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DEVELOPMENT OF ELISA TEST KIT FOR ANTIBODY AGAINST *AVIBACTERIUM PARAGALLINARUM*

Miss Panchita Hongprasertkul

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Veterinary Medicine

Department of Veterinary Medicine

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Thesis Title	DEVELOPMENT OF ELISA TEST KIT FOR ANTIBODY AGAINST *AVIBACTERIUM PARAGALLINARUM*
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พัณณ์ชิตา หงษ์ประเสริฐกุล : การพัฒนาชุดทดสอบอีไลซ่าสำหรับการตรวจวัดแอนติบอดีต่อเชื้อ *เอวิแบคทีเรียม พารากัลลินารุม* (DEVELOPMENT OF ELISA TEST KIT FOR ANTIBODY AGAINST *AVIBACTERIUM PARAGALLINARUM*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: นิวัตร จันทร์ศิริพรชัย, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: วิษณุ วรรณแสวง, 55 หน้า.

ไก่ไข่เพศเมียสายพันธุ์ Babcock 308 จำนวน 100 ตัว ถูกแบ่งกลุ่มแบบสุ่มเป็น 5 กลุ่มๆ ละ 20 ตัว เพื่อใช้เป็นกลุ่มควบคุมผลบวกและผลลบ โดยกลุ่มที่ 1, 2, 3 และ 4 เป็นกลุ่มควบคุมผลบวกที่ให้วัคซีนเชื้อตายทางการค้าชนิดรวมสามซีโรวาร์, และกลุ่มควบคุมผลบวกที่ให้วัคซีนเชื้อตายที่เตรียมเองจากเชื้อ *Avibacterium paragallinarum* ซีโรวาร์ *A* (221), B (0222) และ C (Modesto) ตามลำดับ สำหรับกลุ่มที่ 5 เป็นกลุ่มควบคุมผลลบที่ให้ PBS ตัวอย่างซีรัมควบคุมนำมาใช้ในการคำนวณค่าความไวรับและความจำเพาะของชุดทดสอบอีไลซ่าชนิดอินไดเร็กที่พัฒนาขึ้นใหม่ โดยเก็บตัวอย่างที่ 4 สัปดาห์หลังการทำวัคซีนครั้งแรก ค่า cut-off ของชุดทดสอบอีไลซ่าชนิดอินไดเร็กของแต่ละซีโรวาร์ วิเคราะห์จากตัวอย่างซีรัมควบคุมผลลบที่เก็บก่อนการทำวัคซีนจำนวน 40 ตัวอย่าง ภายใต้สภาวะที่เหมาะสม ค่า cut-off ของซีโรวาร์ A, B และ C ซึ่งคำนวณจากค่าเฉลี่ยการดูดกลืนแสง (OD) ของตัวอย่างซีรัมควบคุมผลลบทั้งหมดบวกสามเท่าของค่าเบี่ยงเบนมาตรฐานได้เท่ากับ 0.334, 0.484 และ 0.678 ตามลำดับ ชุดทดสอบอีไลซ่าชนิดอินไดเร็กที่พัฒนาขึ้นทั้ง 3 ซีโรวาร์ มีประสิทธิภาพในด้านความไวรับเท่ากับ 100% แต่มีความจำเพาะต่ำเท่ากับ 30% เนื่องจากมีการตอบสนองข้ามซีโรวาร์ อย่างไรก็ตามชุดทดสอบอีไลซ่าชนิดอินไดเร็กสำหรับ ซีโรวาร์ A มีการตอบสนองต่อแอนติบอดีของตัวอย่างซีรัมควบคุมผลบวกซีโรวาร์ A (homologous serovar) มากกว่าอีก 2 ซีโรวาร์ (heterologous serovar) อย่างมีนัยสำคัญทางสถิติ *($P < 0.05$)* ในทางตรงข้ามชุดทดสอบอีไลซ่าชนิดอินไดเร็กสำหรับซีโรวาร์ B และ C ไม่พบความแตกต่างของการตอบสนองต่อแอนติบอดีของตัวอย่างซีรัมควบคุมผลบวกซีโรวาร์ A, B และ C สรุปผลจากการทดลองนี้แสดงให้เห็นว่าชุดทดสอบอีไลซ่าชนิดอินไดเร็กที่พัฒนาขึ้นใหม่สามารถเป็นวิธีทางเลือกของการทดสอบทางซีรัมวิทยาเพื่อใช้แยกความแตกต่างระหว่างฝูงไก่สุขภาพดีที่ไม่เคยสัมผัสเชื้อ *A. paragallinarum* และฝูงไก่ที่เคยได้รับวัคซีนและ/หรือติดเชื้อทางธรรมชาติ

ภาควิชา	อายุรศาสตร์	ลายมือชื่อนิสิต .....
สาขาวิชา	อายุรศาสตร์สัตวแพทย์	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
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KEYWORDS: ANTIBODY, AVIBACTERIUM PARAGALLINARUM, CHICKEN, INDIRECT ELISA, INFECTIOUS CORYZA

PANCHITA HONGPRASERTKUL: DEVELOPMENT OF ELISA TEST KIT FOR ANTIBODY AGAINST *AVIBACTERIUM PARAGALLINARUM*. ADVISOR: ASSOC. PROF. DR NIWAT CHANSIRIPORNCHAI, D.V.M., Ph.D., DTBVM, CO-ADVISOR: ASST. PROF. PROF. DR. WISANU WANASAWAENG, D.V.M., Ph.D., DTBVP, 55 pp.

One hundred Babcock 308 female-layer chickens were randomly divided into five groups of 20 each. Groups 1, 2, 3 and 4 were different positive control groups of immunized chickens with commercial trivalent mineral oil vaccine, and prepared bacterins of *Avibacterium paragallinarum* serovars A (221), B (0222) and C (Modesto), respectively. The chickens in Group 5 were assigned as a negative control and immunized with PBS. The serum from Groups 1–5 at 4 weeks after the first vaccination were used to calculate the sensitivity and specificity of the newly developed indirect enzyme-linked immunosorbent assay (I-ELISA) method. Forty negative control sera (taken before vaccination) were used to evaluate the cut-off value of the I-ELISA against each serovar of *A. paragallinarum* under optimal conditions. The cut-off values of serovars A, B and C, calculated by the mean optical density of all the negative sera plus three standard deviations were 0.334, 0.484 and 0.678, respectively. The efficacy of the developed I-ELISA showed 100% sensitivity for all three serovars of coating antigen but with a low specificity of 30% for all three serovars because of the high cross reactivity among serovars. Nevertheless, the serovar A I-ELISA gave a higher response to serovar A antibodies than to the other two heterologous serovars *($P < 0.05$)*. In contrast, the I-ELISA results for B and C did not show any significant difference between the homologous and heterologous serovars. In conclusion, this newly developed I-ELISA could be an alternative method for differentiating between *A. paragallinarum*-free chickens and those that have received either a vaccination and/or a challenge exposure.

Department:	Veterinary Medicine	Student's Signature	.....
Field of Study:	Veterinary Medicine	Advisor's Signature	.....
Academic Year:	2016	Co-Advisor's Signature	.....

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ABBREVIATION

µm	= micrometer
µl	= microliter
CVs	= coefficient of variation
ELISA	= enzyme-linked immunosorbent assay
ERIC	= enterobacterial repetitive intergenic consensus
HagA	= hemagglutinin gene A
HA	= hemagglutinins
HA-HS	= heat stable, trypsin resistant hemagglutinin
HA-HL	= heat labile, trysin resistant hemagglutinin
HA-L	= heat labile, trypsin sensitivity hemagglutinin
HI	= hemagglutination inhibition
IBDV	= infectious bursal disease virus
IBV	= infectious bronchitis virus
I-ELISA	= indirect enzyme-linked immunosorbent assay
IgG	= Immunoglobulin G
kDa	= kilo Dalton
KSCN	= potassium thiocyanate
LPS	= lipopolysaccharide
PCR	= polymerase chain reaction
MG	= *Mycoplasma gallisepticum*
MS	= *Mycoplasma synoviae*
NAD	= nicotinamide adenine dinucleotide
NT	= non treated antigen

OD	= optical density
PBS	= phosphate buffer saline
PM	= *Pasturella multocida*
SDS-PAGE	= sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBE	= tris-borate-EDTA

CHAPTER I
GENERAL INTRODUCTION

Importance and rationale

Infectious coryza is an acute respiratory disease. The disease causes economic loss in poultry industry worldwide because infectious coryza leads to drop in egg production 10-40% in breeder and layer chicken and increase culling rate in broilers (Blackall, 1999). This disease is highly contagious with low mortality rate, so many countries continuously show the evidences of infectious coryza affected chicken. There are reports of the outbreaks in two states of the United States that were potential impact on meat-type chicken (Droual et al., 1990). A study of the 10 infectious coryza outbreaks reported in Morocco were affected to reduce egg production 14-41% and increase mortality 0.7-10% (Mouahid et al., 1989). In 2011, there are still found 20% morbidity and increased 0.1% mortality rate within a week of infectious coryza outbreak which affected 12,500 layer hens in a farm located in Mexico (Soriano-Vargas et al., 2013).

Infectious coryza is caused by *Avibacterium paragallinarum*, Genus *Avibacterium*, Family Pasteurellaceae which is a Gram-negative rod, non-spore forming, facultative anaerobic and non-motile bacterium. The pathogen is classified into 3 antigenic types including A, B and C. *Avibacterium paragallinarum* requires factor V (nicotinamide adenine dinucleotide) for growth. However, some isolates are V-factor-independent *A. paragallinarum*, they have been found for the first time in the Republic of South Africa (Mouahid et al., 1992) and then in Mexico (Garcia et al., 2004). In Thailand, *A. paragallinarum* including serovars A, B and C have been found in both commercial and native chickens despite of having proper vaccination program (Chukiatsiri and Chansiripornchai, 2007). Thitisak et al. (1988) reported a study of village chicken in Thailand that infectious coryza is the most cause of death in chicken less than 2 months old and those over 6 months. Chukiatsiri et al. (2010) reported the first outbreak of infectious coryza disease caused by *A. paragallinarum* serovar B and isolated pathogen from infected layer chickens that showed mucous nasal discharge, facial edema and

conjunctivitis with dropping in egg production in Thailand. Chukiatsiri et al. (2012) also reported 18 isolates which included three serovars from infected chickens with clinical signs.

Serological monitoring is useful to measure antibody against infectious coryza vaccine and to estimate the vaccine efficacy. The monitoring of chicken's immune response against vaccination can be a potential method for disease control and prevention. Whereas, serologic diagnosis of infectious coryza has not been conducted because of short incubation period of the disease. At the present, the hemagglutination inhibition (HI) test is the best available method for detecting infectious coryza antibody titer (Blackall and Hinz, 2008). However, HI method for testing infectious coryza disease is still complicated in step of erythrocyte, antigen and serum preparation. Uncertainty of testing results depend on individual skills and laboratory preparation. Moreover, there is no commercial test kit for HI method. The alternative method is the monoclonal antibody-based blocking enzyme-linked immunosorbent assay (blocking ELISA) (Miao et al., 2000; Sun et al., 2007) but it is mainly used in research. Although blocking ELISA using monoclonal antibody shows high specificity and acceptable sensitivity (Zhang et al., 1999), there are some limitation in testing such as (1) monoclonal antibody have only for Page serovar A and C (2) monoclonal antibody are not commercial available (3) some isolates of *A. paragallinarum* do not react with monoclonal antibody (Blackall, 1999). Regarding to these limitations, the aim of this study was to develop indirect ELISA (I-ELISA) test kit for antibody detection against *A. paragallinarum* serovars A, B and C and to study the efficacy of developed I-ELISA in aspect of sensitivity and specificity. Moreover, we further compared antibody response against infectious coryza to HI assay and analyzed agreement between the two methods.

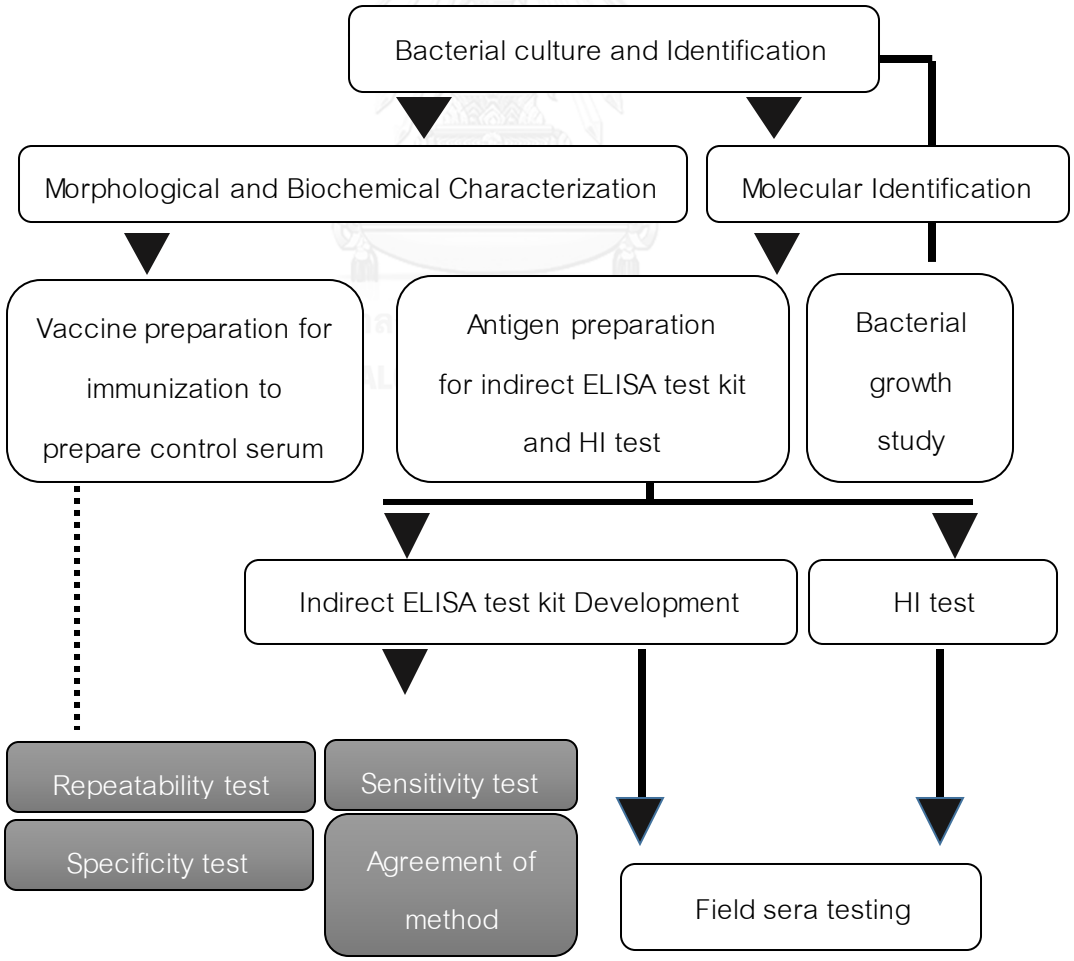
In addition, I-ELISA coated with small amount of protein antigen can be used to test many samples at the same time. It is easily used, saved time and reduced labors because this method is analyzed by computerization, and it can reduce varying factors from technician or sample error such as erythrocyte quality that affected to the results. According to many advantages of I-ELISA test kit, we expected that developed I-ELISA

test for antibody detection against infectious coryza serovar A, B and C may be useful in field of commercial poultry industry and be a good monitoring tool for preventing and controlling of infectious coryza disease.

Objectives of this study

1. To develop I-ELISA test kit for antibody detection against Infectious coryza serovars A, B and C.
2. To study the efficacy of developed I-ELISA in aspect of sensitivity and specificity.
3. To compare antibody response against infectious coryza to HI assay and analyze agreement between the two methods.

Conceptual framework



Overviews of this study

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Expected values

1. Developed I-ELISA test kit to detect antibody against Infectious coryza serovars A, B and C provides good specificity and sensitivity.
2. The method of test kit development will be an alternative way for developing test kit to test antibody against infectious coryza in Thailand.

CHAPTER II
LITERATURE REVIEW

Infectious coryza

Infectious coryza which caused by *Avibacterium paragallinarum* is an acute respiratory disease and sometimes progress to chronic infection within chicken flocks. It affects upper respiratory tract characterized by nasal discharge, facial edema and conjunctivitis. The disease results in poor growth performance of broiler chicken and lead to drop in egg production 10-40% in breeder and layer chickens (Blackall, 1999). The disease is highly contagious but low mortality rate, so many countries continuously still found the evidences of infectious coryza affected chicken. The greater economic loss from infectious coryza may be occurred with co-factors including complicated infection with other pathogens, stress condition, etc. In Argentina, broiler and layer flocks have found some clinical signs such as arthritis and septicemia caused by complicated with *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, *Pasteurella* spp., *Salmonella* spp., and infectious bronchitis virus (Sandoval et al., 1994).

