved at 4°C within 24 hours at the most for investigation by conventional techniques (10% Giemsa and acridine orange) and observation blood parameters.

Hematologic parameters

All blood samples were counted the numbers of erythrocytes and leucocytes using Hemocytometer. All capillary tubes after centrifugation were measured by hematocrit reader. Differential leucocytes were recorded. Percentage of parasitemia was counted from a number of *M. suis* per 2,000 erythrocytes.

Virulence score

Virulence score was given from 0 to 5 based on *M. suis*-like harboring on RBCs surfaces by 10% Giemsa. This method was modified from the previous research in the study on *Eperythrozoon ovis* (*E. ovis*) or *Mycoplasma ovis* (*M. ovis*) in sheep (Littlejohns, 1992) as described in Table 2.

Table 2 Virulence score of Giemsa-stained blood smears

Score	Giemsa-stained slide
0	*M. suis*-like could not be found throughout slide.
1	*M. suis*-like could be seen on only one infected RBC. One infected RBC showed only one suspected *M. suis*.
2	*M. suis*-like could be found more than one infected RBC. Almost RBC showed only one suspected *M. suis*.
3	*M. suis*-like could be observed more than one infected RBC. RBC showed only one or more than one suspected *M. suis*.
4	Suspected *M. suis* characteristic could be found almost infected RBC. RBC showed only one or more than one suspected *M. suis*.
5	Suspected *M. suis* characteristic could be seen every infected RBC. Each RBC showed more than one suspected *M. suis*.

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Conventional techniques

10% Giemsa

A total of 300 thin blood smears were fixed by absolute methanol before air-drying. All slides were stained with 10% Giemsa. 10% Giemsa staining was prepared from stock Giemsa (Merck KGaA, Germany) and buffer pH 7.2 (1.0 g Na_2HPO_4, 0.7 g KH_2PO_4 and 1,000 ml distilled water) as followed by laboratory guidelines of Parasitology unit, Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University, Thailand. *M. suis* showed basophilic pleomorphic characteristics including discoid, rod or ring on RBCs as well as Wright' Giemsa as shown in Figure 4.

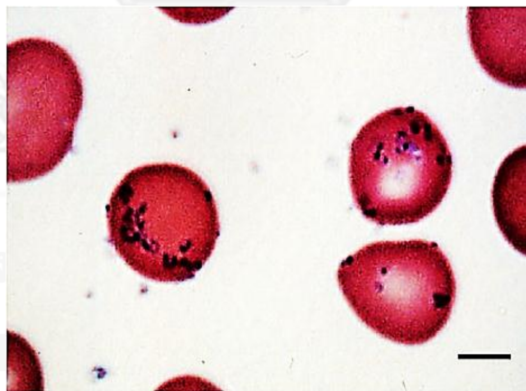

Figure 4 *M. suis* showed basophilic pleomorphic shapes on RBCs surfaces using Romanowsky staining (Messick, 2004).

Acridine orange staining

Three-hundred blood smears were fixed by 10% formalin-saline for 24 hours. Those slides were cleaned by distill water and stained with acridine orange and washed by distill water before detecting by fluorescent microscope. Stock acridine orange was prepared from 1 g of acridine orange (Sigma-Aldrich, Thailand) and 100 ml of 0.1M HCL or 1 V of stock acridine orange and 9 V of 0.1M HCL as described previously (Gainer, 1961; Schreiner et al., 2012). Positive sample was performed bright orange or bright yellow on dark green RBCs surrounding bright yellow WBCs as shown in Figure 5.

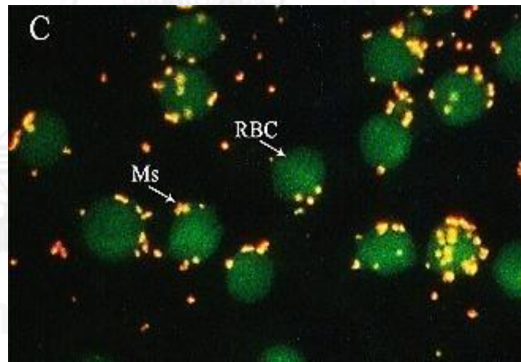

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Figure 5 *M. suis* demonstrated bright orange on green RBC surfaces using acridine orange staining (Groebel et al., 2009).

Conventional polymerase chain reaction (Conventional PCR)

DNA extraction

DNA was extracted from all blood samples according to by manufacturer of DNA extraction commercial kit (Machinary-Nagel, Germany). The quantity of DNA extraction was measured using Nano drop connecting with computer program before used (DNA < 250 ng). DNA was kept in -20 $^{\circ}$C until used for conventional PCR and nested PCR.

Positive and negative controls

5 µl of *M. suis* recombinant DNA (cloned S1) were derived from Prof. Dr. Ryo Harasawa (Emeritus at Iwate University, chair of the board of directors the Iwate Research Center for Wildlife Disease, Nakano, Morioka, Japan) and Dr. Yusaku Watanabe (Mihama Veterinary Clinic, Japan).

Clone S1 was detected band using PCR and confirmed these sequences with GenBank (must be up to 95% for acceptance). Depended on DNA sequences, this clone showed 100% identity to *M. suis* in several strains in GenBank including China (accession number, KC907396). Clone S1 was calculate nanograms (ng) converting to copies/µl. Distilled water and healthy pig blood and Clone S1 were used as negative and positive control, respectively.

Derived primers from Japan

Ana F1 and HemoRII from Japan (Watanabe et al., 2011) were scanned melting temperatures (Tm), 3' and self-complementary and specific matching using Primer-BLAST before used. Tm of primers was 62°C but 56°C (Tm-5) was chosen for annealing step of conventional PCR. Product size of primers showed around 500 bp

Forward (Ana F1): 5'- AGAGTTTGATCCTGGCTCAG-3'

Reverse (HemoRII): 5'-CCTACGCTTCCTTTACGCC-3'

Specificity test for derived primers

Ana F1 and HemoRII were checked specificity with other *Mycoplasma* from swine and feline as shown in Table 3.

Table 3 Bacteria agents for specificity test in derived primers

Host	Organ	Name	Strain	Source
Swine	Lung	*M. hyopneumoniae*	Field	Department of Veterinary
	joint	*M. hyorhinis*	Field	Medicine*
	joint	*M. synoviae*	Field	
Swine	Blood	*M. suis* (recombinant DNA, Positive control)	Morioka	Iwate Research Center for Wildlife Disease, Nakano, Morioka, Japan
Feline	Blood	*M. haemofelis*	Field	Parasitology unit, Department
		M. haemominutum	Field	of Veterinary Pathology*

Note: * Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand

Sensitivity test for derived primers

Clone S1 was measured nanograms using spectrophotometer (Nanodrop) before converting copy numbers according to the manual calculation (Integrated DNA technologies). Clone S1 was diluted by nuclease free water with 10 fold dilution (10^{-1}-10^{-21}) as modified from previously (Hoelzle et al., 2003). Last dilution showing expected band around 500 base pairs was recorded.

Conventional PCR reaction

Ten positive samples (NP1-NP10) based on Giemsa and acridine orange were used for confirmation by conventional PCR.

The total of PCR reaction was performed in 25 μl that was composed of sterile water, 12.5 μl of 2x Gotag green® (Promega®), 0.5 μl in each 10 μM *M. suis*-F (Ana F1) and *M. suis*-R (HemoRII) and 2 μl of DNA template (< 250 ng). PCR reaction was performed in specific condition as accordingly at 94°C initial denaturation for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 56°C for 1 minute and 72°C for 2 minutes at extension step. The final extension step was 72°C for 7 and holding at 4°C minutes using Thermal cycler (Bioer, Little Genius, China).

Expected band was observed under UV light microscope after electrophoresis process at 100V for 30 minutes (Mini-Run GE-100; Hangzhou Bioer Technology Co., LTD). 1.0% agarose gel (Agarose, Low EEO, Molecular Biological Grade) staining gel with 0.4 μl/ml Ethidium bromide (Pacific Science; Biotech) was performed before observing band. Marker ladder (100 base pairs + 1.5 Kilobase pairs) were used for indication molecular weight.

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Development of nested polymerase chain reaction (nested PCR)

Designed outer and inner primers

According to derived primers (Ana F1 and HemoRII), some positive samples using routine methods showed negative based on PCR. So, nested PCR assay was developed to solve this problem. Reverse outer primer (*M. suis* O-R) was designed by 27 strains of *M. suis* submitted in GenBank as shown in **Table 4**. Those sequences were aligned and observed consensus in each position by Multalin program (http://multalin.toulouse.inra.fr/multalin/) together with Bioedit V7.2.5.

Primer3Plus program (www.bioinformatics.nl/primer3plus/) was used for designed inner primers (*M. suis* I-F and *M. suis* I-R) based on Clone S1 sequence. Step of nested PCR assay and concensus position in Multalin as demonstrated in **Figure 6 and Figure 7**, respectively. However, forward primer from Japan (Ana F1 or renamed *M. suis* O-F) was used for outer forward primer in this method (Figure 6).

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Table 4 Twenty–seven strains of *M. suis* for design outer reverse primer

Species	Gene	Source	Country/strain	Genbank/ accession No.
M. suis	16S rRNA	Swine	China/CQ	EU603330
			China/Zhejiang	HQ259257
			China (partial sequence)	DQ346727
			China/ZJ NB01	KC907396
			China/SC	FJ263943
			China/HN	FJ263944
			China (partial sequence)	AY492086
			Japan/Morioka5	AB610847
			Japan/Morioka6	AB610848
			Japan/Morioka8	AB610849
			Switzerland/ 3804	FN984917
			Switzerland/isolate 178	FN391018
			Switzerland/ isolate 93	FN391019
			Switzerland/ isolate 51	FN391020
			Switzerland/ isolate 146/5	FN391021
			Switzerland/ isolate KI	FN391022
			America/Illinois (partial sequence)	AF029394

Species	Gene	Source	Country/strain	Genbank/ accession No.
M. suis	16S rRNA	Wild boar	Switzerland/ isolate Wild boar 128	FN436009
			Switzerland/isolate Wild boar 169	FN436010
			Switzerland/ isolate Wild boar 266	FN436011
			Switzerland/isolate Wild boar 226	FN436012
			Switzerland/isolate Wild boar 65	FN436013
			Switzerland/ isolate Wild boar 39	FN436014
			Switzerland/isolate Wild boar 176	FN436015
			Switzerland/isolate Wild boar 37	FN436016
			Switzerland/isolate Wild boar 74	FN436017
			Switzerland/isolate Wild boar 183	FN436018

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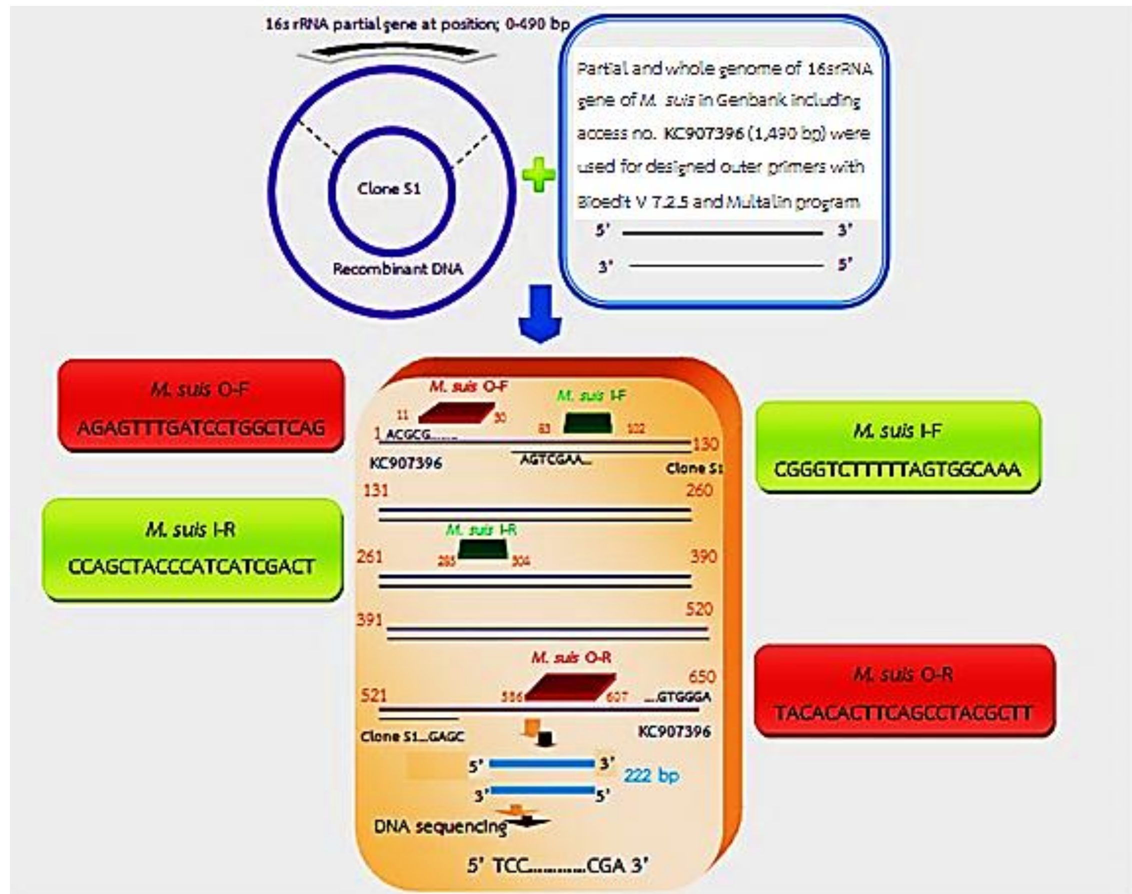

