ปัจจัยความรุนแรงของเชื้อ *Avibacterium paragallinarum* ที่แยกได้จากไก่ในประเทศไทย

นางสาวกฤดา ชูเกียรติศิริ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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VIRULENCE FACTORS OF *AVIBACTERIUM PARAGALLINARUM* ISOLATED FROM CHICKENS IN THAILAND

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กฤดา ชูเกียรติศิริ : ปัจจัยความรุนแรงของเชื้อ *Avibacterium paragallinarum* ที่แยกได้จากไก่ในประเทศไทย (VIRULENCE FACTORS OF *AVIBACTERIUM PARAGALLINARUM* ISOLATED FROM CHICKENS IN THAILAND). อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รศ. น.สพ. ดร. นิวัตร จันทร์ศิริพรชัย, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ศ. น.สพ. ดร. จิโรจ ศศิปรียจันทร์, 120 หน้า

เชื้อ *เอวิแบคทีเรียม พารากัลลินารุม* เป็นเชื้อที่ก่อให้เกิดโรคหวัดหน้าบวมในไก่ ซึ่งเป็นโรคติดเชื้อเฉียบพลันของทางเดินหายใจส่วนต้น และมีความสำคัญทางเศรษฐกิจต่ออุตสาหกรรมการเลี้ยงสัตว์ปีกในหลายๆประเทศทั่วโลก วัตถุประสงค์ในการวิจัยครั้งนี้เพื่อศึกษาซีโรวาร์ของเชื้อ ความไวของเชื้อต่อยาปฏิชีวนะ ความสามารถในการก่อพยาธิสภาพ ความรุนแรงของสารสกัดแคปซูล ไลโปโพลีแซคคาไรด์ และเยื่อเมมเบรนด้านนอกของเชื้อ *เอวิแบคทีเรียม พารากัลลินารุม* ที่แยกได้จากไก่ป่วยในประเทศไทย *เอวิแบคทีเรียม พารากัลลินารุม* จากท้องถิ่นจำนวน 18 ตัวอย่างที่ผ่านการพิสูจน์เชื้อด้วยวิธีปฏิกิริยาลูกโซ่พอลิเมอเรส การจำแนกซีโรวาร์ด้วยวิธีการยับยั้งการตกตะกอนกับเม็ดเลือดแดง พบว่าสามารถแยกได้เป็นซีโรวาร์ A 10 ตัวอย่าง B 5 ตัวอย่าง และ C 3 ตัวอย่าง ผลการทดสอบความไวต่อยาต้านจุลชีพจำนวน 17 ชนิด โดยวิธี disk diffusion พบว่าเชื้อทุกตัวอย่างมีความไวต่อยาอะม็อกซีซิลิน-คลาวูลานิก เอซิด และลินโคสเปคติน เชื้อส่วนมากดื้อต่อสเตรปโตมัยซินและอิริโทรมัยซิน (>90%) เชื้อทุกตัวอย่างดื้อต่อยาคลอกซาซิลิน ลินโคมัยซิน และนีโอมัยซิน การศึกษาการเข้าสู่เซลล์และการอยู่รอดภายในเซลล์แมคโครฟาจ พบความสามารถในการอยู่รอดในแมคโครฟาจของเชื้อแต่ละตัวอย่างมีความแตกต่างกัน แต่ไม่พบว่ามีความสัมพันธ์ที่ชัดเจนกับซีโรวาร์ การทดสอบความรุนแรงของเชื้อโดยการให้เชื้อในไก่ไข่ทดลองเพศเมียอายุ 4 สัปดาห์ โดยใช้เชื้อปริมาตร 400 ไมโครลิตรที่ความเข้มข้น 10^8 cfu ต่อมิลลิลิตร โดยวิธีการหยอดจมูก จากนั้นสังเกตอาการทางคลินิกเป็นเวลา 7 วันและการุณยฆาตเพื่อเพาะแยกเชื้อจากโพรงใต้ตา พบว่าเชื้อท้องถิ่นทั้ง 18 ตัวอย่างสามารถก่อโรคและทำให้ไก่ทดลองแสดงอาการของโรคหวัดหน้าบวม นอกจากนี้ยังสามารถแยกเชื้อจากโพรงใต้ตาได้ภายหลังจากได้รับเชื้อ 7 วัน ความรุนแรงในการก่อโรคของเชื้อแต่ละตัวอย่างไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติ ยกเว้นในกลุ่มที่ได้รับเชื้อ 112179 และ 102984 ซึ่งเป็นซีโรวาร์ A และ B ตามลำดับ พบว่ามีคะแนนความรุนแรงของโรคสูงที่สุด และมีความแตกต่างอย่างมีนัยสำคัญกับกลุ่มที่ได้รับเชื้อ CMU1009 ซึ่งเป็นซีโรวาร์ A จากการศึกษานี้ไม่พบความสัมพันธ์ของซีโรวาร์กับความรุนแรงในการก่อโรค การศึกษาความรุนแรงของสารสกัดแคปซูล ไลโปโพลีแซคคาไรด์ และ เยื่อเมมเบรนด้านนอก โดยการฉีดไข่ฟักพบว่าที่ความเข้มข้น 1000 ไมโครกรัมต่อฟอง สารสกัดไลโปโพลีแซคคาไรด์ทำให้ตัวอ่อนไข่ฟักตายได้ ในขณะที่สารสกัดแคปซูล และเยื่อเมมเบรนด้านนอกไม่ทำให้เกิดการตายของตัวอ่อนไข่ฟัก

ภาควิชา อายุรศาสตร์ ..... นิสิต .....

สาขาวิชา อายุรศาสตร์สัตวแพทย์ ..... อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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KEYWORDS : *Avibacterium paragallinarum* / infectious coryza / virulence / disk diffusion test / extraction

KRIDDA CHUKIATSIRI : VIRULENCE FACTORS OF *AVIBACTERIUM PARAGALLINARUM* ISOLATED FROM CHICKENS IN THAILAND. ADVISOR : ASSOC. PROF. NIWAT CHANSIRIPORNCHAI, Ph.D., CO-ADVISOR : PROF. JIROJ SASIPREEYAJAN, Ph.D., 120 pp.

Avibacterium paragallinarum causes infectious coryza in chickens, an acute respiratory disease that has economic significance in worldwide poultry industry. The objectives of this study were to determine the serovars, antimicrobial sensitivity, pathogenicity, virulence of capsular extraction, lipopolysaccharide and outer membrane protein extraction of *Av. paragallinarum* isolated from Thailand. Eighteen field isolates of *Av. paragallinarum* were confirmed by PCR. Serotyping by a hemagglutination inhibition test, 10 isolates were serovar A, 5 isolates were serovar B, and 3 isolates were serovar C. The susceptibility of the isolates to 17 antimicrobial agents was tested by a disk diffusion method. All isolates were susceptible to amoxicillin-clavulanic acid and lincospectin. Most strains were resistance to streptomycin and erythromycin (>90%). All isolates were resistant to cloxacillin, lincomycin and neomycin. A study of bacterial entry into, and survival within, chicken macrophages showed variation between isolates but no clear connection to serovar. A virulence test was performed by challenging 4 weeks old layers via the nasal route with 400 µl of bacteria (10^8 colony forming units/ml). Clinical signs were observed daily for 7 days and the birds were subjected to a post-mortem necropsy at seven days post-challenge. All 18 field isolates caused the typical clinical signs of infectious coryza and could be re-isolated at 7 days after challenge. There was no significant difference in the clinical scores of the isolates except that two isolates of 112179 and 102984 belonged to serovar A and B, respectively, gave a significantly higher score than isolate CMU1009 (serovar A) did. No correlation between serovar and severity of clinical signs was found. The virulence study of capsule, OMP and LPS extraction at concentration 1000 µg/egg via yolk sac route, LPS of virulent isolates *Av. paragallinarum* could cause chicken embryo death whilst OMP and capsule could not.

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

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

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LIST OF ABBREVIATIONS

µg	= microgram
µl	= microliter
BA	= blood agar
BSA	= bovine serum albumin
C	= degree Celsius
CFB	= chicken fibroblast
CLBA	= columbia blood agar base
CLSI	= clinical laboratory standards institute
CMI	= chicken meat infusion
CP	= capsular polysaccharide
CPE	= cytopathic effect
ELD50	= 50% chicken embryo lethal dose
ERIC	= enterobacterial repetitive intergenic consensus
ELISA	= enzyme-linked immunosorbent assay
FBS	= fetal bovine serum
GA	= glutaraldehyde
hr	= hour
HA	= hemagglutinins
HCl	= hydrochloride
HI	= hemagglutination inhibition
KDO	= 2-keto-3-deoxy-octulonic acid
KSCN	= potassium thiocyanate
L	= liter
LPS	= lipopolysaccharide
mg	= milligram
ml	= milliliter
MEM	= minimum essential medium
MDCK	= madin Darby canine kidney

NAD = nicotinamide adenine dinucleotide (Oxidized form)

NADH = nicotinamide adenine dinucleotide (Reduced form)

NaCl = sodium chloride

NB = nutrient broth

NO = nitric oxide

NOS = nitric oxide synthase

- eNOS = endothelial NOS
- iNOS = inducible NOS
- nNOS = neuronal NOS

NSS = normal saline

OMP = outer membrane protein

PCR = polymerase chain reaction

PBS = phosphate buffer saline

PBST = phosphate buffer saline + 0.5% Tween 20

rHag A = recombinant hemagglutinin protein

SEM = scanning electron microscopy

SDC = sodium deoxycholate

SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SPs = secreted proteins

TBE = tris-borate-EDTA

TEM = transmission electron microscopy

Vero = african green monkey kidney

v/v = volume/volume

w/v = mass/volume

CHAPTER I

GENERAL INTRODUCTION

Avibacterium paragallinarum is a Gram-negative, non-spore forming bacterium which causes infectious coryza, an acute respiratory infection, in chickens (Blackall et al., 2005). The disease has worldwide economic significance and affects both broiler and layer chickens. The clinical signs of this disease are nasal discharge, facial edema, lacrimation, anorexia, diarrhea, retarded growth in young chickens and reduced egg production (10-40%) (Blackall and Soriano, 2008).

The Page scheme, the original scheme to serotype *Av. paragallinarum,* recognizes three serovars - A, B and C (Page, 1962). The Kume scheme recognizes the same serogroups as the Page scheme (termed Kume serogroups A, B and C) but has the capacity to sub-type within Kume serogroups A and C, with four serovars in each serogroup (Kume et al., 1983; Blackall et al., 1990a). There is no cross protection between Page serovars (Blackall and Soriano, 2008). Such a vaccine containing Page serovar A will provide serovar-specific immunity to serovar A and cannot protect against Page serovar C organisms (Blackall and Hinz, 2008). Therefore, commercial vaccines combine many serovars, such as bivalent (serovar A and C) vaccine or trivalent (serovar A, B and C) vaccine. *Av. paragallinarum* has been recognized in Thailand in both commercial and native chickens, with some isolates being obtained from chickens vaccinated according to appropriate programs (Chukiatsiri and Chansiripornchai, 2007). According to the published literature, see the review of Blackall and Soriano (2008), all three Page serovars are widely present in the Americas, Europe and Asia. Some countries are known to have only serovars A and C – Australia and Japan (Blackall and Soriano, 2008). The knowledge gained in serotyping studies provides the necessary information to select the appropriate vaccine due to some apparent field failures of infectious coryza vaccines may be a lack of cross-protection between the vaccine strains and the field strains (Soriano et al., 2004a).

In addition to vaccination, an appropriate selection and application of antibiotics for the treatment and control of coryza is also important. An increase in the resistance to antibiotics by *Av. paragallinarum* has been reported in many countries. The antibiotics

which were used for control of this disease such as neomycin, streptomycin, erythromycin, ampicillin, doxycyclin were found increasing of resistance and moreover the resistance to more than 2 kinds of antibiotic could be found (Poernomo et al., 2000; Chukiatsiri and Chansiripornchai, 2007; Hsu et al., 2007a). Then the antimicrobial susceptibility test may be useful for antibiotic selection.

The pathogenicity of *Av. paragallinarum* varies according to various factors such as passage history of the isolate, the growth conditions and the state of the host (Blackall and Soriano, 2008). The variation in pathogenicity amongst *Av. paragallinarum* isolates has been studied and the results of virulence were difference (Soriano et al., 2004b; Bragg, 2005). In addition, the pathogenicity among NAD-dependent and NAD-independent has also been studied (Horner et al., 1995; Bragg, 2002b). The virulence study has been reported by chicken inoculation for observing the clinical signs and bacterial re-isolation. The other studies that have been used in other bacteria such as invasion to chicken fibroblast (Al-haj Ali et al., 2004; Matter et al., 2011), macrophage entry and survival assays (Kramer et al., 2003a), nitric oxide production assay (Crippen, 2006). The knowledge from virulence and pathogenic study will be useful for severity of disease estimation, treatment, prevention and control planning of this disease.

The virulence factors of *Av. paragallinarum* are not clearly understood. The role of capsule, hemagglutinin, haemocins, lipopolysaccharide and iron-acquistion proteins has been studied (Sandal et al., 2010). An important common mechanism for bacterial pathogenesis is the capacity to adhere to the host cells, leading to colonization and finally infection of the host cells (Finlay and Falkow, 1997). In other gram negative bacteria, some compositions on bacterial cell such as capsule, outer membrane protein, lipopolysaccharide are involving with pathogenicity of bacteria. The studies have been reported such as the invasion to cell culture and chicken embryos inoculation (Smith and Thomas, 1956). The results of these types of studies on virulence mechanisms can provide insight into how pathogens either avoid or resist host defense mechanisms and can provide insight into alternative prevention or control strategies.

Objectives

1. To study the serovar and virulence of fields isolates *Avibacterium paragallinarum* in Thailand.
2. To study the antimicrobial sensitivity of these field isolates.
3. To study the virulence of capsule, lipopolysaccharide and outer membrane protein of some fields isolates *Avibacterium paragallinarum*.

Hypothesis

1. All three Page serovars of *Avibacterium paragallinarum* can be found Thailand and all of them can cause infection and clinical signs in difference levels of virulence.
2. The antimicrobial resistance to several kinds of antibiotics can be occurred especially the antibiotic that frequently used in farms.
3. Lipopolysaccharide and outer membrane can cause the chicken embryonic death whereas capsule cannot.

Conceptual framework

Avibacterium paragallinarum

<u>Virulence test</u>

- Serovar identification
- Electrophoresis (SDS-PAGE)
- 16S rRNA sequencing
- Drug sensitivity test
- *In vitro*
  - Macrophage phagocytosis
  - Invasion assay
  - NO production
- *In vivo*
  - Experimental chicken challenge

<u>Capsule, LPS, OMP study</u>

- Capsule staining and extraction
- LPS extraction
- OMP extraction
- Hemagglutination (HA) test
- *In vitro*
  - Cell culture
- *In vivo*
  - Embryonated eggs inoculation

Expected values

1. The results will provide the information of the serovar of *Av. paragallinarum* that outbreak in Thailand and will be useful for vaccine selection to infectious coryza disease prevention.
2. The results will provide the information of the antimicrobial sensitivity of field isolates *Av. paragallinarum* in Thailand and will be useful for antibiotic selection to infectious coryza disease treatment and control.
3. The results of these types of studies on virulence can provide insight into the pathogenicity of field isolates *Av. paragallinarum* and can provide insight into alternative prevention or control strategies.