1. Epidemiology and transmission

Chicken is natural host for *A. paragallinarum*. The presence of disease in other species is still investigated, so it means that *A.paragallinarum* currently causes infectious coryza in only chickens. In generally, chickens of all ages are susceptible. The study of Byarugaba et al. (2007) observed that three age groups of chickens including 4, 10 and 21 weeks of age had no significant differences in clinical signs and lesions. The clinical signs were assessed numerically according to Bragg (2002). However, the disease is usually more severe in older birds. The incubation period of disease is about 1-3 days and the duration of disease is usually 2-3 weeks, duration may be longer in mature birds with stress condition or presence of concurrent diseases, eg. mycoplasmosis. Chronically ill birds or recovered birds are the problem for infectious coryza disease control because they have been recognized as the main reservoir of infection.

Transmission can be performed by direct contact, airborne droplets and contamination of drinking water. Multiple age flocks should be avoided to minimize a risk for contamination across the flock. So “all-in/all-out” is the proper management to control disease. Infectious coryza is not an egg-transmitted disease. From many literatures, there have been reported that this disease was found in both intensive production systems and in village-type production system. The disease frequently occurs on intensive chicken farms, especially large-scale egg production complex and breeding farms. Whereas, Zaini and Kanameda (1991) and Lin et al. (1996) reported that indigenous chickens were also susceptible to *A. paragallinarum*.

2. Etiology

Infectiuos coryza is an acute respiratory disease caused by *Avibacterium paragallinarum* (Blackall et al., 2005), previously known as *Haemophillus paragallinarum*. In 1930s, *H. gallinarum* was identified to be the causative agent of infectious coryza due to the requirement of both X (hemin) and V (NAD) factors for growth *in vitro*. Later from 1960s, the organisms isolated from infected chicken of infectious coryza required only V-factor and have been termed *Haemophillus paragallinarum* (Page, 1962). However, some isolates did not require V-factor for growth called NAD-independent, they have been found at the first time in the Republic of South Africa (Mouahid et al., 1992) and then in Mexico (Garcia et al., 2004). In 2005s, *H. paragallinarum* was changed to be in a new genus which called *Avibacterium* likewise other organisms that were previously recognized in genus *Pasteurella* including *A. avium*, *A. gallinarum*, and *A. volantium* (Blackall and Hinz, 2008). *A. volantium*, *A. avium*, and *Avibacterium* sp. taxon A are not causing the disease.

Characteristics

A. paragallinarum is Gram negative, non-motile and non-spore forming. Morphology of pathogen is short rod, coccobacilli (1-3 µm in length and 0.4 – 0.8 µm in width) and tendency to filamentous after bacterial culture for 24 hours. The bacterium may become fragments and indefinite forms due to degeneration within 48-60 hours (Yamamoto, 1991).

Colonies of *A. paragallinarum* show dewdrop shape, non-hemolysis and satellitic growth pattern on blood agar which cross streak with *Staphylococcus* spp. (e.g. *S. aureus* (Bragg et al., 1997), *S. hyicus* (Blackall and Reid, 1982) or *S. epidermidis* (Page, 1962)) as a feeder. *A. paragallinarum* is commonly grown in condition with 5% carbon dioxide, and it can also grow anaerobically. Moreover, the organisms can grow in range of minimum and maximum temperature between 25 - 45 ^{o}C, and the optimum temperature for growth are around 37-38 ^{o}C (Blackall and Soriano, 2008).

Biochemical properties of *A. paragallinarum* are including the ability to reduce nitrate to nitrite and ferment glucose without the formation of gas, oxidase activity, the presence of the enzyme alkaline phosphatase, and a failure to produce indole or hydrolyse urea (Blackall, 2008). The biochemical properties of NAD dependent and NAD independent *A. paragallinarum* are the same. *A. paragallinarum* can be differentiated from other species of genus *Avibacterium* by using catalase test that is presented in Table 1

Susceptibility to chemical and physical agents

A. paragallinarum is inactivated easily outside the host. Exudate or tissue from infected chickens are still infectious at 37°C for 24 hours and may be up to 48 hours; at 4°C, exudate remains infectious for several days. The organism is heat-sensitive, at temperatures of 45-55°C, Haemophilus is killed within 2-10 minutes (Blackall et al., 1997).

Table 1 Biochemical properties of the species within the genus *Avibacterium*

Property	*A. gallinarum*	*A. paragallinarum*	*A. voluntium*	*A. avium*	*A. species A.*
Catalase	+[A]	-	+	+	+
Symbiotic growth	-	V	+	+	+
Growth in Air	+	-	+	+	+
Acid form					
L-arabinose	-	-	-	-	+
D-galactose	+	-	+	+	+
Lactose	V	-	V	-	-
D-mannitol	-	+	+	-	V
Maltose	+	V	+	-	V
D-sorbitol	-	+	V	-	-
Trehalose	+	-	+	+	+
ONPG[B]	V	-	+	-	V

[A] + = positive (>90%), - = negative (>90%), V = variable reaction

[B] ONPG = ß-Galactosidase

Source: Blackall et al. (1997)

3. Strain classification

Serovar classification and protective characteristics

Firstly, Page (1962) classified *A. paragallinarum* by plate agglutination test using bacterial whole cells and chicken antisera into serovars A, B and C. Then, hemagglutination inhibition (HI) test has been commonly used for serotyping because of higher efficiency to identify the Page serovar (Blackall et al., 1990). Three Page serovars reveal distinct immunovars since no-cross protection occurs among serovars. The Kume schemes based on hemagglutination-inhibition test firstly recognized into serogroup I, II and III using potassium thiocyanate-treated and-sonicated cells, rabbit hyperimmune serums, and glutaraldehyde-fixed chicken erythrocytes (Kume et al., 1983). They further tested with cross absorption studies to divide subgroup within the Kume serogroups. Then, there are classified to nine serovars including A-1, A-2, A-3, A-4, B-1, C-1, C-2, C-

3, C-4. Later, there have been changed to serogroup A, B and C corresponding the Page schemes as presented in Table 2 (Blackall et al., 1990). The Page schemes have been commonly used than the Kume schemes due to more complexity and technical demand. The definitive study about cross protection within Kume serogroups A and C have not been conducted; however, cross protection within Kume serogroups are still accepted theory. Soriano et al. (2004a) reported serovars A-1, A-2 and A-3 are excellent cross protection while serovars A-1 and A-4 are almost good cross protection. Vaccination with Kume serovars C-1, C-2 and C-3 can provide protection against serovar C-4 challenge.

Table 2 Serotyping of *A. paragallinarum* compared between the original and new nomenclature for the Kume schemes.

Reference	Original scheme (Kume)		New scheme (Blackall)	
isolates	Serogroup	Serovar	Serogroup	Serovar
221	I	HA-1	A	A-1
2043	I	HA-1	A	A-2
E-3C	I	HA-1	A	A-3
HP14	I	HA-1	A	A-4
H-18	II	HA-1	C	C-1
Modesto	II	HA-1	C	C-2
SA-3	II	HA-1	C	C-3
HP60	II	HA-1	C	C-4
2571	III	HA-1	B	B-1

Source: Blackall et al. (1990)

Recently, occurrence of "variant serovar" have challenged a concept of cross protection within the Page serovars and the Kume serogroups. Around 40% of the Page serovar A isolates were not recognized by specific monoclonal antibody, it might expect that these isolates were different from typical serovar A (Terzolo et al., 1993). In addition, an antigenic diversity in Page serovar B showed that bivalent vaccine including serovar A and C can provide protection against only Page serovar B strain Spross but not against two South African isolates of Page serovar B. Also, cross protection within various strains of Page serovar B are only partial (Yamaguchi et al., 1991). Although, there have been some evidences of variant serovars occurring that may lead to vaccine failure, no reports presented definitive examination of vaccination/challenge studies to support these suggestions. Further investigation should be conducted for more information.

Many countries still found the evidences of infectious coryza with distribution of different Page serovars in each area. There have been reported Page serovar A in China (Chen et al., 1993); serovar C in in Taiwan (Lin et al., 1996); serovars A and B in Germany (Hinz, 1973); serovars A and C in Australia (Blackall and Eaves, 1988). Moreover, all of serovars A, B and C have been widely found in Europe, Americas and Asia (Blackall and Soriano, 2008). In Thailand, *A. paragallinarum* including serovars A, B and C have been found in both commercial and native chickens despite of having proper vaccination programs (Chukiatsiri and Chansiripornchai, 2007; Chukiatsiri et al., 2010; Chukiatsiri et al., 2012). The investigation to differentiate the serovar of *A. paragallinarum* isolates can be useful to choose appropriated vaccine in each area around the world.

<u>Molecular techniques</u>

Molecular techniques are an alternative method to be used for subtyping of *A. paragallinarum*. DNA fingerprinting by restriction endonuclease analysis (Blackall et al., 1991), ribotyping (Miflin et al., 1997) and multilocus enzyme electrophoresis (Bowles et al., 1993) have been performed. Another technique which has been studied for subtyping of *A. paragallinarum* was Enterobacterial repetitive intergenic consensus (ERIC) PCR. This method is based on the polymerase chain reaction technique using long sequence primers which hybridize with sufficient affinity to chromosomal DNA sequences at low

annealing temperatures. Because ERIC sequence is a highly conserve region in each species, ERIC-PCR can differentiate among species or strains. It has been conducted in previous studies for typing with other members of the genus *Haemophilus* as *H. Somnus* (Appuhamy et al., 1997), *H. influenzae* (Gomez-De-Leon et al., 2000) and *H. parasuis* (Rafiee et al., 2000). According to Soriano et al. (2004c), nine Kume serovars reference strains of *A. paragallinarum* showed different ERIC patterns, but it cannot be used for serotyping new field isolates because the patterns are not specific to serovar. In addition, a hemagglutinin of *A. paragallinarum*, HagA, was identified and sequenced by Hobb et al. (2002). They expected that there should have a high variation in the amino acid sequence of HagA since hemagglutinin protein is the main antigen for serotyping. In fact, a study of Hobb et al. (2002) showed a little variation in the amino acid sequence of 11 reference strains. Later, genetic differences of the hemagglutinin gene between serovars A and C have been investigated (Amal et al., 2012). Then, Sakamoto et al. (2012a) developed a multiplex PCR and PCR-RFLP method for serotyping of *A. paragallinarum* which used HMTp210 gene, an outer membrane protein, is a major protective antigen of *A. paragallinarum*. Based on the results of Sakamoto et al. (2012a)' s study, the newly developed PCR and PCR-RFLP methods provided high accuracy for rapid serotyping of *A. paragallinarum*. However, another study of multiplex PCR was showed poor performance with both reference strains as well as the field isolates (Morales-Erasto et al., 2014). Until now, there are not any molecular techniques for completely serotyping. Further investigations are need for molecular assay development.

4. Pathogenicity and virulent factor

The variations of pathogenicity depend on many factors including passage history of the isolate and the state of the host. Bacterial cultures of *A. paragallinarum* can be propagated and increased their pathogenicity by bacterial inoculation into five to seven days old chicken embryonated eggs. There have been studies about pathogenicity in varying serotype or strains for many years. The virulence of nine reference strains of Kume serovars investigated by Soriano et al. (2004b) showed that serovar C-1 led to highest clinical signs than C-4 and the strains of serovars A-1, A-4, C-1, C-2 and C-3 showed

higher virulence than serovars A-2, A-3, B-1 and B-4. In addition, another study presented that the NAD-independent isolates may commonly cause airsacculitis than the NAD-dependent *A. paragallinarum* isolates (Horner et al., 1995).

The virulence of bacteria is associated with mechanism for adherence, colonization and infection to host cells. A range of factors has been associated with the pathogenicity of *A. paragallinarum* attending to hemagglutinin (HA), capsule, outer membrane protein and lipopolysaccharide (LPS), iron-acquisition proteins, production of toxins and other factors. As literature review, hemagglutinin is a main structure related to the antigenicity, pathogenicity and immunogenicity (Soriano and Terzolo, 2004). This fact points that the specific hemagglutinin receptors located in both erythrocytes and respiratory epithelial cells, so it can be proved that hemagglutinin is the important adhesion of *A. paragallinarum.* Previous studies have revealed that a non-hemagglutinating variant strains are unable to produce infection and unable to induce immunology (Yamaguchi et al., 1993) and chicken inoculated with fractionated hemagglutinin can protect from *A. paragallinarum* challenge (Iritani et al., 1981). From these examinations indicated that the hemagglutinin of *A. paragallinarum* plays an important role in both pathogenicity and protective immunity.

Another virulent factor is capsule which has been associated with adhesion, colonization and bacterial cell protection from immunity of host such as phagocytosis or the chicken serum bactericidal activity (Sawata et al., 1984). Moreover, it has been suggested that capsule is the important factor for morphological changes inducing lesions (Sawata and Kume, 1983; Sawata et al., 1985). And toxin released from capsular organisms during *in vivo* multiplication was responsible for the clinical disease (Kume et al., 1984).

5. Pathogenesis

A. paragallinarum enters to nasal cavity by mainly intranasal route. The pathogen is not invasive to ciliated mucosa of the upper respiratory tract but adheres to mucosa. The appearance of clinical signs and lesions are occurred by toxic substances releasing during bacterial colonization (Kume et al., 1984). The organisms can migrate into lower

respiratory tract in case of co-infection with other diseases and/or synergism by immunosuppression.

6. Clinical signs

Infectious coryza prominently affects to upper respiratory tract which causes an acute respiratory inflammation characterized with many signs including nasal discharge, facial swelling, lacrimation and conjunctivitis. In severe case shows severe swelling of one or both infraorbital sinuses with edema of the surrounding tissue, which may close one or both eyes. Swollen wattles may be evident, particularly in males. Rales may be heard in birds with infection of the lower respiratory tract. The swelling usually relieves within 10-14 days but swelling can remains with complicated infection for months. Moreover, infected chickens show anorexia, diarrhea, decreased feed and water consumption that decrease growth performance in broiler flocks and reduce egg production in laying flocks (Blackall et al., 1997). Although, the disease causes more impact in mature/older birds which especially found evidence in breeder or layer flocks, there have been reported a swollen head-like syndrome associated with *A. paragallinarum* in broilers in the absence of avian pneumovirus (Droual et al., 1990). In Argentina, outbreaks have been observed unique clinical signs such as arthritis and septicemia in both broiler and layer flocks detected with other pathogens complication (Sandoval et al., 1994). And these cases can be isolated *A. paragallinarum* from other tissues other than respiratory sites such as the liver, kidney and tarsus. In chronic and complicated illness with other bacteria may be found in flocks, and they are still reservoirs and difficult to control the disease.

Infection is usually characterized by low mortality and high morbidity. Complicating factors such as poor environment, inadequate nutrition, complicated with other pathogens making more severity and prolonged disease result in increased mortality (Sandoval et al., 1994).

7. Pathology

A. paragallinarum produces an acute catarrhal inflammation of mucous membranes of nasal passages and sinuses, and frequently causes a catarrhal conjunctivitis and subcutaneous edema of face and wattles (Blackall and Soriano, 2008). In chronic or complicated cases, lesions may become more severe which presents yellowish and consolidated exudate, and can reach to impact lower respiratory tract such as pneumonia and airsacculitis (Soriano and Terzolo, 2004). The histopathologic response of respiratory organs consists of disintegration and hyperplasia of mucosal and glandular epithelia and edema and hyperemia with infiltration of heterophils, macrophages and mast cells.

8. Diagnosis

Isolation and identification of causative agent

The basic information for diagnosis are based on clinical signs, morbidity and mortality, a history of infectious coryza outbreaks in previous flocks or in surrounding areas, vaccination program, etc. Moreover, the isolation of a catalase-negative bacterium showing satellitic growth on blood agar which is cross streaked with *Staphylococcus epidermidis* (Page, 1962) or *S. hyicus* (Blackall and Reid, 1982) or *S. aureus* as a “feeder” and incubated at 37°C in candle jar is common bacterial diagnosis of this disease. Specimens for bacterial cultures should be used swab that is inserted deep into the sinus cavity particularly in infraorbital sinus. Although, opportunity of *A. paragallinarum* migration into lower respiratory tract is rarely found, tracheal and air sac can be swabbed. Isolation of NAD-independent *A. paragallinarum* does not require the addition of a nurse colony or V (NAD) factor. The biochemical identification is an important tool for differentiation among the species of genus *Avibacterium*. However, carbohydrate fermentation tests are sometimes difficult to interpret due to individual strain variation.

The polymerase chain reaction (PCR) that is specific for *A. paragallinarum* has been developed by using HPG-2 primer (Chen et al., 1996). The HPG-2 PCR assay is also specific for both NAD-dependent and NAD-independent *A. paragallinarum*, so it is

beneficial in South Africa where the diagnostic test of infectious coryza disease is confused by presence of NAD-independent isolates (Miflin et al., 1999). This method has been used to identify *A. paragallinarum* from nasal swab samples and confirm the colonies on agar.