Figure 6 Several steps of designed primers and nested PCR reaction for detection *M. suis*

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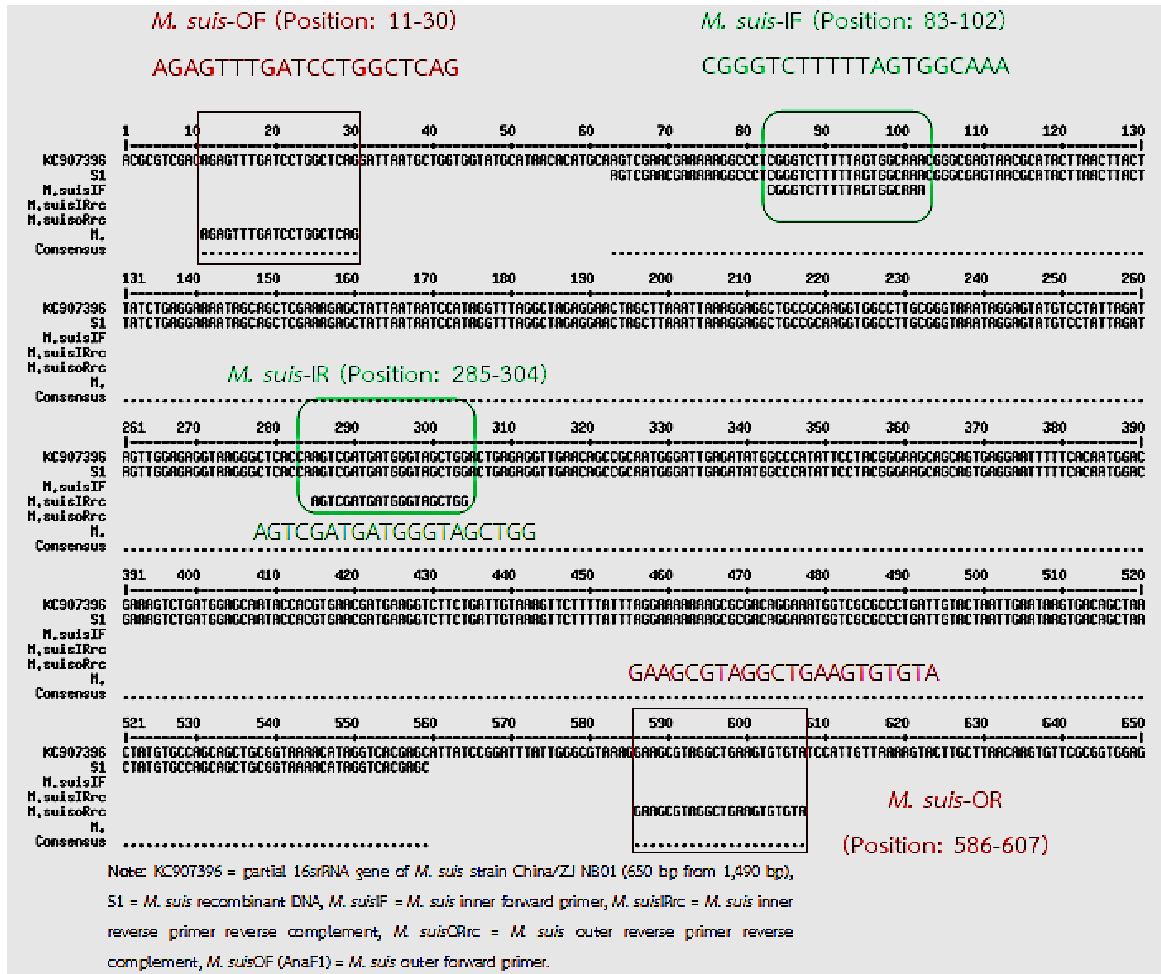

Figure 7 Example of China strain (accession number KC907396) out of 27 and Clone S1 were scanned the correct position for design outer primer (reverse) by Multalin.

Owing to limitation of design new primers, only outer primers (*M. suis* O-F and *M. suis* O-R) were checked specificity with other *Mycoplasma* in GenBank including *M. hyopneumoniae* (access no., Y00149), *M. hyorhinis* (access no., NR_103932), *M. hyosynoviae* (access no., NR_044811), *M. haemofelis* (access no., AF178677) and *M. haemominutum* (access no., AY150981). Outer and inner primers could not amplify those agents based on Primer-BLAST. However, those primers and inner primers were checked other agents as manifested in **Table 3** before used. Outer and inner primers showed melting temperature (Tm) at 61°C using OligoCalc (Oligonucleotide Properties Calculator). But, 56°C (Tm-5) was chosen for annealing step in nested PCR.

Outer forward (*M. suis* O-F): 5'- AGAGTTTGATCCTGGCTCAG-3'

Outer reverse (*M.suis* O-R): 5'- TACACACTTCAGCCTACGCTTC-3'

Product size of outer primers showed at around 597 base pairs

Inner forward (*M. suis* I-F): 5'-CGGGTCTTTTTAGTGGCAAA-3'

Inner reverse (*M. suis* I-R): 5'- CCAGCTACCCATCATCGACT-3'

Product size of inner primers was observed at 222 base pairs

Specificity and sensitivity tests for designed primers

Accordingly, outer and inner primers were interpreted specificity with other *Mycoplasma* as shown in **Table 3** such as *M. hyopneumoniae*, *M. hyorhinis*, *M. hyosynoviae*, (swine) *M. haemofelis* and *M. haemominutum* (feline). Only designed inner primers were tested sensitivity as described previously in **8.3.** Last dilution showing 222 bp was recorded.

Nested PCR reaction

Nested PCR was carried out in the first step using outer primers and the second step using inner primers as described in below.

Step one: 1 µl of DNA, PCR Master Mix, sterile water and outer primers (*M. suis* O-F; *M.suis* O-R) were mixed together in total 12.5 µl as below.

Distilled water (N.A.)	4.75	µl
1.0 µM 2x Gotag green	6.25	µl
0.2 µM *M. suis*-O-F (Ana F1)	0.25	µl
0.2 µM *M. suis*-O-R	0.25	µl
DNA sample (< 250 ng)	1.00	µl

PCR reaction using Thermal cycler (Bioer, Little Genius, China) was performed in specific condition including initial denaturation, denaturation, annealing, extension and final extension steps as followed in below.

Step	Temperature (°C)	Time (min)	Cycles
1. Initial denaturation	94°C	5	X 1
2. Denaturation	94°C	1	X 35
3. Annealing	56°C	1	X 35
4. Extension	72°C	2	X 35
5. Final extension	72° C	7	X 1
6. Holding	4° C	∞	

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Step two: 2 µl of PCR product from the first step, PCR Master Mix and inner primers (*M. suis* I-F; *M. suis* I-R) was composed of this in each one reaction/one DNA sample in total 25 µl.

Distilled water (N.A.)	8.50	µl
1.0 µM 2x Gotag green	12.5	µl
0.2 µM *M. suis*-I-F	0.50	µl
0.2 µM *M. suis*-I-R	0.50	µl
DNA sample (< 250 ng)	2.00	µl

PCR reaction was performed in specific condition as accordingly at 94°C initial denaturation for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 56°C for 1 minute and 72°C for 2 minutes at extension step as the mentioned above in **Step one**. The final extension step was 72°C for 5 minutes and holding at 4°C using Thermal cycler (Bioer, Little Genius, China).

Expected band were observed under UV light microscope after electrophoresis process (100V for 30 minutes) (Mini-Run GE-100; Hangzhou Bioer Technology Co., LTD). 1.0% agarose gel (Agarose, Low EEO, Molecular Biological Grade) was strained with Ethidium bromide (Pacific Science; Biotech) before observing expected band. Marker ladder (100 base pairs + 1.5 Kilobase pairs) was used for indicating molecular weight.

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DNA sequencing

50 µl of PCR products from positive samples in conventional PCR (Clone S1 and NP1) and nested PCR (CH1, CM1, NP1, NP4, NP5, NP7, NP12, NP13, NP17, P1 and Si1) was purified as followed by commercial purified PCR product (Macherey – Nagel, Pacific science) before sequencing (1st BASE Laboratories Sdn Bhd, Malaysia). Those sequences were analyzed and compared identity to *M. suis* in several strains based on GenBank in NCBI website (http: //www. ncbi. nlm. nih. gov/genbank/).

Descriptive and statistical analysis

Results of 10% Giemsa, acridine orange, virulence scores, conventional PCR and nested PCR were interpreted by descriptive analysis and graphs. Comparison of hematologic parameters and % parasitemia between negative and positive samples were analyzed using Mann Whitney U test (Sigma Stat 3.1). Cohen's kappa (*k*) of 10% Giemsa and acridine orange compared to nested PCR were analyzed by Cohen's kappa coefficient calculator in website: http://project.astyleplus.net/lesson2_2.html. and described previously (Landis and Koch, 1977).

Sensitivity, specificity, validity and reliability of Giemsa, acridine orange and nested PCR were calculated as described in previously (Baron, 1994) and analyzed by clinical calculators online (http://vassarstats.net/clin1.html) and SISA online statistical software (http://www.quantitativeskills.com/sisa/distributions/binomial.htm).

CHAPTER IV

RESULTS

Clinical signs of suspected pigs

All blood samples from eleven open-air swine farms in Thailand mostly showed revealed *M. suis* infection. For instance, post-weaning pigs mostly showed mild to moderate anemia, growth retardation and compatible with respiratory disorders (Figure 8). However, some pigs manifested normal clinical signs including healthy gilt in sensitivity swine herds.

Figure 8 Twelve weeks fattening pigs (black arrow) in open-air swine farm at Ratchaburi province of Thailand manifested anemia and low growth performance.

10% Giemsa

23 out of 300 blood samples (7.67%) were positive to Giemsa microscopy. Suspected *M. suis* harboring on RBCs surfaces manifested basophilic discoid and coccoid arranged in single and chain-like as illustrated in Figure 9.

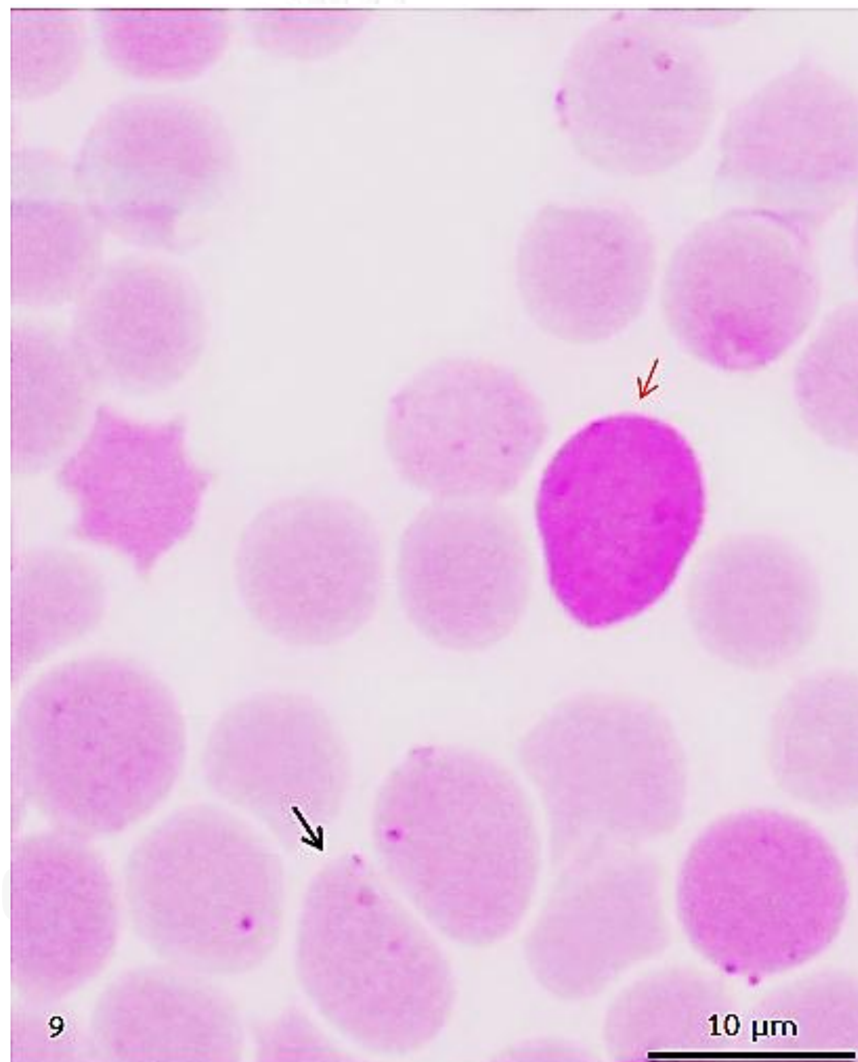

Figure 9 Positive blood samples showed basophilic discoid characteristics arranging in chain-like on erythrocytes surfaces (black arrow) with reticulocyte (hallmark of anemic signs) (red arrow) in 10% Giemsa (Bar = 10 µm).

Acridine orange staining

45 out of 300 blood samples (15%) were positive to acridine orange. Suspected *M. suis* showed dark yellow discoid shapes on dark green erythrocytes surface as manifested in Figure 10.