CHAPTER II

LITERATURE REVIEW

1. *Avibacterium paragallinarum*

Avibacterium paragallinarum (Blackall et al., 2005) or previously known as *Haemophilus paragallinarum*, is a cause of infectious coryza (IC). In 1930, the causative agent of IC was classified as *H. gallinarum* due to the requirement of both X (haemin) and V (nicotinamide adenine dinucleotide; NAD) factors for *in vitro* growth (Blackall and Soriano, 2008). Later, in 1962, Page reported the organisms that isolated from infectious coryza cases required only V-factor for growth and are termed *Haemophilus paragallinarum* (Page, 1962). The strains which need V-factor for growth are called NAD-dependent, whereas the strains which not need V-factor is first found in South Africa in 1990 (Mouahid et al., 1992) and recently in Mexico (Garcia et al., 2004), are called NAD-independent. In 2005, a polyphasic taxonomic study has concluded that the species *H. paragallinarum* is not a member of genus *Haemophilus* and then it is allocated to new genus, *Avibacterium*, along with several other chicken-associated members of the bacterial family *Pasteurellaceae* (Blackall et al., 2005). These other members are *Av. avium*, *Av. gallinarum* and *Av. volantium*. All the bacteria are previously recognized in the genus *Pasteurella* (Blackall and Hinz, 2008). *Av. avium*, *Av. volantium* and *Avibacterium* species A are non-pathogenic.

Characteristics

Morphology and staining

Av. paragallinarum is a Gram-negative, non spore forming, nonmotile and capsulated rod shaped or coccobacilli bacterium (1-3 mm x 0.4-0.8 mm) with a tendency to morphological change after an incubation period of more than 24 hr (Blackall and Soriano, 2008).

Growth requirement

Most isolates of *Av. paragallinarum* are required V-factor (NAD-dependent) for growth but not for X factor. The isolates which not require V-factor are called NAD-independent (Blackall, 2008), they have been found in the Republic of South Africa and Mexico. The organism is commonly grown in an atmosphere of 5% carbon dioxide;

however, they also can grow under reduced oxygen tension or anaerobically (Rimler, 1979) for example, in candle jar. The minimum and maximum temperatures of growth are 25 and 45 C, respectively, the optimal range being 34-42 C (Blackall and Soriano, 2008) but is commonly grown at 37-38 C.

In vitro growth, V-factor is necessary for NAD-dependent isolates. *Staphylococcus* spp. (e.g. *S. aureus* (Bragg et al., 1997), *S. hyicus* (a normal inhabitant of the skin of chickens) (Blackall and Reid, 1982) or *S. epidermidis* (Page, 1962)) are commonly used as "feeder" in blood agar plates (BA). In enrichment media, NAD (20-100 µg/ml media) which is the oxidized form or NADH (1.56-25 µg/ml media) which is the reduced form of NAD (Page, 1962) is used. The other requirements include sodium chloride (NaCl) (1.0-1.5%) and for chicken serum (1%) is required by some isolates (Blackall and Soriano, 2008).

Colony morphology

Colonies of *Av. paragallinarum* are typically tiny (0.3 mm after 24 hr of growth) with a dewdrop shape and non-hemolytic (Blackall and Soriano, 2008). On BA plates that cross streak with *Staphylococcus* spp. as V-factor feeder, the NAD dependent isolates are show satellitic growth pattern (Figure 2-1). The colonies become smaller with increasing distance from the nurse culture. Whereas the NAD-independent isolates produce small colonies (1-2 mm) that do not show satellitic growth and the colonies very similar to *Ornithobacterium rhinotracheale*. Colonies of *Av. avium*, *Av. volantium* and *Avibacterium* species A can also show satellitic growth but are typically bigger than NAD dependent *Av. paragallinarum* (Blackall, 2008).

Figure 2-1 Characteristic satellitic growth patterns of *Av. paragallinarum* when grow nearly a feeder culture (*S. aureus*).

Biochemical properties

All species within the genera *Avibacterium* are Gram-negative, non-motile and negative colony hemolysis, including the ability to ferment glucose without the formation of gas and reduce nitrate to nitrite. The other uniform characteristics are oxidase activity, the presence of the enzyme alkaline phospatase and a failure to produce indole or hydrolyse urea or gelatin (Blackall, 2008). From the biochemical properties of *Avibacterium* spp. (Table 2-1), *Av. paragallinarum* can be isolated from other members by catalase test which is catalase-negative whereas others are catalase-positive. The biochemical properties of NAD dependent and NAD independent *Av. paragallinarum* are the same.

Table 2-1 Biochemical properties of the species within the genera *Avibacterium* (Blackall, 2008).

Property	*Avibacterium gallinarum*	*Avibacterium paragallinarum*	*Avibacterium volantium*	*Avibacterium avium*	*Avibacterium species A.*
Catalase	+[B]	-	+	+	+
Symbiotic growth	-	V	+	+	+
Growth in Air	+	-	+	+	+
Acid from					
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

Preferred culture media and substrates

Artificial media

Many kinds of artificial media can be used for isolation or as enrichment media of *Av. paragallinarum*. All solid media for *Av. paragallinarum* growth have to incubate under 5% CO_2 or if CO_2 incubators are not available, candle jars can be used; whereas liquid media can be incubated at normal condition. For isolation, blood agar (BA) is commonly used for observation of the satellitic growth pattern. The medium is prepared from a dehydrated base such as Bacto-tryptose-blood-agar base (Difco, Detroit, Mich) and enrichment with 5% erythrocytes (Blackall, 2008). The V factor feeder such as *S. hyicus* is streaked onto the blood agar plate to excrete adequate V factor to support the growth of *Av. paragallinarum*.

The maintenance medium for *Av. paragallinarum* is described by Terzolo et al. (1993). The compositions of this media are Columbia blood agar base (CLBA) (Microbiology Systems, Sparks, Maryland, USA) with 7% equine hemolyzed blood that is prepared by holding fresh equine blood at 56 C for 40 min with occasional stirring. The lyzed equine blood can be stored at – 20 C (Blackall, 2008). The plates are incubated at 37 C in the microaerophilic atmosphere for 24-48 hr. CLBA can be transport *Av. paragallinarum* isolates for up to 15 days and can be kept longer in room temperature or 37 C. Another transport media is Ames Transport medium (without charcoal), it can yield positive cultures for up to 8 days at transport temperatures of either 25 C or room temperature (Bragg et al., 2004).

Many enrichment media that can be used for the growth for *Av. paragallinarum* are varying, the examples are showed as in Table 2-2 and 2-3. Some media are complicated to prepare but also give a good yield of *Av. paragallinarum* whereas some media may be more suitable for a small scale research laboratory.

Table 2-2 The solid media can be used for growing *Av. paragallinarum*.

Media	Composition	Supplements
BHI[A]	Brain heart infusion agar	5% Chicken serum, 0.01% NADH
Chocolate agar	Blood agar base, 5% Defibrinated sheep blood	0.0025% NADH
CMI[B]	90% Chicken meat infusion, 0.5% Vitamin assay casamino acid (Difco), 0.5% Bacto-soytone (Difco), 0.5% NaCl and 1.5% Bacto-agar (Difco)	5% Chicken serum, 5% Fresh yeast extract and 0.0025% NADH
GC	GC agar base (Oxoid) and 2% solution of soluble powder (Oxoid)	Vitox supplement (Oxoid, Basingstoke, UK)
HMM[C]	1% polypeptone (BBL), 1% biosate peptone (BBL), 0.24% beef extract, 0.005% para-aminobenzoic acid, 0.005% nicotinamide, 0.1% starch, 0.05% glucose, 0.9% NaCl, 0.23% leptospira base Ellinghausen-MaCullaugh-Johnson-Harris (EMJH) (Difco) and 2% Noble agar (Difco)	0.0025% NADH and 1% Chicken serum
TM/SN[D]	1% biosate peptone (BBL), 1% NaCl, 0.1% Starch, 0.05% Glucose and 1.5% Noble agar (Difco)	5% Oleic albumin complex, 1% Chicken serum, 0.0005% Thiamine and 0.0025% NADH

[A]BHI = Brain heart infusion (Wu et al., 2011)

[B]CMI = Chicken meat infusion (Iritani et al., 1976)

[C]HMM = Haemophilus maintenance medium (Blackall, 2008; Rimler, 1979)

[D]TM/SN = supplemented test medium agar (Blackall and Reid, 1982)

Table 2-3 The liquid media can be used for growing *Av. paragallinarum*.

Media	Composition	Supplements
NB[A]	Nutrient broth	0.01% NADH
CMI[B]	90% Chicken meat infusion,0.5% Vitamin assay casamino acid (Difco), 0.5% Bacto-soytone (Difco) and 0.5% NaCl	5% Chicken serum, 5% Fresh yeast extract and 0.0025% NADH
HMB[C]	1% polypeptone (BBL), 1% biosate peptone (BBL), 0.24% beef extract, 0.005% para-aminobenzoic acid, 0.005% nicotinamide, 0.1% starch, 0.05% glucose, 0.9% NaCl and 0.23% leptospira base Ellinghausen-MaCullaugh-Johnson-Harris (EMJH) (Difco)	0.0025% NADH and 1% Chicken serum
TMB[D]	1% biosate peptone (BBL), 1% NaCl, 0.1% Starch and 0.05% Glucose	5% Oleic albumin complex, 1% Chicken serum, 0.0005% Thiamine and 0.0025% NADH

[A]NB = Nutrient broth (Jacobs et al., 1992)

[B]CMI = Chicken meat infusion (Iritani et al., 1976)

[C]HMM = Haemophilus maintenance medium (Rimler, 1979; Blackall, 2008)

[D]TM/SN = supplemented test medium agar (Blackall and Reid, 1982)

Chicken embryos

Five to seven day old chicken embryonated eggs can be used to propagate and increasing the pathogenicity of *Av. paragallinarum*. The eggs are inoculated via yolk sac route and large amounts of organism are present in egg yolk after overnight incubation. The infected egg yolk always contains *Av. paragallinarum* approximately 0.5-5 x 10^8 cfu/ml (Jacobs et al., 1992). The egg yolk can be harvested and preserved in -70 C freezer (or lower) or keep as lyophilized (Blackall, 2008). The dead embryos may be found hemorrhage on body surface (Figure 2-2).

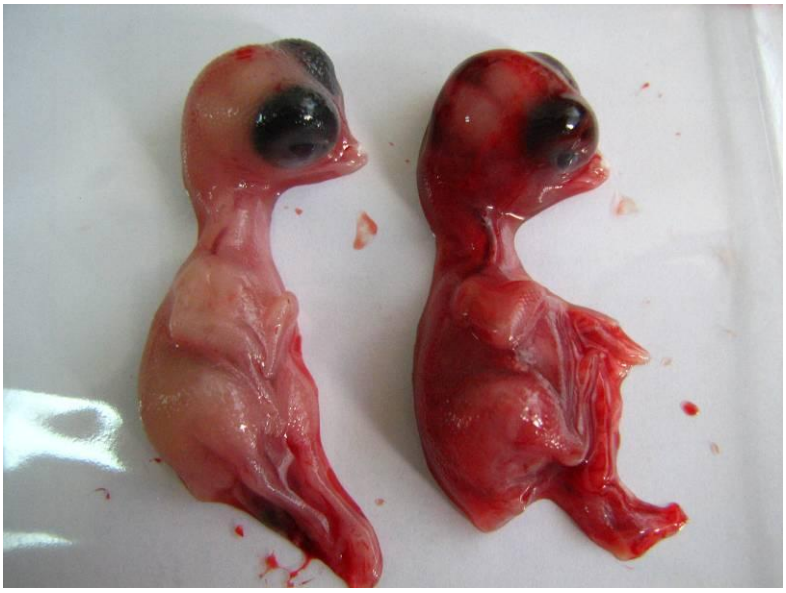

Figure 2-2 Eleven days old chicken embryos are inoculated with *Av. paragallinarum*. The hemorrhage on body surface is found (right) compare with sterile PBS inoculated control (left).

Chicken inoculation

Four weeks old or more of susceptible chicken is appropriate to use for *Av. paragallinarum* challenge. The chicken inoculation via intranasal inoculation or intraorbital sinus frequently uses for diagnosis, vaccine study, virulence study and so on. Normally, the appearance of the typical clinical signs of infectious coryza would be found 24-48 hr after challenge, however; the number of viable organisms or pathogenecity of organism are involved. For diagnosis, a second passage may be required in some cases to produce the typical rapid onset of clinical signs (Blackall, 2008).

Serovar classification and protection

The Page (1962) and the Kume (1983) schemes are two mainly serotyping schemes for *Av. paragallinarum*. The Page scheme is the most widely used serotyping based on an agglutination test. Three serovars (A, B and C) have been recognized. The Kume scheme is serotyping based on hemagglutination inhibition (HI) test and firstly recognized to serogroup I, II and III. Later, The nomenclature of the Kume scheme has been changed from serogroup I, II and III to serogroup A, C and B, respectively

(Blackall et al., 1990a) to relate with the Page scheme. Within the Kume serogroups, there are classified to nine serovars (A-1, A-2, A-3, A-4, B-1, C-1, C-2, C-3 and C-4) by using monoclonal antibody. The comparison of the original and newly proposed nomenclature for the Kume serotyping scheme for *Av. paragallinarum* are showed as in Table 2-4. However, the Page scheme has been widely used than Kume scheme due to the technically demanding of Kume scheme is more complicated. Recently the newly variant type B is described by Jacobs et al. (2003). The new type is discovered by isolating the isolates from IC outbreak in farms that vaccinated with commercial vaccine containing serovar A, B and C in US, Ecuador, Argentina and Zimbabwe. The isolates are serovar B but give a weak protection for reference B vaccine (Jacobs et al., 2003). At this time the HI test with serovar specific antiserum is the best available test for serotyping.

Table 2-4 The comparison of the original and newly proposed nomenclature for the Kume serotyping scheme for *Av. paragallinarum* (Blackall et al., 1990a).

Reference isolates	Original scheme (Kume)		New scheme (Blackall)	
	Serogroup	Serovar	Serogroup	Serovar
221	I	HA-1	A	A-1
2403	I	HA-2	A	A-2
E-3C	I	HA-3	A	A-3
HP14	I	HA-8	A	A-4
H-18	II	HA-4	C	C-1
Modesto	II	HA-5	C	C-2
SA-3	II	HA-6	C	C-3
HP60	II	HA-9	C	C-4
2671	III	HA-7	B	B-1

There are limitations of cross protection among 3 serovars of Page scheme. Such a vaccine containing Page serovar A will providing serovar-specific immunity to serovar A and cannot protect against Page serovar C organism (Blackall and Hinz, 2008). For Kume serogroups, the cross protection within serogroups are reported by Soriano et al. (2004a). Within serogroup A, serovars A-1, A-2 and A-3 are excellent cross

protection while between serovars A-1 and A-4 are almost good cross protection. The cross protection within serogroups C is highly strain dependent. The good cross protection (> 80% protection) is only found in Kume serovars C-1, C-2 and C-3 vaccine that are provided protection against serovar C-4 challenge and among serovars C-1 and C-3. In addition, the good cross protection across Kume serogroups are found only between serovar C-4 vaccine to serovar B-1 challenge (70% protection). This study thus broadly confirmed the widely accepted dogma that serogroups A, B and C represent three distinct immunovars. The hemagglutination-inhibition antibody levels generally show the same trend as with the cross-protection results (Blackall and Soriano, 2008).

Av. paragallinarum of serovars A, B and C has been recognized in Thailand in both commercial and native chickens, with some isolates being obtained from chickens vaccinated according to appropriate programs (Neramitmansuk and Neramitmansuk, 1985; Chukiatsiri et al., 2009; Chukiatsiri et al., 2010). According to the published literature, see the review of Blackall and Soriano (2008), all three Page serovars are widely present in the Americas, Europe and Asia. Some countries are known to have only serovars A and C – Australia and Japan (Blackall and Soriano, 2008). To know the serovar of *Av. paragallinarum* isolates that outbreak in each country is benefit for vaccine selection. Moreover some failures of field infectious coryza vaccination may be due to a lack of cross-protection between the vaccine strains and the field strains (Soriano et al., 2004a).