Serological detection

Serological monitoring test is potential method to detect immune response against vaccination, evaluate the prevalence of disease (Noormohammadi et al., 2002) and diagnose the disease for separating infected chickens out of a flock to reduce bacterial shedding. At the present, hemagglutination inhibition (HI) test is the best available method for detecting infectious coryza antibody titers (Blackall and Hinz, 2008). Three form of HI assays have been recognized including simple, extracted and treated HI tests. First, the simple HI test by Iritani et al. (1977) is properly basic method to evaluate antibodies titer response only against serovar A from both infected and vaccinated chickens. It is based on whole bacterial cells of Page serovar A of *A. paragallinarum* and fresh chicken erythrocytes. Second, the extracted HI test by Sawata et al. (1982) has been validated mainly for the detection of antibodies to Page serovar C organisms. It is based on KSCN-extracted sonicated of *A. paragallinarum* and glutaraldehyde-fixed chicken erythrocytes. This test is capable for serovar-specific antibodies detection in Page serovar C vaccinated chickens, but antibodies detection of chickens infected with serovar C is not quite appropriate (Yamaguchi et al., 1988). Third, the treated HI test by Yamaguchi et al. (1989) has been used to detect antibodies in chicken vaccinated with Page serovars A, B and C, but higher titers level has been found in only serovars A and C vaccinated chickens (Yamaguchi et al., 1991). The test is based on hyaluronidase-treated whole bacterial cells of *A. paragallinarum* and formaldehyde-fixed chicken erythrocytes. From these methods have been shown different techniques in step of erythrocyte and antigen preparation, may result from the variation of hemagglutinin in each serovar. Sawata et al. (1982) described that there are three possible hemagglutinin, termed HA-HL, a heat labile, trysin resistant hemagglutinin, HA-HS, a heat stable, trypsin resistant hemagglutinin and HA-L, a heat labile, trypsin sensitivity hemagglutinin. As literature reviews, HA-L

antigen located at outer membrane protein and was marked by capsule, whereas HA-HL and HA-HS hemagglutinin was marked by HA-L hemagglutinin. In addition, previous study of Sawata et al. (1982) reported that serotype specificity of hemagglutination was correlated with HA-L hemagglutinin. This report indicated that HA-L is the variant specific hemagglutinin while HA-HL and HA-HS is the common hemagglutinins among bacterial strains. Previously, Sawata et al. (1979) classified *A. paragallinarum* by the agglutination test using specific HA-L hemagglutinin, designated as HA-L1, HA-L2, and HA-L3; Sawata's serotype 1 (Page serovar A) strains contained HA-L1 and HA-L3, and the serotype 2 (Page serovar C) strains had HA-L2 and HA-L3. The variation of a heat labile, trypsin sensitivity hemagglutinin (HA-L) appear to be related with the ability of hemagglutinating activity in each serotype, and there must be prepared for antigen and red blood cells in different conditions. Kume et al. (1980) reported that non-treated (NT) antigen prepared from serotype 1 strains was found to agglutinate freshly collected chicken erythrocytes (RBC) and induce hemagglutination inhibition (HI) antibodies, whereas NT antigen prepared from serotype 2 lacked these properties. Moreover, Sawata et al. (1982) reported that antigens treated with potassium thiocyanate (KSCN) and sonication of serotype 2 had ability to agglutinate with glutaraldehyde-fixed chicken erythrocytes (GA-fixed RBC). In addition, the sample with an HI titer of 1:5 or higher was interpreted as a positive result, because previous studies have shown that vaccinated chickens with a HI ratio of greater or equal to1:5 can protect from *A. paragallinarum* challenge (Sawata et al., 1982). However, the efficacy of HI assay is based on properly used method. And, HI method for testing infectious coryza is still complicated in step of erythrocyte, antigen and serum preparation. Uncertainty of testing results depends on individual skills and method of preparation. The alternative method is monoclonal antibody-based blocking enzyme-linked immunosorbent assay (blocking ELISA) (Miao et al., 2000; Sun et al., 2007) which is highly specific with acceptable sensitivity (Zhang et al., 1999). They reported that the sensitivity of blocking ELISA against infectious coryza for serovar A and serovar C were 78.7% and 64.7%, respectively, and the specificity of blocking ELISA against infectious coryza for serovar A and C were 99.7% and 99.8%,

respectively (Zhang et al., 1999). But, this technique is mainly used in research and not in commercial *in situ* based testing because monoclonal antibodies are not generally available, complicated to prepare and expensive cost. There are only monoclonal antibodies for Page serovar A and C and some isolates of *A. paragallinarum* are not react with monoclonal antibodies, so it is possible that some cases are undetectable by blocking ELISA. As the examples, the problems of some isolates of *A. paragallinarum* serovar A in Brazil and Argentina have been not recognized by monoclonal antibodies (MAb $E_5C_{12}D_{10}$) (Terzolo et al., 1993; Blackall et al., 1994). While, isolates of Kume serovar C-4 in Australia did not react with serovar C monoclonal antibody (Blackall et al., 1990). As above description, there are not a knowledge about correlation between ELISA titer and protection. The limitations of the HI test and blocking ELISA assay means that they are not routinely used for antibody detection after vaccination or infection. So, this study considered using whole *A. paragallinarum* bacterial cells of each respective serovar to separately coat the I-ELISA plates to detect the antibody response, because I-ELISA is easily used, saves time and reduces the required labor due to being compatible with computerized analysis. Although, I-ELISA has already been developed with good efficacy using a subunit of the serovar-specific regions of HMTp210, an outer membrane protein (Sakamoto et al., 2012b), whole bacterial cells may provide a more comprehensive antigenicity than this subunit.

9. Differential diagnosis

Infectious coryza must be differentiated from other diseases, such as chronic respiratory disease, chronic fowl cholera, fowl pox, ornithobacterosis, swollen head syndrome, and hypovitaminosis A, which can produce similar clinical signs (Blackall, 2008). When the disease shows high mortality and takes a prolonged course, other bacteria or virus should be also considered.

10. Treatment

Various antibiotics can be used for infectious coryza treatment such as spectinomycin, streptomycin, danofloxacin, enrofloxacin, flumequine, sulfonamides and trimethoprim (Blackall and Hinz, 2008). Erythromycin and oxytetracycline are also usually beneficial. And several new-generation antibiotics (eg, fluoroquinolones, macrolides) are active against infectious coryza. Although, various antimicrobial drugs are useful for reducing the severity and course of infection, drug resistance in *A. paragallinarum* particularly occurs when treatment is discontinued and the carrier state is not eliminated (Blackall and Soriano, 2008). There have been reported about drug resistant occurrence in many countries (Blackall, 1988; Chukiatsiri and Chansiripornchai, 2007; Hsu etal., 2007). In 2007, around 72% of *A. paragallinarum* isolates contained plasmid pYMH5 which were encoded in functional streptomycin, sulphonamides, kanamycin and neomycin resistance genes (Hsu et al., 2007). *A. paragallinarum* isolated from the outbreak in Thailand have presented resistance to amoxicillin, erythromycin, sulfamethoxazole-trimethoprim and doxycycline (Chukiatsiri and Chansiripornchai, 2007). While Chukiatsiri et al. (2012) reported that a high level of resistance to lincomycin and erythromycin were found, and all isolates were resistant to cloxacillin and neomycin. In addition, Noonkhokhetkong et al. (2013) who studied antimicrobial susceptibility by broth microdilution method also reported a high prevalence of the resistance MIC pattern to oxytetracyclin, doxycycline, streptomycin, ciprofloxacin, erythromycin and sulfamethoxazole-trimethoprim. So, drug sensitivity test should be conducted due to increasing problems of drug resistance.

11. Control

Infectious coryza can be generally controlled by strict biosecurity, good sanitation, avoidance of multiage farms and depopulation. Because infected or recovery birds are reservoir of the disease which need to be eliminated out of flock. In addition, vaccination is another good measure for disease control. Commercial vaccine, typically based on killed *A. paragallinarum* (bacterin), are widely available around the world (Blackall, 1999). Since no-cross protection occur among serovars, the use of more than one serovars in

the vaccines is required. Bivalent vaccine containing 2 strains of Page serovars A and C have been recently used, based on the belief that Page serovar B was not a true serovar and that serovar A and C based vaccines provided cross-protection. Trivalent vaccine containing serovars A, B and C are now available to provide comprehensive protection due to the confirmed existence of Page serovar B as a true serovar with pathogenicity (Jacobs et al., 1992). In addition, tetravalent vaccine is added the strains of serovar variant-B (Jacobs et al., 2003). However, the efficacy of vaccination depends on choosing an appropriate vaccine that contains the serovars/ strains presenting in the target population of each country. The international commercial vaccines are produced upon recognized strains on the standard, so local variation is not sufficient to justify adding or removing strains. According to some researches have suggested that such international vaccines are not providing protection against the local variants of *A. paragallinarum* (Bragg et al., 1996; Terzolo et al., 1997). Vaccination failure is caused by no cross protection between vaccine strain and field strain (Chukiatsiri et al., 2009).

The inactivated vaccine using whole cell bacterial must contain at least 10^8 colony-forming units/ml to be effective. There are several adjuvants used in vaccine preparation such as aluminium hydroxide gel, mineral oil and saponin. An aluminium hydroxide adjuvant vaccine can induce high and rapid antibody titer, thus it is proper for the first immunization (Morein et al., 1996). Whereas, an oil adjuvant vaccine is appropriate for booster because it can slowly release antigen inducing prolonged immune response than the aluminium hydroxide adjuvant vaccine (Morein et al., 1996).

The enzyme-linked immunosorbent assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) is a common laboratory technique which is used to detect and quantify specific antibodies and antigens (proteins, peptides, hormones, etc.) in a sample (Gan and Patel, 2013). The basic principle of ELISA is to use an enzyme to detect the antigen-antibody binding. In some assays the conjugate containing either an antigen or antibody that has been labeled with an enzyme is then added to the plate. The enzyme converts a colorless substrate (chromogen) to a colored product, indicating the presence of antigen-antibody binding.

The color develops in the presence of bound enzyme and the optical density is read with an ELISA plate spectrophotometer. The advantages of the assay are rapid, simple to perform and easily automated. Moreover, this biochemical technique is one of the most sensitive and reproducible technologies available. There are several types of ELISA including direct ELISA, indirect ELISA, sandwich ELISA, competitive ELISA.

Indirect ELISA

Indirect ELISA assay involve attachment of the specific antigen to a solid phase (walls of a microtiter plate), followed by a solution of non-reacting protein such as bovine serum albumin to block any areas of the wells not coated with the antigen. The primary antibody, which binds specifically to the antigen, is then added, followed by a labeled enzyme-conjugated secondary antibody. The addition of an enzyme substrate-chromogen reagent causes color to develop. This color is directly proportional to the amount of bound sample antibody. The more antibody present in the sample, the stronger the color development in the test wells (Gan and Patel, 2013). This format is suitable for determining total specific antibodies level in samples. The advantages of indirect ELISA are high sensitivity, flexibility and cost saving. On the other hand, cross-reactivity may occur with secondary antibody, resulting in non-specific signal.

CHAPTER III
MATERIALS AND METHODS

Part 1: Bacterial culture and identification

1.1 Bacterial culture

1.1.1 *A. paragallinarum* serovars A (221) and C (Modesto) were received from Bacteriology section, National Institute of Animal Health, Bangkok, Thailand, and *A. paragallinarum* serovar B (0222) was received from Queensland Primary Industries and Fisheries, Animal Research Institute, Australia.

1.1.2 The isolates were cultured on blood agar and cross streaked with *Staphylococcus aureus* that is v-factor source for *A. paragallinarum* growth.

1.1.3 Plates were incubated with candle jar at 37°C for 24-48 hours. The bacterial colonies showed satellite growth.

1.1.4 Single colony of *A. paragallinarum* was sub-cultured on chocolate agar plates which were GC agar base (Oxoid, Cambridge, UK) supplemented with 2% w/v soluble hemoglobin powder and vitox as a feeder to increase growth. All incubations with candle jar were at 37°C for 24- 48 hours.

1.1.5 The *A. paragallinarum* colonies on GC agar plate were picked into supplemented test medium (TMB broth) (Blackall and Reid, 1982) and incubated for 18 hours in 37° C shaking incubator to obtain higher quantity of pathogens for antigen preparation process.

1.2 Increasing the pathogenicity of *A. paragallinarum*

1.2.1 *A. paragallinarum* were incubated into the yolk sac of 5-to-7-day-old chicken embryonic egg to propagate and enhance pathogenicity of pathogen.

1.2.2 The infected eggs which were incubated overnight showed hemorrhagic dead embryos and increasing large amounts of bacterium in egg yolk.

1.2.3 The infected yolks were harvested and preserved in -80°C freezer or kept as lyophilized (Blackall, 2008) and can be cultured in agar or broth.

1.3 Morphological and biochemical characterization

Results of Morphological and biochemical test showed in Table 3

Table 3 Morphological and biochemical properties of *A. paragallinarum*

Test	Result	Test	Result
Colony hemolysis	-	CO_2 requirement	+
Morphology	gram negative rod	X-factor requirement	-
Nitrate reduced	+	V-factor requirement	+
Indole	-	Delta-ALA utilization	+
Catalase	-		

Source: Chukiatsiri and Chansiripornchai (2007)

1.3.1 The characteristic of bacterium showed non-hemolysis and satellite colonies that require v-factor from *S. aureus* for growth.

1.3.2 The bacterium was characterized by Gram's staining that consisted of 4 basic steps after fixing bacterial culture smear as (1) applying crystal violet for 1 minutes to a heat-fixed smear of a bacterial culture and rinse out (2) adding iodide, which binds to crystal violet and traps it in the cell for 1 minutes and rinse out (3) decolorizing with 95% alcohol solution for 5-10 seconds and rinse out. (4) counterstaining with safranin for 30 seconds and rinse out. Bacterial cells were differentiated and characterized by light microscopy.

1.3.3 The bacterial colonies were tested with catalase test to isolate *A. paragallinarum* from other members of the genera *Avibacterium*. Colonies were put into the glass slide, and hydrogen peroxide was dropped on these colonies. The results showed that *A. paragallinarum* is catalase-negative whereas others are catalase-positive.

1.3.4 *A. paragallinarum* showed porphyrin test-positive. Gamma-aminolevulinic acid hydrochloride (ALA) 0.5 ml were prepared in microcentrifuge tube, then bacterial colonies from GC agar were scraped into 1.5 ml microcentrifuge tube and incubated at 37°C for 24 hours. Color after dropping 1-2 drop of Kovac's reagent was observed. If the color changed into pink, it can interpret as porphyrin test-positive.

1.4 Molecular characterization

The PCR amplification step using primer HPG-2 as followed previous study with some modification was conducted (Chen et al., 1996).

1.4.1 Growing *A. paragallinarum* on GC agar was scraped by using 1 µl disposable loop into 200 µl sterile PBS (phosphate buffered saline) in a microcentrifuge tube. The sample was vortexed and heated on water bath at 98 °C for 5 minutes. After that, the sample was centrifuged in a benchtop microfuge at 17,000xg for 5 minutes. The DNA template was in supernatant that prepared for experiments. The positive control which is commercial vaccines were extracted by DNA TRAP kit.

1.4.2 DNA extraction of positive control sample was conducted as according to the protocol of DNA Technology Laboratory, Kasetsart University Kamphaengsaen.

a) Briefly, 0.5 µl of commercial vaccine was loaded into 1.5 ml microcentrifuge tube, then added 800 µl of extraction buffer II, mixed and incubated at 65 °C for 1 hour.

b) The suspension was centrifuged in a benchtop microfuge at 17,000xg for 10 minutes.

c) The supernatant was transferred to tube, and then added 400 µl of trapping buffer, mixed and centrifuged at 17,000xg for 10 seconds.

d) The supernatant was removed and 500 µl of washing buffer I was added, mixed and centrifuged at 17,000xg for 10 seconds.

e) The supernatant was removed and 500 µl of washing buffer II was added, mixed and centrifuged at 17,000xg for 10 seconds.

f) The supernatant was removed and 100 µl of elution buffer was added, mixed, and incubated at 65 °C for 30 minutes for separating DNA.

g) After that, the suspension was centrifuged at 17,000xg for 10 seconds.

h) Finally, the DNA template was in supernatant.

1.4.3 The species-specific oligonucleotide primers for HPG-2 PCR (Chen et al., 1996) (N1 5' TGA GGG TAG TCT TGC ACG CGA AT 3'; R1 5' CAA GGT ATC GAT CGT CTC TCT ACT 3') were used to identify *A. paragallinarum* that provided the PCR product size approximately 500 base pairs.

1.4.4 Each 25 µl reaction mix was contained PCR master mix 10 µl (Gotaq® green master mix; Promega, WI, USA), N1 primer (10 pmol/ µl) 2 µl, R1 primer (10 pmol/ µl) 2 µl, H_20 10 µl and DNA template1 µl. The nuclease free water and commercial vaccines were used as negative control and positive control, respectively.

1.4.5 PCR reaction was performed by using MJ Mini Thermal Cycler (Biorad, USA). The PCR product was heated in pre-denature step at 98°C for 2 min 30 sec and 25 cycles of denature step 94 °C for 1 min, annealing step 65 °C for 1 min and extension step 72°C 2 min. The last step was final extension 72 C 10 min. The amplified PCR products, mixing 5 µl PCR product with 2 µl loading buffer were analyzed by gel electrophoresis in 1.4% agarose gel and using 0.5 X TBE buffer to be a running buffer in Wide Mini-Sub® Cel GT Horizontal Electrophoresis (Biorad, USA)at 100 volt for 40 min. Then the gel was stained by 1% ethidium bromide for 20 min and visualized under UV light with Gel documentation.

Part 2: Bacterial growth study

The *A. paragallinarum* colonies on GC agar plate were picked into supplemented test medium (TMB broth) and incubated in 37°C shaking incubator for 24 hours. One hundred microliters of bacterial suspension was sub-cultured into 10 ml of TMB broth for bacterial growth experiment. Bacterial cell count was conducted in triplicate at 3, 6, 9, 12, 15, 18, 24, 30, 36 and 48 hours of incubation periods. Then, number of bacterial cells at various times were plotted into a graph for growth curve analysis of *A. paragallinarum*. Bacterial growth study was conducted to find proper bacterial harvesting time.