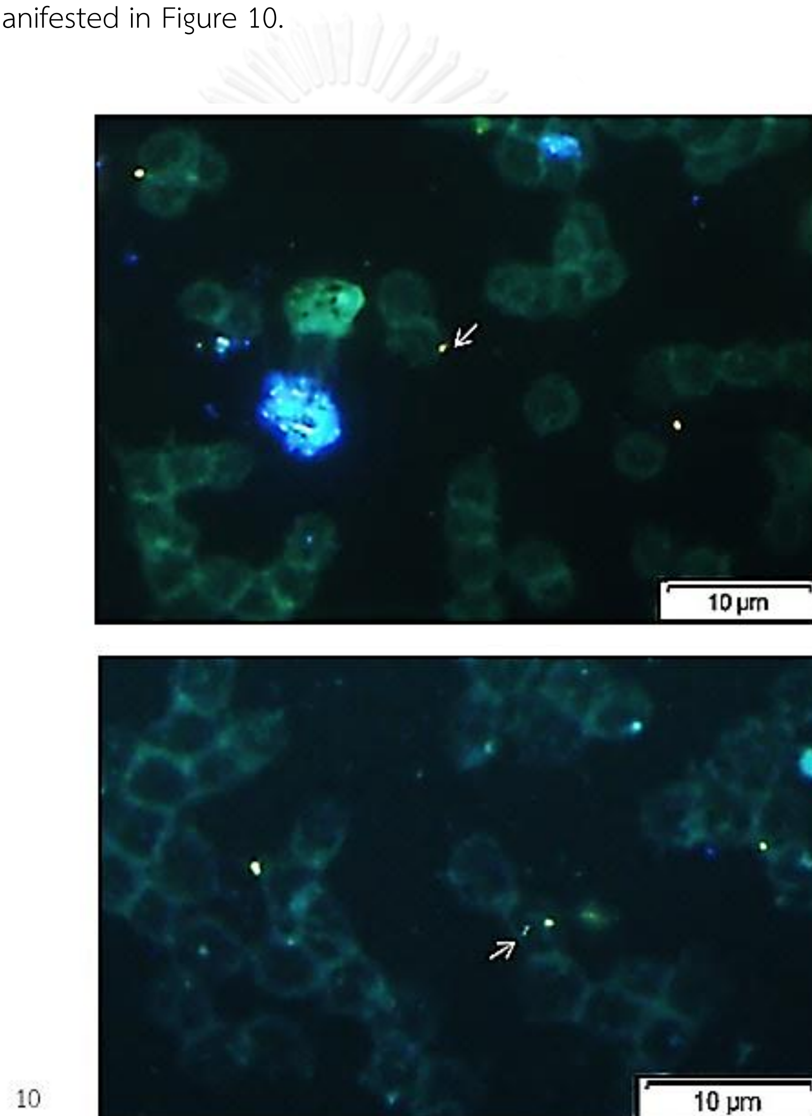

Figure 10 Suspected *M. suis* showed bright yellow discoid on dark green RBC (white arrow) in acridine orange staining (Bar = 10 μm).

Copy number calculation

Calculation nanograms of clone S1 (8.04 x n g/µl) converted to copy number as followed by this manual formula in this below:

$$\text{Numbers of copies (molecule)} = \frac{A \times 6.0221 \times 10^{23} \text{ molecule/mole}}{(N \times 649 \text{ g/mole/bp}) \times 10^{9} \text{ng/g}}$$

A = the amount of amplicon (g/µl)

N = length of total dsDNA amplicons (640 g/mole = average mass of 1bp of dsDNA)

** Total N = pGEM-T + PCR product size (3,015 +496 base pairs)

= 3,511 base pairs or plasmid weight = 2.28×10^{6} g/mole and 2.059×10^{6} g/mole

= 6.0221×10^{23} copies

$$\textbf{Clone S1} = \frac{8.04 \times 10^{-9} \text{g/µl} \times 6.0221 \times 10^{23} \text{ molecule/mole}}{2.059 \times 10^{6} \text{ g/mole}}$$

= 2.351×10^{9} copies/µl

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Conventional PCR

Specificity test

Derived primers (AnaF1 and HemoRII) from Japan could amplify DNA of the other *Mycoplasma* such as *M. haemominutum*, *M. haemofelis, M. hyorhinis*, *M. hyopheumoniae* and *hyosynoviae* (Lane 3- Lane 6) showing expected band around 500 base pairs (bp) as well as Clone S1 (Lane 8). However, *M. hyopneumoniae* showed negative band (Lane 7) as illustrated in Figure 11.

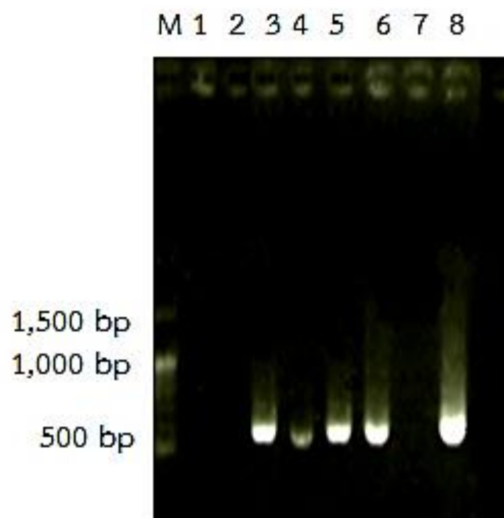

Figure 11 Amplification of 16srRNA in *Mycoplasma* isolated from blood in feline, respiratory tract and joint in swine, respectively using derived primers (AnaF1 and HemoRII) represented expected band approximately 500 bp. Left to right: M = 100+1,500 bp DNA ladder, Lane 1 = sterile water, Lane 2 = non-infected pig blood, Lane 3 = *M. haemominutum*, Lane 4 = *M. haemofelis*, Lane 5 = *M. hyorhinis*, Lane 6 = *M. hyosynoviae*, Lane 7 = *M. hyopneumoniae*, Lane 8 = Clone S1 (Positive control).

Sensitivity test

Derived primers (AnaF1 and HemoRII) from Japan could amplify DNA of Clone S1 (Lane 1-Lane 5) in dilution at least 10^{-15} or 2.351×10^{-15} copies/µl (Lane 5) as shown in Figure 12.

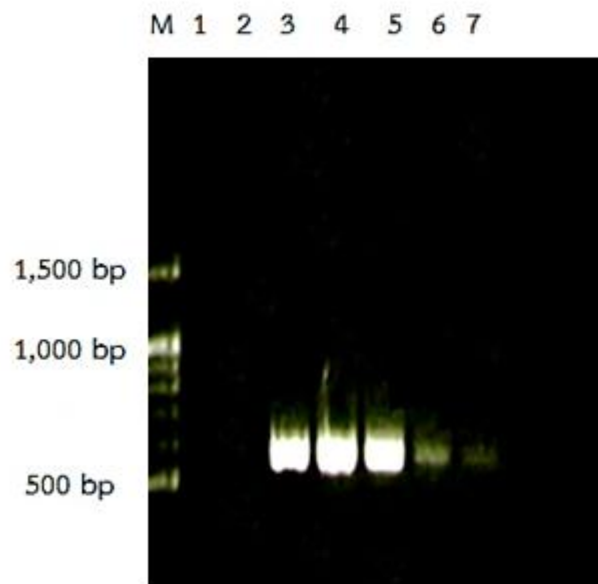

Figure 12 Amplification of 16srRNA gene of *M. suis* recombinant DNA (Clone S1) diluting with nuclease water in dilution at 10^{-1}-10^{-21} using derived primers (AnaF1 and HemoRII) represented expected band around 500 bp. Left to right: M = 100+1,500 bp DNA ladder, Lane 1 = sterile water (negative), Lane 2 = non-infected band (negative), Lane 3 = Clone S1 at 10^{-3} (500 bp), Lane 4= Clone S1 at 10^{-6} (500 bp), Lane 5 = Clone S1 at 10^{-9} (500 bp), Lane 6 = Clone S1 at 10^{-12} (500 bp), Lane 7 = Clone S1 at 10^{-15} (500 bp).

Blood samples from open-air swine farms

Only one samples (NP1, Lane 4) showed expected band abruptly 500 bp compared to the size of PCR product of Clone S1 as shown in Figure 13. DNA sequencing of NP1 manifested 100% identity to *M. suis* strain Morioka 8 (accession number, AB610849) from Japan.

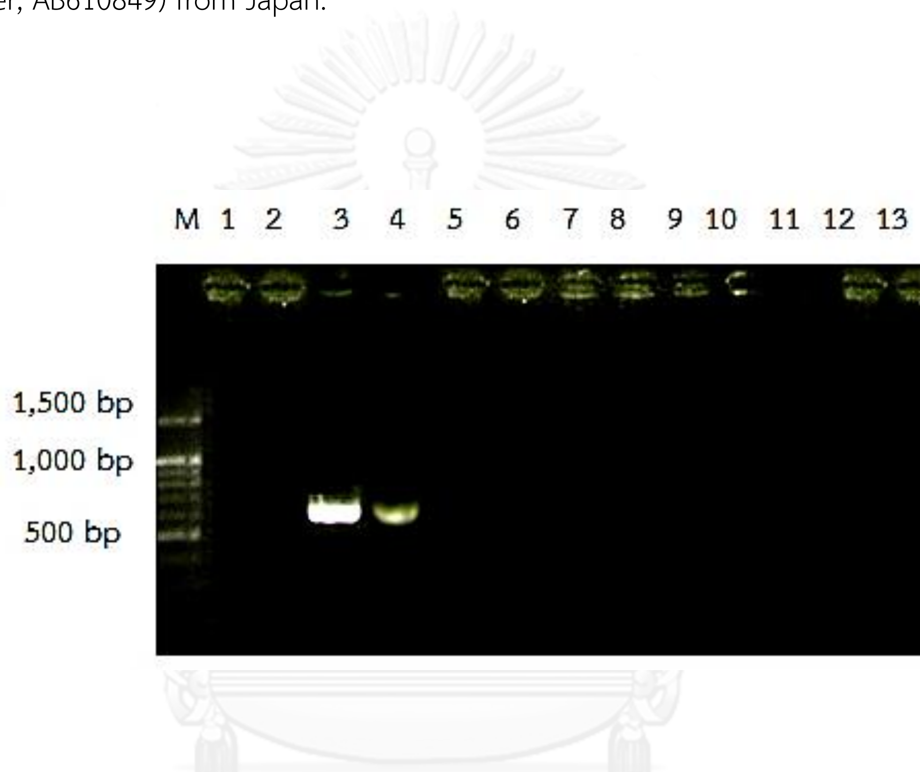

Figure 13 Amplification of 16srRNA in ten blood samples (NP1-NP10) derived from swine open-farm at Nakhon Prathom (NP) province of Thailand using derived primers (AnaF1 and HemoRII) compared to expected size to *M. suis* recombinant DNA. Left to right: Lane 1 = sterile water, Lane 2 = non-infected pig, Lane 3 = Clone S1 (Positive control), Lane 4 = NP1 (500 bp), Lane 5–Lane 13 = NP2-NP10 (Negative band).

Nested PCR

According to positive samples by 10% Giemsa and acridine orange in some samples (NP2 –NP10), they showed negative band after conventional PCR using derived primers. Hence, the detector designed outer primers and inner primers amplify *M. suis* DNA in those samples with nested–PCR. Designed primers were investigated both specificity (6.2.1) and sensitivity (6.2.2) tests before used.

Specificity test of designed primers

Designed outer primers (*M. suis*-OF*, M. suis*–OR) could amplified different band patterns of *Mycoplasma* including *M. haemominutum* (Lane 5; 600 bp) and NP1 as positive sample after conventional PCR confirmation (Lane 17; 600 bp), *M. haemofelis* (Lane 7; 800 bp). *M. hyorhinis* (Lane 9), *M. hyopneumoniae* (Lane 11) and *M. hyosynoviae* (Lane 13) showed negative band (Figure 14).

Based on inner primers (*M. suis*-IF, *M. suis*-IR), *M. haemominum* (Lane 6), *M. haemofelis* (Lane 8), *M. hyorhinis* (Lane 10), *M. hyopneumoniae* (Lane 12), Clone S1 (Lane 16) and NP1 (Lane 18) showed around 222 bp. However, *M. haemominutum* showed approximately 700 bp using inner primers that could differentiate between *M. suis* and *M. haemominutum* (Figure 14).

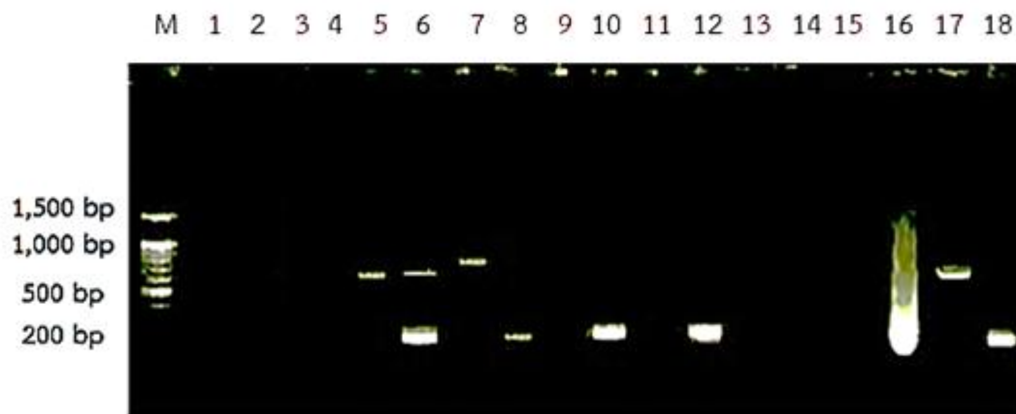

Figure 14 Amplification of 16srRNA of *Mycoplasma* from feline and swine using designed primers. Left to right: M = 100+1,500 bp DNA ladder, Lane 1 = sterile water (Outer primers; negative), Lane 2 = sterile water (Inner primers; negative), Lane 3 = non-infected pig blood (Outer primers; negative), Lane 4 = non-infected pig blood (Inner primers; negative) Lane 5 = *M. haemominutum* (Outer primers; 600 bp), Lane 6 = *M. haemominutum* (Inner primers; 700 and 222 bp), Lane 7 = *M. haemofelis* (Outer primers; 800 bp), Lane 8 = *M. haemofelis* (Inner primers; 222 bp), Lane 9 = *M. hyorhinis* (Outer primers; negative), Lane 10 = *M. hyorhinis* (Inner primers; 222 bp), Lane 11 = *M. hyopneumoniae* (Outer primers; negative), Lane 12 = *M. hyopneumoniae* (Inner primers; 222 bp), Lane 13 = *M. hyosynoviae* (Outer primers; negative), Lane 14 = *M. hyosynoviae* (Inner primers; 222 bp), Lane 15 = Clone S1 (Outer primers; data not shown), Lane 16 = Clone S1 (Inner primers; 222 bp), Lane 17 = NP1 (Outer primers; 600 bp), Lane 18 = NP1 (Inner primers; 222 bp).