Serotyping by molecular technique

At present, molecular studies of *Av. paragallinarum* are limit. No molecular technique for serotyping is available. Some molecular techniques have been tried to serotype for *Av. paragallinarum* such as Enterobacterial repetitive intergenic consensus (ERIC) PCR or 16S rRNA gene. ERIC PCR assay is based on the use of long sequence primers at low annealing temperatures. It has been used for molecular typing purposes in some other members of the genus *Haemophilus* such as *H. somnus*, *H. influenza* and *H. parasuis*. ERIC PCR cannot apply for *Av. paragallinarum* typing. According to the results, although it can be classified the nine Kume serovars to different patterns but cannot be used for classified field isolates because the patterns are not specific to serovar (Soriano et al., 2004c).

16S ribosomal RNA (16S rRNA) is a component of the 30S subunit of prokaryotic ribosomes that contain 1,542 nucleotides in length. PCR sequencing of the gene coding for 16S rRNA is the method that can be used for some bacterial identification. Also this technique also can be applied to *Avibacterium* spp. (Blackall et al., 2005; Mendoza-Espinoza et al., 2008). The satellitic colonies of *Avibacterium* spp. can be identified as *Av. paragallinarum*, *Av. avium*, *Av. volantium* or *Av*. species A by using 16S rRNA sequencing in the case that biochemical test cannot used. Christensen et al. (2009) also used the 16S rRNA sequencing to differentiate unclassified avian haemophili.

Pathogenicity

The pathogenicity of *Av. paragallinarum* varies according to various factors such as passage history of the isolate, the growth conditions and the state of the host (Blackall and Soriano, 2008). The variation in pathogenicity amongst *Av. paragallinarum* isolates has been studied. The virulent study of nine reference strains of Kume serovars revealed that the highest clinical signs score was obtained for serovar C-1, while the lowest clinical signs score was obtained for serovar C-4. Overall the strains of serovars A-1, A-4, C-1, C-2 and C-3 showed higher virulence than the strains for serovar A-2, A-3, B-1 and C-4 (Soriano et al., 2004b). Whereas Bragg (2005) tested the virulence of 4 South African serovars that is, serovar A-1, B-1, C-2 and C-3 and found serovar C-3 is highly virulent. Serovar C-2 is less virulent than serovar C-3 but is substantially more virulent than serovars A-1 or B-1. The pathogenicity among NAD-dependent and NAD-independent has been studied. Horner et al. (1995) suggested that the NAD-independent *Av. paragallinarum* isolates may cause air-sacculitis more commonly than the NAD-dependent isolates. Bragg (2002b) reported that the NAD-independent isolates were less virulent when compared to the virulence of the NAD-dependent isolates from the same serovar.

Virulent factors of *Av. paragallinarum*

An important mechanism of bacterial pathogenesis is capable of adhering to the host cells, leading to colonization and finally infection of the host cells. In addition, the amount of colonization and even virulence of the microorganisms are also influenced by

the initial adherences of bacteria to the host cells (Finlay and Falkow, 1997). Mechanisms that used by *Av. paragallinarum* are included the use of a capsule, LPS, hemagglutinatinin (HA), production of toxins and different speculated proteins involved in adherence of the bacteria to the host cell (Sandal et al., 2010). However, information on the factors associated with the virulence of *Av. paragallinarum* is limited.

Structure and function of gram negative bacteria cell wall

Both structure and chemistry of Gram negative cell walls are more complex than Gram positive cell walls (Figure 2-3). Structurally, a Gram negative cell wall contains two layers external and the cytoplasmic membrane. The peptidoglycan layer is very much thinner, which accounts for only 5% to 10% of the Gram negative cell wall by weight and lies between the cytoplasmic membrane and a second membrane known as the outer membrane (Figure 2-3), which is unique to Gram negative bacteria. The inner side of the outer membrane consists mainly of phospholipid whilst the outer side is composed of lipopolysaccharide (LPS) (Wilson et al., 2002). The area between the external surface of the cytoplasmic membrane and the internal surface of the outer membrane is referred to the periplasmic space. This space is a compartment containing a variety of hydrolytic enzymes, which are important to the cell for the breakdown of large macromolecules for metabolism, i.e. proteases, phosphatases, lipases, nucleases and carbohydrate-degrading enzymes.

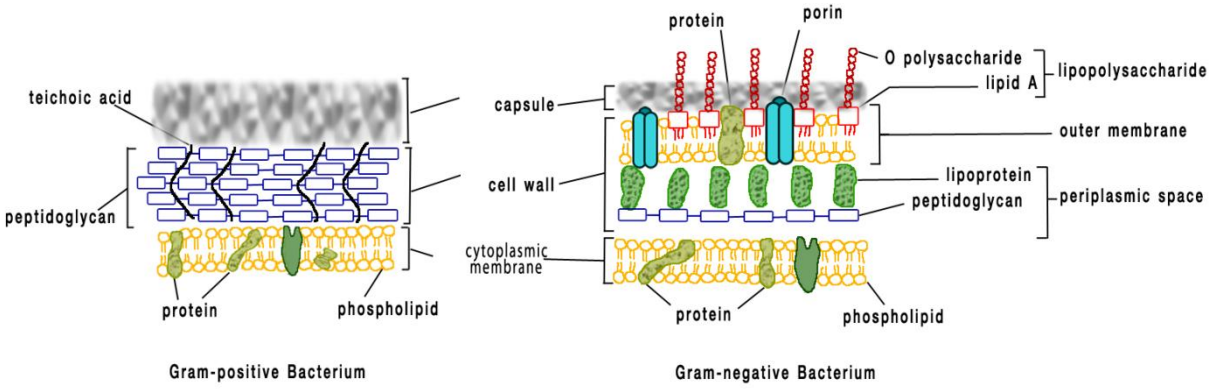

Figure 2-3 Comparative structure between Gram positive and Gram negative bacteria cell wall.

The factors that probably associated with the virulence of *Av. paragallinarum*

1. Capsule

A capsular polysaccharide (CP) has been found in many bacterial species, especially invasive pathogens. A capsule composed of high molecular weight polysaccharide that forms a dense thick coat outside of the bacterial cells. The important functions of capsule involving bacterial cell protection, adhesion to the host cells, protect the bacteria from phagocytosis and also against the bactericidal action of complementation (Quinn et al., 2002a; Wilson et al., 2002). In the process of phagocytosis, the phagocytic cells will attach and ingest the microorganisms, either spontaneously or with the aid of opsonins. The opsonins, such as complement component C3b and immunoglobulin (IgG), are substances that enhance phagocytosis by mechanism that called opsonization. The IgG and C3b ligands on the bacteria attach to phagocytic cells through specific receptors that are IgG receptor and C3b receptor on phagocytic cells and the bacteria will be uptake (Dziarski, 2007). Capsules of some bacteria can protect the bacteria from phagocytosis by several mechanism especially preventing formation of C3 convertase that will split C3 complement to C3a and C3b. The opsonin or capsules of some organisms simply cover the C3b that will bind to the bacterial surface and prevent the C3b receptor on phagocytic cells from contact with the C3b. In addition, the capsule is not a good immunogen. Antibodies to the capsule are not protective against the disease.

According to the literature reviews, the capsule of *Av. paragallinarum* has also been associated with colonization and has been suggested to be the key factor in the lesions associated with infectious coryza (Sawata and Kume, 1983; Sawata et al., 1985). The chickens was inoculated nasally with encapsulated and non-encapsulated organisms and then the histological, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were used for examine histological changes of the nasal mucosa. The chickens were received encapsulated *Av. paragallinarum* showed marked loss of cilia and microvilli, infiltration of leukocytes and deposition of a mucopurulent substance. The microvillous cells were particularly destroyed and infiltrated of the mast cells into the lamina propria of the mucous membrane was observed. In addition, numerous encapsulated organisms were found near cilia and on microvilli and the capsule appeared to mediate attachment of organisms to the cilia. The results are different in the chickens that inoculated with non-encapsulated. The histological was found a very little change of the nasal mucosa and a few colonization of

non-encapsulated organism near cilia. Overall, the colonization of encapsulated organism may play the important role to induce the morphological changes that saw in the nasal mucosa and the adhesion of organisms to nasal mucosa are important to the virulence of bacteria.

Rimler et al. (1977) supposed that the presence of hyaluronic acid which found in most of their isolates could play a role as a virulence factor. Kume and Sawata (1984) found that highly encapsulated variants of serotype I organism lost their virulence when treated with hyaluronidase. Other evidence that hyaluronic acid may play a role in virulence was obtained by Sawata et al. (1978). However some bacteria which do not have hyaluronic acid are still pathogenic indicating that other antigens must also play some role in virulence. The other research has been suggested that a toxin released from capsular organisms during *in vivo* multiplication was responsible for the clinical disease (Kume et al., 1984).

2. Hemagglutinin (HA)

Adhesion is one of the most important factors influenced effective infection. Finlay and Falkow (1997) reviewed the common adhesion proteins that can be divided into two classes of adhesions : afimbrial and fimbrial. Hemagglutinins (HA) is one of the afimbrial adhesions that can be found in some bacteria eg. *E. coli*, *Bordetella pertussis* and *Haemophilus influenza* (Sandros and Tuomanen, 1993). An hemagglutinin structure of *Av. paragallinarum*, is mainly related to the antigenicity, pathogenicity and immunogenicity (Soriano and Terzolo, 2004). In addition, they are important for serotyping (Blackalll, 1989). Yamaguchi et al. (1993) revealed the hemagglutinins of *Av. paragallinarum* play an important role in both the pathogenicity as well as protective immunity. Mutant strains which were not expressing any hemagglutinin could act as non-pathogenic and non-immunogenic. While, chickens inoculated with the fractionated HA were protected from challenge exposure with *Av. paragallinarum* (Iritani et al., 1980). Takagi et al. (1993) purified the hemagglutinin from *Av. paragallinarum* and immunized the chickens with this purified hemagglutinin by intramuscular route. The hemagglutinin purification showed a good immunogen and high titers of anti-HA antibodies indicating protection against infectious coryza. Wu et al. (2011) reported the hypervariable region of the hemagglutinin. The experimental chickens that were immunized with recombinant proteins containing the hypervariable region revealed protection against challenge

infection with *Av. paragallinarum* of the homologous serovar. The immunological properties of a functional recombinant hemagglutinin protein (rHagA) from *Av. paragallinarum* were investigated by using them as the immunogen for vaccination (Hsu et al., 2007b). The result was found this rHagA subunit vaccine can protect 71% of immunized chickens against homologous strain challenge and all vaccinated chickens are not showed any clinical signs of coryza.

Another function of HA is adhesion to mucosal membrane. Sawata et al. (1979) reported that the L and HA-L antigens located in the outer membrane were responsible for adherence to the sinus mucosal surface. Hobb et al. (2002) indicated that HA is considered the most important of the known virulence attributes due to its participation in tissue adhesion. The other experiments from Fernández et al. (2000b) was found the complete loss of the hemagglutinating activity and adherence ability of *Av. paragallinarum* to epithelial cells when this bacterium was absorption either with rabbit or chicken sera or with tracheal washing from immunized chickens (Soriano and Terzolo, 2004).

3. Iron-Acquisition proteins

The secretion of some substances from bacteria for uptake the essential nutrients from host is one of pathogenicity mechanisms. Iron is an essential micronutrient for bacterial growth that participates in many biological processes requiring electron transfer. In many spp. of bacteria, iron is also related to their pathogenicity. Iron is not freely available in the host; most of them are found intracellularly eg. in ferritin or heme. Whilst extracellular iron in serum and in other body fluids is bound to the high-affinity iron- binding glycoproteins transferrin and lactoferrin. Bacterial pathogens use various systems for acquiring essential iron *in vivo* (Abascal et al., 2009).

Ogunnariwo and Schryvers (1992) informed that *Av. paragallinarum* was capable to acquire the iron from chicken and turkey transferrins, whereas two strains of *Av. avium* were unable. The response to iron deficiency in *Av. paragallinarum* has been found the expression of four iron-regulated outer-membrane proteins of 53, 62, 66 and 94 kDa. The 53 and 94 kDa proteins were further purified and identified as specific receptors for chicken or turkey ovotransferrin (Ogunnariwo and Schryvers, 1996), suggesting its participation in iron acquisition. Another report from Abascal et al. (2009)

that was experimented in an iron-restricted culture medium. *Av. paragallinarum* isolate expressed the 60, 68 and 93 kDa outer-membrane proteins that were identified as transferring receptors and iron transport proteins. The study of iron-restriction-induced protein could lead to an understanding of the responses of *Av. paragallinarum* to survive in an iron-restricted environment on host mucosal surfaces.

4. Outer membrane and Lipopolysaccharide (LPS)

The bacterial outer membrane is found in only gram-negative bacteria that containing asymmetric bilayer structure. The inner leaflet consists mainly of phospholipids, whereas outer leaflet is composed of lipopolysaccharide (LPS) (Wilson et al., 2002).

Lipopolysaccharides (LPS) are unique and abundant glycolipids found in the outer leaflet of the gram-negative outer membrane. The 3 importance components of LPS are lipid A, core polysaccharide and O-specific antigen. A glycolipid (lipid A) is responsible for the endotoxic properties of LPS because it stimulates the production of inflammatory mediators and can potentially lead to sepsis. Core polysaccharide contains a characteristic sugar acid, 2-keto-3-deoxy-octulonic acid (KDO) and other sugars including a heptose. An antigenic polysaccharide composed of a chain of repeating oligosaccharide subunits (O antigenic side chain) and a major antigenic determinant (antibody-combining site) of the Gram-negative cell wall resides in this O polysaccharide. Variations in sugar content of the O polysaccharide contribute to the wide variety of antigenic types of *Salmonella* and *E. coli* and presumably other strains of Gram-negative species and then it can be used for serotyping of Gram-negative cells. (Quinn et al., 2002b; Wilson et al., 2002)

According to the literatures, LPS is responsible for endotoxin effect and can stimulate B cell and induce cytokine release (Wilson et al., 2002). Effect of endotoxin depends on the amount of LPS present in the circulation and may be influenced by previous exposure to the toxin. In low concentrations, endotoxin elicits fever through the release of endogenous pyrogens. Whereas high doses induce disseminated intravascular coagulation associated with hypotension and shock (Quinn et al., 2002a). The LPS are reviewed as virulence factor in many bacterial species (Cryz Jr et al., 1984; Kimura and Hansen, 1986).

5. Other factors

The plasmid pYMH5 that is the first multidrug-resistance plasmid are reported in *Av. paragallinarum* isolate (Hsu et al., 2007a). Plasmid pYMH5 encoded functional streptomycin, sulfonamide, kanamycin and neomycin resistance genes. The spread of antibiotic-resistance gene between bacteria may occur by plasmid transfer. In addition, the putative virulence plasmid pA14 and haemocin-like activity are also found indicated two possible mechanisms might be responsible for the pathogenesis (Hsu et al., 2007a).

Av. paragallinarum releases extracellular membrane vesicles (MVs) that contain immunogenic proteins, proteases, putative RTX proteins, hemagglutinin and nucleic acids into the medium. The release of these structures can damage host tissues because of virulence factors that contains in or associated with MVs (Ramón Rocha et al., 2006). The secretion of 110-kDa putative RTX protein from *Av. paragallinarum* was reported (Mena-Rojas et al., 2004; Pérez-Márquez et al., 2008). Pathogenicity of *Av. paragallinarum* secreted proteins (SPs) containing 110-kDa (a putative RTX protein) and 120 kDa against to specific pathogen-free chicken embryos was reported the mortality of chicken embryos approximately 90-100% when SPs from *Av. paragallinarum* isolates were inoculated via yolk sac route. While there was not observed the mortality when SPs from *Av. avium* were inoculated. It may be due to *Av. paragallinarum* SPs could contain toxins responsible for the embryo deaths (Pérez-Márquez et al., 2008). Other reviews, Rivero-García et al. (2005) informed the presence of secreted metalloproteases of *Av. paragallinarum* isolates able to degrade chicken immunoglobulin G (IgG) partially.