Part 3: Antigen preparation

3.1 Antigen preparation

Antigen for coating plate of developed I-ELISA and using in HI test was prepared as same as previously described method (Sun et al., 2007) with some modification.

3.1.1 The bacterial cells were cultured in GC agar base, harvested bacterium in a centrifuge tube and washed for three times in sterile PBS. The cells were centrifuged for washing at 1,400 x g for 30 min.

3.1.2 The antigens were treated with potassium thiocyanate-saline solution (0.5M KSCN+0.425M NaCl; pH 6.3) and stirred at 4˚C for 2 hours.

3.1.3 The bacterial suspension was sonicated for 4 min 4 sec (program: pulse on 40 off 10, Ampl 40%). The sonicated bacterial cells were harvested, washed for 3 times in PBS and resuspended in PBS containing 0.01% (v/v) thimerosal.

3.1.4 Lastly, the bacteria were tested by hemagglutination before stock storage, and the antigens were kept at 4 ˚C for 4 days before testing.

3.2 Protein concentration measurement

The bacterial suspensions preparing for I-ELISA were calculated for antigen protein concentration by using Qubit® Protein Assay Kits (Qubit® 2.0 Fluorometer, Invitrogen, USA).

3.2.1 The Qubit® working solution was made by diluting the Qubit® protein reagent 1:200 in Qubit® protein buffer.

3.2.2 190 µl of Qubit® working solution was loaded into each of the tubes used for standards.

3.2.3 10 µl of each Qubit® standard was added to appropriate tube, then mixed by vortex 2-3 seconds. Be careful not to create bubbles.

3.2.4 Three standard reagents were calibrated before sample examination.

3.2.5 Qubit® working solution was loaded into individual assay tubes so that the final volume in each tube after adding sample was 200 µl.

3.2.6 Each samples were added to the assay tubes containing the correct volume of Qubit® working solution, then mixed by vortex 2-3 seconds. The final volume in each tube should be 200 µl.

3.2.7 All tubes were allowed to incubate at room temperature for 15 min.

3.2.8 On the Home Screen of the Qubit® 2.0 Fluorometer, pressed Protein. The Standards Screen was displayed and selected to run a new calibration or to use the last calibration.

3.2.9 For running a new calibration, then inserted Standard#1, Standard#2 and Standard#3, respectively in the Qubit®2.0 Fluorometer.

3.2.10 The Sample Screen was displayed, then inserted a sample tube into the Qubit®2.0 Fluorometer.

3.3 Protein analysis and determination of molecular mass SDS-Polyacrylamide Gel Electrophoresis (PAGE)

The SDS- Polyacrylamide Gel Electrophoresis (PAGE) was conducted to determine protein molecular weights and recheck purity of protein antigen according to their electrophoretic mobility. The protocol was slightly modified from Laemmli (1970).

3.3.1 SDS-PAGE was performed by loading approximately 100 µg of protein in 4% stacking gel and 12% separating gel according to Laemmli (1970)'s protocol. The composition of gel showed in Table 4 and the gel mixer were prepared, mixed and poured in a gel caster.

3.3.2 When the separating gel polymerized, prepared the stacking gel mixture and poured it to the top. The comb was inserted to make wells for loading protein. The comb was removed when the gel polymerized.

Table 4 Composition of separating gel and stacking gel

Separating gel (12% gel)

Solution	Volume (ml)
Distilled water	2.83
40% Acrylamide mix (Bio-rad, PA, USA)	2.912
1.5M Tris (pH 8.8)	2.5
10% SDS	0.10
10% Ammonium persulphate	0.05
TEMED (Bio-rad, PA, USA)	0.005

Stacking gel (4% gel)

Solution	Volume (ml)
Distilled water	2.92
40% Acrylamide mix (Bio-rad, PA, USA)	0.48
2% Bis-Acrylamide	0.26
0.5M Tris (pH 8.8)	1.26
10% SDS	0.05
10% Ammonium persulphate	0.025
TEMED (Bio-rad, PA, USA)	0.005

3.3.3 The loading samples were prepared by mixing 10 µl of sample with 10 µl of loading buffer and heated on AccuBlock™ Digital Dry Bath (Labnet International, USA) at 95°C for 10 min. After that the standard marker (Precision Plus Protein™ Standard, Biorad, USA) was loaded 5 µl to the first well and loading samples were loaded 10 µl of to the next well also.

3.3.4 Electrophoresis was performed at a constant voltage (200 V) for 45 min. The gel was stained with 0.25% Coomassie Brillant Blue for 1 hour and destained until protein band appearance.

Part 4: Animals in experimental study

One hundred female- layer chickens (Babcock 308) were obtained from a commercial hatchery (Kerd Charoen, Chachoengsao, Thailand). The chickens were raised in the animal experimental facility, Faculty of Veterinary Science, Nakorn Pathom, Thailand until 12 weeks of age. The guidelines and legislative regulations of Chulalongkorn University, Bangkok, Thailand, on the use of animals for scientific purposes were followed as certified in permission no. 1431025. Feed and water was provided ad lib. At 13 weeks old, all chickens were randomly divided into five groups of 20 each to produce a positive sera control for a commercial trivalent mineral oil vaccine (Coryza Oil-3®, Zoetis, Animal Health, Campinas), prepared bacterins of *A. paragallinarum* serovars A, B and C, and a negative control, respectively (Table 5). Before vaccination, the HI test was performed on all the chickens to confirm their *A. paragallinarum*-free status.

Table 5 Immunization design

Groups		Immunization design
1	Positive serum control	commercial trivalent mineral oil vaccine (Coryza Oil-3®, Zoetis, Animal Health, Campinas)
2	Positive serum control	Prepared bacterin Serovar A (221)
3	Positive serum control	Prepared bacterin Serovar B (0222)
4	Positive serum control	Prepared bacterin Serovar C (Modesto)
5	Negative serum control	PBS

Part 5: Immunization design and vaccine preparation

Positive control sera in Group 1 was obtained from immunized chickens with Coryza Oil-3® that contained *A. paragallinarum* at 10^8 colony forming units (CFU)/ml, while the positive control sera in Groups 2, 3 and 4 was obtained from chickens immunized with prepared bacterins of *A. paragallinarum* serovar A (221), B (0222) and C (Modesto), respectively. Each strain was grown for 18 hours in supplemented test medium broth (TMB) collected by centrifugation at 1,400 x g, 30 min using a high speed centrifuge (Eppendorf, Germany) and then washed three times in pH 7.2 phosphate-buffered saline (PBS). The bacterial cells were rechecked for contamination and resuspended to a final concentration of 10^9 CFU/ml. The number of bacteria was ascertained by bacterial cell counts. The bacterial cells were then inactivated with 0.2% (w/v) formalin for 48 h and prepared with Freud's complete adjuvant for the first immunization and Freud's incomplete adjuvant for the second immunizations (antigen: adjuvant (w/w) ratio of 1:1). The vaccines were given as a 0.5-ml intramuscular breast injection at two weeks apart. All vaccinated birds in each group were separated in each experimental room. Chickens in Group 5 were assigned as the negative control and immunized with PBS. Blood samples were collected at 0, 2, 4, 6 and 8 weeks after the first vaccination. Forty serum samples taken before vaccination (at 13 weeks old) presented no antibody response against *A. paragallinarum* by the HI test and were used to determine the cut-off value of the developed ELISA kit.

Part 6: The development of I-ELISA

6.1 Establishment and optimization of I-ELISA

The checkerboard titration of I-ELISA was performed by adjustment of antigen concentration level for coating plate, serum dilution and conjugate concentration (Goat anti-chicken IgG (H+L) horseradish peroxidase, KPL, USA)

6.1.1 The coating antigen of each serovar was serially diluted by two fold (from 25 to 0.78 µg/ml).

6.1.2 The positive and negative sera were optimized by two-fold dilution from 1:50 to 1:800 in same plate but separated serovars.

6.1.3 The optimal concentration of antigen and serum dilution were considered by the highest positive to negative ratio values (using with 1:100 of fixed conjugate concentration).

6.1.4 Then the optimal conjugate concentration was chosen from 1:500, 1:1000 and 1:2000 in accordance with manufacturing recommendation.

6.1.5 All samples were tested in duplicate and measured at wavelength OD_{405}. The highest positive to negative ratio values was considered optimal.

6.2 Validation of the method

The repeatability, sensitivity and specificity tests were used to validate a new method that can be equivalently effective for testing samples compared with commonly used method.

6.2.1 The 40 negative control sera were used to evaluate cut-off value of developed I-ELISA against each serovar of *A. paragallinarum* under optimal conditions. These cut-off values were calculated following the mean of the total negative OD value plus three standard deviation (SDs) (Shen et al., 2015). The results were considered as positive for the serum sample when OD value was higher than cut-off.

6.2.2 The repeatability of I-ELISA against each serovar of *A. paragallinarum* was evaluated by using coated antigens from same and different batches to test with positive

(n=3) and negative (n=3) serum samples. Coefficient of variation (CVs) were considered from testing with the intra-assay and inter assay three times.

6.2.3 The specificity evaluation was performed by antigenic cross reactivity test using reference positive control serum against infectious bronchitis virus (IBV), infectious bursal disease virus (IBDV), *Mycoplasma gallisepticum* (MG), *Mycoplasma synoviae* (MS) and *Pasturella multocida* (PM) (Synbiotics Corporation, USA). In addition, antigenic cross reactivity tests were also tested by different serovar of *A. paragallinarum*.

6.2.4 The results from all control serum samples at 4 weeks after the first immunization were used to calculate sensitivity and specificity of newly developed method. The sera from Group 1 were defined as positive to all serovars, while the sera from Groups 2, 3 and 4 were defined as homologous positive to serovar A, B and C, respectively. We defined the sera from Group 5 as negative to all serovars.

6.2.5 The concordance between I-ELISA and HI assays was considered from agreement rate ([true matched positive samples + true matched negative samples/ total samples] x100)

6.3 Developed I-ELISA

6.3.1 A 96-well microplate (SPL Lifescience, Korea) was coated with 100 µl per well of sonicated cells of either serovar A (221), B (0222) or C (Modesto) for detection antibodies against *A. paragallinarum* serovars A, B and C, separately. The antigen was adjusted to approximately 25 µg protein /ml in 0.05M carbonates-bicarbonate, pH 9.4 (Sigma, CA), incubated overnight at 4°C and then washed three times with 300 µl of washing buffer.

6.3.2 The plates were added with 300 µl blocking solution (KPL, USA) for one hour to minimize non-specific interaction and washed three times with washing buffer.

6.3.3 After that, 100 µl of each diluted serum samples were transferred into duplicate well. The plates were incubated for 30 minutes at room temperature.

6.3.4 After washing with washing buffer for three times, 100 µl of HRP-conjugated goat anti-chicken IgG diluted 1000-fold with milk diluent solution (KPL, USA) were added and then incubated for 30 minutes.

6.3.5 Following washing with washing buffer for three times, the 100 µl of ABTS Peroxidase Substrate (KPL, USA) was added to each well and incubated for 7 minutes.

6.3.6 Finally, the stop solution was added and the ODs were measured at 405 nm with ELISA reader (Biotek Instrument, USA).

Part 7: Antibody response detecting by hemagglutination-inhibition (HI) test

All samples were tested by HI method in according to Chukiatsiri et al. (2009) with some modification. The sample with higher or equal 1:5 of HI titer was interpreted as positive result. Because previous study showed that vaccinated chicken with greater or equal 1:5 of HI titer can be protected from *A. paragallinarum* challenge (Sawata et al., 1982).

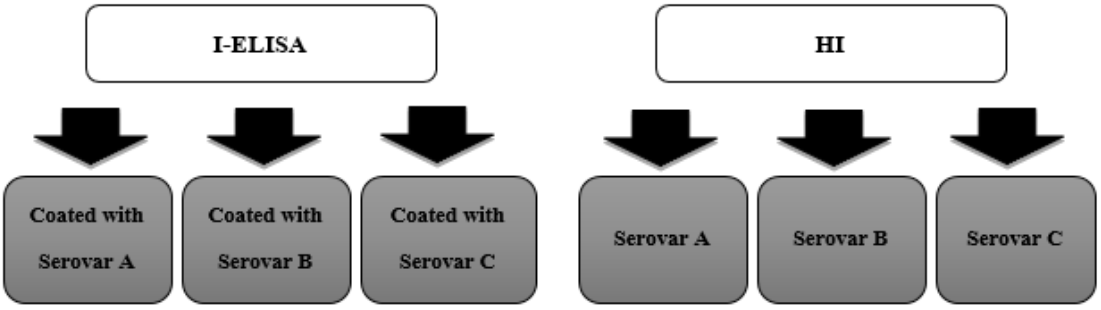
7.1 The sera were absorbed by 10% (V/V) glutaraldehyde (GA)-fixed chicken red blood cells for 2 hours at room temperature. The supernatant of sera had a final dilution of 1:5 that were used to test HI by two-fold serial dilution with BSA-PBS (0.1% bovine serum albumin in PBS) from1:5 to 1:5120. Each diluted serum contain 25 µl of volume.

7.2 The bacterial antigen was treated with KSCN and sonicated were used for HI test. Then, 25 µl of the antigen contained 4 hemagglutinating units was added to each well. The mixture was shaken and incubated at room temperature for 30 minutes.

7.3 Finally, 50 µl of 1% (V/V) glutaraldehyde (GA)-fixed chicken red blood cells was added and the mixture was shaken and incubated for 30 minutes before reading the HI titer. The highest dilution that completely inhibited hemagglutination was identified HI titer.

Part 8: Comparison of developed I-ELISA and HI for detecting antibody against *A. paragallinarum* in field study

Serum samples were received from commercial breeder chickens that were known the vaccination program. All chickens were immunized with trivalent infectious coryza killed vaccine 2 times at 6-8 weeks of age and 14-15 weeks of age. There has no history of infectious coryza outbreaks in this farm before. Total of forty-five sera were collected from nineteen-weeks-old chickens of three houses in a farm. Then, all sera were tested by developed I-ELISA compared with HI test.



CHAPTER IV
RESULTS

Part 1: Bacterial culture and identification

1.1 Bacterial culture

1.1.1 *A. paragallinarum* serovars A (221) and C (Modesto) were received from Bacteriology section, National Institute of Animal Health, Bangkok, Thailand, and *A. paragallinarum* serovar B (0222) was received from Queensland Primary Industries and Fisheries, Animal Research Institute, Australia. Then, the bacteria were recovered on blood agar and cross streaked with *Staphylococcus aureus*. The bacterial cultures with candle jar at 37°C and 5%CO_2 for 24 - 48 hours showed small dewdrop satellite colonies around factor V feeder given from *S. aureus* with no hemolytic characteristics in blood agar. (Figure 1)

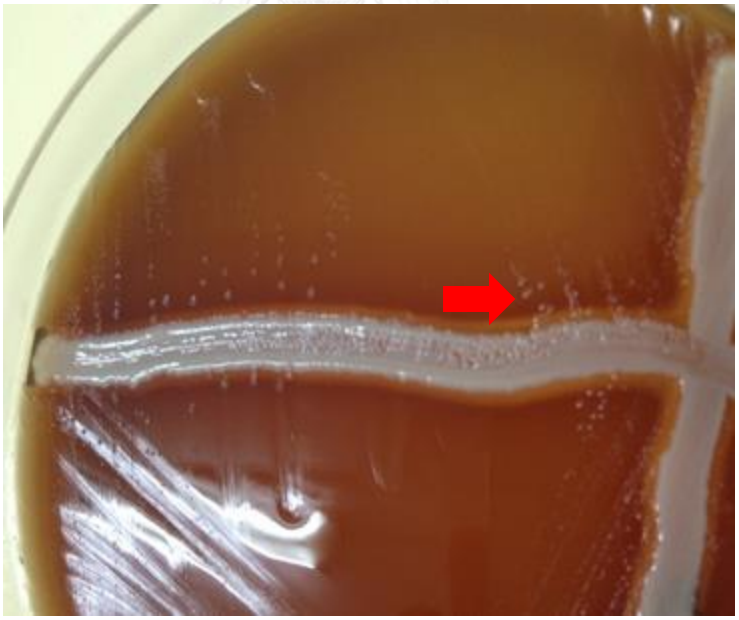


Figure 1 The isolates of *A. paragallinarum* showed satellitic growth on blood agar plate with *S. aureus* streak

1.1.2 After single colony of *A. paragallinarum* was sub-cultured on GC agar base supplemented with 2% w/v soluble hemoglobin powder and vitox as a feeder to increase growth and then incubated in candle jar at 37°C for 24-48 hours, the small dewdrop

colonies with approximately 1-2 mm. of diameter were seen as opaque on GC agar. (Figure 2)

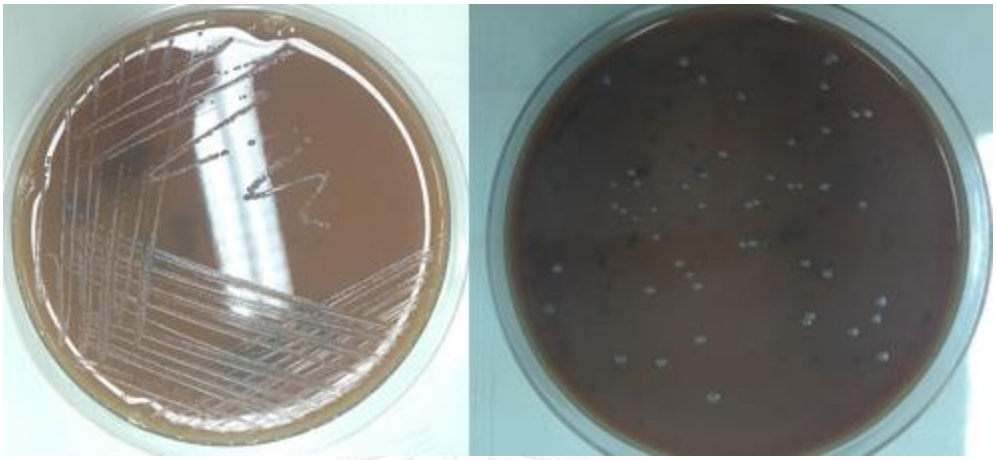


Figure 2 The morphology of *A. paragallinarum* colonies on GC agar base

1.2 Increasing the pathogenicity of *A. paragallinarum*

The infected eggs which were incubated overnight showed hemorrhagic dead embryos (Figure 3). The infected yolk was harvested and preserved in -80˚C freezer and can be cultured in agar or broth. The embryonated eggs can be used for increasing the pathogenicity and increasing large amounts of bacterium in egg yolk.