Sensitivity test of designed inner primers

Designed inner primers (*M. suis*-IF, *M. suis*-IR) could amplify DNA of positive control (Clone S1) in dilution at least 10^{-6} or 2.351 x 10^{-6} copies/µl as manifested in Figure 15.

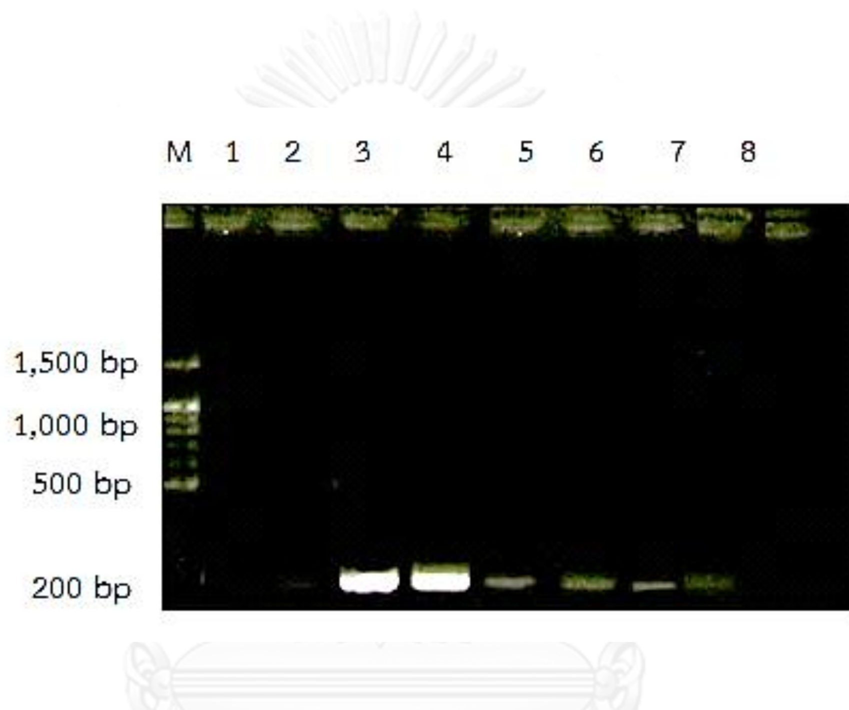

Figure 15 Amplification of 16srRNA gene of *M. suis* recombinant DNA (Clone S1) diluting with nuclease water in dilution at 10^{-1}-10^{-21} using designed primers (*M. suis*-OF, *M.suis*–OR; *M. suis*-IF, *M. suis*-IR). Left to right: M = 100+1,500 bp DNA ladder, Lane 1 = sterile water (negative), Lane 2 = non-infected pig (negative), Lane 3 = Clone S1 at 10^{-1} (222 bp), Lane 4 = Clone S1 at 10^{-2} (222 bp), Lane 5 = Clone S1 at 10^{-3} (222 bp), Lane 6 = Clone S1 at 10^{-4} (222 bp), Lane 7 = Clone S1 at 10^{-5} (222 bp), Lane 8 = Clone S1 at 10^{-6} (222 bp).

Survey *M. suis* in swine farms in Thailand

70 out of 300 samples (23.33%) were positive to *M. suis* using nested PCR. Three out of 70 samples showed around 600 bp after using outer primers (*M. suis* O-R) and 222 bp using inner primers (*M. suis* I-R) was observed. The remaining samples (67/300) manifested 222 bp using inner primers and showed negative in outer primers. Five positive samples were exemplified as demonstrated in Figure 16.

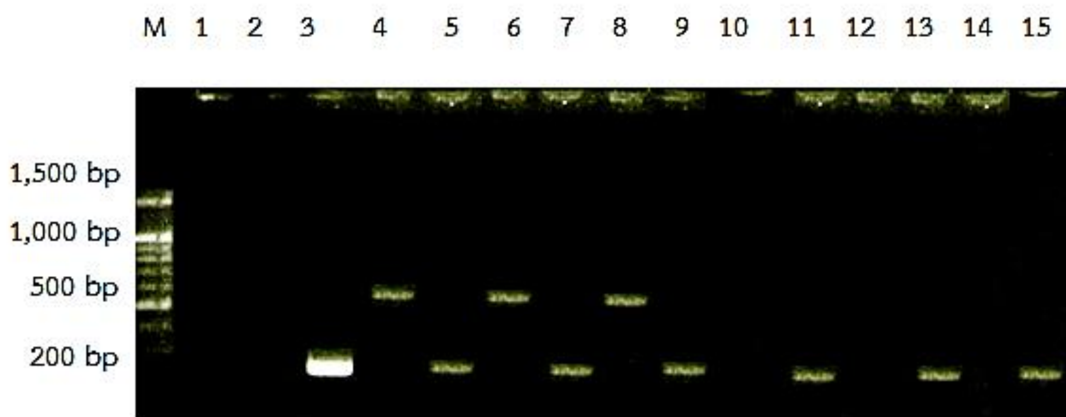

Figure 16 Amplification of 16srRNA in five blood samples (CH1, Si1, NP7, CM1 and P1) from swine farms using derived primers (*M. suis*-OF*, M.suis*–OR; *M. suis*-IF, *M. suis*-IR). Outer primers (O) and inner primers (I) represented at 600 and 222 bp, respectively. Left to right: Lane 1 = sterile water (Negative), Lane 2 = non-infected pig (Negative), Lane 3 = Clone S1 (Positive control) (I; 222 base pairs), Lane 4 = Positive *M. suis* using derived primers (NP1) as positive control (O; 600 bp), Lane 5 = NP1 (I; 222 bp), Lane 6 = CH1 (O; 600 bp), Lane 7 = CH1 (I; 222 bp), Lane 8 = Si1 (O; 600 bp), Lane 9 = Si1 (I; 222 bp), Lane 10 = NP7 (O; negative), Lane 11 = NP7 (I; 222 bp), Lane 12 = CM1 (O; negative), Lane 13 = CM1 (I; 222 bp), Lane 14 = P1 (O; negative) and Lane 15 = P1 (I; 222 bp).

Comparison between Giemsa microscopy, acridine orange and nested PCR

Among three methods for investigating *M. suis*, 7.7% (23/300) pigs were *M. suis*-positive by Giemsa microscopy. 15.0% (45/300) pigs were positive to *M. suis*. 23.3% (70/300) pigs were positive by nested PCR assay. False positive and false negative results could be found in both conventional methods. In Giemsa, those showed 73.9% (95% CI, 61.7 to 83.4) and 2.2% (95% CI, 0.8 to 5.3), respectively. False positive and false negative of acridine orange showed 15.2 (95% CI, 6.8 to 29.5) and 91.3% (95% CI, 87.0 to 94.4). Relationship of positive results between those techniques was demonstrated in Figure 17.

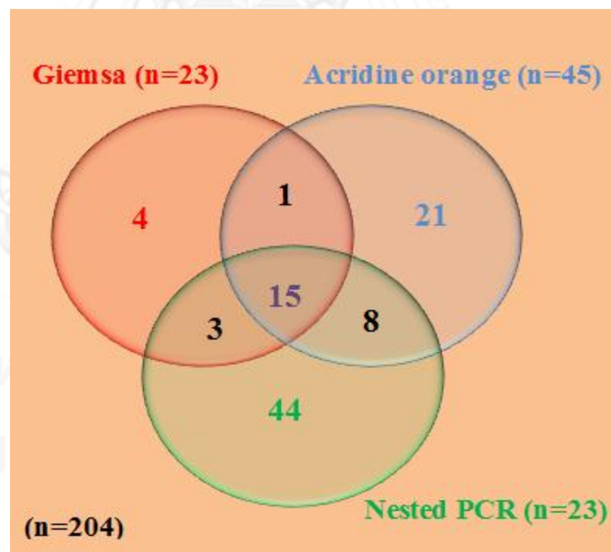

Figure 17 Venn diagram displaying the relationship of positive results based on Giemsa, acridine orange and nested PCR.

Agreement, sensitivity, specificity, accuracy and reliability of Giemsa microscopy, acridine orange and nested PCR

Using nested PCR as reference the sensitivity, specificity, accuracy and reliability of Giemsa microscopy were 78.3%, 81.6%, 41.3% and 25.5%, with fair agreement (k = 0.312) to nested PCR. Those of acridine orange showed 24.1%, 85.6%, 35.5% and 14.0%, respectively, with slightly compliance (k = 0.077) as illustrated in Table 5.

Table 5 Sensitivity, specificity, validity and reliability in 10% Giemsa, acridine orange using nested PCR as reference

Techniques	Tests (%)			
	Sensitivity (95% CI)	Specificity (95% CI)	Validity (95% CI)	Reliability (95% CI)
1. 10% Giemsa	78.3 (55.8-92.0)	81.6 (76.4-86.0)	41.3 (37.1-45.8)	25.5 (21.0-30.2)
2. Acridine orange	24.1 (11.0-43.9)	85.6 (80.7-89.5)	35.5 (31.3-41.3)	14.0 (8.2-21.7)

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Hematologic parameters and percentages of parasitemia

Hematocrit and erythrocytes values of positive samples were significantly lower than negative blood samples (*P* value ≤ 0.05). % parasitemia, monocytes and eosinophil had significantly higher than negative samples (*P* value ≤ 0.05). Leucocytes counts, band neutrophil, basophil and neutrophil of positive and negative samples had not showed significantly different as illustrated in Table 6.

Table 6 Hematologic parameters and parasitemia

Blood parameters	Nested PCR Positive (n=70) Mean ± SD*	Nested PCR Negative (n=230) Mean ± SD*	Hematologic references**	*p*
1. Hematocrit (%)	30.96 ± 6.70[a]	35.56 ± 6.76[b]	32.5 -44.2	0.001[ab]
2. Erythrocytes (10^6/µl)	2.99 ± 1.74[a]	3.61 ± 1.80[b]	5.79-7.52	0.003[ab]
3. Leucocytes (10^3/µl)	15.23 ± 7.15	16.10 ± 7.66	13-24	0.079
4. Differentiated leucocytes count (%)				
4.1 Band neutrophil	0	0.13 ± 0.76	0-3	0.700
4.2 Basophil	0.49 ± 0.85	0.60 ± 1.23	0.5-1	0.797
4.3 Eosinophil	4.39 ± 3.53[a]	1.68 ± 2.98[b]	1-4	0.001[ab]
4.4 Neutrophil	19.59 ± 15.24	21.67 ± 16.21	40-60	0.379
4.5 Lymphocyte	65.93 ± 13.89[a]	72.89 ± 16.29[b]	20-40	0.001[ab]
4.6 Monocyte	9.61 ± 5.34[a]	3.05 ± 3.27[b]	2-8	0.001[ab]
5. Parasitemia (%)	0.06 ± 0.25[a]	0[b]	-	0.001[ab]

Note: * SD = Standard deviation, ** Hematological reference ranges according to the previous publications (Miller et al., 1961), [ab] *P* value ≤ 0.05 were noted as significant (Mann-Whitney U test).

Based on nested PCR, *M. suis* could be observed during showing anemia sign and non-anemia. However, *M. suis* in this study was not only investigated during anemia but also non-anemia phase as shown in Table 7.

Table 7 Relation of swine health status and the result of nested PCR assay

Status	Positive (n = 70)		Negative (n = 230)	
	Anemia	Non-anemia	Anemia	Non-anemia
Samples	63 (90%)	7 (10%)	174 (75.65%)	56 (24.35%)

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Virulence score

Virulence score of all blood samples in this study was shown in below.