Nitric oxide production

Nitric oxide (NO) is an important physiological messenger and effector molecule in many biological systems. There are affecting vascular tone, neurotransmission and host defense (Bredt and Snyder, 1994; Dawson and Dawson, 1995). NO is produced in a variety of tissues by nitric oxide synthase (NOS) that based on the location and the mechanism of regulation, 3 isoforms of NOS have been identified. They are neuronal NOS (nNOS, also termed NOS I), inducible NOS (iNOS, also termed NOS II) and endothelial NOS (eNOS, also termed NOS III) (Chi et al., 2003). NO can also function in a cytotoxic role by generating highly reactive compounds during its oxidation to nitrite and nitrate and by binding to metalloenzymes in bacteria leading to bacterial death

(Lancaster Jr, 1992). The enzyme responsible for cytotoxic nitric oxide production from macrophages is inducible nitric oxide synthase or iNOS which express only after activation by stimulants such as endotoxins or cytokines (Crippen et al., 2003; Hibbs, 1988). The chicken cell lines have previously been used to study nitric oxide production in avian species and the nitrite concentrations in supernatants can be quantified as an indirect means to measure nitric oxide production by the cells (Crippen, 2006).

2. Disease

Infectious coryza

Infectious coryza is an acute respiratory disease in chickens. The disease has worldwide economic recognition and causes infections in both broiler and layer flocks. Chickens are the natural host for *Av. paragallinarum*. It can be found in both industrial and native chickens (Byarugaba et al., 2007). Chickens of all ages are susceptible (Blackall and Soriano, 2008) but the disease is usually severe in older chickens. This disease can be transmitted by contaminating in drinking water through nasal discharge, airborne route or direct contacts but not egg transmission. Chronic illness or healthy carrier birds are the main reservoirs of infectious coryza infections. The disease outbreaks are frequently occurred during the fall and winter. The predisposing causes that may promote the disease such as high level of ammonia, climate change and overpopulation. The incubation period of infectious coryza is short; the clinical sign can be occurred within 24-48 hr after intranasal or intrasinus inoculation with either cultures or exudates. Susceptible birds exposed by contacting infected cases usually have observed the clinical signs within 24-72 hr. In the absence of a concurrent infection, the disease can be recovered within 10 days for mild forms and 2-3 wk for severe forms (Blackall and Hinz, 2008). The mortality rate of IC is low but the morbidity rate is high. The older chickens can found a high mortality as shown by an outbreak in California where the total mortality reached to 48% (Blackall and Soriano, 2008). The other factors can be involved to the severity and duration of the disease such as poor managements, inadequate nutrition, parasitism or co-infection with other respiratory tract pathogens (infectious bronchitis virus, laryngotracheitis virus, *Mycoplasma gallisepticum*, *Escherichia coli* or *Pasteurella* sp.) (Blackall and Hinz, 2008).

Pathogenesis

Av. paragalliarum enters to the nasal cavity via nasally as the main route and adheres to the ciliated mucosa of the upper respiratory tract. The capsule and the hemagglutination antigen play an important role in the colonization. After that the inflammatory process will occur such as cytokine release and leukocyte infiltration. The association with production of lesions in the mucosa and appearance of the clinical signs are due to the toxic substances that are released from the bacteria during proliferation. In addition, the capsule may act as a natural defense substance that is against the bactericidal activity of complement via the alternative pathway.

Normally, *Av. paragallinarum* is a non invasive bacterial agent with a strong tropism for ciliated cells. If co-infection with other infectious agents and/or synergies by immunosuppression are occurred, the migration of *Av. paragallinarum* into the lower respiratory tract (lungs, air sac) may be found (Blackall and Hinz, 2008).

Clinical signs

Infectious coryza is regarded as a disease limited to the upper respiratory tract (Reid and Blackall, 1984), but infection in the lower respiratory tract maybe occurred when synergism with other respiratory tract pathogens. The clinical signs include a seromucoid nasal discharge, ocular discharge and swelling of infraorbital sinuses. In severe cases marked conjunctivitis with closed eyes, swollen of face or wattles and difficulty in breathing can be seen (Blackall and Hinz, 2008). Other clinical signs that can be found such as diarrhea, decreased feed and water consumption, retarded growth in younger chickens and reduced egg production 10-40% (Eaves et al., 1989; Blackall and Hinz, 2008).

Pathology

The pathological changes have been found approximately 20 hr after infection and the severity is increased till the highest level at 7-10 days post infection. Gross pathology may have catarrhal to fibrinopurulent inflammation of the nasal passages, infraorbital sinus and conjunctivae (Blackall and Hinz, 2008). There is frequently a catarrhal conjunctivitis and subcutaneous edema of face and wattles (Blackall and

Soriano, 2008). However, very virulent strains have also been described as causing lesions of pneumonia, airsacculitis and arthritis (Soriano and Terzolo, 2004).

Histological changes may be found marked loss of cilia and microvilli, cell edema, degeneration and desquamation of mucosal and glandular epithelium, infiltration of leukocytes and deposition of mucopurulent substances that can be seen and are followed by infiltration of mass cells into the lamina propria of the mucous membrane (Blackall and Hinz, 2008). In chickens with experimentally induced coryza has been reported to be an acute catarrhal inflammation of the mucous membranes of the nasal passages, infra-orbital sinuses and trachea.

Diagnosis

The history of a rapid spreading disease, clinical signs and lesions may allow to be considered and confirmed by cultural isolation and identification for *Av. paragallinarum* diagnosis (Blackall and Hinz, 2008). Sample collection can be swabbed from infraorbital sinus or from fresh nasal exudates in live birds. The infraorbital sinus can be incised by sterility the skin over their sinuses seared with a heated spatula and the skin are incised with scalpel blade. Another method can be done by cutting in the middle of beak from lateral (behind the nostrils). Fresh nasal exudates are swabbed by pressing on the sinus and the sampling is the best done with a small sterile loop that just touches the surface of the fresh exudates. After that the sample are directly inoculated on to a blood agar plate and cross-streaked with a feeder organism such as *Staphylococcus epidermidis*. The trachea and air sac can be swabbed, although *Av. paragallinarum* is less frequently isolated from these areas. *Av. paragallinarum* is a fragile organism that does not survive outside of chickens' bodies for more than 5 hr (Blackall, 2008). Ames Transport medium (without charcoal) can be used as transport media for *Av. paragallinarum*, it can yield positive cultures for up to 8 days at transport temperatures of either 25 C or room temperature (Bragg et al., 2004). The isolated organism can be identified by biochemical test or by confirmatory polymerase chain reaction (PCR) with HPG-2 primer (Chen et al., 1996) that specific to both NAD dependent and independent isolates of *Av. paragallinarum*. This PCR test is now routinely performed for the identification of *Av. paragallinarum* in nasal swabs and for confirmation of *Av. paragallinarum* grown in the laboratories (Miflin et al., 1999).

Differential diagnosis

Infectious coryza disease must be differentiated from other diseases such as chronic respiratory disease, chronic fowl cholera, fowl pox, ornithobacteriosis (due to *O. rhinotracheale*), swollen head syndrome (associated with avian pneumovirus) and hypovitaminosis A, which can produce similar clinical signs (Blackall, 2008). If the mortality rate is high and the duration of disease takes a long time, the co-infection of *Av. paragallinarum* with other bacteria or virus should be considered.

Serological detection

The best available method for detecting infectious coryza antibody titer at this time is the hemagglutination inhibition (HI) test (Blackall and Hinz, 2008). The other method is using the monoclonal antibody-based blocking enzyme-linked immunosorbent assay (ELISA) (Miao et al., 2000; Sun et al., 2007) but it is not commonly used. The HI test is done using the method as described by Eaves et al. (1989). The tested sera are absorbed non-specific hemagglutinin using glutaraldehyde (GA) fixed chicken erythrocyte and the HI test is done using KSCN treated serovar-specific antigen.

Treatment

Antimicrobial drugs that recommend for infectious coryza such as spectinomycin, streptomycin, danofloxacin, difloxacin, enrofloxacin, flumequine, sulfonamides and trimethoprim (Blackall and Hinz, 2008). Erythromycin and oxytetracycline are also commonly used (Blackall and Soriano, 2008). However, the drug resistance in *Av. paragallinarum* could occur (Blackall, 1988; Chukiatsiri and Chansiripornchai, 2007; Hsu et al., 2007a), therefore the antimicrobial susceptibility test may be useful. The study of antibiotic resistance mechanism in *Av. paragallinarum* is limited. But normally, the antimicrobial resistance can be divided into 2 types. The first is natural resistance or intrinsic resistance that could be found in some bacteria such as enterococci bacteria resistant to cephalosporins, *Pasteurella aeruginosa* resistant to co-trimoxazole. Another one is acquired resistance such as reduces permeability or uptake, alteration of the drug target, enzymes production to destroy antimicrobial agents (Tenover, 2006).

After 5-7 days of the treatment, if the chickens are continuously treated with appropriate antimicrobial drugs the clinical signs should be disappeared completely. The recovery from clinical signs takes shorter than the recovery for egg productions (Blackall and Hinz, 2008).

Control

The disease outbreak can be reduced by improving management measures such as good biosecurity, good sanitation, depopulation and avoidance of multiage farms. Depopulation the infected or recovery flock is necessary for elimination the agent from a farm because these birds remain a reservoir of the bacterial agent. The building should be cleaned, disinfected and abstained for at least 1 week before introducing new chickens (Blackall and Hinz, 2008; Blackall and Soriano, 2008; Chukiatsiri et al., 2009).

Vaccination

Because the protection across serovars is limited, the use of more than one serovars in the vaccine is required. The vaccine must consist of at least 10^8 colony-forming units per dose per strain. Mostly, the vaccines are inactivated whole cells containing the different adjuvants and contained different strains up to the company. Adjuvants used in vaccine preparation are also different in the use of such elements as aluminium hydroxide gel, mineral oil or saponin, where each adjuvant has advantages and disadvantages. An aluminium hydroxide gel adjuvant vaccine is appropriate for the 1^{st} injection since antibody titer production is high and increases rapidly (Morein et al., 1996) and there is very little local reaction when compared with oil adjuvant that usually causes swelling or granulomatous (Reid and Blackall, 1987). An oil adjuvant vaccine is appropriate as a booster, which will slowly release antigen and directly respond to antigens presenting macrophages resulting in having a longer effect in antibodies than the aluminium hydroxide gel adjuvant vaccine (Morein et al., 1996; Deguchi et al., 1998; Fukanoki et al., 2000).

The types of commercial vaccine, bivalent vaccine contain 2 strains of different serovars that are serovars A and C, while trivalent vaccine contains all 3 strains of Page serovars (A, B and C). The tetravalent vaccine is added the strain of serovar variant-B. To choose vaccine is upon a serovar of *Av. paragallinarum* that spreads out in each

country. The one causing failure in vaccination occurs because there is no cross protection between vaccine strain and field strain (Chukiatsiri et al., 2009). Thus an autogenous bacterin should be used if a commercial bacterin is unable to induce a protective immunity (Blackall and Hinz, 2008).

CHAPTER III

MATERIALS AND METHODS

Part 1: The study of Thai field isolates of *Avibacterium paragallinarum*

1. Bacterial isolation and identification

1.1 Culture media

1.1.1 Blood agar plate with *Staphylococcus hyicus* as a feeder colony was used for observing satellite growth.

1.1.2 TM/SN agar supplemented with NADH (Sigma, MO, USA), thiamine HCl, chicken serum and O-A complex (Merck, Damstadt, Germany) (Blackall and Reid, 1982) was used for *Av. paragallinarum* isolation, disk diffusion test and total plate count.

1.1.3 TMB broth (Blackall and Reid, 1982) was used as the final growth medium for the antigen preparation and challenge preparation.

1.1.4 GC agar base with 2% w/v soluble hemoglobin powder and supplement with Vitox (Oxoid, Cambridge, UK) was used for *Av. paragallinarum* growth and initial growth for inoculate CMI broth.

1.1.5 CMI (chicken meat infusion) broth (Iritani et al., 1976) was used for *Av. paragallinarum* growth for protein extraction. All agar plates were incubated under 5% CO_2 at 37 C.

1.2 Bacteria

1.2.1 Eighteen isolates of *Av. paragallinarum* were obtained from chickens showing the typical clinical signs of infectious coryza. Twelve isolates were provided from the Bacteriology section, National Institute of Animal Health (NIAH) and six isolates were collected from Avian Health Research Unit (AHRU), Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. The isolates from NIAH were collected from the outbreaks at different times and places, but no accurate records have been kept. The AHRU isolates (IR1, 211108, CMA0509, F1CM0709, CHS0809 and CMU1009) were collected from the outbreaks during 2006-2009 in different vaccinated flocks from

various provinces of Thailand including Chaiyaphum, Chonburi, Chiangmai, Chiangmai, Chachoengsao and Chiangmai, respectively.

1.2.2 The bacteria were growth on TM/SN and blood agar, the latter with a nurse culture of *S. hyicus*. TM/SN was used as enrichment media. The colonies of NAD-dependent *Av. paragallinarum* should be shown satellite growth on blood agar plate. The suspected colonies were growth on GC agar to get higher amount of bacteria and identified as *Av. paragallinarum* by a species-specific polymerase chain reaction (PCR) test as explained below. The *Av. paragallinarum* isolates were kept in Mist. desiccans at – 70 C until used.

1.3 Polymerase chain reaction (PCR)

The PCR protocol was performed as described previously (Chen et al., 1996) with some modification.

1.3.1 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 for *Av. paragallinarum* identification that provided product size approximately 500 base pairs.

1.3.2 The DNA template was prepared by growing *Av. paragallinarum* on GC agar overnight, harvested by using 1 µl disposable loops and scraped from the surface into 200 µl sterile PBS (phosphate buffered saline) in a microcentrifuge tube. The sample was vortexed and heated on AccuBlock ™ Digital Dry Bath (Labnet international, Inc., USA) at 98 C for 5 min. After that, centrifuged in a benchtop microfuge at 17,000 x g for 5 min and the supernatant were collected for use as DNA template. This preparation could be frozen at -20 C and used for experiments.

1.3.3 Each 50 µl reaction mix was contained 2x PCR Master mix 25 µl (Gotaq® green master mix; Promega, WI, USA), N1 primer (10 pmol/µl) 2 µl, R1 (10 pmol/µl) primer 2 µl, H_2O 20 µl and DNA template 1 µl. The nuclease free water was used as negative control and *Av. paragallinarum* strain 221 was used as positive control.

1.3.4 The PCR was conducted by heating at 98 C for 2 min 30 sec and 25 cycles of denaturation at 94 C for 1 min, annealing at 65 C for 1 min and extension at 72 C for 2 min with a final extension step of 10 min at 72 C. The amplified PCR products were analyzed in 0.7% agarose gel by using TBE (Tris-borate-EDTA) (Bio basic inc.,

Markham, ON, Canada) as running buffer in electrophoresis (Power Pac™ Basic, Biorad, USA) at 80 volt for 30 min. The gel was stained with ethidium bromide at concentration 0.1125 % for 20 min and visualized under UV light.

2. *Avibacterium paragallinarum* classification

2.1 Serotyping

All isolates were typed according to the Page scheme (1962) using a hemagglutination-inhibition (HI) method with specific antisera as previously described with some adaptations (Eaves et al., 1989).

2.1.1 The antigen preparation was started by growing each isolated bacteria on GC agar and then harvested, washed with PBS by centrifugation at 13,000 x g for 2 min and treated with potassium thiocyanate-saline solution (0.5 M KSCN + 0.425 M NaCl; pH 6.3) (Sigma-aldrich, MO, USA) by resuspending the bacteria in KSCN-NaCl solution at a 5 McFarland turbidity and stirred at 4 C for 2 hr. Then, the bacterial suspension from above were sonicated for 4 min 40 sec (program: pulse on 40 off 10, Ampl 40%) for 2 times (Vibra Cell™, Sonics). The sonicated bacteria were harvested, washed for 3 times with PBS and resuspended in PBS containing 0.01% (v/v) thimerosal and kept at 4 C for 4 days before examination.