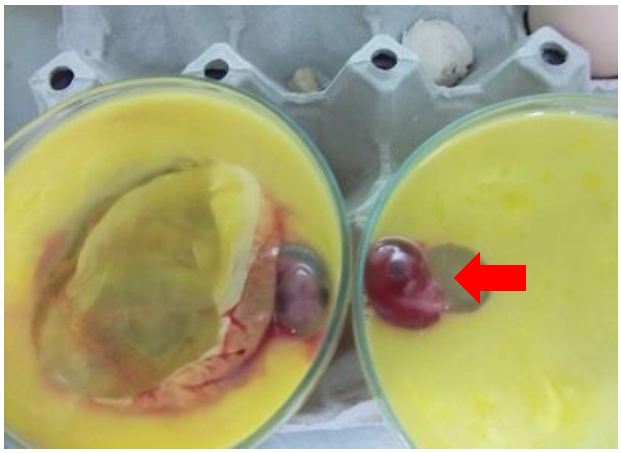


Figure 3 Hemorrhagic dead embryos (right) were compared with normal embryo (left).

1.3 Morphological and biochemical characterization

The bacterium was found Gram negative, rod shape, non-motile on Gram's staining. The results of catalase test showed that *A. paragallinarum* is catalase-negative. *A. paragallinarum* showed porphyrin test-positive. The morphological characterization of *A. paragallinarum* which were older cultured than 24-48 hours could be seen pleomorphism and filamentous shape. The cells were also showed fragment or indefinite forms because of degeneration under culture 48-72 hours complied with Yamamoto (1991). (Figure 4)

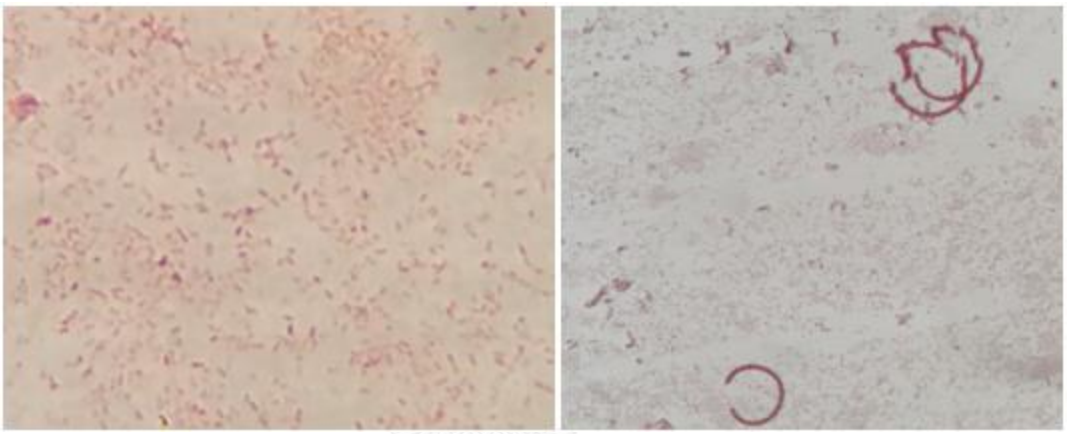


Figure 4 *A. paragallinarum* showing Gram negative, rod shape (left), pleomorphism and filamentous shape with fragments (right). (Gram's stains 100x)

1.4 Molecular characterization

The PCR amplification using primer HPG-2 was used to identify *A. paragallinarum.* The specific PCR product size was approximately 500 base pairs as same as positive control sample (Figure 5)

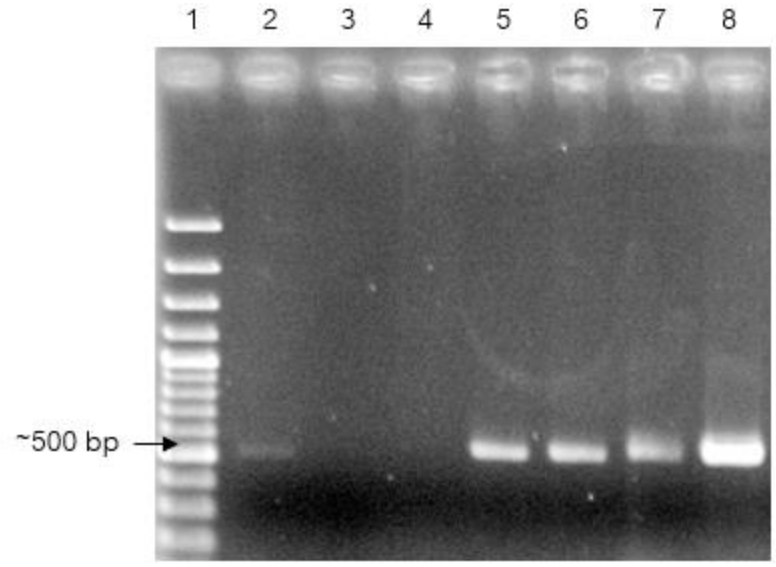


Figure 5 Amplification of HPG-2 for identification of *A. paragallinarum*
Lane1: 100-bp marker, Lane 2: positive control sample (Coryza Oil-3®, Zoetis, Animal Health, Campinas), Lane 3, 4: negative control samples, Lane 5: *A. paragallinarum* serovar A (221), Lane 6: *A. paragallinarum* serovar B (0222), Lane 7:*A. paragallinarum* serovar C (Modesto), Lane 8: positive control sample.

Part 2: Bacterial growth study

The bacterium was cultured in TMB broth for bacterial growth experiment. Bacterial cell count was conducted in triplicate at 3, 6, 9, 12, 15, 18, 24, 30, 36 and 48 hours of incubation periods. The number of bacterial cells were plotted into a graph for growth curve analysis of *A. paragallinarum* (Figure 6). Bacterial cells grew up to 10^7-10^8 CFU/ml at 9 to 48 hours of incubation periods which were the suitable time for harvesting organisms. However, our study harvested bacteria for process of vaccine preparation at 18 hours of incubation period.

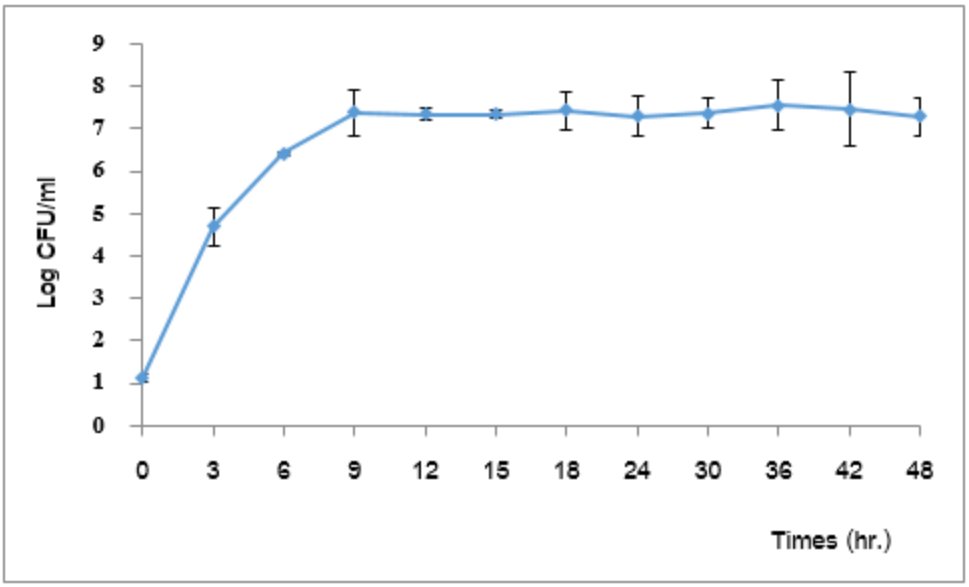


Figure 6 Growth curve of *A. paragallinarum*

Part 3: Antigen preparation

3.1 Antigen preparation

The bacterial cells were cultured in GC agar base, harvested in a centrifuge tube and washed for three times in sterile PBS. Then, antigen for coating plate of developed I-ELISA was prepared as same as previously described method (Sun et al., 2007). In addition, the total amount of bacterium which were tested by hemagglutination were about 2^7-2^9 for stock storage before use.

3.2 Protein concentration measurement

The bacterial suspension preparing from method (3.1) was brought to measure protein concentration by using Qubit® Protein Assay Kits (Figure 7). Calibration method have already done by insertion of Standard#1, Standard#2 and Standard#3, respectively in the Qubit®2.0 Fluorometer. The antigen protein concentration of all serovars was showed in according to Table 6 that were used for preparing developed I-ELISA batch 1, 2 and 3.

Table 6 The antigen protein concentration for preparing developed I-ELISA batch 1, 2 and 3.

serovar	Protein concentration Batch 1 (µg/ml)	Protein concentration Batch 2 (µg/ml)	Protein concentration Batch 3 (µg/ml)
A 221	498	500	475
B 0222	455	500	498
C Modesto	498	495	520

Figure 7 Protein concentration measurement by using Qubit® Protein Assay Kits (Qubit® 2.0 Fluorometer)

3.3 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The bacterial suspensions preparing for I-ELISA were checked protein purity by SDS-PAGE. Protein profiles of three serovars *A paragallinarum* were presented nearly identical pattern with three visible band groups divided between 60-100 kDa, 40-55 kDa and 25-38 kDa that were similar to Amal et al. (2012) (Figure 8).

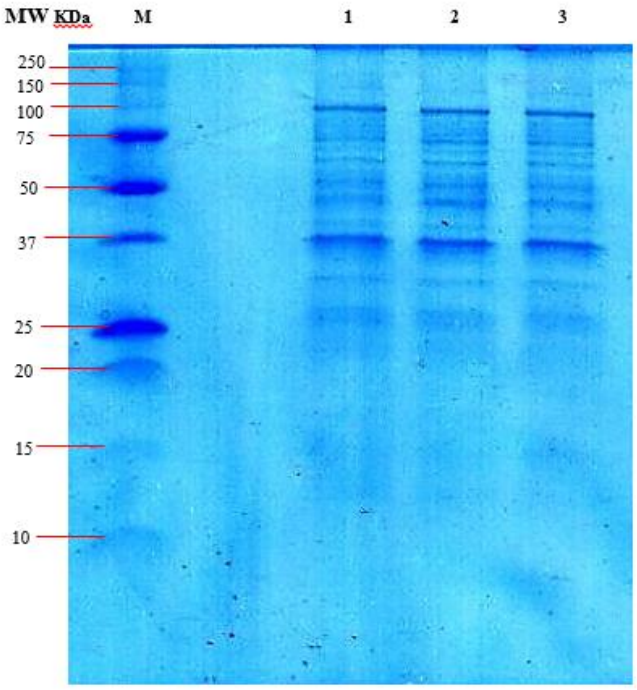


Figure 8 SDS-PAGE analysis of *A. paragallinarum*. Lane 1: *A. paragallinarum* serovar A (221), Lane2: *A. paragallinarum* serovar B (0222), and Lane3: *A. paragallinarum* serovar C (Modesto).

Part 4: Vaccine preparation for immunization

The cells of *A. paragallinarum* were cultured in TMB broth for 18 hours to increase amount of bacterium. Total bacterial cells were counted by Bacterial plate count (Table 7).

Table 7 *A. paragallinarum* serovar A, B and C for preparing inactivated vaccine.

Serovar	Total bacterial cells (cfu/ml)	
	1st vaccine	2nd vaccine
A 221	5.7×10^9	5.9×10^9
B 0222	4.9×10^9	5.0×10^9
C Modesto	4.5×10^9	5.6×10^9

Part 5: Development of I-ELISA

5.1 Establishment and optimization of I-ELISA

The optimal concentration of antigen, goat anti-chicken IgG conjugate and serum of developed I-ELISA serovar A (221) were 25 µg/ml, 1:1000 and 1:800, respectively (Table 8 and 9). In addition, developed I-ELISA serovars B (0222) and C (Modesto) were optimized by using 25 µg/ml of antigen, 1:1000 of goat anti-chicken IgG conjugate and 1:400 of serum dilution (Table 10, 11, 12 and 13). The most different OD values between positive and negative serum samples were considered the optimization.

Table 8 The highest P/N values corresponding to optimal antigen serovar A (221) concentration and serum dilutions (using 1:100 of conjugate dilution)

Dilutions of positive and negative sera	Antigen dilution (µg/ml)					
	25	12.5	6.25	3.125	1.56	0.78
1 : 50	1.118	1.119	1.192	1.227	1.4	1.206
1 : 100	1.584	1.437	1.437	1.469	1.501	1.446
1 : 200	2.323	1.962	1.741	1.56	1.679	1.445
1 : 400	2.899	2.293	1.916	1.605	1.805	1.465
1 : 800	2.898	3.667	2.597	1.786	1.56	1.549

Table 9 The highest P/N values corresponding to optimal antigen serovar A (221) concentration and serum dilutions (using various dilution of conjugate in accordance with manufacture's recommendation)

Dilutions of positive and negative sera	Antigen dilution (µg/ml)								
	25	12.5	6.25	25	12.5	6.25	25	12.5	6.25
P (1:400)	2.475	2.371	2.121	1.812	1.423	1.61	1.426	0.925	0.773
N (1:400)	0.293	0.292	0.278	0.188	0.163	0.183	0.137	0.143	0.157
P/N (1:400)	8.447	8.120	7.629	9.638	8.73	8.798	10.409	6.469	4.924
P (1:800)	2.514	2.280	1.893	2.012	1.298	1.169	1.395	0.925	0.596
N (1:800)	0.247	0.207	0.215	0.138	0.145	0.137	0.113	0.117	0.116
P/N (1:800)	10.178	11.014	8.805	14.58	8.952	8.533	12.35	7.91	5.138
	Conjugate 1:500			Conjugate 1:1000			Conjugate 1:2000		

Table 10 The highest P/N values corresponding to optimal antigen serovar B (0222) concentration and serum dilutions (using 1:100 of conjugate dilution)

Dilutions of positive and negative sera	Antigen dilution (µg/ml)					
	25	12.5	6.25	3.125	1.56	0.78
1 : 50	1.147	1.188	1.296	1.283	1.347	1.238
1 : 100	1.335	1.757	1.775	1.700	1.688	1.616
1 : 200	1.649	2.007	1.594	1.609	1.441	1.337
1 : 400	1.498	2.030	1.673	1.416	1.282	1.335
1 : 800	1.519	1.700	2.078	1.614	1.441	1.335

Table 11 The highest P/N values corresponding to optimal antigen serovar B (0222) and serum dilutions (using various dilution of conjugate with manufacture's recommendation)

Dilutions of positive and negative sera	Antigen dilution (µg/ml)								
	25	12.5	6.25	25	12.5	6.25	25	12.5	6.25
P (1:400)	2.612	2.154	1.671	2.098	1.172	0.879	1.365	0.606	0.535
N (1:400)	0.279	0.275	0.259	0.177	0.178	0.169	0.16	0.155	0.151
P/N (1:400)	9.362	7.833	6.452	11.853	6.584	5.201	8.531	3.910	3.543
P (1:800)	2.528	2.063	1.56	1.857	0.973	0.981	1.27	0.563	0.538
N (1:800)	0.247	0.287	0.252	0.173	0.171	0.161	0.129	0.115	0.123
P/N (1:800)	10.234	7.188	6.190	10.734	5.690	6.093	9.845	4.896	4.374
	Conjugate 1:500			Conjugate 1:1000			Conjugate 1:2000		

Table 12 The highest P/N values corresponding to optimal antigen serovar C (Modesto) concentration and serum dilutions (using 1:100 of conjugate dilution)

Dilutions of positive and negative sera	Antigen dilution (µg/ml)					
	25	12.5	6.25	3.125	1.56	0.78
1 : 50	1.408	1.486	1.503	1.482	1.495	1.437
1 : 100	2.045	2.148	2.163	2.144	2.158	1.966
1 : 200	2.910	2.734	2.411	2.455	2.202	2.110
1 : 400	3.434	2.662	1.981	3.035	1.716	2.212
1 : 800	3.320	2.262	1.729	2.841	1.410	2.037

Table 13 The highest P/N values corresponding to optimal antigen serovar C (Modesto) and serum dilutions (using various dilution of conjugate in accordance with manufacture's recommendation)

Dilutions of positive and negative sera	Antigen dilution (µg/ml)								
	25	12.5	6.25	25	12.5	6.25	25	12.5	6.25
P (1:400)	2.388	1.285	1.045	1.457	0.574	0.63	0.883	0.463	0.440
N (1:400)	0.334	0.394	0.363	0.25	0.202	0.199	0.187	0.176	0.167
P/N (1:400)	7.150	3.261	2.879	5.828	2.842	3.166	4.722	2.631	2.635
P (1:800)	2.107	1.061	0.871	1.25	0.531	0.453	0.763	0.334	0.318
N (1:800)	0.368	0.383	0.358	0.306	0.251	0.216	0.2	0.204	0.218
P/N (1:800)	5.726	2.770	2.433	4.085	2.116	2.097	3.815	1.637	1.459
	Conjugate 1:500			Conjugate 1:1000			Conjugate 1:2000		

Remark: 1:500 of conjugate dilution was not chosen to use because OD values of negative control serum were showed high values.