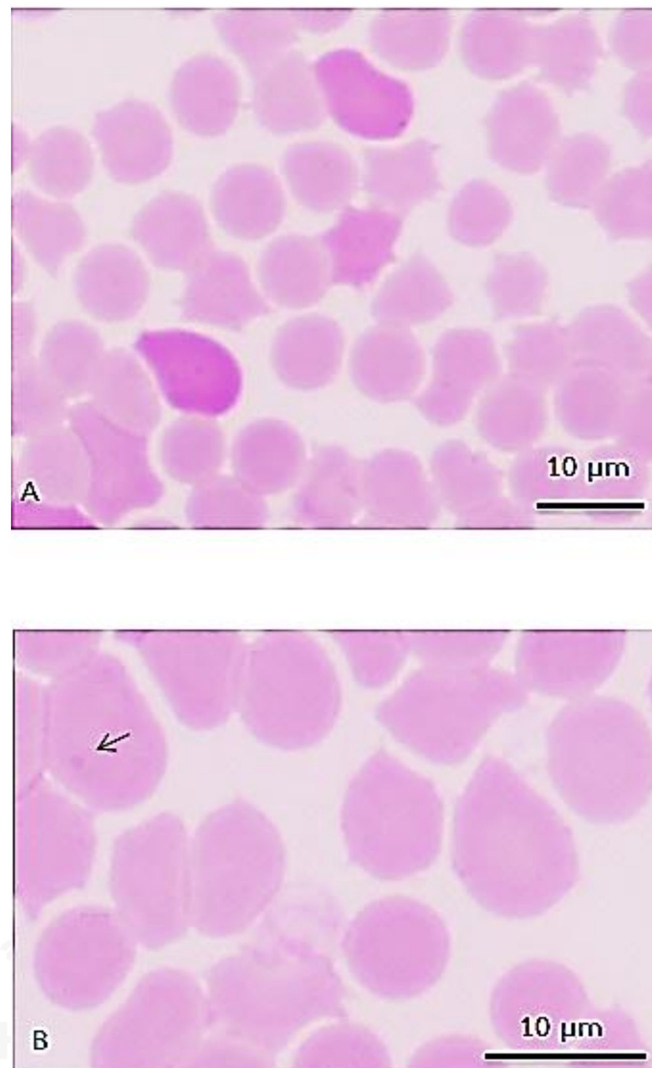

Figure 18 Virulence score (0-5) of Giemsa-stained blood smear. (**A**) suspected *M. suis* could not be found on RBCs surfaces as score 0 (bar = 10 µm) and (**B**) only one *M. suis*-like harboring on one infected erythrocyte was observed as score 1 (black arrow) (bar = 10 µm).

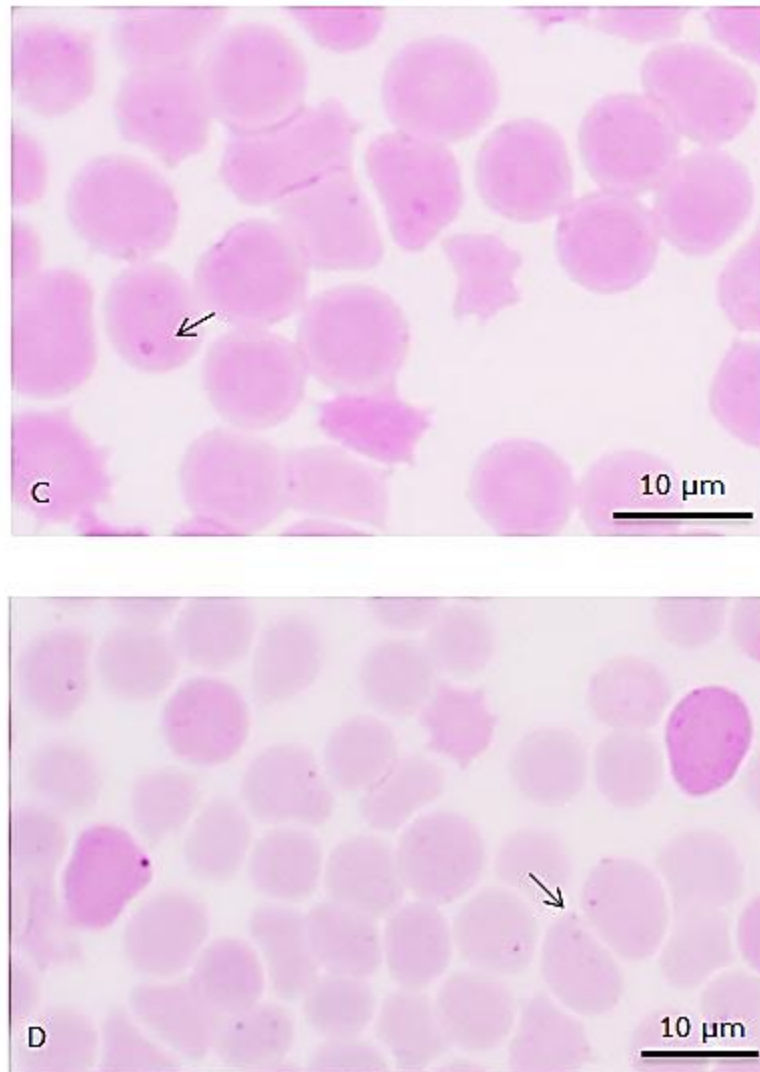

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Figure 19 Virulence score (0-5) of Giemsa-stained blood smears. (**C**) only one *M. suis*-like on more than one infected RBCs was seen as score 2 (black arrow) (bar = 10 μm) and (**D**) more than one *M. suis*-like harboring on infected erythrocytes was observed as score 3 (black arrow) (bar = 10 μm).

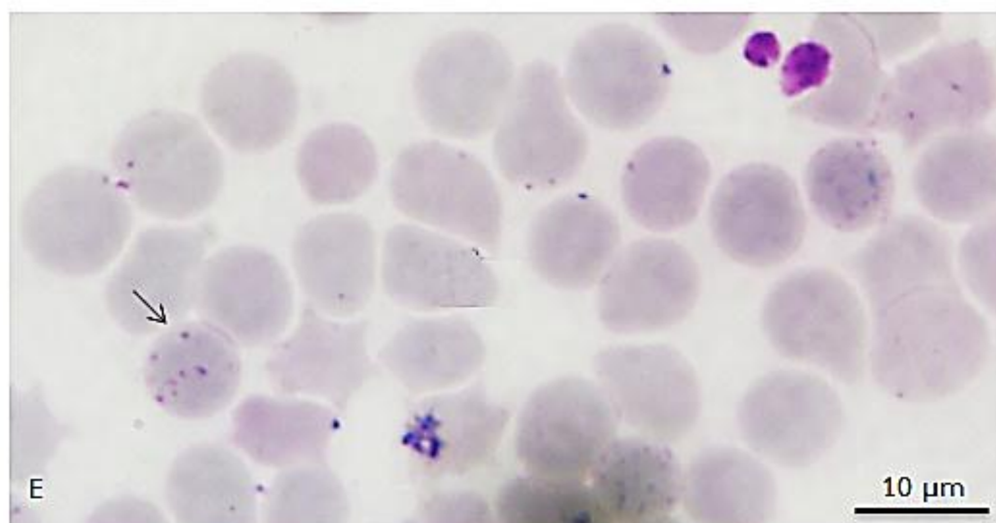

Figure 20 Virulence score (0-5) from Giemsa-stained blood smears. (**E**) more than one or one suspected *M. suis* (black arrow) on almost infected RBCs was found as score 4 (bar = 10 μm).

Note: Every RBC was infected by *M. suis* as score 5 in this study was not observed.

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In virulence score, score 0 was observed up to 52 samples. Score 1 was found in six blood samples. Score 2 was observed in 10 samples. Only one blood sample was seen in score 3 and score 4, respectively. Score 5 in this study was not observed. Bar graph of virulence score was shown in Figure 21.

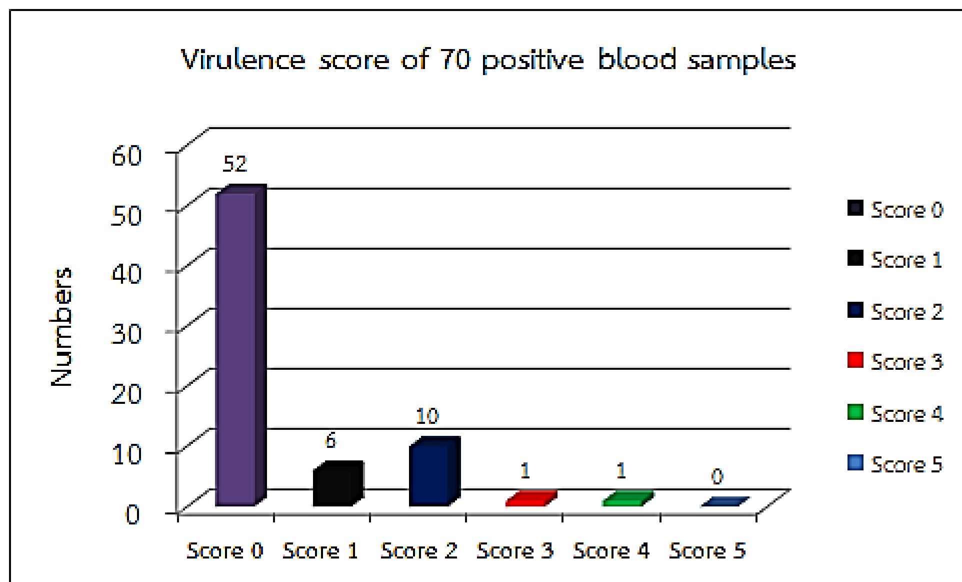

Figure 21 Bar graph of virulence score (0-5) in seventy positive blood samples based on nested PCR. Score 0 could be observed most up to 52 samples. Score 1 was seen in 6 samples. Score 2 was seen in 10 samples. Score 3 was observed in 1 sample. Score 4 was seen in 1sample. Score 5 was not found.

CHAPTER V
DISCUSSION

This present study is the first study confirmation existence of *M. suis* in swine farms in Thailand by molecular detection including conventional PCR and nested PCR. In Thailand, little is known about prevalence and existence of this bacterial agent due to source-limited of molecular assays. Consequently, affirmation of this agent in this present study was determined based on molecular affirmation and DNA sequencing that displayed 100% identity to *M. suis* by conventional PCR using derived primers from Japan and 99% identity to *M. suis* with nested PCR using designed new primers. Those molecular methods could confirm this bacterial agent exactly existed in swine farms in Thailand especially open-air farm which was one of the best criteria for blood collection of suspected pigs in this study.

Generally, the criteria of *M. suis* infection in swine are anemia and growth retardation in piglets, low growth performance in fattening pigs and reproductive disorders in sows and gilts including abortion, long estrous cycles, pyrexia and maternal behaviors changing in some cases (Messick, 2004; Hoelzle, 2008). In this research, suspected pigs that directly consistent to *M. suis* infection such as anemia, low growth performance, delay estrous cycles, high fever and co-infection with other swine respiratory disorders (Hoelzle, 2008) were collected blood for identification via routine techniques and molecular assays.

In this study, 63 out of 70 samples (90%) of anemic pigs were positive to *M. suis* and 7 samples out of 70 (10%) were positive of healthy pigs (unapparent clinical signs) by nested PCR using designed primers. Based on this result, anemic sign was one of the criteria of this disease for blood selection. However, only this sign could not indicate those samples were infected from this bacterial agent.

Moreover, clinical signs and hematologic parameters are not enough to insist blood samples are positive or negative to *M. suis* after identification by molecular methods. Thereby, several sensitive and specific diagnostic tests have been continually established for early diagnosis and rapid treatment. Even through, *M. suis* had ever been indicated existing in small scale batches at Nakhon Pathom province of Thailand since 1980s using only conventional techniques including Giemsa under inoculated splenectomized pigs (Luengyosluechakul and Nithiuthai, 1989). But, those techniques could not accurately confirm this agent because ambiguous characteristics were observed. Despite, this agent is a neglect bacterial agent in swine herds in Thailand over 20 years due to difficulty investigation by only conventional techniques and non-typically clinical signs and insufficient rapid molecular tests.

Additionally, conventional techniques are limited to identify this agent in swine farms in Thailand especially during very low parasitemia based on antibiotics therapy. In other countries, this agent was documented in many publications revealed pig production losses and established molecular tests worldwide over 70 years (Splitter, 1950; Heinritzi, 1990; Gwaltney et al., 1993; Messick et al., 1999; Yang et al., 2000; Wu et al., 2006; Ritzmann et al., 2009; Yuan et al., 2009; Hoelzle et al., 2010a; Huang et al., 2012; Watanabe et al., 2012).

Diagnosis of this agent in current was followed as four core concepts including conventional techniques (Giemsa, Wright-Giemsa, Diff quick and acridine orange), clinical signs of splenectomized pigs after agent inoculation, serological diagnostic assay (ELISA and IHA) and molecular identification (conventional PCR, nested PCR and real-time PCR) (Messick, 2004; Hoelzle, 2008). At present, PCR and real-time PCR were accepted as gold standard for investigation *M. suis* correctly and promptly.

Based on positive samples (NP1-NP10) by routine methods, all samples were positive to *M. suis* by nested PCR using designed new primers manifesting approximately 222 bp. Conversely, only one sample (NP1) out of ten was positive to this agent by conventional PCR using derived primers from Japan illustrating around 500 bp. From this result, it is possibly involved in several factors including sensitivity of primers, overload DNA samples, error techniques and existence of various *M. suis* strains in Thailand.

Nested PCR is highly sensitive and specific assay to detect interested genes or agents by the reasons of the second steps of product amplification and reducing non-specific band depended on two pairs of specific primers known as external primers (outer) and internal primers (inner). This technique is a better to differentiate interested agent from others in the level of species than conventional PCR but contamination has been still concerned as this assay.

In this study, the sensitivity of derived primers from Japan was higher than inner primers of designed new primers but outer primers were not detected sensitivity test because of limitation of recombinant DNA (positive control). Based on designed outer primers, they were selected derived forward primer and designed reverse primer based on 16S rRNA gene that showed position was far from the sequence of recombinant DNA.