2.1.2 The HI test was done by diluting specific antiserum in round bottomed microtitre trays in a doubling dilution series using BSA-PBS (0.1% bovine serum albumin in PBS) to give final dilutions of 1/2-1/512 (each well contained 50 µl of each diluted serum). The antigen (50 µl adjusted to contain 4 HA units) was added to each well. The mixture was shaken well and left to stand for 20 min at room temperature. Lastly, 50 µl of 0.5% (v/v) GA-fixed chicken erythrocyte was added and shaken well. The mixture was left to stand for 30 min at room temperature before reading.

2.1.3 The maximum serum dilution completely inhibiting hemagglutination was regarded as the HI titer. The isolate was assigned to the serovar corresponding to the antiserum that gave the highest titer.

2.1.4 *Av. paragallinarum* strains 221, 0222 and Modesto were used as antigen control for serovar A, B and C, respectively.

2.2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE was used to separate the proteins according to their electrophoretic mobility. The protocol was followed as previously described (Blackall and Yamamoto, 1989) with some adaptations.

2.2.1 Each isolate of *Av. paragallinarum* were growth overnight in CMI broth and the bacteria were collected by centrifugation at 5000 x g for 20 min and then washed with NSS (normal saline solution) for 3 times.

2.2.2 SDS-PAGE was performed in 12% (w/v) acrylamide gel with 4% (w/v) stacking gels and approximately 100 µg of protein was loaded per well. The protocol was followed as previously described (Laemmli, 1970). The composition of separating gel and stacking gel were prepared as below.

Separating gel (12 % gel)

Solution	Volume (ml)
distilled water	3.345
30% acrylamide mix (Bio-rad, PA,USA)	4
1.5M Tris (pH 8.8)	2.5
10% SDS	0.1
10% Ammonium persulphate	0.05
TEMED (Bio-rad, PA,USA)	0.005
Total volume	10

Stacking gel (4% gel)

Solution	Volume (ml)
distilled water	3
30% acrylamide mix (Bio-rad, PA,USA)	0.66
0.5M Tris (pH 6.8)	1.26
10% SDS	0.05
10% Ammonium persulphate	0.025
TEMED (Bio-rad, PA,USA)	0.005
Total volume	5

2.2.3 The solution for separating gel were gently mixed, poured and allowed to polymerize in a gel caster. After that the stacking gels were prepared and a comb was placed to create the wells. The comb was removed when the gel was polymerized.

2.2.4 The loading samples were prepared by mixing 10 µl of sample with 10 µl of loading buffer and then heated on AccuBlock™ Digital Dry Bath (Labnet international, Inc., USA) at 100 C for 10 min. After that the standard marker (Prestained SDS-PAGE Standards, Broad Range; Bio-Rad, PA, USA) was loaded 5 µl in the first well and 10 µl of the loading samples were loaded.

2.2.5 Electrophoresis was performed at a constant voltage (120 V) in the Protean II electrophoresis cell (BioRad Laboratories, Richmond, CA) for 50 min. The separated proteins were visualized by staining with 0.25% Coomassie Brilliant Blue R-250 for 15 min and destaining with destain solution 2-3 times.

2.3 The molecular characterization by using 16S Ribosomal RNA Restriction Analysis

PCR Amplification

The eleven field isolates were used in this study. They were included with 4 isolates of serovar A, 4 isolates of serovar B and 3 isolates of serovar C. The 16S rRNA gene sequencing was performed as described previously (Blackall et al., 2005) with some modifications. The primers that used for amplified the full length of 16S rRNA were followed.

27F: 5' GAG TTT GAT CCT GGC TCA G 3'

1525R : 5' AAG GAG GTG (AT)TC CA(AG) CC 3'

The reaction mixture was composed of 50 µl 2x Gotaq® green master mix (Promega, Madison, WI, USA), 29 µl of DNase free water, 8 µl (10 pmol/ml) of each primer and 5 µl of DNA template. The PCR was conducted by heating at 94 C for 5 min and 35 cycles of denaturation at 94 C for 1 min, annealing at 52 C for 1 min and extension at 72 C for 1 min with a final extension step of 10 min at 72 C. All 100 µl of amplified PCR products were analyzed in 1 % agarose gel by using TBE (Tris-borate-EDTA) (Biobasic inc., Markham, ON, Canada) as running buffer in electrophoresis (Power Pac™ Basic, Biorad, USA) at 100 volt for 50 min. The gel was stained with

ethidium bromide at concentration 0.5 µg/ml for 20 min and visualized under UV light. The product was approximately 1500 base pairs.

PCR product purification

The product was purified by cutting from the gel and then using the Wizard SV Gel and PCR Clean-Up system (Promega, Madison, WI, USA) as the protocols suggested by the manufacturer. The purified products were sequenced in both of the forward and the reverse direction by commercial services (First Base, Selangor, Malaysia). The sequence specific primers which used for sequence analysis were showed as below.

27F: 5' GAG TTT GAT CCT GGC TCA G 3'

519R: 5' GWA TTA CCG CGG CKG CTG 3'

530F: 5' GTG CCA GCM GCC GCG G 3'

787R: 5' CTA CCA GGG TAT CTA AT 3'

907R: 5' CCG TCA ATT CMT TTR AGT TT 3'

926F: 5' AAA CTY AAA KGA ATT GAC GG 3'

1114F: 5' GCA ACG AGC GCA ACC C 3'

1392R: 5' ACG GGC GGT GTG TRC 3'

1525R: 5' AAG GAG GTG WTC CAR CC 3'

Sequence and phylogenetic analysis

The partial nucleotide sequences of the 16S rRNA gene of 11 Thai *Av. paragallinarum* field isolates were assembled, aligned and compared with published reference strains *Av. paragallinarum* sequences deposited in the GenBank database. The first time, they were compared with published 16S rRNA of *Av. paragallinarum* sequences deposited in the GenBank database using a BLAST search via the National Center of Biotechnology Information. Sequence identities by BLAST analysis were included in the alignment and phylogenetic construction. The multiple sequence alignments and determination of the nucleotide and amino acid identities were performed using BioEdit version 7.0.5.2 (Hall, 1999). A phylogenetic tree of the nucleotide sequences was constructed with the neighbor-joining method using MEGA version 4 (Tamura et al., 2007). The bootstrap values were determined from 1000 replicates of the original data. The 16S rRNA gene sequences of the 11 *Av. paragallinarum* isolates were submitted to the GenBank database. The other 16S rRNA

gene sequences from the GenBank database which were used for comparison or phylogenetic analysis in this study as below.

- AvP strain HP105 (Australia)
- AvP strain SA7177 (South Africa)
- AvP strain Modesto serovar C (USA)
- AvP strain HP107 (Australia)
- AvP strain SCVY-1 (China)
- AvP strain 0083 serovar A (USA)
- AvP strain 0222 serovar B (USA)
- AvP strain NCTC 11296 serovar A (German)
- AvP strain NCTC 11296T serovar A (German)

3. Virulence study of field isolates *Av. paragallinarum*

3.1 Cell culture invasion assay

Chicken fibroblast (CFB) cells were used for an invasion assay by a previously described method (Al-haj Ali et al., 2004) with some modifications.

3.1.1 CFB was prepared from 9 to 10 days old specific antibody negative, embryonated chicken eggs (Kasetsart University, Bangkok, Thailand) by strictly sterile technique. The embryonated egg was sprayed with 70% ethanol. The shell was opened and the embryo was removed. The embryos were placed in petri dish containing PBS and then head, limbs and viscera organ were removed. The bodies were transfer to new beaker and were chopped with sterile scissors. After that sterile PBS was added and left to stand until the tissues went down to the bottom, then the supernatant was removed. The tissues were washed with 0.25% trypsin, left to stand about 1-2 min and the supernatant was discarded. The cells were trypsinized by adding warm 0.25% trypsin and gently stirred for 10 min, left to stand and the supernatant was collected into centrifuge tube with fetal bovine serum (FBS), this step was done twice. The second supernatant was collected, the cells were filtered through sterile gauze and cells were centrifuged at 1,500 x g for 10 min at room temp., washed twice with PBS. The cells were counted and resuspended.

3.1.2 The cells were suspended in Minimum essential medium (MEM) (Gibco, Auckland, New Zealand) and the number of cells was adjusted to 1×10^6 cells/ml. After that, the cells were cultured in 24 well-plate with MEM supplemented with 50 µg/ml of gentamicin and 5% FBS under 5% CO_2 at 37 C for 24 hr. After incubation, the cell monolayer was washed 3 times with sterile PBS.

3.1.3 Each bacterial isolate was growth overnight on TMB at 37 C and diluted with MEM without antibiotic and serum to give a final concentration to 10^8 CFU/ml. The mixture was added for 1 ml per well. The test was done in duplicate plates. The first plate was incubated for 3 hr and the other plate was incubated for 6 hr. After incubation in a 5% CO_2 incubator at 37 C for 3 or 6 hr, the medium was removed for the nitric oxide production assay (see below).

3.1.4 After end of the incubation time, the cells were washed with PBS for 3 times and 1 ml of MEM with 200 µg/ml of gentamicin was added to kill extracellular bacteria. After 1 hr incubation, the medium was removed and the cells were washed 3 times with PBS. Sodium deoxycholate (SDC) (Sigma-aldrich, MO, USA) (1 ml of a 0.1% dilution in nutrient broth) was added to each well and left for 1 hr (at 37 C) to lyse the CFB cells.

3.1.5 The number of intracellular *Av. paragallinarum* was then counted by preparing 10-fold serial dilutions and spread plating onto TM/SN agar. The samples were duplicated and incubated under 5% CO_2 at 37 C for 24 hr. This experiment was repeated 3 times, the average results were recorded as percentage of bacteria invasion.

3.1.6 The effect of 0.1% SDC to the survival of *Av. paragallinarum* was tested by incubating the bacteria with nutrient broth (NB) containing 0.1% SDC for 1 hr, 3 hr and 6 hr. After end of the incubation time, total plate counted was done as described above. The number of bacterial counts from incubation time at 1, 3 and 6 hr were nearly that mean there was no effect from 0.1% SDC to the survival of the bacteria.

3.2 Macrophage phagocytosis

Chicken macrophage cell line (HD 11) developed by a viral transformation with myelocytomatosis virus (MC-29) (Beug et al., 1979), was used for this experiment. The entry and survival assay was performed as described previously (Kramer et al., 2003) with some modifications.

3.2.1 HD 11 cells were cultured in RPMI 1640 (Gibco, Auckland, NewZealand) supplemented with 10% FBS and 10 µM 2-mercaptoethanol (Bio-rad, PA, USA). The number of cells was adjusted to $1x10^6$ cells/ml and cultured in 24 well culture plates under 5% CO_2 at 37 C for 48 hr. After incubation, the monolayer cells were washed 3 times with sterile PBS.

3.2.2 The bacteria which were growth as described in cell culture invasion assay were diluted with RPMI 1640 without antibiotic and serum to give a final concentration to 10^8 CFU/ml. The mixture was added for 1 ml per well and incubated in 5% CO_2 incubator at 37 C for 1 hr and then media were removed for nitric oxide production assay. The test was done in duplicate plate.

3.2.3 The plate were washed with PBS for 3 times and added with 1 ml of RPMI 1640 with 200 µg/ml, 1 hr of gentamicin for extracellular bacteria killing while the viability of intracellular bacteria was not affected (Vaudaux and Waldvogel, 1979). To confirm that all extracellular bacteria were killed, the culture medium was taken after gentamicin treatment and plated on TM/SN agar. After incubation in the presence of the gentamicin, the media were removed and the cells were washed with PBS for 3 times to remove gentamicin.

3.2.4 One ml of RPMI 1640 medium supplemented with 5% FBS was added and the cells were incubated under 5% CO_2. At various time points (1, 4, 12, 24 and 48 hr post-inoculation), the number of intracellular *Av. paragallinarum* were counted by lysis of the HD 11 cells with 0.1% SDC in NB and spread plating of 10-fold dilutions in PBS onto TM/SN agar in duplicate. This experiment was repeated 3 times, the average results were recorded as CFU/ml.

3.2.5 Another experiment was performed as above; exception that the bacteria were incubated with the HD 11 cells for 3 hr and without re-incubation. The cells were washed with gentamicin and lysed for total plate count immediately after the 3 hr incubation.

3.3 Nitric Oxide (NO) production assay

The Griess reaction system was used for NO production measurement as previous described in the technical bulletin for Griess reagent system (Promega, Madison, WI, USA) with some modifications. After incubation, 300 µl of media was removed from each well and placed into 24 well culture plates. An equal volume of 1% sulfanilamide (Sigma, MO, USA) in 5% phosphoric acid (Merck, Damstadt, Germany) was added, the plates were incubated in dark place for 10 min at room temperature. After that 300 µl of 0.1% naphthylenediamine (Sigma, MO, USA) was added and left to stand at room temperature for 10 min. The purple/magenta color was formed

immediately and the samples were measured at 550 nm absorbance using GENESYS 20 visible spectrophotometer (Thermo Fisher Scientific Inc., USA) within 30 min. The results were calculated as molar amounts of nitrite by comparison with a standard curve using sodium nitrite (Merk, Damstadt, Germany). The preparation of nitrite standard reference curve was followed as explained in the technical bulletin for Griess reagent system (Promega, Madison, WI, USA).

3.4 Pathogenicity test using experimental chickens

The pathogenicity test was done in experimental chickens for observation and evaluation of the clinical signs and mortality of difference fields isolates of *Av. paragallinarum*.

3.4.1 Each isolate was taken from storage, thawed and inoculated into the yolk sac of 6 to 7 days old chicken embryonic eggs to enhance pathogenicity. After 24 hr, the infected yolk sac was harvested and cultured in TMB broth for 18 hr in a 37 C shaking incubator.

3.4.2 The 4 weeks old, female-layer chickens, Babcock 308, obtained from a commercial hatchery were divided into 19 groups of 10 each in separated room with own ventilation system. They were divided into 2 experiments, first was done with 10 groups of serovar A isolates, second was done with 6 groups of serovar B isolates and 3 groups of serovar C isolates. The negative control group was included in each experiment. The equipment such as lab coats, gloves and boots were used separately in each room. Moreover, negative control group was used to ensure that there were no cross contamination between groups. All chickens were fed *ad lib* on commercial layer feed (Betagro, Thailand).

3.4.3 Each group was challenged with one field isolate by intranasal inoculation of 0.2 ml of the overnight TMB into both sinuses, a total of approximately $2x10^7$ colony forming units (CFU) per side. The negative control group was challenged with sterile TMB broth.

3.4.4 All the chickens were examined daily after challenge for the presence of the clinical signs of coryza such as nasal discharge and facial swelling. The scoring of clinical signs was recorded as describe previously (Soriano et al., 2004b):

0 - No sign

1 - Nasal discharge or slight facial swelling

2 - Nasal discharge or moderate facial swelling

3 – Abundant nasal discharge or several facial swelling

4 – As in 3 but including swelling of wattles

3.4.5 A test for the presence of *Av. paragallinarum* from infraorbital sinuses culture was done at 7 days after challenge. The chickens were euthanized with 150 mg/kg of barbiturate intravenously at 7 days post challenge. The left and right infraorbital sinuses were swabbed and cultured for *Av. paragallinarum* on TM/SN agar and BA which crosses streaked with *S. hyicus*. Chickens were considered infected if they showed clinical signs of coryza or *Av. paragallinarum* could be cultured.

4. Disk diffusion susceptibility test

The disk diffusion test was performed as recommended by the Clinical Laboratory Standards Institute (CLSI, 2008) with some modifications. They were used for antimicrobial susceptibility screening. The reference strain that was used as the quality control strain was the recommended *Escherichia coli* (ATCC 25922). TM/SN agar was used for this test instead of Muller Hinton agar and the inhibition zones of antimicrobial discs against *E. coli* (ATCC 25922) were performed in standard range according to CLSI manual.