4.2 Validation of the method

4.2.1 Forty negative control sera were tested duplicate by developed I-ELISA coated serovars A, B and C antigens under the determined optimal conditions. The range of OD values and a mean OD value were 0.111-0.343 and 0.184 against serovar A, 0.174-0.423 and 0.274 against serovar B, 0.213-0.590 and 0.360 against serovar C. The cut-off values of serovars A, B and C calculated by the mean OD value of negative sera plus 3SD were 0.334, 0.484 and 0.678, respectively (Table 14).

Table 14 The OD values of 40 negative control serum samples including OD range values and OD mean values. The cut-off values were calculated by mean OD + 3 (SD)

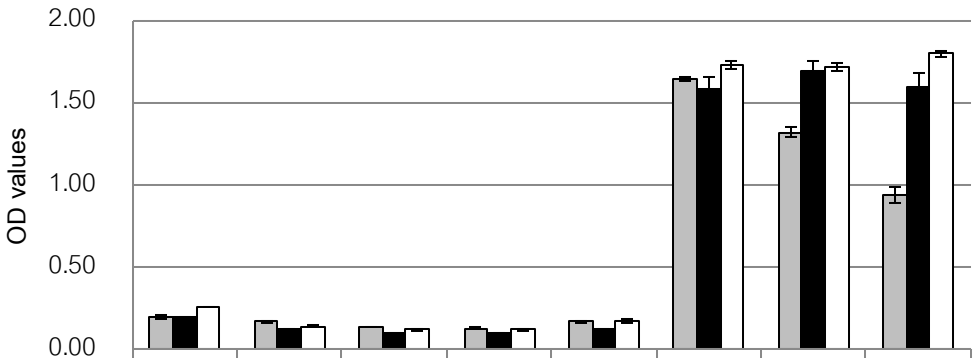
serovar	The range of OD values	The mean OD values	The standard deviation (SD)	Cutoff values
A 221	0.111-0.343	0.184	0.050	0.334
B 0222	0.174-0.423	0.274	0.070	0.484
C Modesto	0.213-0.590	0.360	0.106	0.678

4.2.2 Repeatability of the in-house kit was assessed by determining average CV of the same batch and different batch of antigen. Most of CV values were not higher than 10% when calculated of the same and different batches from both of positive and negative sera (Table 15). There were only two CV values calculated from different batches of serovars B and C that were slightly higher than 10% (12.92% and 11.51%, respectively).

Table 15 The repeatability of the I-ELISA coated A. paragallinarium serovars A, B and C antigens were assessed by determining the average CVs of the same and different batches. (positive samples (n=3) and negative samples (n=3))

I-ELISA		Range of CV values (n=6)	Mean CV value
Same batch	Serovar A	1.57–9.78%	5.45%
	Serovar B	1.11–9.16%	3.67%
	Serovar C	0.14–7.62%	2.85%
Different batch	Serovar A	1.22–8.69%	4.1%
	Serovar B	3.87–12.92%	6.15%
	Serovar C	1.42–11.52%	6.28%

4.2.3 The specificity of the developed I-ELISA test kits was evaluated by testing with reference sera against IBDV, IBV, PM, MG and MS (Synbiotics Corporation, USA). The OD values for the IBDV, IBV, PM, MG and MS-positive reference samples ranged from 0.13 ± 0.004 to 0.20 ± 0.014 for serovar A, 0.10 ± 0.002 to 0.20 ± 0.004 for serovar B and 0.12 ± 0.005 to 0.26 ± 0.002 for serovar C (Table 16 to 18). Thus, all the OD values from the other pathogens were less than the cut-off values for all three serovars, giving a high specificity for *A. paragallinarum* (Figure 9). On the other hand, the OD values of the *A. paragallinarum*-positive samples tested with the different I-ELISA coated serovar antigens were all higher than the cut-off values (Figure 9).



	MG	MS	PM	IBV	IBD	serovar A	serovar B	serovar C
Plate A	0.20	0.17	0.14	0.13	0.17	1.65	1.32	0.94
Plate B	0.20	0.12	0.10	0.10	0.12	1.59	1.70	1.60
Plate C	0.26	0.14	0.12	0.12	0.17	1.73	1.72	1.80

Figure 9 Specificity of the developed I-ELISA coated *A. paragallinarum* serovar A, B and C antigens were tested with positive control serum against IBD, IB, PM, MG and MS, and *A. paragallinarum* serovars A, B and C.

Table 16 The OD values of the MG, MS, PM, IBV and IBD-positive samples tested with I-ELISA coated *A. paragallinarum* serovar A

Control serum	OD values
MG	0.200 ± 0.014
MS	0.167 ± 0.007
PM	0.136 ± 0.003
IBV	0.129 ± 0.004
IBD	0.168 ± 0.008
Serovar A	1.652 ± 0.012
Serovar B	1.318 ± 0.032
Serovar C	0.943 ± 0.049

Table 17 The OD values of the MG, MS, PM, IBV and IBD-positive samples tested with I-ELISA coated *A. paragallinarum* serovar B

Control serum	OD values
MG	0.204 ± 0.004
MS	0.116 ± 0.004
PM	0.098 ±.0.005
IBV	0.096 ± 0.002
IBD	0.124 ± 0.001
Serovar A	1.587 ± 0.073
Serovar B	1.698 ± 0.050
Serovar C	1.597 ± 0.083

Table 18 The OD values of the MG, MS, PM, IBV and IBD-positive samples tested with I-ELISA coated *A. paragallinarum* serovar C

Control serum	OD values
MG	0.261 ± 0.002
MS	0.142 ± 0.004
PM	0.120 ± 0.005
IBV	0.115 ± 0.009
IBD	0.169 ± 0.011
Serovar A	1.732 ± 0.029
Serovar B	1.723 ± 0.023
Serovar C	1.798 ± 0.018

4.2.4 The sensitivity and specificity of new method I-ELISA coated with *A. paragallinarum* antigens were 100% and 36.67% for serovar A, 100% and 31.67% for serovar B and 100% and 31.67% for serovar C. (Table 19, 20, 21). While, agreement rate between I-ELISA and HI assays for serovar A, B and C were 59%, 53% and 59% (Table 22, 23, 24)

Table 19 The sensitivity and specificity of developed I-ELISA coated *A. paragallinarum* serovar A (221) antigen.

In-house ELISA	Control serum		Total
	Positive results	Negative results	
Positive results	40 (a)	38 (b)	78
Negative results	0 (c)	22 (d)	22
Total	40 (a+c)	60 (b+d)	100

%Sensitivity: a / (a+c) x 100 = 100%

%Specificity: d / (b+d) x 100 = 36.67%

%False positive: b / (b+d) x 100 = 63.33%

%False negative: c / (a+c) x 100 = 0%

Table 20 The sensitivity and specificity of developed I-ELISA coated *A. paragallinarum* serovar B (0222) antigen

In-house ELISA	Control serum		Total
	Positive results	Negative results	
Positive results	40 (a)	41 (b)	81
Negative results	0 (c)	19 (d)	19
Total	40 (a+c)	60 (b+d)	100

%Sensitivity: a / (a+c) x 100 = 100%

%Specificity: d / (b+d) x 100 = 31.67%

%False positive: b / (b+d) x 100 = 68.33%

%False negative: c / (a+c) x 100 = 0%

Table 21 The sensitivity and specificity of developed I-ELISA coated *A. paragallinarum* serovar C (Modesto) antigen.

In-house ELISA	Control serum		Total
	Positive results	Negative results	
Positive results	40 (a)	41 (b)	81
Negative results	0 (c)	19 (d)	19
Total	40 (a+c)	60 (b+d)	100

%Sensitivity: a / (a+c) x 100 = 100%

%Specificity: d / (b+d) x 100 = 31.67%

%False positive: b / (b+d) x 100 = 68.33%

%False negative: c / (a+c) x 100 = 0%

Table 22 The agreement rate of developed I-ELISA coated *A. paragallinarum* serovar A (221) antigen compared with HI assay.

In-house ELISA	Hemagglutination inhibition test		Total
	Positive results	Negative results	
Positive results	37 (a)	41 (b)	78
Negative results	0 (c)	22 (d)	22
Total	37 (a+c)	63 (b+d)	100

% agreement: (a+d)/(a+b+c+d) x100 = 59%

Table 23 The agreement rate of developed I-ELISA coated *A. paragallinarum* serovar B (0222) antigen compared with HI assay.

In-house ELISA	Hemagglutination inhibition test		Total
	Positive results	Negative results	
Positive results	34 (a)	47 (b)	81
Negative results	0 (c)	19 (d)	19
Total	34 (a+c)	66 (b+d)	100

% agreement: (a+d)/(a+b+c+d) x100 = 53%

Table 24 The agreement rate of developed I-ELISA coated *A. paragallinarum* serovar C (Modesto) antigen compared with HI assay.

In-house ELISA	Hemagglutination inhibition test		Total
	Positive results	Negative results	
Positive results	40 (a)	41 (b)	81
Negative results	0 (c)	19 (d)	19
Total	40 (a+c)	60 (b+d)	100

% agreement: (a+d)/(a+b+c+d) x100 = 59%

Part 6: Comparison of developed I-ELISA and HI for detecting antibody against *A. paragallinarum* in experimental study

Comparison of antibody response between newly developed I-ELISA and HI assays was shown in Table 25. The results of control sera received from immunized chickens at 2 weeks after the second vaccination revealed that the positive rates for I-ELISA test coated serovars A, B and C antigens were almost 100% of all positive control groups. Antisera to each serovar were positive in heterologous test. On the other hand, the positive rates for HI test were presented only homologous test. The HI tests of serovars A, B and C were positive of 85, 70 and 100%, respectively. However, only one negative control sample was positive (5% of positive rates) to serovars B and C by I-ELISA test. All negative control sera found negative by HI test. In addition, the average OD values results of control sera received from immunized chickens at 2 weeks after the second vaccination in the homologous and heterologous serovar I-ELISA was revealed in Table 26.

Moreover, our study investigated the proportion of positive samples (%) and HI titer examined with the HI test of the *A. paragallinarum* serovar A, B and C of all control serum samples which were collected at 0, 2, 4, 6 and 8 weeks after the first vaccination (Figure 10).

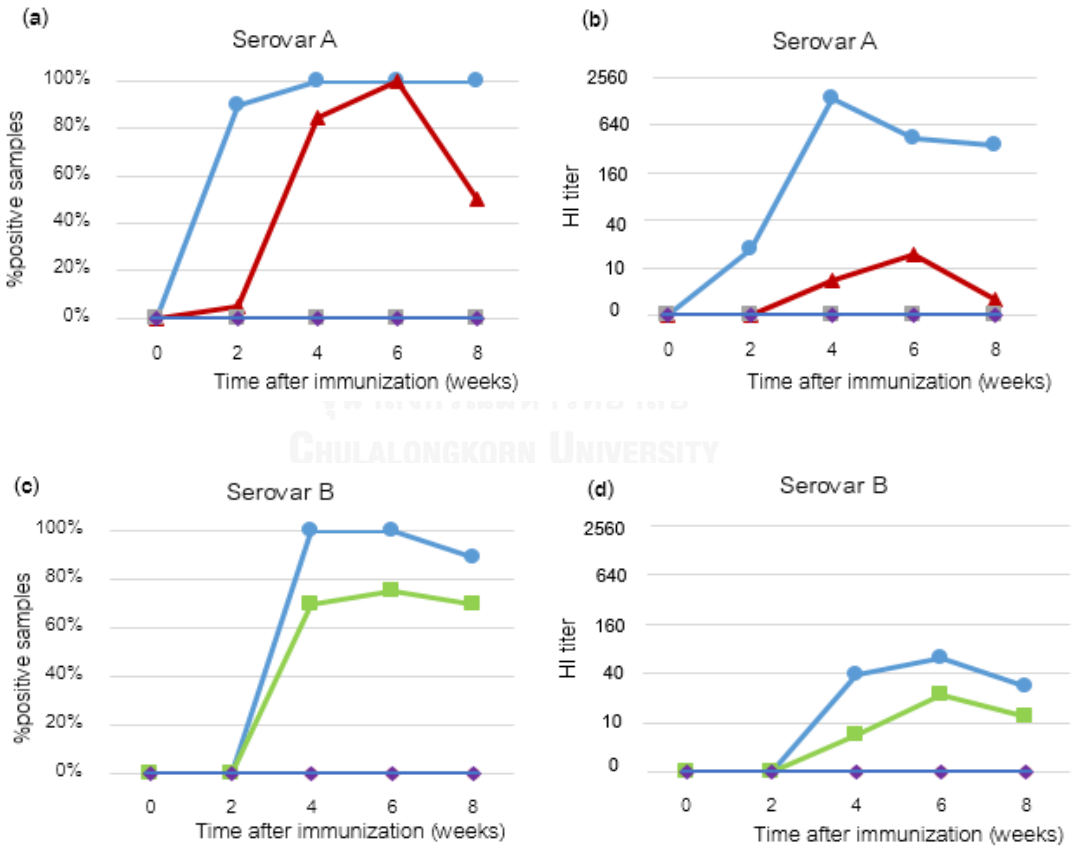
Table 25 Positive rate of samples from positive serum control and negative serum control group when testing with developed I-ELISA and HI test. (number of each group = 20 samples)

	Serum control group				
Method	Coryza Oil-3®	A 221	B 0222	C Modesto	Negative
I-ELISA coated serovar A	100%	100%	100%	90%	0%
HI + serovar A antigen	100%	85%	0%	0%	0%
I-ELISA coated serovar B	100%	100%	100%	100%	5%
HI + serovar B antigen	100%	0%	70%	0%	0%
I-ELISA coated serovar C	100%	100%	100%	100%	5%
HI + serovar C antigen	100%	0%	0%	100%	0%

Table 26 Average OD values in the homologous and heterologous serovar I-ELISA (number of each group = 20 samples)

Serum control group	Antigen coated on the plate		
	Serovar A	Serovar B	Serovar C
Serovar A (221)	1.193 ± 0.156 [a]	1.447 ± 0.158 [a]	1.501 ± 0.115 [a]
Serovar B (0222)	0.702 ± 0.230 [b]	1.615 ± 0.229 [ab]	1.566 ± 0.246 [a]
Serovar C (Modesto)	0.561 ± 0.204 [b]	1.299 ± 0.286 [ac]	1.580 ± 0.210 [a]
Negative	0.128 ± 0.016 [c]	0.289 ± 0.112 [d]	0.408 ± 0.153 [b]

Remark: Data are shown as the mean ± SD, derived from independent repeats. Different superscripts within same column indicate significant differences ($P < 0.05$)



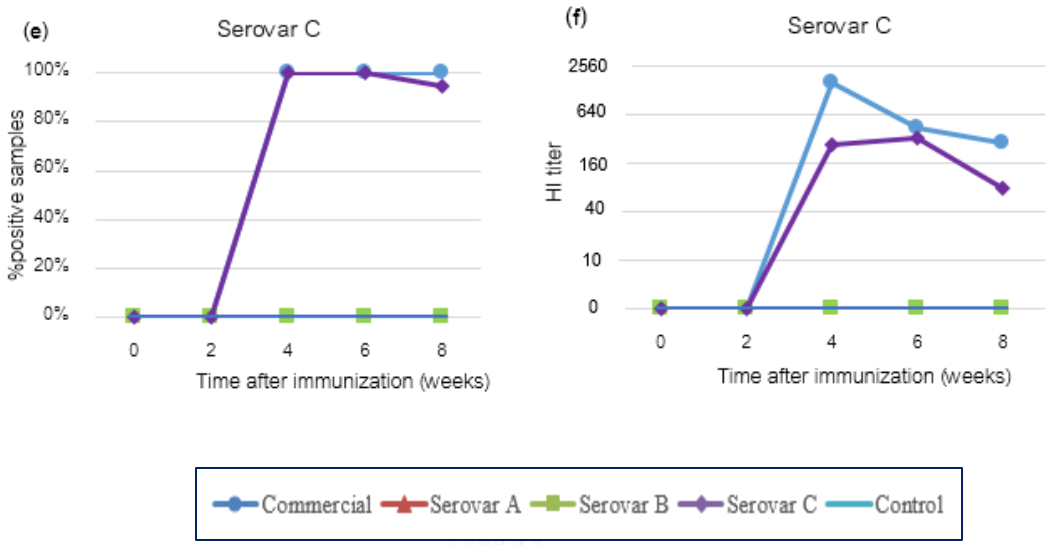


Figure 10 The (a, c, e) proportion of positive samples (%) and (b, d, f) HI titer examined with the HI test of the *A. paragallinarum* serovar (a, b) A, (c, d) B and (e, f) C. Positive and negative control serum samples were divided into the five groups of commercial vaccine 1 (Group 1), prepared bacterin serovar A (Group 2), prepared bacterin serovar B (Group 3), prepared bacterin serovar C (Group 4) and negative control (Group 5).