However, nested PCR is one of the techniques for increasing a quantity of interested genes or agents due to two steps PCR. Hence, it is possible to increase DNA in first step (not mentioned sensitivity). Derived PCR product from the first step was amplified during the second step of nested PCR that was enough to get a high yield DNA for detection this agent in all samples. Based on nested PCR assay, 3 samples out of 70 positive blood samples showed expected band approximately 600 bp in first step and the final step manifesting 222 bp was seen. The remaining displayed only final step around 222 bp.

However, DNA sequencing of those eleven samples showed 99% identity to *M. suis* in various strains submitted to GenBank. According to those problems, several factors causing unapparent band in first step (outer primer) were noted including technique errors and various strains of *M. suis*. However, technique errors by human were solved by repeating process and those outer primers were checked by several programs. However, the results were the same. So, it is possible this bacterial agent exists in swine farms in Thailand to date may be more than one strain even if the data of *M. suis* strains have never been documented.

However, a quantity of *M. suis* DNA in blood sample is one of the factors that affect to detect this agent by conventional techniques. For instance, acridine orange staining could detect *M. suis* on RBCs in stained-blood smears up to 40% due to abundant DNA (around 10^5-10^6 *M. suis* copies number/ml blood) using real-time PCR as reference (Ritzmann et al., 2009). Conversely, little DNA (less than 10^5 *M. suis* copies number/ml blood) in sample could reduce capability of those techniques to examine *M. suis* harboring on erythrocytes. Furthermore, sensitive and specific primers are also very important to investigate this minimal DNA in samples (Messick, 2004; Hoelzle, 2008).

Besides, sensitivity and specificity of primers are always checked before used for investigation. In this study, 16S rRNA gene was selected for designed primers because this gene could differentiate several bacteria in the level of strains and was so stable. 16S rRNA mostly used for study on phylogenetic analysis and taxonomic nomenclature categorization of several bacteria because of similarity of multifamily genes or operons (Clarridge, 2004; Janda and Abbott, 2007). In addition, approximately 1,500 bp of 16S rRNA are better information elucidating about interested bacteria (Clarridge, 2004; Janda and Abbott, 2007).

Among the techniques for investigation using nested PCR as reference, 10% Giemsa showed sensitivity higher than acridine orange (78.3% versus 24.1%) and, while, it showed specificity lower than acridine orange (81.6% versus 85.6%). Those results directly related to the results of detection in blood samples. However, those results reveled routine methods contrasted to the previous publications noted that acridine orange were easily to differentiate this agent and host cells based on color difference (Messick, 2004; Hoelzle, 2008). In spite of human experience, it may be also a factor. (Messick, 2004; Hoelzle, 2008).

Nested PCR was so sensitive to prove *M. suis* especially very low virulence scores (Score 0-1) and investigate existence of this agent in Thailand up to 23.3% more than 10% Giemsa (7.7%) and acridine orange (15%), respectively. In addition, nested PCR could confirm negative blood samples based on Giemsa microscopy and acridine orange procedures were positive. Thereby, nested PCR in this study was a highly sensitive and specific to rapidly detect this agent in particular carrier pigs.

False negative and false positive also were observed from those routine methods which also affected to decrease of specificity and sensitivity of their tests. However, the result of this study was directly linked to the previous publications noted that molecular techniques were highly specificity and sensitivity for its investigation (Messick, 2004; Hoelzle, 2008).

In the past, Giemsa microscopy and acridine orange were used for scanning this agent harboring on erythrocytes worldwide because of easy, convenient and cheap. However, those techniques are limited to determine this agent particularly very low sensitivity and specificity during very low parasitemia. Moreover, infection periods (less than 1 day) and needing laboratory experts are also needed to confirm ambiguous results from background staining (Messick, 2004; Hoelzle, 2008).

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Interestingly, some publications noted that *M. suis* is an intracellular bacteria based on the results of their studies by quantitative real-time PCR, confocal laser scanning microscopy (CLSM), scanning electron microscopy (SEM) and transmission electron microscopy (TEM) (Groebel et al., 2009). However, entire genomes of two strains of *M. suis* do not insist that this agent is intracellular bacteria because some genes or proteins revealed those reason are not existed (Guimaraes et al., 2011; Oehlerking et al., 2011).

Depended on this result of agreement between methods, 10% Giemsa showed fair agreement to nested PCR assay (k = 0.312) and acridine orange showed slightly compliance to nested PCR (k= 0.077). Hence, Giemsa could be used instead of nested PCR in some cases because a Giemsa result was directly related to the result of nested PCR. However, false positive and false negative could be seen in Giemsa. Moreover, Giemsa is limited to detect correct agent especially during very low parasitemia and early infection (less than one day) (Messick, 2004; Hoelzle, 2008) before used.

In this present study, nested PCR assay could solve the problems reveled misclassification from color precipitation of those routine techniques. As a result, nested PCR in this study was more sensitive tool for investigation this agent and was a highly effective to prove *M. suis* in carrier pigs in farms. Carrier pig is the main source for its distribution between pigs that mostly shows normal signs affecting on eradication. Unfortunately, conventional techniques are limited to insist *M. suis* exists in those pigs. That cause this agent was taken for granted from swine farmers and had circulated in farms continuously via insect vectors and recycle needles. (Messick, 2004; Hoelzle, 2008).

Furthermore, this agent was observed in suckling pigs, nursery pigs, fattening pigs and gilts (see in: Appendix A). Almost pigs showed revealed *M. suis* infection including anemia, low growth performance and co-infection with other respiratory and enteric disorders.

However, detector also could investigate existence of *M. suis* by nested PCR from those pigs. But some pigs causing anemia showed negative (174/230 or 75.65%). Hence, only anemia is not typically sign of detection *M. suis*. Interestingly, gilts were also positive to *M. suis* (Appendix A). Therefore, they were possibly to transmit this agent to neonatal pigs and caused sick piglets (Messick, 2004; Hoelzle, 2008; Guimaraes et al., 2011; Oehlerking et al., 2011). However, the study aimed to identify *M. suis* existence in swine farms in Thailand with conventional and molecular techniques. Hence, detectors had concentrated on confirmation existence of *M. suis* in Thailand and had not described much more about the details of swine managements in each farms and drugs treatment.

In this study, some farms used antibiotic drug including cyclin in feed stuffs showed positive results by nested PCR in nursery pigs. So, those results were related to the previous publication recorded that antibiotics could not eliminate this agent in blood system (Messick, 2004; Hoelzle, 2008; Guimaraes et al., 2011; Oehlerking et al., 2011). Despite, those drugs can reduce severe symptoms. Early diagnosis can help swine farmers to eradicate carrier pigs and prevent this agent among farms (Messick, 2004; Hoelzle, 2008; Guimaraes et al., 2011; Oehlerking et al., 2011). However, infection periods and time tables for treatment in each farm was not to be clear. Therefore, prevention is needed to be done especially carrier gilts in some farms.

Based on hematologic parameters, hematocrit value and erythrocytes counts of positive blood samples had significantly lower than negative blood samples ($p \le 0.05$). This result was directly linked to the previous publication that reported positive blood samples showed hematocrit and RBCs counts lower than negative blood samples (Ritzmann et al., 2009). According to anemia, this sign is one of the key symptoms after *M. suis* infection as other swine diseases. Hence, rapid detection can be needed to answer before effective treatment (Messick, 2004; Hoelzle, 2008; Guimaraes et al., 2011; Oehlerking et al., 2011).

Generally, *M. suis* can replicate and rapidly develop on erythrocytes surface in three forms known as immature, juvenile and mature forms by budding. Erythrocytes membrane during early infection had not been still deformation due to immature forms. Later, large forms of *M. suis* can destroy cell membrane because it embed in deep cup-like on RBCs surfaces (Zachary and Basgall, 1985) for requiring nutrients for its survival (Messick, 2004; Hoelzle, 2008; Guimaraes et al., 2011; Oehlerking et al., 2011).

In addition, host immune modulating in particular IgM and IgG during infection is noted for RBCs destroying and finally causing anemia and hypoglycemia (Messick, 2004; Hoelzle, 2008; Guimaraes et al., 2011; Oehlerking et al., 2011). At present, several concepts of pathogenesis are involved in parasitic behaviors, genome characteristics, host immunity and program cell death signals. Those factors can answer anemia process by alterations of RBCs membrane and adjacent based on developing stages and taking biomolecules from host. Later, host immune responses eliminated infected RBCs and they are destroyed at spleen region (Messick, 2004; Hoelzle, 2008; Guimaraes et al., 2011; Oehlerking et al., 2011).

However, monocytes and eosinophil of positive blood samples in this study showed significantly higher than negative blood samples ($p \leq 0.05$). This result contrasted to the previous studies had ever been noted that differentiated leucocytes had not showed significantly difference between positive and negative blood samples (Henderson et al., 1997; Ritzmann et al., 2009). Monocyte cell is generally played important role in phagocytosis, inflammatory response and iron metabolisms (Doan, 1954). However, many factors can cause monocytosis also recorded such as chronic inflammation, stress responses, immune mediated disease, necrosis and viral infection (Ziegler-Heitbrock et al., 2010).

In this study, health status of swine during blood collection consistent with *M. suis* infection such as growth retardation, pyrexia, anemia and compatible with respiratory diseases (Hoelzle, 2008; Dent et al., 2013). Health status of the suspected pigs showed acute and chronic stages that related to monocytosis. However, negative blood samples also showed monocytes parameters higher than reference as well as positive samples. Consequently, monocytes values could not indicate *M. suis* infection in pigs.

Lymphocyte in this study showed higher than reference but it had significantly lower than negative blood samples ($p \leq 0.05$). Basically, its function is activation host immune responses against many agents as monocytes (Doan, 1954). Moreover, this cell is associated with viral infection as neutropenia particularly influenza virus. This study showed percentages of lymphocytes of positive and negative blood samples higher than reference and percentages of neutrophil was lower. Therefore, suspected pigs in this study manifesting respiratory disorders may be possibly infected virus or others. However, detector focused on only detection of *M. suis* thus other diseases in farms could not be proved.

Surprisingly, eosinophil of positive samples had significantly higher than negative blood samples ($p \leq 0.05$) which contrasted to the former studies (Henderson et al., 1997; Ritzmann et al., 2009). They had been reported that differentiated leucocytes count had not showed significantly difference between positive and negative blood samples (Henderson et al., 1997; Ritzmann et al., 2009) whereas, eosinophil of negative blood samples showed normal. In general, the function of eosinophil is activation of hypersensitivity reaction and parasites infection involvement especially nematodes (Doan, 1954).

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Based on eosinophil result, positive pigs may be involved in parasitic infection due to farm characteristics and biosecurity. However, no document reported about eosinophilia after *M. suis* infection under experiments and swine fields. As a consequent, blood parameters of *M. suis* isolated in Thailand must be continue study under inoculated splenectomized SPF pigs. Besides, this study was not determined actually blood parameters from only *M. suis* infection because suspected pigs for blood collection in this study could infected one or more than one disease.

Parasitemia in this study was very low (0.06±0.25%) that directly deal with the results of virulence score (0-4) showed low score (score 0) most. Basically, *M. suis* inoculated in splenectomized pigs mostly showed very high parasitemia more than 90% that manifesting *M. suis* more than one on every RBCs surfaces (Guimaraes et al., 2011). In this study, virulence scores 4 had been found only one sample out of all positive blood samples (1/70). Depended on nested PCR results, inner primers could amplified a quantity of DNA at least 2.351×10^{-6} copies/µl thus the quantities of *M. suis* DNA in positive blood samples indicated virulence score 0 was more than 2.351×10^{-6} copies/µl. Consequently, *M. suis* in swine herds in Thailand in update is very low virulence based on virulence score and parasitemia results of this study.

In conclusion, this present study is the first confirmation existence of *M. suis* in swine farms in Thailand by molecular techniques including conventional PCR and nested PCR. Additionally, development of highly sensitive nested PCR in this study could be used in laboratory diagnosis in Thailand as specific diagnostic assay for *M. suis* investigation in terms of control and eradication carrier pigs and early effective treatment in the future.

Conclusions

1. This study is the first confirmation exactly existence of *M. suis* in swine farms in Thailand by molecular techniques including conventional PCR and nested PCR.

2. Development of sensitive nested PCR diagnostic assay could be used as alternative tool in laboratory diagnosis in Thailand to solve the problems of misclassification in conventional techniques and rapidly to detect carrier pigs for eradiation.

3. Blood parameters (Hematocrit and erythrocytes) in this study of positive samples showed significantly lower than negative blood samples that directly linked to anemia. Only those parameters could not confirm *M. suis* infection. Moreover, exactly parameters of positive samples should be done under *M. suis* inoculated in splenectomized SPF pigs in the further research. Besides, *M. suis* in swine farms in Thailand to date is low virulence based on virulence score and parasitemia results.

Advantages of the study

1. Existence of *M. suis* in this study can be made swine farmers, veterinarian and researchers realized for carrier eradication and prevention this agent in farms to reduce pig production losses.

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2. Current scientific information in this present study can be used for fundamental data for the further study in the near future.