4.1 A bacteria suspension from an overnight culture in TMB was prepared in PBS at a 0.5 McFarland turbidity standard and spread over the surface of TM/SN agar plates. The 24 antimicrobial disks, all from Oxoid (Basingstoke, UK) except for tylosin which was obtained from HuvePharma (Bangkok, Thailand), were placed on the agar surface using a multi-disk dispenser (Oxoid, Basingstoke, UK). After incubation under 5% CO_2 condition at 37 C for 24 hr, inhibition zones were measured with a vernier caliper and the zones were recorded in millimeters.

4.2 The susceptibility category (sensitive, intermediate or resistant) was determined by comparing the zone of inhibition with the zone diameter breakpoint as recommended by CLSI (CLSI, 2008) exceptional some antibiotics which CLSI interpretative standard criteria are not presently available. The zone diameter breakpoint of tetracycline has been used for doxycyclin and oxytetracyclin. Following CLSI recommendation, the zone diameter breakpoint of clindamycin and oxacillin has been

used for lincomycin and cloxacillin, respectively. The others were interpreted according to the company recommended (HuvePharma and Oxoid). The lists of antibiotic disks that used in this study and zone diameter interpretive standards are shown in Table3-1.

Table. 3-1 Antimicrobial lists and zone diameter interpretive standards.

Antimicrobial agent	Disk content	Zone Diameter (mm)		
		S	I	R
Amoxycillin[A]	25 µg	≥18	14-17	≤13
Amoxy-clavulanic acid[B]	20+10 µg	≥ 18	14-17	≤ 13
Ampicillin[B]	10 µg	≥ 17	14-16	≤ 13
Cefotaxime[A]	30 µg	≥ 23	15-22	≤ 14
Ceftiofur[B]	30 µg	≥ 21	18-20	≤ 17
Ciprofloxacin[A]	5 µg	≥ 21	16-20	≤ 15
Cloxacillin[B]	5 µg	≥ 13	11-12	≤ 10
Doxycyclin[B]	30 µg	≥19	15-18	≤ 14
Enrofloxacin[B]	5 µg	≥ 23	17-22	≤ 16
Erythromycin[B]	15 µg	≥ 23	14-22	≤ 13
Fosfomycin[A]	50 µg	≥ 18	12-17	≤ 11
Furazolidone[A]	15 µg	≥ 19	15-18	≤ 14
Gentamicin[B]	10 µg	≥ 16	13-15	≤ 12
Lincomycin[B]	2 µg	≥ 21	15-20	≤ 14
Lincospectin[A]	10 µg	≥ 20	17-19	≤ 16
Nalidixic acid[A]	30 µg	≥ 19	14-18	≤ 13

Neomycin[B]	30 µg	≥ 17	13-16	≤ 12
Norfloxacin[A]	10 µg	≥ 17	13-16	≤ 12
Oxytetracyclin[B]	30 µg	≥19	15-18	≤ 14
Penicillin[A]	10 units	≥ 22	12-21	≤ 11
Sulfamethoxazole-trimethroprim[B]	23.75+1.25 µg	≥ 16	11-15	≤ 10
Spectinomycin[B]	100 µg	≥ 14	11-13	≤ 10
Streptomycin[B]	10 µg	≥ 15	12-14	≤ 11
Tylosin[C]	150 µg	≥ 26	23-25	≤ 22

[A] zone diameter breakpoint as recommended by Oxoid company (Basingstoke, UK)

[B] zone diameter breakpoint as recommended by CLSI (2008)

[C] zone diameter breakpoint as recommended by HuvePharma company (Bangkok, Thailand)

5. Statistical analysis

The clinical sign scores were analyzed by using the Kruskal Wallis test while the Wilcoxon test was used for pair-wise comparison between challenge groups.

Part 2: The study of capsule, lipopolysaccharide and outer membrane protein of *Avibacterium paragallinarum*

1. Bacteria

The most virulence isolates of 3 field *Av. paragalinarum* that were used for study of capsule, lipopolysaccharide and outer membrane protein, were selected from each serovar. They were inoculated into the yolk sac of 6 to 7 days old chicken embryonic eggs to enhance pathogenicity. After 24 hr, egg yolk was collected and growth on GC agar and then incubated overnight at 37 C. The bacteria from GC agar were inoculated into CMI broth and incubated in 37 C shaking incubator for 18 – 24 hr. The whole bacteria that used for extraction were collected by centrifugation at 5000 x g for 20 min and washed with PBS for 3 times.

2. Extraction

2.1 Capsule identification and extraction

The capsule identified using india ink capsule stain was performed as described previously (Breakwell et al., 2009). The isolates that presented the capsule were extracted by using NaCl extraction as described previously (Rosendal et al., 1986). Briefly, the whole bacteria from 2 liters CMI broth were harvested and washed with PBS for 2 times using centrifugation at 10000 x g for 10 min. From 2 liters of CMI broth could harvest 0.1 g of bacterial pellet. The bacterial pellets were resuspended in 40 ml of 2.5% NaCl and agitated at 56 C for 1 hr and then centrifuged at 17000 x g for 20 min. The supernatant was dialyzed against 0.85% NaCl solution containing 0.01% thimerosal for 48 hr. The capsule extracts were harvested by centrifugation at 17000 x g for 30 min at room temperature. After weighted, the capsules were dissolved in 0.85% NaCl solution.

2.2 Lipopolysaccharide (LPS) extraction

The whole bacteria were prepared as above in capsule extraction protocol. The lipopolysaccharide was extracted by using LPS extraction kit (iNtRON Biotechnology Inc., Kyungki-Do, Korea) as the protocols suggested by the manufacturer. The LPS extracts were dissolved in 10mM Tris-HCl buffer (pH 8.0) with 2 min boiling and were kept at 4 C until used.

2.3 Outer membrane protein (OMP) extraction

The whole bacteria were prepared as above in capsule extraction protocol. The outer membrane protein was extracted as previously described (Blackall et al., 1990b).

2.3.1 The bacteria were resuspended in 1 ml of cold (4 C) 10 mM HEPES (*N*-2-hydroxyethylpiperzine-*N'*-2-ethanesulfonic acid; pH 7.4) and were sonicated (program: pluse on 40 off 10, Ampl 40%) with an ultrasonic cell disruptor (Vibra Cell™, Sonics). The debris and unbroken cells were removed by centrifugation at 14000 x g, 4 C for 2 min and the supernatant were removed and centrifuged again for 30 min at 4 C.

2.3.2 The gel-like pellet was resuspended in 200 µl 10 mM HEPES and 200 µl of 10mM HEPES containing 2% (v/v) Triton X-100 was added. The suspension was left to stand for 30 min at room temperature with occasional shaking. After that, the suspension was centrifuged at 14000 x g, 4 C for 30 min and washed without resuspension in 500 µl 10 mM HEPES and the pellet was resuspened in 10 mM HEPES. The OMP extraction was kept at -20 C until used.

3. Hemagglutination (HA) test

Each 10 mg/ml of capsule, LPS or OMP extractions were used for HA test. The test was started with dilution the antigen extraction in round bottomed microtitre trays in a doubling dilution series using BSA-PBS (0.1% bovine serum albumin in PBS) to give final dilutions of 1/2-1/512 (each well contained 50 µl of each diluted serum). Then 50 µl of 0.5% (v/v) GA-fixed chicken erythrocyte was added and shaken well. The mixture was left to stand for 30 min at room temperature before reading. The minimum antigen dilution completely showed hemagglutination was regarded as the HA titer.

4. *In ovo* and *in vitro* virulence study

The sample extracts were weighed and resuspened to give a final concentration at 10 mg/ml. Each sample was prepared 10-fold serial dilutions from 10 mg/ml to 10 µg/ml and then was used for virulence testing in embryonated egg and cell cultures.

4.1 *In ovo* study

Eleven days old chicken embryonated eggs were used for *in ovo* study of the sample extracts to observe the chicken embryo death at 4 dilutions per sample (10 mg, 1 mg, 100 µg and 10 µg). Six embryonated eggs per group were inoculated with 0.1 ml

of each sample extract per eggs via yolk sac route using 23 gauge needles. The inoculated eggs were incubated for 7 days and were observed twice daily for the chicken embryo death. The mortality of embryonated eggs of each isolate was recorded and the 50% chicken embryo lethal dose (ELD_{50}) was determined by the method of Reed and Muench (1938).

4.2 *In vitro* study

Vero (African green monkey kidney) cell lines and MDCK (Madin Darby canine kidney) cell lines were used in this study. The protocol was used as method for tissue culture infective dose 50 ($TCID_{50}$) calculations (Ahamed et al., 2004) with some adaptations.

4.2.1 Vero cell and MDCK cell were cultured in MEM (Gibco, Auckland, New Zealand) supplemented with 5% FBS and 0.11g/L sodium pyruvate and 1% L-glutamine. The number of cells was adjusted to 5 x 10^5 cells/ml and cultured in 96 well culture plates under 5% CO_2 at 37 C for 24 hr.

4.2.2 After incubation, the monolayer cells were washed 3 times with sterile PBS. One hundred and eighty microliters of MEM supplemented with 2% FBS was added per well and 20 µl of 10 mg/ml sample extract was added in first column and make a ten-fold serial dilution to the last column. The negative control were also performed by using PBS, 0.85% NaCl solution or Tris-HCl buffer (pH 8.0) as sample control upon the type of sample extracts. The plates were incubated under 5% CO_2 at 37 C for 96 hr with twice daily observed for the gradual development of cytopathic effect (CPE).

4.2.3 After 4 days of incubation, the cells were fixed with 4% formalin in PBST (PBS+0.5% tween 20) for 25 min and followed by washing with PBST for 3 times. One percent crystal violet was used for staining and the cells were washed with distilled water until excess violet was removed.

4.2.4 The cells were observed the morphological changes and destruction of the whole monolayer cells using inverted microscope and data were reported as the minimum dilution that found more than 50% whole cell destruction .

CHAPTER IV

RESULTS

Part 1: The study of Thai field isolates of *Avibacterium paragallinarum*

1. *Avibacterium paragallinarum* classification

Thai Field isolates

The bacterial isolates were isolated from chickens which were found clinical signs of upper respiratory disease. Twenty-five isolates could grow under 5% CO_2 condition and showed satellitic growth (Figure 4-1) on blood agar plate nursing with *S. hyicus*. The HPG-2 PCR analysis was used to identify *Av. paragallinarum*. The size of amplicon was approximately 500 base pairs (Figure 4-2). Eighteen isolates were positive while other 7 isolates were negative. Later, some of the negative and positive isolates were amplified by 16S rRNA primers and sequenced.

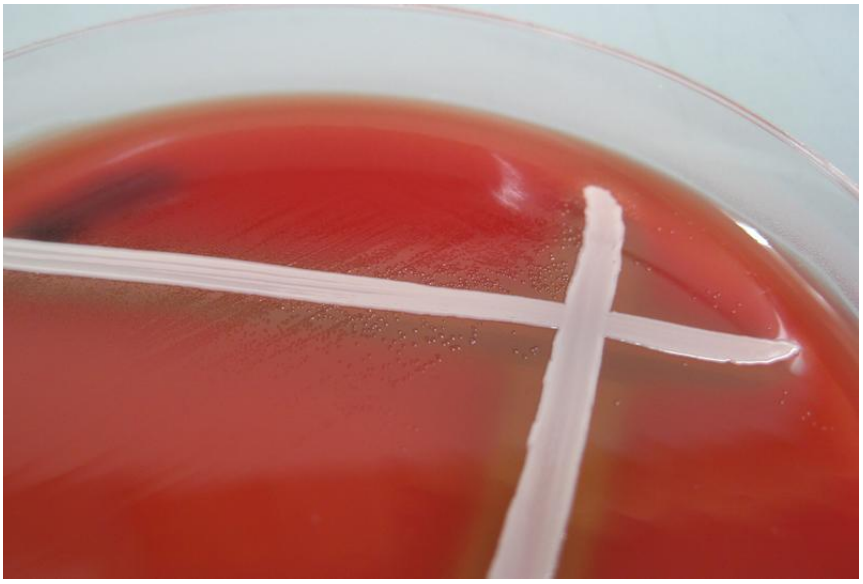

Figure 4-1 Satellitic growth of *Avibacterium* spp. on blood agar with *S. hyicus* cross streak.

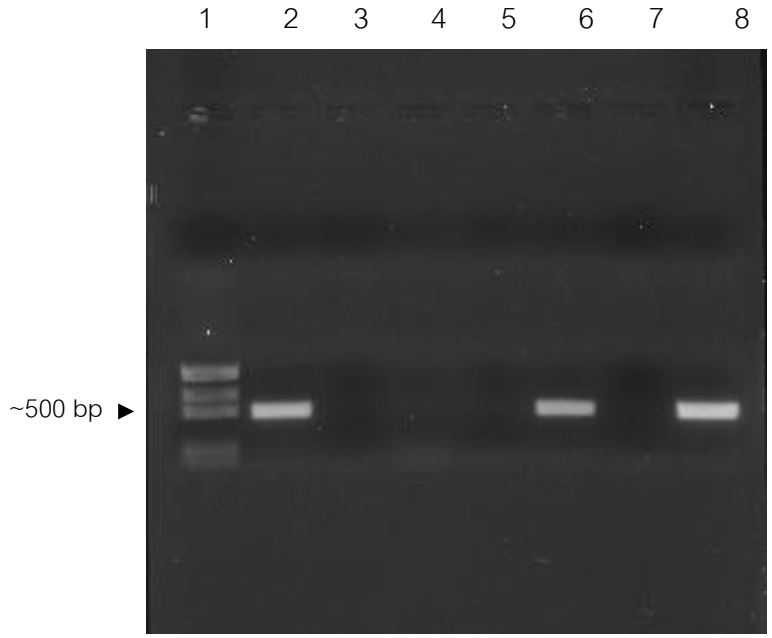

Figure 4-2 Amplification of HPG-2 for detection of *Av. paragallinarum*.
Lane1: 100-bp marker, Lane2: Positive control, Lane3: Negative control, Lane4, 5, 7: Field isolates (Non *Av. paragallinarum*), Lane6, 8: Field isolates (*Av. paragallinarum*)

Serotyping

The eighteen field isolates of *Av. paragallinarum* were serotyped by HI test with specific antiserum (Figure 4-3). Ten, five and three isolates were belonged to serovars A, B and C, respectively.

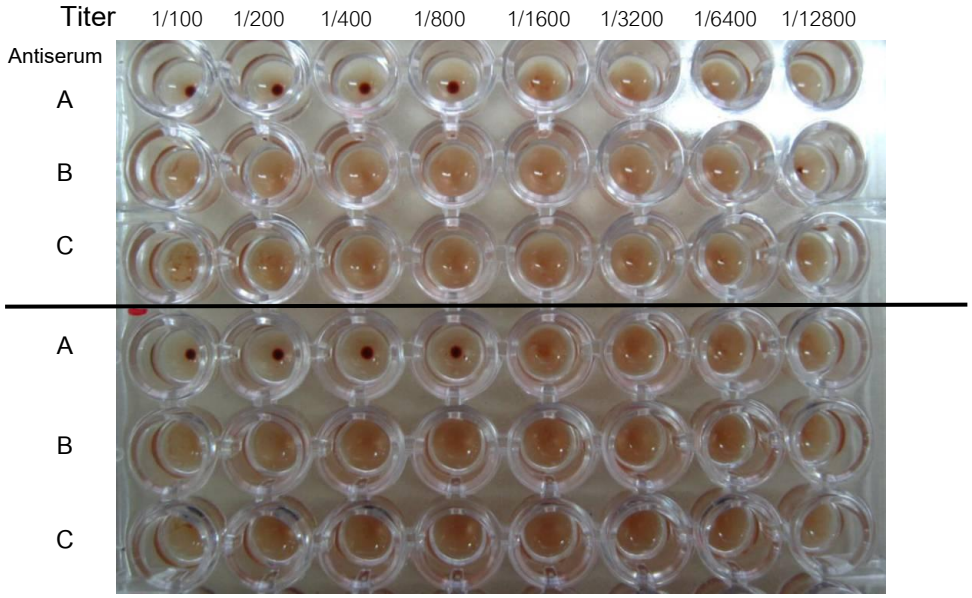

Figure 4-3 Hemagglutination inhibition test with serovar specific antiserum.
The 3 upper rows were 112179 isolate and 3 lower rows were IR1 isolate. Both of them belonged to serovar A.