** Remark: HI titer level are diluted serum from 1/5, 1/10, 1/20, 1/40, 1/80, 1/160, 1/320, 1/640, 1/1280 and 1/2560.

Part 7: Comparison of developed I-ELISA and HI for detecting antibody against *A. paragallinarum* in field sera

The results of antibody response detection against infectiuos coryza disease were tested against commercial breeder chicken sera. The chickens which were immunized following vaccination program with trivalent infectious coryza killed vaccine 2 times. Nineteen-weeks-old chickens were collected serum from three houses in a farm. All forty-five serum samples were tested antibody response by developed I-ELISA compared with HI test. The results showed that all samples were detected 100% of positive when testing by I-ELISA coated with serovars A and B. And 97.78% of positive results were detected against serovar C. However, these samples were detected 91.11%, 17.78% and 55.56% of positive results when testing by HI test serovars A, B and C, respectively. The detail of OD range values and OD mean values of I-ELISA were presented in Table 27.

Table 27 The OD range values and OD mean values of commercial breeder chicken sera which were tested with I-ELISA coated with either serovar A, B and C antigen.

Serovar	Developed I-ELISA			Mean HI titer
	OD range values	OD mean values	Cut-off	
serovar A (221)	0.421-1.723	1.108	0.334	133.67
serovar B (0222)	0.586-1.755	1.296	0.484	2.22
serovar C (Modesto)	0.655-1.750	1.356	0.678	7.89

CHAPTER V
DISCUSSION AND CONCLUSIONS

Serological tests for infectious coryza based upon the HI test and blocking ELISA have been performed for many years (Iritani et al., 1977; Yamaguchi et al., 1988; Zhang et al., 1999). However, these methods still have the limitation of being a complicated method with uncertain interpretation and unavailable commercial monoclonal antibodies. The limitations of the HI test and blocking ELISA assay means that they are not routinely used for antibody detection after vaccination or infection. So, we considered using whole *A. paragallinarum* bacterial cells of each respective serovar to separately coat the I-ELISA plates to detect the antibody response, because I-ELISA is easily used, saves time and reduces the required labor due to being compatible with computerized analysis. Although, I-ELISA has already been developed with good efficacy using a subunit of the serovar-specific regions of HMTp210, an outer membrane protein (Sakamoto et al., 2012b), whole bacterial cells may provide a more comprehensive antigenicity than this subunit. For this study, whole *A. paragallinarum* bacterial cells of each of the three serovars showed a similar protein pattern to each other and accorded to previous report (Amal et al., 2012), and so these bacterial antigen preparations were likely to be suitable for I-ELISA plate coating (no contamination).

From the higher cut-off values for serovars B and C, the I-ELISA showed that reaction with serovars B and C had more variation than serovar A, which is possibly due to nonspecific immune-reactivity. No antigenic cross-reactivity of the antisera against other chicken pathogens (IBDV, IBV, PM, MG and MS) was detected, whereas cross-reactivity of each antiserum to the other two *A. paragallinarum* serovar antigens was seen in all three cases. Thus, these I-ELISA tests are likely to be used in the detection of *A. paragallinarum* infections but not in discriminating between serovars. The average CV of the same and different antigen batches was mostly < 10% and so of a good repeatability. The efficacy of the I-ELISA showed 100% sensitivity in all coating serovars, but the specificity of all serovars of I-ELISA was low (approximately 30%) because of the high

cross-reactivity among the three *A. paragallinarum* serovars. This is in contrast to previous studies on monoclonal antibody based blocking ELISA that reported serovar specificity (Zhang et al., 1999; Sun et al., 2007). The validation tests indicated that the new I-ELISA was highly sensitive to detect an antibody response against *A. paragallinarum* from immunized chickens, both of natural infection and vaccination, compared to *A. paragallinarum*-free chickens. However, the serovar A I-ELISA showed a slightly higher specificity (36.7%) than the other two serovars (31.7%), and gave significantly higher OD values against serovar A than against the other two heterologous serovars ($P<0.05$). In contrast, but the I-ELISA results for B and C did not show any significant difference between the homologous and heterologous serovars (Table 27). It is interesting that the serovar A I-ELISA can distinguish the serovar A antibody response from the other two serovars (B and C). Perhaps the serovar A antigen had a more specific-immunoreactivity than serovars B and C.

We did not compare the I-ELISA method to the HI test in terms of the sensitivity and specificity because the HI test is not the best comparable method since HI negative results do not always equate to no antibody response. For example, chickens inoculated with the ACΔ5-1 fusion peptide had no detectable antibodies in the HI test but were protected from infectious coryza challenge (Sakamoto et al., 2012b; Sakamoto et al., 2013). Indeed, protective immunity was not only induced by the hemagglutinin protein (HA), but also by the outer membrane protein (Tokunaga et al., 2005; Noro et al., 2007; Noro et al., 2008). These reasons account for the agreement rate for the I-ELISA and HI assay of around 60%. Comparison between the I-ELISA and HI assays from serum samples at 2 weeks after the second immunization with the prepared bacterins revealed that the HI test showed a higher serovar specific antibody response than the whole cell I-ELISA due to the genetic differences in the HA gene between serovars A and C (Amal et al., 2012). In addition, other explanations have included the (1) post-translational modifications of the HA protein, (2) variations in the expression or sequence of other proteins, such as the steric hindrance of HA and (3) multiple HA (Kume et al., 1983).

There have been few studies on the HI antibody response against serovar B. This study reported here examined the HI titer response pattern against three serovars of *A. paragallinarum* (Figure 10). The sera in commercial Group 1 showed a similar pattern to that reported before (Yamaguchi et al., 1988), where the serovar A-HI titer was rapidly detected at 2 weeks after the first vaccination. The serovar C-HI titer in Group 1 was detected later than the serovar A-HI titer, in agreement with a previous report (Sun et al., 2007), where the positive serovar C titer was first detected at 3 weeks post-challenge. The serovar B-HI titer pattern in Group 1 was detected at 2 weeks, peaked at 4 weeks after the second immunization and then declined, similar to that previously reported (Yamaguchi et al., 1991), where the HI titer level of serovar B was lower than that of serovars A and C at all times. The HI titers in the prepared bacterins (Groups 2, 3 and 4) showed a serovar specific positive rate of more than 70% and were higher than a 1:5 HI titer at 2 weeks after the second vaccination. From the findings, it can be expected that these prepared bacterins were effective at inducing protection against infectious coryza.

This field study has presented a comparison of developed I-ELISA and HI for detecting antibody against *A. paragallinarum*. Almost 100% positive results detected by I-ELISA against all three serovars indicated that chicken flock in this farm had antibody response against infectious coryza serovars A, B and C because of immunization with trivalent vaccine. There has been no history of infectious coryza outbreaks in this farm before, it is expected that immune response was induced by vaccination. Since developed I-ELISA has high cross reactivity among serovars, the OD values among serovars are not different. The finding suggested that I-ELISA test cannot be used to differentiate between serovar of *A. paragullinarum* which is vaccinated and/or infected in chicken flock. In addition, there is no knowledge about correlation between I-ELISA titer and protection, so the positive results of I-ELISA cannot evaluate the ability of vaccination program in aspect of protection. On the other hand, HI test were shown 91.11%, 17.78% and 55.56% of positive results against serovar A, B and C, respectively. The pattern of immune response complied with previous studies have shown that percentage of positive result and HI titer level in serovar A are always higher than serovars B and C (Yamaguchi

et al., 1991; Fernández et al., 2005). From HI titer finding in our study, we can imply that this flock of chicken is protected from infectious coryza serovar A challenge, whereas it may still have the risk for serovar B and C outbreaks.

In conclusion, the developed I-ELISAs were unable to clearly differentiate among the three serovars of *A. paragallinarum*, but they could be used to distinguish between *A. paragallinarum* -free healthy chickens and immunized chickens which are from vaccination or from infection, and so will be used in pathogen surveillance and monitoring of any immune response against infectious coryza disease. Further studies are required to find the cut-off values for determining the protective antibody response or using a specific subunit protein for coating the ELISA plates to eliminate the non-specific effects among the different serovars in the whole bacterial cell ELISA.

REFERENCES

Amal M, Soliman YA and Shafey SM. 2012. Molecular characterization of *Avibacterium paragallinarum* strains used in evaluation of coryza vaccine in Egypt. J Am Sci. 8(3): 253-263.

Appuhamy S, Parton R, Coote JG and Gibbs HA. 1997. Genomic fingerprinting of *Haemophilus somnus* by a combination of PCR methods. J Clin Microbiol. 35(1): 288-291.

Blackall PJ. 1988. Antimicrobial drug resistance and the occurrence of plasmids in *Haemophilus paragallinarum*. Avian Dis. 32(4): 742-747.

Blackall PJ. 1999. Infectious coryza: overview of the disease and new diagnostic options. Clin Microbiol Rev. 12(4): 627-632.

Blackall PJ. 2008. Laboratory manual for the isolation, identification, and characterization of avian pathogens. In: Infectious coryza. 5th ed. (eds.). Jacksonville: American Association of Avian Pathologists. 6-19.

Blackall PJ and Eaves LE. 1988. Serological classification of Australian and South African isolates of *Haemophilus paragallinarum*. Aus Vet J. 65(11): 362-363.

Blackall PJ and Hinz K-H. 2008. Infectious coryza and related diseases In: Poultry Diseases 6th ed. Pattison M, McMullin PF, Bradbury JM and Alexander DJ (eds.). Edinburgh: W.B. Saunders. 155-159.

Blackall PJ and Reid GG. 1982. Further characterization of *Haemophilus paragallinarum* and *Haemophilus avium*. Vet Microbiol. 7(4): 359-367.

Blackall PJ and Soriano VE. 2008. Infectious coryza and related bacterial infection. In: Disease of Poultry. 12th ed. Saif Y (eds.). US: Wiley-Blackwell. 789-803.

Blackall PJ, Christensen H, Beckenham T, Blackall LL and Bisgaard M. 2005. Reclassification of *Pasteurella gallinarum*,[*Haemophilus*] *paragallinarum*, *Pasteurella avium* and *Pasteurella volantium* as *Avibacterium gallinarum* gen. nov., comb. nov., *Avibacterium paragallinarum* comb. nov., *Avibacterium avium*

comb. nov. and *Avibacterium volantium* comb. nov. Int J Syst Evol Microbiol. 55(1): 353-362.

Blackall PJ, Eaves LE and Morrow CJ. 1991. Comparison of *Haemophilus paragallinarum* isolates by restriction endonuclease analysis of chromosomal DNA. Vet Microbiol. 27(1): 39-47.

Blackall PJ, Eaves LE and Rogers DG. 1990. Proposal of a new serovar and altered nomenclature for *Haemophilus paragallinarum* in the Kume hemagglutinin scheme. J Clin Microbiol. 28(6): 1185-1187.

Blackall PJ, Matsumoto M and Yamamoto R. 1997. Infectious coryza. In: Diseases of poultry. 10th ed. Calnek B, Barnes H, Beard C, Reid W, McDougald L and Saif Y (eds.). Ames: Iowa State University Press. 179-190.

Blackall PJ, Silva EN, Yamaguchi T and Iritani Y. 1994. Characterization of isolates of avian haemophili from Brazil. Avian Dis. 38(2): 269-274.

Bowles R, Blackall PJ, Terzolo H and Sandoval VE. 1993. An assessment of the genetic diversity of Australian and overseas isolates of *Heamophillus paragallinarum* by multilocus enzyme electrophoresis. XII World Poultry Congress, Sydney. 148.

Bragg RR. 2002. Virulence of South African isolates of *Haemophilus paragallinarum*. Part 2: Naturally occurring NAD-independent field isolates. Onderstepoort J Vet Res. 69(2): 171-175.

Bragg RR, Coetzee L and Verschoor JA. 1996. Changes in the incidences of the different serovars of *Haemophilus paragallinarum* in South Africa: a possible explanation for vaccination failures. Onderstepoort J Vet Res. 63(3): 217-226.

Bragg RR, Greyling JM and Verschoor JA. 1997. Isolation and identification of NAD-independent bacteria from chickens with symptoms of infectious coryza. Avian Pathol. 26(3): 595-606.

Byarugaba DK, Minga UM, Gwakisa PS, Katunguka-Rwakishaya E, Bisgaard M and Olsen JE. 2007. Virulence characterization of *Avibacterium paragallinarum* isolates from Uganda. Avian Pathol. 36(1): 35-42.

Chen X, Miflin JK, Zhang P and Blackall PJ. 1996. Development and application of DNA probes and PCR tests for *Haemophilus paragallinarum*. Avian Dis. 40(21): 398-407.

Chen X, Zhang P, Blackall P and Feng W. 1993. Characterization of *Haemophilus paragallinarum* isolates from China. Avian Dis. 37(2): 574-576.

Chukiatsiri K and Chansiripornchai N. 2007. Case report: An outbreak of infectious coryza in a layer farm. J Thai Vet Med Assoc. 58(3): 98-107.

Chukiatsiri K, Chansiripornchai N and Chotinun S. 2010. An outbreak of *Avibacterium paragallinarum* serovar B in a Thai layer farm. Thai J. Vet. Med. 40(4): 441-444.

Chukiatsiri K, Sasipreeyajan J, Blackall PJ, Yuwatanichsampan S and Chansiripornchai N. 2012. Serovar identification, antimicrobial sensitivity, and virulence of *Avibacterium paragallinarum* isolated from chickens in Thailand. Avian Dis. 56(2): 359-364.

Chukiatsiri K, Sasipreeyajan J, Neramitmansuk W and Chansiripornchai N. 2009. Efficacy of autogenous killed vaccine of *Avibacterium paragallinarum*. Avian Dis. 53(3): 382-386.

Droual R, Bickford AA, Charlton BR, Cooper GL and Channing SE. 1990. Infectious coryza in meat chickens in the San Joaquin Valley of California. Avian Dis. 34(4): 1009-1016.

Fernández R, Colindres H, Velásquez Q, Soriano V and Blackall P. 2005. Protection conferred by bivalent and trivalent infectious coryza bacterins against pre valent serovars of *Avibacterium* (*Haemophilus*) *paragallinarum* in Mexico. Avian Dis. 49(4): 585-587.

Gan SD and Patel KR. 2013. Enzyme immunoassay and enzyme-linked immunosorbent assay. J Invest Dermatol. 133(9): e12.

Garcia AJ, Angulo E, Blackall PJ and Ortiz AM. 2004. The presence of nicotinamide adenine dinucleotide-independent *Haemophilus paragallinarum* in Mexico. Avian Dis. 48(2): 425-429.

Gomez-De-Leon P, Santos JI, Caballero J, Gomez D, Espinosa LE, Moreno I, Piñero D and Cravioto A. 2000. Genomic variability of *Haemophilus influenzae* isolated from Mexican children determined by using enterobacterial repetitive intergenic consensus sequences and PCR. J Clin Microbiol. 38(7): 2504-2511.

Hinz K-H. 1973. Report on the differentiation of Haemophillus isolates from chickens.I. Note: cultural and biochemical studies. Avian pathol. Journal of the WVPA. 2(3): 211-229.

Hobb RI, Tseng HJ, Downes JE, Terry TD, Blackall PJ, Takagi M and Jennings MP. 2002. Molecular analysis of a haemagglutinin of *Haemophilus paragallinarum*. Microbiology. 148(Pt 7): 2171-2179.

Horner RF, Bishop GC, Jarvis CJ and Coetzer THT. 1995. NAD (V-factor)-independent and typical *Haemophilus paragallinarum* infection in commercial chickens: A five year field study. Avian Pathol. 24(3): 453-463.

Hsu Y-M, Shieh HK, Chen W-H, Sun T-Y and Shiang J-H. 2007. Antimicrobial susceptibility, plasmid profiles and haemocin activities of *Avibacterium paragallinarum* strains. Vet Microbiol. 124(3): 209-218.

Iritani Y, Iwaki S, Yamaguchi T and Sueishi T. 1981. Determination of types 1 and 2 hemagglutinins in serotypes of *Hemophilus paragallinarum*. Avian Dis. 25(2): 479-483.

Iritani Y, Sugimori G and Katagiri K. 1977. Serologic response to *Haemophilus gallinarum* in artificially infected and vaccinated chickens. Avian Dis. 21(1): 1-8.