3. Nested PCR diagnostic assay in this study was developed for detection *M. suis* in swine farms in Thailand.

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APPENDIX

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APPENDIX A

Table A1 300 pigs from swine farms at six provinces of Thailand during 2012-2014 using nested PCR assay

Farms	Province	Ages (weeks)	Number	Positive	Negative
1.	Chachoengsao	12	4	0	4
		13	6	0	6
		14	5	0	5
		15	2	0	2
		16	4	0	4
		17	4	0	4
		19	3	0	3
		20	2	0	2
2.	Chiang Mai	11	40	13	27
3.	Nakhon Pathom	11	13	3	10
		10	8	1	7
4.	Nakhon Pathom	14	18	18	0
5.	Prachinburi	8	15	1	14
6.	Prachinburi	10	30	6	24
		20	30	6	24
7.	Ratchaburi	4	11	1	10
		8	10	2	8
		12	11	1	10
		16	5	3	2
		20	5	1	4

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Farms	Province	Ages (weeks)	Number	Positive	Negative
8.	Ratchaburi	10	4	0	4
		16	4	0	4
		22	4	0	4
9.	Ratchaburi	14	2	0	2
		15	3	0	3
		18	5	0	5
		20	4	0	4
10.	Ratchaburi	4	13	0	13
		6	5	0	5
		12	5	0	5
		13	7	0	7
11.	Sisaket	5	16	13	3
		31	1	0	1
		33	1	1	0
Total			**300**	**70**	**230**

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APPENDIX B

DNA Sequences of positive blood sample based on conventional PCR

Clone S1: 495 base pairs

AGTCGAACGA AAAAGGCCCT CGGGTCTTTT TAGTGGCAAA CGGGCGAGTA
ACGCATACTT AACTTACTTA TCTGAGGAAA ATAGCAGCTC GAAAGAGCTA
TTAATAATCC ATAGGTTTAG GCTAGAGGAA CTAGCTTAAA TTAAAGGAGG
CTGCCGCAAG GTGGCCTTGG GGTAAATAGG AGTATGTCCT ATTAGATAGT
TGGAGAGGTA AGGGCTCACC AAGTCGTGAT GGGTAGCTGG ACTGAGAGGT
TGAACAGCCG CAATGGGATT GAGATATGGC CCATATTCCT ACGGGAAGCA
GCAGTGAGGA ATTTTTCACA ATGGACGAAA GTCTGATGGA GCAATACCAC
GTGAACGATG AAGGTCTTCT GATTGTAAAG TTCTTTTATT TAGGAAAAAA
AGCGCGACAG GAAATGGTCG CGCCCTGATT GTACTAATTG AATAAGTGAC
AGCTAACTAT GTGCCAGCAG CTGCGGTAAA ACATAGGTCA CGAGC

NP1: 543 base pairs

AGGCCCTCGG GTCTTTTTAG TGGCAAACGG GCGAGTAACG CATACTTAAC
TTACTTATCT GAGGAAAATA GCAGCTCGAA AGAGCTATTA ATAATCCATA
GGTTTAGGCT AGAGGAACTA GCTTAAATTA AAGGAGGCTG CCGCAAGGTG
GCCTTGCGGG TAAATAGGAG TATGTCCTAT TAGATAGTTG GAGAGGTAAG
GGCTCACCAA GTCGATGATG GGTAGCTGGA CTGAGAGGTT GAACAGCCGC
AATGGGATTG AGATATGGCC CATATTCCTA CGGGAAGCAG CAGTGAGGAA
TTTTTCACAA TGGACGAAAG TCTGATGGAG CAATACCACG TGAACGATGA
AGGTCTTCTG ATTGTAAAGT TCTTTTATTT AGGAAAAAAA GCGCGACAGG
AAATGGTCGC GCCCTGATTG TACTAATTGA ATAAGTGACA GCTAACTATG
TGCCAGCAGC TGCGGTAAAA CATAGGTCAC GAGCATTATC CGGATTTATT
GGGCGTATGC TTAACAAGTG TTCGCGGTGG AGATTACACT TCT

APPENDIX C

DNA sequences of positive blood sample based on nested PCR

CH1: 224 base pairs

TCCAGCTACC CATCATCGAC TTGGTGAGCC CTTACCTCTC CAACTATCTA

ATAGGACATA CTCCTATTTA CCCGCAAGGC CACCTTGCGG CAGCCTCCTT

TAATTTAAGC TAGTTCCTCT AGCCTAAACC TATGGATTAT TAATAGCTCT

TTCGAGCTGC TATTTTCCTC AGATAAGTAA GTTAAGTATG CGTTACTCGC

CCGTTTGCCA CTAAAAAGAC CCGA

CM1: 224 base pairs

TCCAGCTACC CATCATCGAC TTGGTGAGCC CTTACCTCTC CAACTATCTA

ATAGGACATA CTCCTATTTA CCCGCAAGGC CACCTTGCGG CAGCCTCCTT

TAATTTAAGC TAGTTCCTCT AGCCTAAACC TATGGATTAT TAATAGCTCT

TTCGAGCTGC TATTTTCCTC AGATAAGTAA GTTAAGTATG CGTTACTCGC

CCGTTTGCCA CTAAAAAGAC CCGA

NP1: 224 base pairs

TCCAGCTACC CATCATCGAC TTGGTGAGCC CTTACCTCTC CAACTATCTA

ATAGGACATA CTCCTATTTA CCCGCAAGGC CACCTTGCGG CAGCCTCCTT

TAATTTAAGC TAGTTCCTCT AGCCTAAACC TATGGATTAT TAATAGCTCT

TTCGAGCTGC TATTTTCCTC AGATAAGTAA GTTAAGTATG CGTTACTCGC

CCGTTTGCCA CTAAAAAGAC CCGA

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NP4: 224 base pairs

TCCAGCTACC CATCATCGAC TTGGTGAGCC CTTACCTCTC CAACTATCTA

ATAGGACATA CTCCTATTTA CCCGCAAGGC CACCTTGCGG CAGCCTCCTT

TAATTTAAGC TAGTTCCTCT AGCCTAAACC TATGGATTAT TAATAGCTCT

TTCGAGCTGC TATTTTCCTC AGATAAGTAA GTTAAGTATG CGTTACTCGC

CCGTTTGCCA CTAAAAAGAC CCGA

NP5: 224 base pairs

TCCAGCTACC CATCATCGAC TTGGTGAGCC CTTACCTCTC CAACTATCTA

ATAGGACATA CTCCTATTTA CCCGCAAGGC CACCTTGCGG CAGCCTCCTT

TAATTTAAGC TAGTTCCTCT AGCCTAAACC TATGGATTAT TAATAGCTCT

TTCGAGCTGC TATTTTCCTC AGATAAGTAA GTTAAGTATG CGTTACTCGC

CCGTTTGCCA CTAAAAAGAC CCGA

NP7: 224 base pairs

TCCAGCTACC CATCATCGAC TTGGTGAGCC CTTACCTCTC CAACTATCTA

ATAGGACATA CTCCTATTTA CCCGCAAGGC CACCTTGCGG CAGCCTCCTT

TAATTTAAGC TAGTTCCTCT AGCCTAAACC TATGGATTAT TAATAGCTCT

TTCGAGCTGC TATTTTCCTC AGATAAGTAA GTTAAGTATG CGTTACTCGC

CCGTTTGCCA CTAAAAAGAC CCGA

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NP12: 224 base pairs

TCCAGCTACC CATCATCGAC TTGGTGAGCC CTTACCTCTC CAACTATCTA

ATAGGACATA CTCCTATTTA CCCGCAAGGC CACCTTGCGG CAGCCTCCTT

TAATTTAAGC TAGTTCCTCT AGCCTAAACC TATGGATTAT TAATAGCTCT

TTCGAGCTGC TATTTTCCTC AGATAAGTAA GTTAAGTATG CGTTACTCGC

CCGTTTGCCA CTAAAAAGAC CCGA

NP13: 224 base pairs

TCCAGCTACC CATCATCGAC TTGGTGAGCC CTTACCTCTC CAACTATCTA
ATAGGACATA CTCCTATTTA CCCGCAAGGC CACCTTGCGG CAGCCTCCTT
TAATTTAAGC TAGTTCCTCT AGCCTAAACC TATGGATTAT TAATAGCTCT
TTCGAGCTGC TATTTTCCTC AGATAAGTAA GTTAAGTATG CGTTACTCGC
CCGTTTGCCA CTAAAAAGAC CCGA

NP17: 224 base pairs

TCCAGCTACC CATCATCGAC TTGGTGAGCC CTTACCTCTC CAACTATCTA
ATAGGACATA CTCCTATTTA CCCGCAAGGC CACCTTGCGG CAGCCTCCTT
TAATTTAAGC TAGTTCCTCT AGCCTAAACC TATGGATTAT TAATAGCTCT
TTCGAGCTGC TATTTTCCTC AGATAAGTAA GTTAAGTATG CGTTACTCGC
CCGTTTGCCA CTAAAAAGAC CCGA

P1: 224 base pairs

TCCAGCTACC CATCATCGAC TTGGTGAGCC CTTACCTCTC CAACTATCTA
ATAGGACATA CTCCTATTTA CCCGCAAGGC CACCTTGCGG CAGCCTCCTT
TAATTTAAGC TAGTTCCTCT AGCCTAAACC TATGGATTAT TAATAGCTCT
TTCGAGCTGC TATTTTCCTC AGATAAGTAA GTTAAGTATG CGTTACTCGC
CCGTTTGCCA CTAAAAAGAC CCGA

Si1: 224 base pairs

TCCAGCTACC CATCATCGAC TTGGTGAGCC CTTACCTCTC CAACTATCTA
ATAGGACATA CTCCTATTTA CCCGCAAGGC CACCTTGCGG CAGCCTCCTT
TAATTTAAGC TAGTTCCTCT AGCCTAAACC TATGGATTAT TAATAGCTCT
TTCGAGCTGC TATTTTCCTC AGATAAGTAA GTTAAGTATG CGTTACTCGC
CCGTTTGCCA CTAAAAAGAC CCGA

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Description	Max score	Total score	Query cover	E value	Ident	Accession
Mycoplasma suis strain ZJNB01 16S ribosomal RNAgene, partial sequence	409	409	99%	7e-111	99%	KC907396.1
Mycoplasma suis gene for 16S ribosomal RNA, partial sequence, strain: Morioka6	409	409	99%	7e-111	99%	AB610848.1
Mycoplasma suis gene for 16S ribosomal RNA, partial sequence, strain: Morioka8	409	409	99%	7e-111	99%	AB610849.1
Mycoplasma suis gene for 16S ribosomal RNA, partial sequence, strain: Morioka5	409	409	99%	7e-111	99%	AB610847.1
Mycoplasma suis KI3806 complete genome	409	409	99%	7e-111	99%	FQ790233.1
Mycoplasma suis partial 16S rRNAgene, isolate KI	409	409	99%	7e-111	99%	FN391022.1
Mycoplasma suis partial 16S rRNAgene, isolate 146/5	409	409	99%	7e-111	99%	FN391021.1
Mycoplasma suis str. Illinois strain Illinois 16S ribosomal RNA, complete sequence	403	403	99%	3e-109	99%	NR_103930.1
Mycoplasma suis str. Illinois, complete genome	403	403	99%	3e-109	99%	CP002525.1
Eperythrozoon suis 16S ribosomal RNA gene, partial sequence	403	403	99%	3e-109	99%	AF029394.1
Eperythrozoon suis 16S ribosomal RNA gene, partial sequence	403	403	99%	3e-109	99%	U88565.1
Mycoplasma suis strain CQ 16S ribosomal RNAgene, partial sequence	337	337	98%	3e-89	94%	EU603330.1
Mycoplasma suis strain SC 16S ribosomal RNAgene, partial sequence	309	309	98%	7e-81	92%	FJ263943.1

Figure C1: DNA sequences of positive samples (CH1, Si1, NP7, CM1, P1, NP1, NP4, NP5, NP12, NP13 and NP17) in swine farms in Thailand showed 99% identity to several strains of *M. suis* in GenBank including Morioka6 (accession no, AB610848).