SDS-PAGE

The protein profiles of 18 field *Av. paragallinarum* isolates were revealed by SDS-PAGE. The protein bands were found between 18 kDa to 121 kDa (Figure 4-4 to 4-7) and clearly found 10 bands at molecular weight approximately 18, 23, 28, 33, 38, 44, 51, 60, 103, 121 kDa. The protein profiles between serovar A, B and C were found the same clear bands and could not identify the different between serovars.

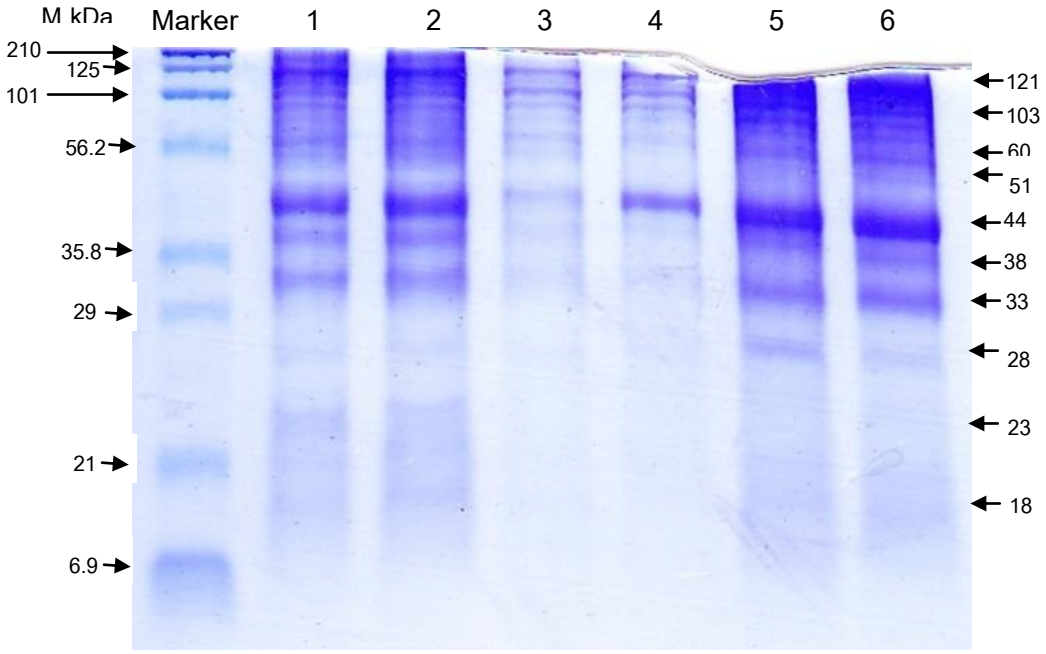

Figure 4-4 SDS-PAGE of 2 reference strains (serovar A) and 4 field isolates of *Av. paragallinarum* serovar A. Lane 1: 221 (reference serovar A), Lane 2: 083 (reference serovar A), Lane 3: 98, Lane 4: 423, Lane 5: 746, Lane 6: 102090

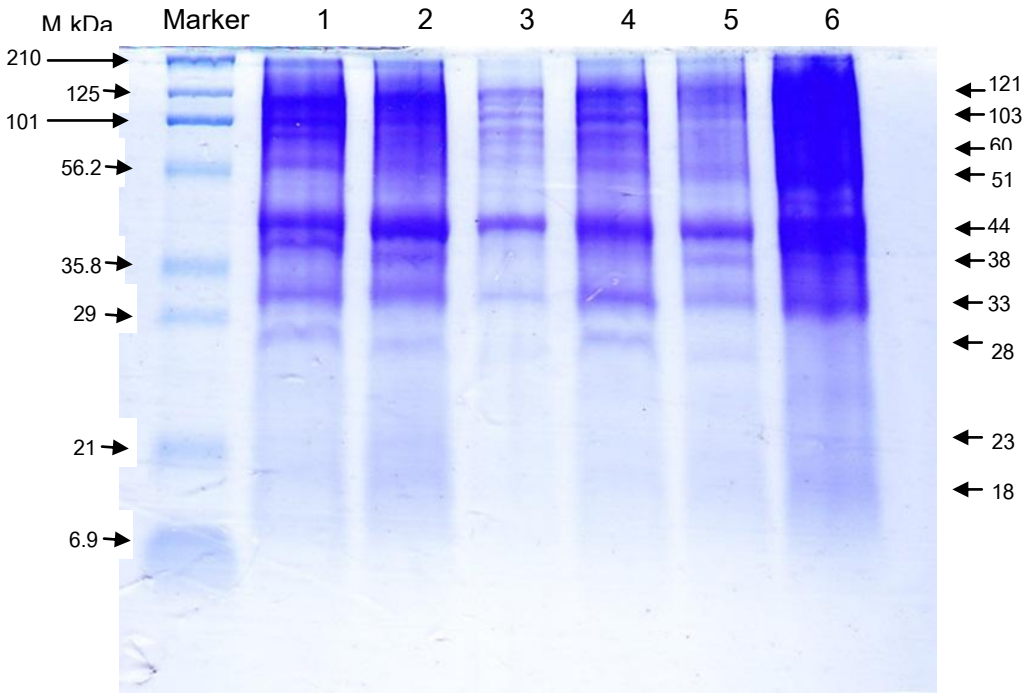

Figure 4-5 SDS-PAGE of 6 field isolates of *Av. paragallinarum* serovar A. Lane 1: 111492, Lane 2: 112179, Lane 3: B1E1, Lane 4: IR1, Lane 5: CHS0809, Lane 6: CMU1009

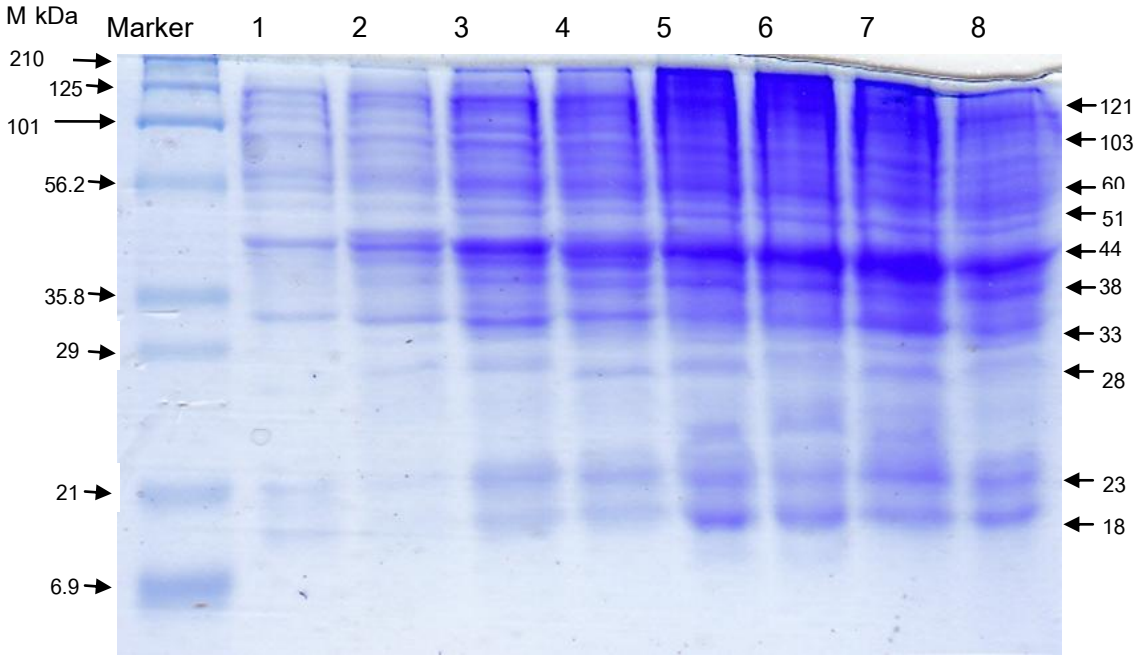

Figure 4-6 SDS-PAGE of 3 reference strains (serovarB) and 5 field isolates of *Av. paragallinarum* serovar B. Lane 1: 0222 (reference serovar B), Lane 2: Spross (reference serovar B), Lane 3: 48 (reference serovar variant-B), Lane 4: 1687, Lane 5: 102984, Lane 6: 211108, Lane 7: CMA0509, Lane 8: F1CM0809

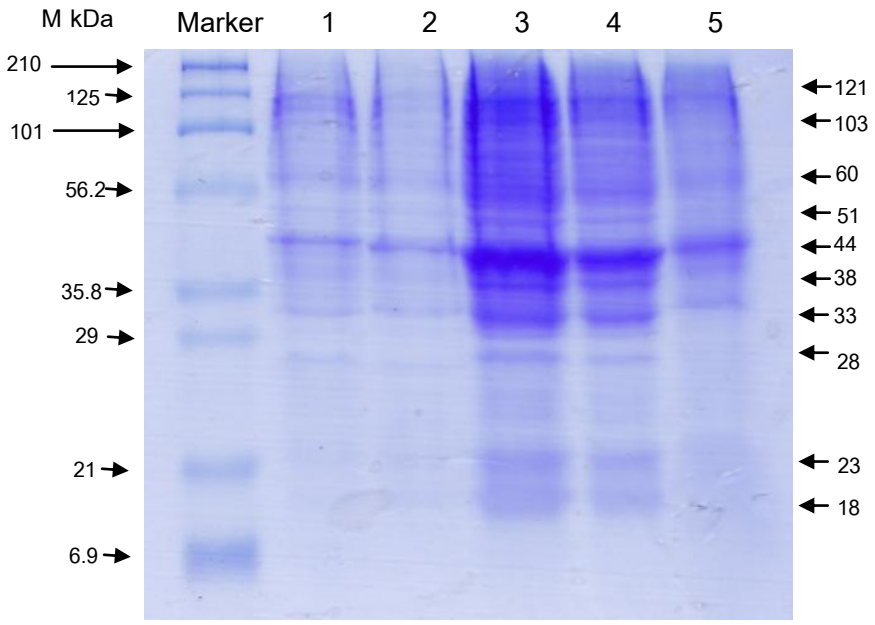

Figure 4-7 SDS-PAGE of 5 isolates of *Av. paragallinarum* serovar C

Lane 1: Modesto (reference serovar C), Lane 2: H-18 (reference serovar C), Lane 3: 102943, Lane 4: 102947, Lane 5: 115757

16S rRNA sequencing

Ten isolates of *Av. paragallinarum* and five isolates of non *Av. paragallinarum* were done 16S rRNA sequencing. The 10 isolates of PCR positive belonged to *Av. paragallinarum* by using BLAST (The Basic Local Alignment Search Tool) search in National Center for Biotechnology Information. Whereas the 5 isolates that were negative with HPG-2 primer, 3 of them were *Av. volantium* and *Av. avium.*

The identities between 16S rRNA nucleotide sequences of the 10 *Av. paragallinarum* isolated in Thailand and the strains deposited in GenBank database were performed. The results showed that they had 99-100% nucleotide identity among the Thai isolates and they had 97.7-99.5% nucleotide identity with the strains deposited in GenBank database. Phylogenetic relationships based on the nucleotide sequences of 16S rRNA were constructed (Figure 4-8). The results revealed that the ten of *Av. paragallinarum* isolated in Thailand were clustered into two distinct groups, group 1 containing 8 isolates clustered together with strain HP 105 isolated in Australia and group 2 containing 2 isolates clustered with reference strain Modesto and strain HP 107

in Australia. For Thailand field isolates, within group 1 could be found all 3 serovars (A, B and C) while group 2 found both isolates were serovar A.

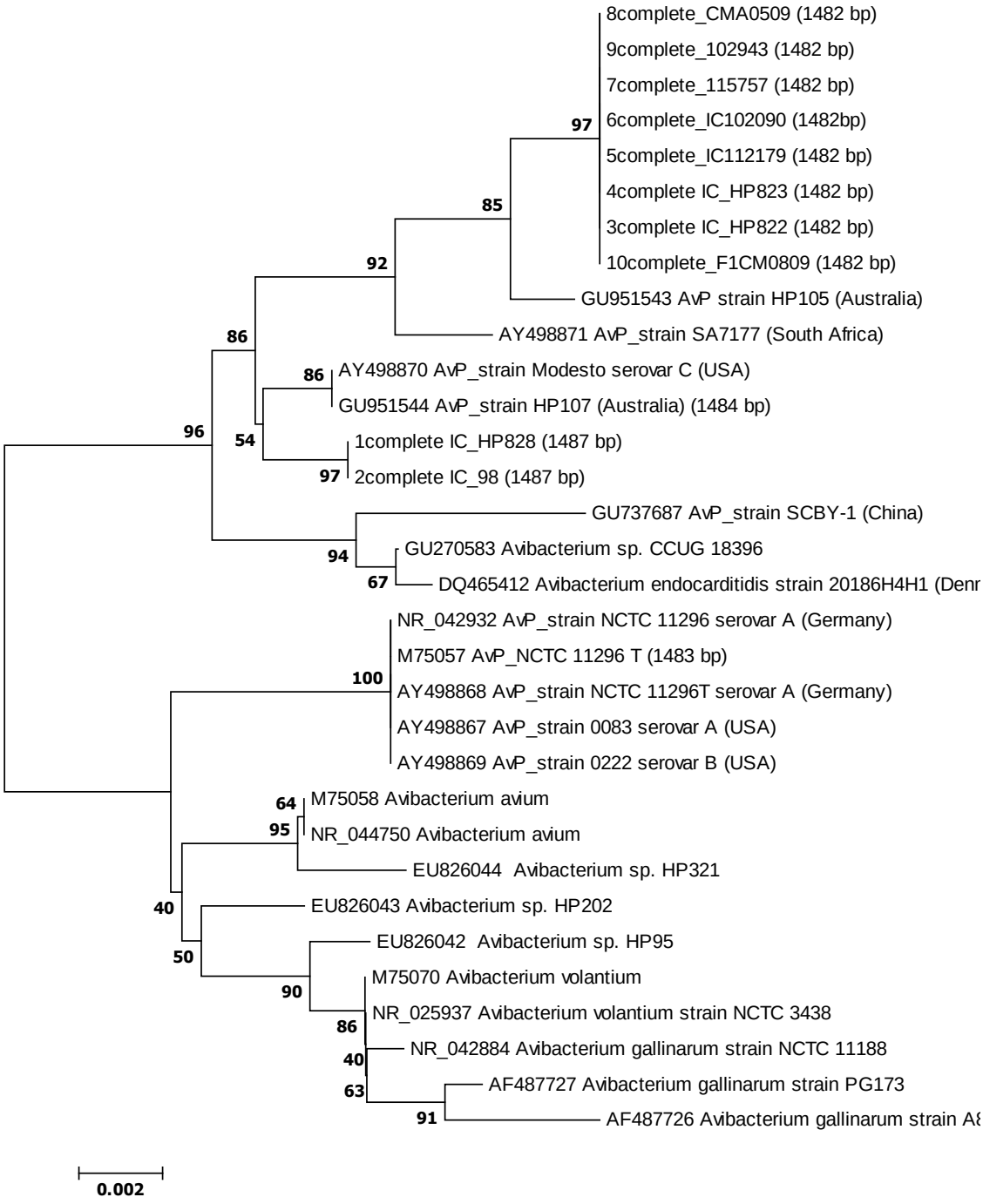

Figure 4-8 Phylogenetic tree of *Av. paragallinarum* isolated from chickens in Thailand, group1 (filled circle) and group2 (filled diamond). A tree was constructed using neighbour-joining method of MEGA4 software based on the nucleotide sequences of 16S rRNA.