Jacobs AAC, Cuenen W and Storm PK. 1992. Efficacy of a trivalent *Haemophilus paragallinarum* vaccine compared to bivalent vaccines. Vet Microbiol. 32(1): 43-49.

Jacobs AAC, van den Berg K and Malo A. 2003. Efficacy of a new tetravalent coryza vaccine against emerging variant type B strains. Avian Pathol. 32(3): 265-269.

Kume K, Sawata A and Nakai T. 1983. Serologic and immunologic studies on three types of hemagglutinin of *Haemophilus paragallinarum* serotype 1 organisms. Jap J Vet Sci. 45(6): 783-792.

Kume K, Sawata A and Nakai T. 1984. Clearance of the challenge organisms from the upper respiratory tract of chickens injected with an inactivated *Haemophilus paragallinarum* vaccine. Jap J Vet Sci. 46(6): 843-850.

Kume K, Sawata A and Nakase Y. 1980. Immunologic relationship between Page's and Sawata's serotype strains of *Haemophilus paragallinarum*. Am J Vet Res. 41(5): 757-760.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. nature. 227: 680-685.

Lin J, Shyu C, Yamaguchi T and Takagi M. 1996. Characterization and pathogenicity of *Haemophilus paragallinarum* serotype C in local chickens of Taiwan. J Vet Med Sci. 58(10): 1007-1009.

Miao D, Zhang P, Gong Y, Yamaguchi T, Iritani Y and Blackall P. 2000. The development and application of a blocking ELISA kit for the diagnosis of infectious coryza. Avian Pathol. 29(3): 219-225.

Miflin JK, Chen X and Blackall PJ. 1997. Molecular characterization of isolates of *Haemophilus paragallinarum* from China by ribotyping. Avian Pathol. 26(1): 119-127.

Miflin J, Chen X, Bragg RR, Welgemoed J, Greyling J, Horner R and Blackall P. 1999. Confirmation that PCR can be used to identify NAD-dependent and NAD-independent *Haemophilus paragallinarum* isolates. Onderstepoort J Vet Res. 66(1): 55-57.

Morales-Erasto V, de Jesús Posadas- Quintana J, Fernández-Díaz M, Saravia LE, Martínez-Castañeda JS, Blackall PJ and Soriano-Vargas E. 2014. An evaluation of serotyping of *Avibacterium paragallinarum* by use of a multiplex polymerase chain reaction. J Vet Diagn Invest. 26(2): 272-276.

Morein B, Villacrés-Eriksson M, Sjölander A and Bengtsson KL. 1996. Novel adjuvants and vaccine delivery systems. Vet Immunol Immunopathol. 54(1): 373-384.

Mouahid M, Bisgaard M, Morley AJ, Mutters R and Mannheim W. 1992. Occurrence of V-factor (NAD) independent strains of *Haemophilus paragallinarum*. Vet Microbiol. 31(4): 363-368.

Mouahid M, Bouzoubaa K and Zouagui Z. 1989. Chicken infectious coryza in Morocco: epidemiological study and pathogenicity trials. Actes Inst Agron Vet (Maroc). 9: 11-16.

Noonkhokhetkong T, Chukiatsiri K, Sasipreeyajan J and Chansiripornchai N. 2013. Determination of Antimicrobial Susceptibility, Antimicrobial Resistance Genes and in vivo Testing of Antimicrobial Susceptibility of *Avibacterium paragallinarum*. Thai J. Vet. Med. 43(4): 525-531.

Noormohammadi AH, Jones JF, Underwood G and Whithear KG. 2002. Poor systemic antibody response after vaccination of commercial broiler breeders with *Mycoplasma gallisepticum* vaccine ts-11 not associated with susceptibility to challenge. Avian Dis. 46(3): 623-628.

Noro T, Oishi E, Kaneshige T, Yaguchi K, Amimoto K and Shimizu M. 2008. Identification and characterization of haemagglutinin epitopes of *Avibacterium paragallinarum* serovar C. Vet Microbiol. 131(3): 406-413.

Noro T, Yaguchi K, Amimoto K and Oishi E. 2007. Identification and expression of a gene encoding an epitope that induces hemagglutination inhibition antibody to *Avibacterium paragallinarum* serovar A. Avian Dis. 51(1): 84-89.

Page LA. 1962. Haemophilus infections in chickens. I. Characteristics of 12 Haemophilus isolates recovered from diseased chickens. Am J Vet Res. 23: 85-95.

Rafiee M, Bara M, Stephens CP and Blackall PJ. 2000. Application of ERIC PCR for the comparison of isolates of *Haemophilus parasuis*. Aus Vet J. 78(12): 846-849.

Sakamoto R, Baba S, Ushijima T, Kino Y, Honda T, Mizokami H and Sakaguchi M. 2013. Development of a recombinant vaccine against infectious coryza in chickens. Res Vet Sci. 94(3): 504-509.

Sakamoto R, Kino Y and Sakaguchi M. 2012a. Development of a multiplex PCR and PCR-RFLP method for serotyping of *Avibacterium paragallinarum*. J Vet Med Sci. 74(2): 271-273.

Sakamoto R, Sakai AT, Ushijima T, Imamura T, Kino Y, Honda T and Sakaguchi M. 2012b. Development of an enzyme-linked immunosorbent assay for the measurement of antibodies against infectious coryza vaccine. Avian Dis. 56(1): 65-72.

Sandoval VE, Terzolo HR and Blackall PJ. 1994. Complicated infectious coryza outbreaks in Argentina. Avian Dis. 38: 672-678.

Sawata A and Kume K. 1983. Relationships between virulence and morphological or serological properties of variants dissociated from serotype 1 *Haemophilus paragallinarum* strains. J Clin Microbiol. 18(1): 49-55.

Sawata A, Kume K and Nakai T. 1984. Susceptibility of *Haemophilus paragallinarum* to bactericidal activity of normal and immune chicken sera. Jap J Vet Sci. 46(6): 805-813.

Sawata A, Kume K and Nakase Y. 1979. Antigenic structure and relationship between serotypes 1 and 2 of *Haemophilus paragallinarum*. Am J Vet Res. 40(10): 1450-1453.

Sawata A, Kume K and Nakase Y. 1982. Hemagglutinin of *Haemophilus paragallinarum* serotype 2 organisms: occurrence and immunologic properties of hemagglutinin. Am J Vet Res. 43(7): 1311-1314.

Sawata A, Nakai T, Kume K, Yoshikawa H and Yoshikawa T. 1985. Lesions induced in the respiratory tract of chickens by encapsulated or nonencapsulated variants of *Haemophilus paragallinarum*. Am J Vet Res. 46(5): 1185-1191.

Shen Y, Cheng A, Wang M, Chen S, Jia R, Zhu D, Liu M, Sun K, Yang Q and Chen X. 2015. Development of an indirect ELISA method based on the VP3 protein of duck hepatitis A virus type 1 (DHAV-1) for dual detection of DHAV-1 and DHAV-3 antibodies. J Virol Methods. 225: 30-34.

Soriano VE and Terzolo H. 2004. Epizootiology, prevention and control of infectious coryza. Jap. J. Vet. Sci. 35: 261-279.

Soriano VE, Garduno ML, Tellez G, Rosas PF, Suarez-Guemes F and Blackall PJ. 2004a. Cross-protection study of the nine serovars of *Haemophilus paragallinarum* in the Kume haemagglutinin scheme. Avian Pathol. 33(5): 506-511.

Soriano VE, Longinos GM, Fernández RP, Velásquez QE, Ciprián CA, Salazar-García F and Blackall PJ. 2004b. Virulence of the nine serovar reference strains of *Haemophilus paragallinarum*. Avian Dis. 48(4): 886-889.

Soriano-Vargas E, Sanchez-Morales J, Morales-Erasto V and Garcia-Sanchez A. 2013. Isolation and identification of NAD-independent *Avibacterium paragallinarum* (serovar C-1) from growing layer hens with infectious coryza. Vet Mexico. 44(1): 57-62.

Soriano VE, Téllez G, Hargis BM, Newberry L, Salgado-Miranda C and Vázquez JC. 2004c. Typing of *Haemophilus paragallinarum* strains by using enterobacterial repetitive intergenic consensus-based polymerase chain reaction. Avian Dis. 48(4): 890-895.

Sun H, Miao D, Zhang P, Gong Y and Blackall P. 2007. A comparison of a blocking ELISA and a haemagglutination inhibition assay for the detection of antibodies to *Avibacterium* (*Haemophilus*) *paragallinarum* in sera from artificially infected chickens. Biologicals. 35(4): 317-320.

Terzolo HR, Paolicchi FA, Sandoval VE, Blackall PJ, Yamaguchi T and Iritani Y. 1993. Characterization of isolates of *Haemophilus paragallinarum* from Argentina. Avian Dis. 37(2): 310-314.

Terzolo HR, Sandoval VE and Pondal FG. 1997. Evaluation of inactivated infectious coryza vaccines in chickens challenged by serovar B strains of *Haemophilus paragallinarum*. Avian Pathol. 26(2): 365-376.

Thitisak W, Janviriyasopak O, Morris RS, Srihakim S and van Kruedener R. 1988. Causes of death found in an epidemiological study of native chickens in Thai villages. Acta Vet Scand Suppl. 84: 200-202.

Tokunaga E, Sakaguchi M, Matsuo K, Hamada F and Tokiyoshi S. 2005. Polypeptide for *Haemophilus paragallinarum* and process for preparing the same. United State Patent No.6,919,080 B2.

Yamaguchi T, Blackall PJ, Takigami S, Iritani Y and Hayashi Y. 1991. Immunogenicity of *Haemophilus paragallinarum* Serovar B Strains. Avian Dis. 35(4): 965-968.

Yamaguchi T, Iritani Y and Hayashi Y. 1988. Serological response of chickens either vaccinated or artificially infected with *Haemophilus paragallinarum*. Avian Dis. 32: 308-312.

Yamaguchi T, Iritani Y and Hayashi Y. 1989. Hemagglutinating activity and immunological properties of *Haemophilus paragallinarum* field isolates in Japan. Avian Dis. 33: 511-515.

Yamaguchi T, Kobayashi M, Masaki S and Iritani Y. 1993. Isolation and characterization of a *Haemophilus paragallinarum* mutant that lacks a hemagglutinating antigen. Avian Dis. 37(4): 970-976.

Yamamoto R. 1991. Infectious coryza. In: Diseases of poultry. 9th ed. (eds.). Iowa State University Press, Ames, Iowa. 186-195.

Zaini MZ and Kanameda M. 1991. Susceptibility of the indigenous domestic fowl (Gallus gallus) to experimental infection with *Haemophilus paragallinarum*. J Vet Malaysia. 3: 21-24.

Zhang P, Blackall PJ, Yamaguchi T and Iritani Y. 1999. A monoclonal antibody-blocking enzyme-linked immunosorbent assay for the detection of serovar-specific antibodies to *Haemophilus paragallinarum*. Avian Dis. 43: 75-82.

APPENDIX

Media

Blood agar

Ingredients	500 ml	1000 ml
Blood agar base (Oxoid®, England)	40 g	80 g
Distilled water	500 ml	1000 ml
Sheep blood	25 ml	50 ml

1. Dissolve blood agar base in distilled water.
2. Sterilize by using autoclave at 121°C for 15 minutes.
3. Pour the sheep blood into the media, when their temperature is decrease around 45-50 °C
4. Gently mix the media to be homogenous solution before pouring into sterile petri dishes.

GC agar base

Ingredients	500 ml
GC agar base (Oxoid®, England)	18 g
Soluble haemoglobin (Oxoid®, England)	5 g
Vitox (Oxoid®, England)	1 vial

1. Dissolve 18 g of GC Agar Base in 240 ml of distilled water and sterilize by autoclaving at 121°C for 15 minutes.
2. Dissolve 5 g of 2% solution of Soluble Haemaglobin Powder by adding 250 ml of distilled water and sterilize by autoclaving at 121°C for 15 minutes.
3. Aseptically add the Vitox solution and sterile haemoglobin solution into sterile GC agar base media when their temperature is decrease around 45-50 °C.
4. Gently mix the media to be homogenous solution before pouring into sterile petri dishes.

TMB broth

(1) Ingredients for TMB.

Ingredients	1000 ml	500 ml
Biosate Peptone (BBL,USA)	1 g	5 g
NaCl	1 g	5 g
Starch (Merck, Germany)	0.1 g	0.5 g
Glucose (Merck, Germany)	0.05 g	0.25 g
Yeast extract (Merck, Germany)	0.05 g	0.25 g
Distilled water	100 ml	500 ml

1. Accurately weigh (+/-0.05 g) all of the dry ingredient from table 1.

2. Add distilled water to required volume and mix the media to be homogenous solution.

3. Adjust the pH to 7.5 using NaOH solution.

4. Sterilize by using autoclave at 121 °C for 15 minutes.

(2) Supplements for TM/SN agar

Ingredients	1000 ml	500 ml
1% NADH (Sigma, USA)	0.25 ml	1.25 ml
0.05% Thiamine HCl[A]	1 ml	5 ml
Heat Inactivated Chicken Serum (56 °C, 30 min.)	1 ml	5 ml
O-A Complex[B]	5 ml	25 ml

5. Aseptically combine the supplements required as following in table 2 in sterile broth and then gently mix the media to be homogenous solution.

6. Check sterility before using.

[A]0.05% Thiamine solution

1. Accurately weigh 0.05 g of thiamine into a 200 ml beaker and add 100 ml of RO water and dissolve thiamine.

2. Sterilize the solution by using a 0.22 µm filter and a 50 ml syringe.

3. Store the solution at 4 °C

** The solution always dispense aseptically in the biohazard cabinet.

[B]Oleic acid-Albumin (O-A) complex

1. Make a sodium oleate solution by combining 0.3 ml of oleic acid with 25 ml of 0.05N NaOH.
2. Make an albumin solution by dissolving 23.75 g of bovine albumin fraction V (Merck, Germany) in 475 ml of normal saline.
3. Prepare O-A complex by adding 25 ml of sodium oleate solution to albumin solution.
4. Adjust the pH to 6.8
5. Sterilize the solution by using a 0.22 µm filter in amounts of 100 ml.
6. Incubate in 37 °C water bath overnight and incubate in 56 °C water bath for 30 minutes.
7. Store the solution at 4 °C

** The solution always dispense aseptically in the biohazard cabinet.

Solusion for SDS Page

Alsever's solution Volume 1 liter

Dextrose	20.5	g
Sodium citrate	8	g
Citric acid	0.55	g
NaCl	4.2	g
Distilled water add to	1,000	ml

Dissolve all chemical substances in distilled water and sterilize by autoclaving at 121 °C for 10 minutes.

Fixed chicken erythrocyte 10%

Alsever's solution	30	ml
PBS	500	ml
Chicken blood	30	ml
Glutaraldehyde	4	ml
1%thimerosal	1	ml

1. Bleed chicken into Alsevers solution at 50:50 final ratios and mix gently.
2. Centrifuge the mixture at 3,000xg for 7 min, 4 ^{o}C.
3. Remove buffy coat and supernatant and wash chicken erythrocytes 3 times with sterile PBS.
4. Estimate Pack cell volume and add PBS to the final concentration of erythrocytes is 10% and mix with cold4 % glutaraldehyde in PBS at 1:1 final ratio.
5. Stirrer the mixture overnight at 4 ^{o}C on magnetic stirrer.
6. Then centrifuge at 3,000 xg for 10 min and wash with PBS 3 times.
7. Dilute the glutaraldehyde fixed erythrocytes with PBS to 10% final concentration and add thiomersal to the final concentration of 0.01%
8. Store at 4 ^{o}C until use.

1.5 M Tris (pH 8.8) for separating gel Volume 100 ml

Tris (1.5M)	18.171	g
2 Na EDTA (0008M)	0.296	g

Dissolve in 80 ml of distilled water, adjust pH to 8.8 with HCl and adjust volume to 100 ml with distilled water.

0.5 M Tris (pH 6.8) for stacking gel Volume 100 ml

Tris (0.5M)	6.285	g
2 Na EDTA (0008M)	0.296	g

Dissolve in 80 ml of distilled water, adjust pH to 6.8 with HCl and adjust volume to 100 ml with distilled water.

Running buffer 5X (pH 8.75) Volume 500 ml

Tris (0.25M)	15.14	g
2 Na EDTA	1.86	g
Glycine (1.9M)	71.32	g
SDS	2.5	g

Dissolve Tris, EDTA, Glycine, SDS in 400 ml of distilled water, adjust pH to 8.75 and dilute to 500 ml with distilled water.

Loading buffer Volume 100 ml

SDS (2%)	2	g
Tris (0.625%)	0.75	g
Glycerol	33	ml
Bromphenol blue	0.1	g

Dissolve SDS, Tris, Glycine, Glycerol in distilled water and adjust volume to 100 ml with distilled water.

Staining solution Volume 100 ml

Stock coomassie blue[A]	22.5	ml
Absolute methanol	22.5	ml
H_2O	45	ml
Glacial acetic acid	10	ml

Mix all of substances and adjust to 100 ml with distilled water.

Stock coomassie blue (0.5%)

Coomassie blue R250	0.5	g
Absolute methanol	100	ml

Dissolve coomassie blue in absolute methanol and filter with Whatman® No.1, keep in bottle for stock.

Destain solution Volume 100 ml

Absolute methanol	45	ml
H_2O	45	ml
Glacial acetic acid	10	ml

VITA

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