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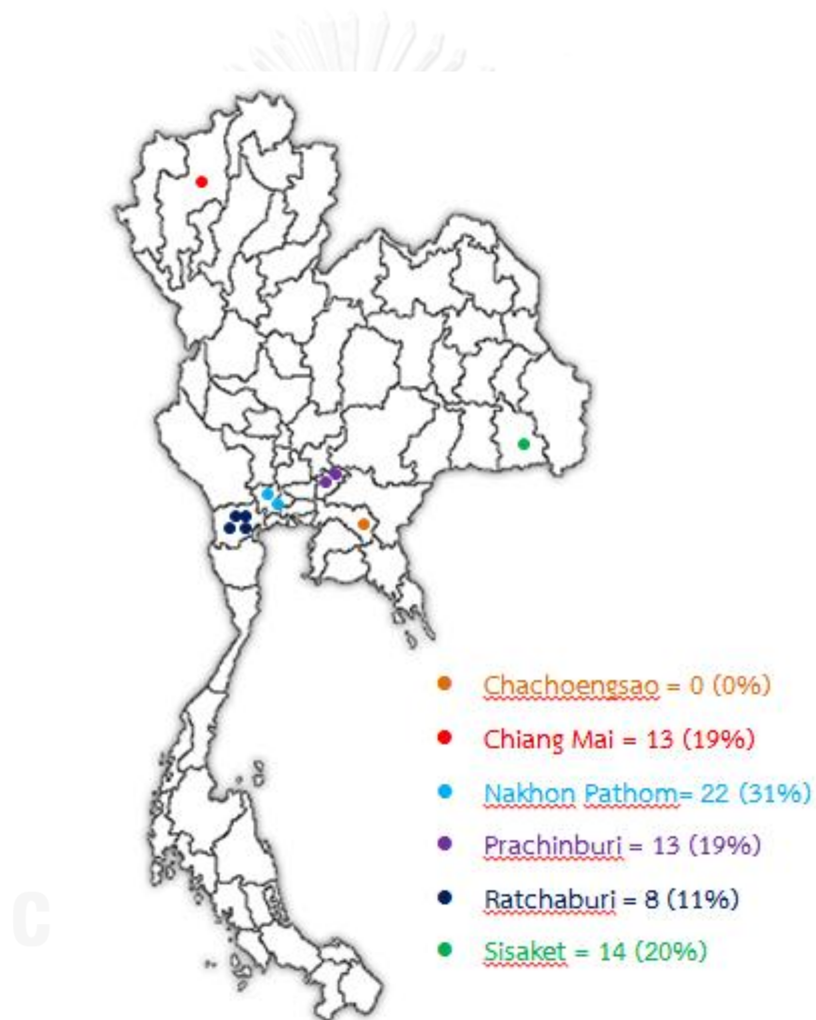
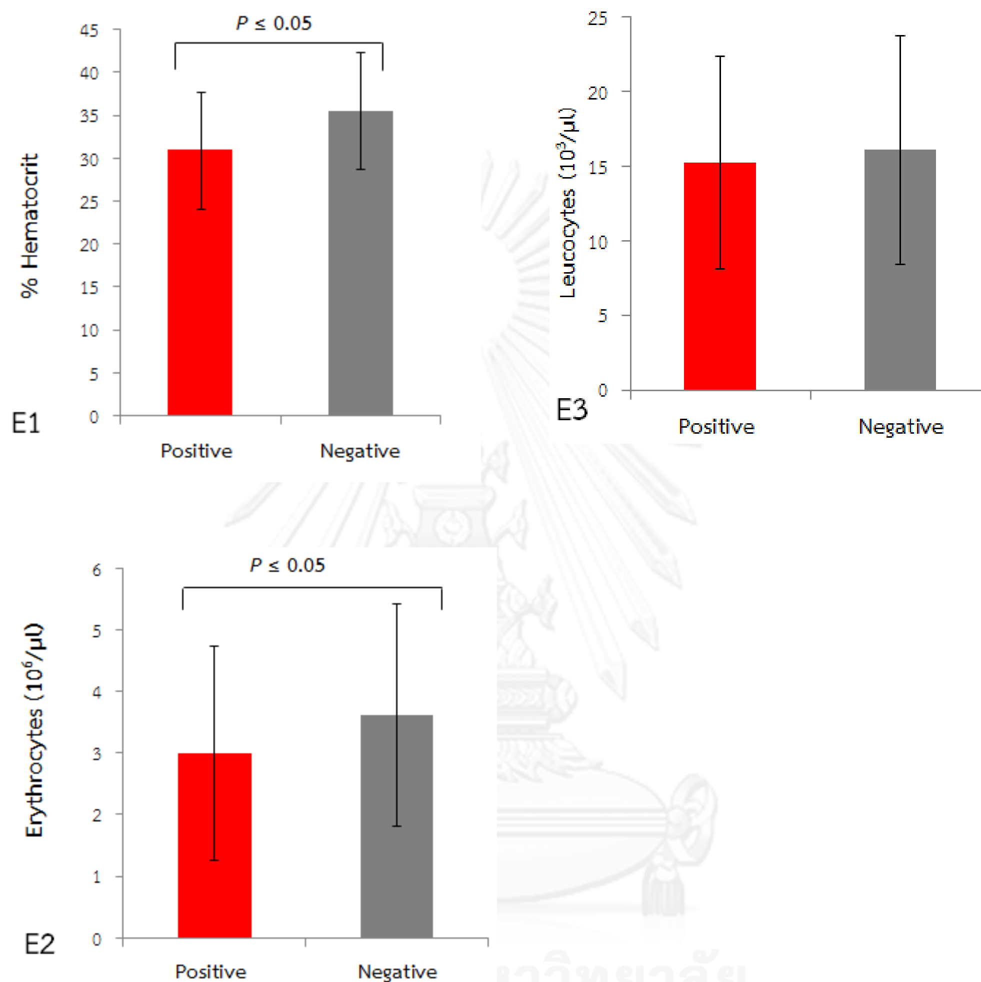
APPENDIX D

Three-hundred suspected pigs derived from commercial swine farms at six provinces of Thailand during 2012-2014. Seventy samples showed *M. suis*-positive by nested PCR.



Note: ●= Numbers of suspected open-air swine farms in six provinces of Thailand such as Chachoengsao, Chiang Mai, Nakhon Pathom, Prachinburi, Ratchaburi and Sisaket provinces

APPENDIX E



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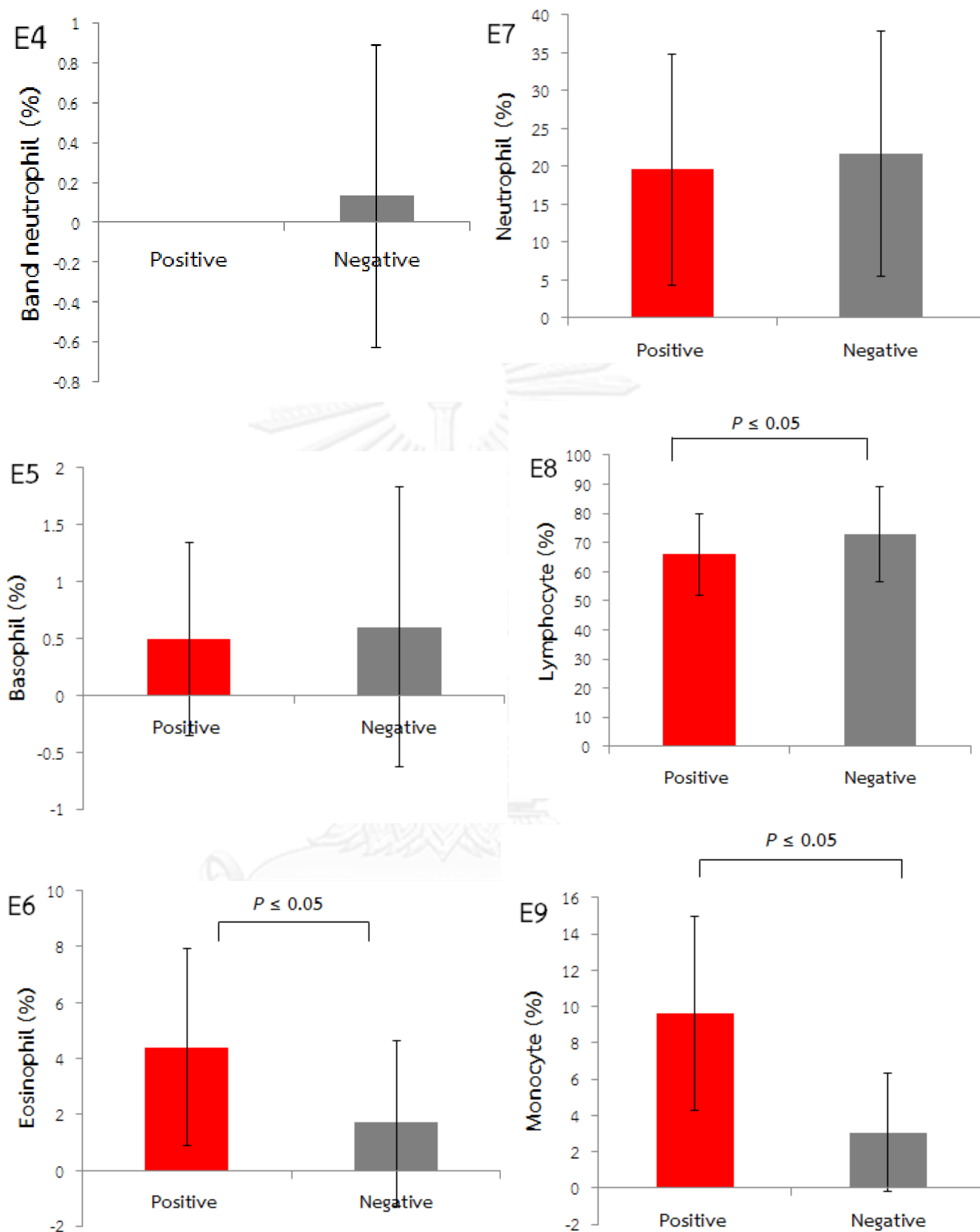
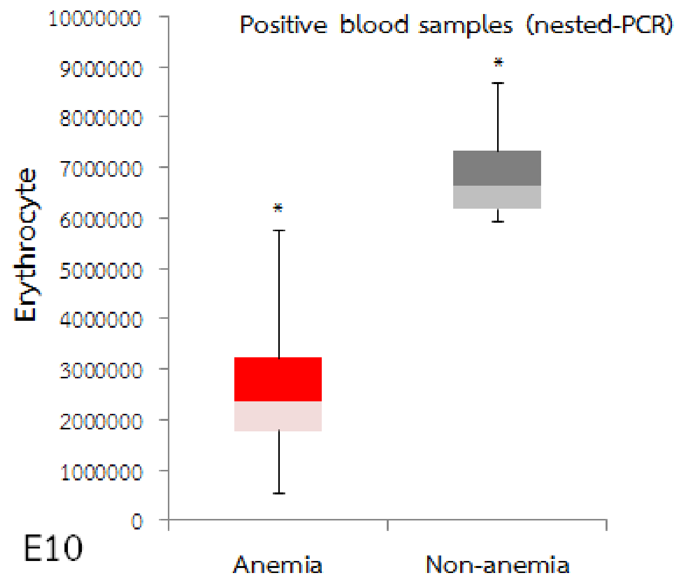
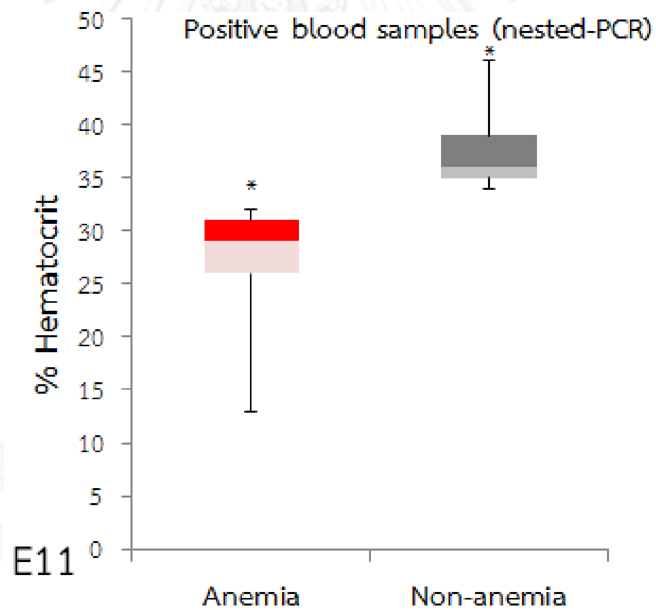
Mean ± standard deviation (SD) of hematocrit (%) (E1) and Erythrocytes (10^6/µl) (E2) values of positive samples showed significantly lower than negative blood samples ($P \leq 0.05$). Leucocytes values of positive and negative samples had not showed significantly different (E3).



Mean ± SD of band neutrophil (E4), basophil (E5) and neutrophil (E7) of positive and negative samples had not showed significantly different. Mean ± SD of eosinophil (E6) and monocyte (E9) of positive samples showed significantly higher than negative ($P \leq 0.05$). Mean ± SD of lymphocyte of positive samples had showed significantly lower than negative samples ($P \leq 0.05$) (E8).





Box plot analysis of *M. suis* positive blood samples in both non-anemia and anemia. Both erythrocytes count (E10) and hematocrit (%) (E11) of anemia group showed significantly lower than non-anemia blood samples (p value ≤ 0.109) using *t*-test analysis. Boxes represented the 25th and 75th quartiles.

VITA

My name is Ruangurai Kitchodok. My birthday is 27th January 1988 and birthplace is Hatyai district, Songkhla province of Thailand. I graduated from Hatyaiwittayalai Somboonkulkunya high school in 2005. In secondary school, I got some of certificates and rewards in competition among students in south regions of Thailand including Chemistry branch of the Promotion of Academic Olympic and Development of Science Education Foundation under the Patronage of Her Royal Highness Princess Galyani Vadhana Krom Luang Naradhiwas Rajanagarindra (POSN) in 2004 and Designed new rocket against gravity in rocket competition of National Science and Technology Development (NSTDA) in 2005.

Later, I got second-class honor (GPAX = 3.53) in branch of Doctor of Veterinary Medicine (D.V.M.), Chulalongkorn University, Bangkok, Thailand in 2011. In my university' lifetime, I had joined the VIV Asia 2009 at Bitech, Bangna, Bangkok, Thailand for interpreter and exhibitors in British pig association under cooperation between department live stock in England and England embassy in Thailand. Besides, I ever had published my senior project as poster presentation in the topic on porcine epidemic diarrhea (PED) villi height per crypt depth ratio in small intestine of experimental porcine epidemic diarrhea (PED) virus–inoculated piglets in ICVS 2012, Thailand. Afterwards, I derived scholarship (Chulalongkorn University graduate scholarship to commemorate the 72nd anniversary of his majesty king Bhumibol Adulyadej scholarship from Chulalongkorn University) from Chulalongkorn University for two years for pursuing in master degree level.

During my research, I presented some parts of my thesis to exhibit as three poster presentations. Firstly, Existence of Mycoplasma suis infection in Thai swine farms at IPVS, Vietnam, 2013. Secondly, Mycoplasma suis is coming back to Thai swine farms by using conventional techniques and confirming with molecular identification Thai swine farms, Swine conference, Bangkok, Thailand, 2014. Lastly, Development of nested-PCR assay for identification of a neglect swine hemoplasmosis (Mycoplasma suis) in Thai swine farms at swine conference, Bangkok, Thailand, 2014.

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