2. Virulence study of field isolates *Av. paragallinarum*

Cell culture invasion assay

Chicken fibroblast cells (Figure 4-9) were used for invasion assay. All of 18 isolates could not invade chicken fibroblast cells at 3 and 6 hr after incubation. Similar results were obtained even when the incubation time was increased to 12 hr and 24 hr. After 3 and 6 hr incubation, there were not found any morphological change of infected chicken fibroblast cells when compare with negative control.

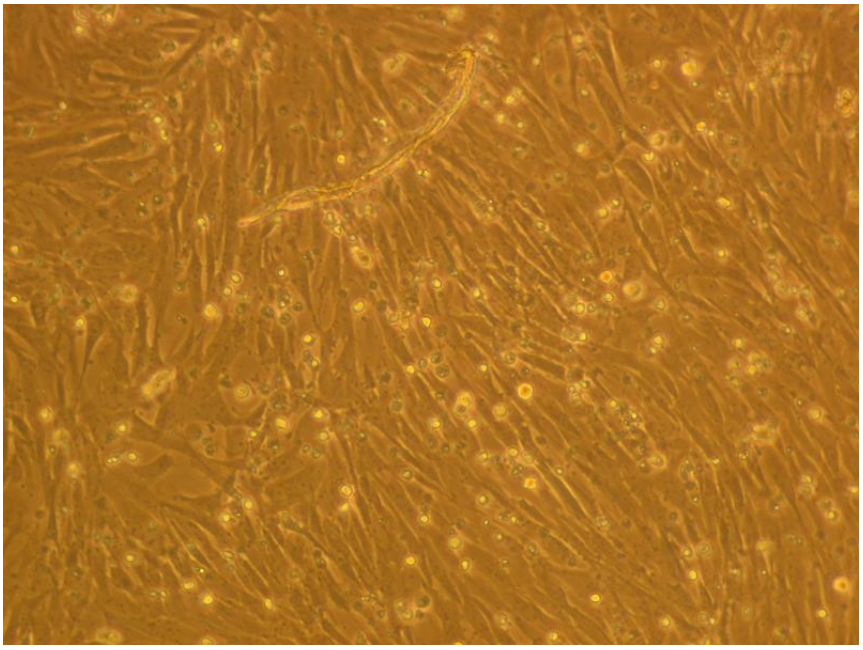

Figure 4-9 The spindle-shaped morphology of chicken fibroblast cells. After infect with *Av. paragallinarum* at 3 hr, they were no found any morphological changes.

Macrophage phagocytosis

The HD 11 cell lines (Figure 4-10) were used for survival study by infected with *Av. paragallinarum*. The survival bacteria were proved by total plate count on TM/SN agar (Figure 4-11). The differences were found among the isolates in terms of their survival inside the HD 11 cell line. A total of eight isolates could not be found inside the HD 11 cells even when the incubation time was increased to 3 hr (Table 4-1). In the 1 hr incubation experiment, the highest amount of bacteria within HD 11 cells were found at 1 hr after reincubation, with eight isolates giving counts above 100 (Table 4-1). With 4 hr re-incubation, only two isolates had counts above 100. Only one isolate was positive at the 12 hr level and then at a low level (Table 4-1). No isolates yielded a detectable viable count with 24 and 48 hr re-incubation.

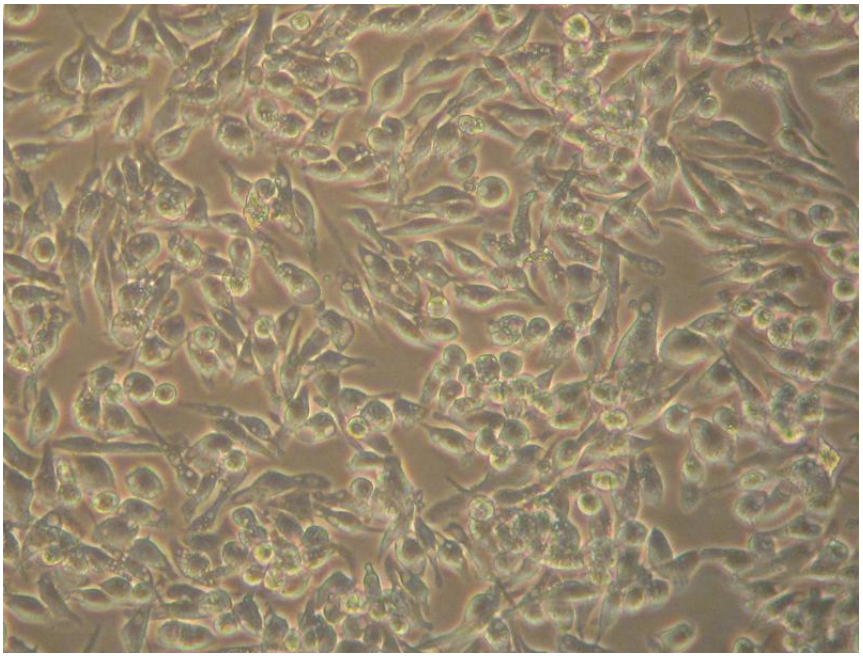

Figure 4-10 The normal morphology of HD 11 cells.

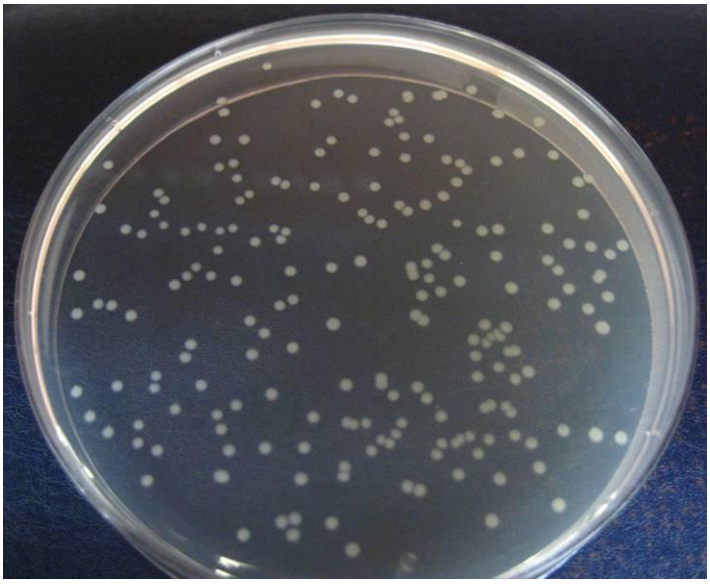

Figure 4-11 The total plate count was performed on TM/SN agar.

Table 4-1 The intracellular survival of field isolates of *Av. paragallinarum* in the HD 11 cell line and NO production

Serovar	Isolate	HD 11 Phagocytosis (cfu/ml) Incubate 3 hr	NO production (μM)	HD 11 Phagocytosis (cfu/ml) Incubate 1 hr		
				Reincubate 1 hr	Reincubate 4 hr	Reincubate 12 hr
A	98*	<10	13	<10	<10	<10
	423	<10	6	20	<10	<10
	746	<10	<1	<10	<10	<10
	102090*	$1.62x10^4$	>100	$4.2x10^3$	<10	<10
	111492	$6.2x10^2$	>100	20	<10	<10
	112179*	$3.3x10^3$	>100	$1.45x10^3$	<10	<10
	B1E1	<10	12	<10	<10	<10
	IR1*	<10	2	<10	<10	<10
	CHS0809	<10	11	<10	<10	<10
	CMU1009	<10	5	<10	<10	<10
B	1687	$2.3x10^4$	28	$3x10^2$	<10	<10
	102984	$1.11x10^5$	>100	$6.6x10^3$	$1.8x10^2$	<10
	211108*	$8.1x10^3$	>100	$1.3x10^3$	<10	<10
	CMA0509*	$6.5x10^3$	>100	20	<10	<10
	F1CM0809*	<10	2	40	10	<10
C	102943*	$2.25x10^5$	>100	$1x10^2$	<10	<10
	102947*	$1.76x10^4$	84	$4.9x10^2$	<10	<10
	115757*	$2.94x10^3$	>100	$4.3x10^3$	$3.8x10^3$	70

***** The isolates were used for 16S rRNA sequencing

NO production

Nitric oxide production assay was tested after incubation between bacteria and chicken fibroblast cells or HD 11 cell line. In the fibroblast cells, all isolates showed very low levels of NO (<1 μM). In the HD 11 cell line, there were nine isolates that gave NO levels of above 80 μM with these isolates also being those that showed higher intra-cellular survival levels (Table 4-1).

Pathogenicity test using experimental chickens

The clinical signs were scored as Figure 4-12. All field isolates of *Av. paragallinarum* caused clinical signs in the challenged chickens. A range of the clinical signs were seen including depression, ruffled feathers, prostration, ocular discharge, conjunctivitis and foamy exudates at the medial canthus of the eye (Figure 4-13), mucous or serous nasal discharge (Figure 4-13), infraorbital sinus swelling or swollen head. The clinical signs started at 2 days after challenge. Some isolates showed the highest clinical score at 2 days post challenge while some isolates revealed the highest clinical sign score at 4 days after challenge but the scores were not related to the serovar. The occurrence of swollen of infraorbital sinuses disappeared within 4-5 days after challenge while nasal discharge could be found until day 7.

From the clinical sign scores, isolate 102984 was shown the highest average score (1.5±1.18) while isolate CMU1009 was shown the lowest average score (0.6±0.52) (Table 4-2). Isolates 112179 (serovar A) and 102984 (serovar B) had significantly higher average clinical sign scores ($P < 0.05$) than the isolate CMU1009 but were not different from the other isolates. Seven days after challenge, the infraorbital sinuses were examined, almost chickens found serous or mucous nasal discharge but some chickens were found mucopurulent or caseous exudates (Figure 4-13) without contamination by observing on BA plate. The *Av. paragallinarum* was re-isolated from 90 to 100% of the chickens (Table 4-2).

Figure 4-12 The scoring of clinical signs was recorded. Score 0 – no clinical sign, (**A**) 1 - nasal discharge or slight facial swelling, (**B**) 2 - nasal discharge or moderate facial swelling, (**C**) 3 – abundant nasal discharge or several facial swelling and (**D**) 4 – as in 3 but including swelling of wattles

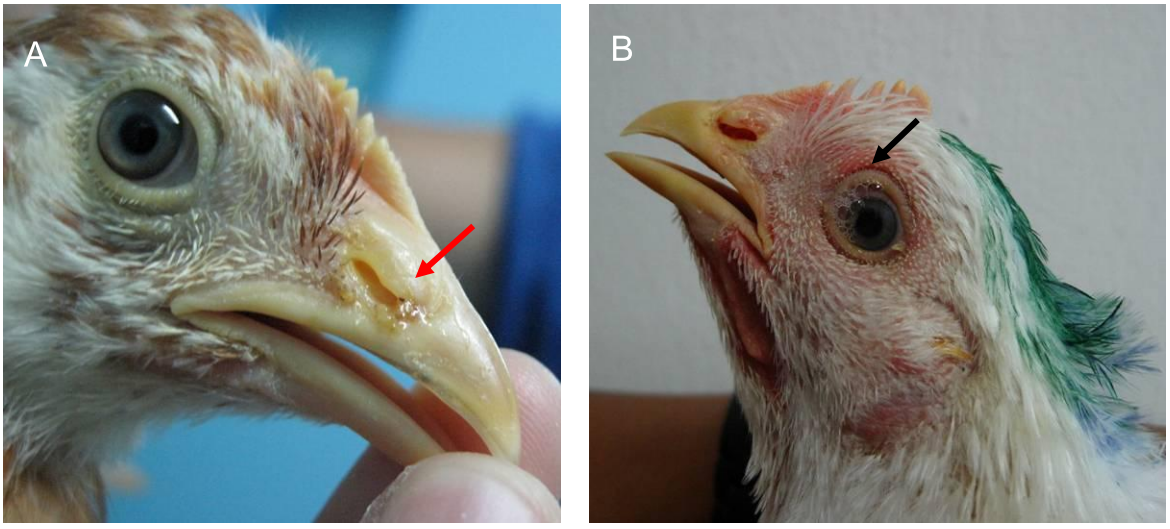

Figure 4-13 Other clinical signs. (**A**) The serous nasal discharge and (**B**) foamy exudates at the medial canthus of the eye.

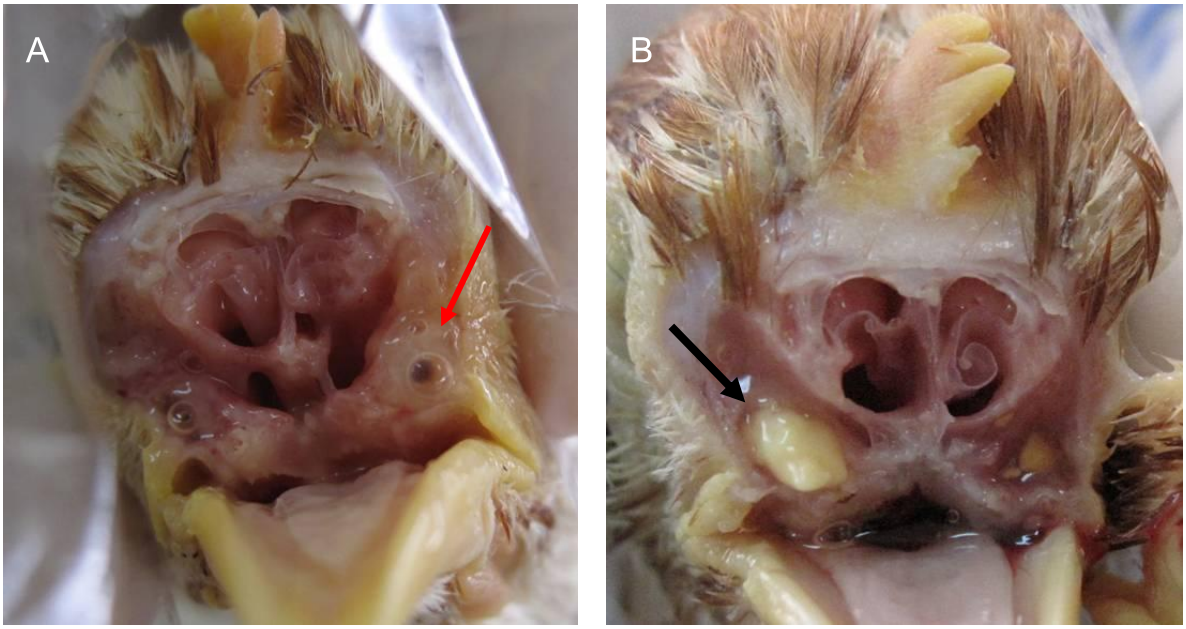

Figure 4-14 The nasal discharge might be found mucopurulent (**A**) or caseous exudate (**B**).

Table 4-2 Results of virulence tests of field isolates of *Av. paragallinarum* in experimental chickens.

Serovar	Isolate	Average clinical score[A]	Lowest score	Highest score	No. of chickens positive for indicated criterion		% infection
					Clinical signs	Re-isolation	
A	98*	0.9±0.32^{ab}	0	1	9/10	10/10	100
	423	1.0±0.00^{ab}	1	1	10/10	10/10	100
	746	1.0±0.00^{ab}	1	1	10/10	10/10	100
	102090*	0.9±0.32^{ab}	0	1	9/10	10/10	100
	111492	0.8±0.42^{ab}	0	1	8/10	10/10	100
	112179*	1.4±0.70^{a}	1	3	10/10	10/10	100
	B1E1	0.7±0.48^{ab}	0	1	7/10	10/10	100
	IR1*	0.9±0.88^{ab}	0	3	7/10	10/10	100
	CHS0809	0.7±0.48^{ab}	0	1	7/10	8/10	80
	CMU1009	0.6±0.52^{b}	0	1	6/10	9/10	90
B	1687	0.9±0.31^{ab}	0	1	9/10	10/10	100
	102984	1.5±1.18^{a}	0	4	9/10	10/10	100
	211108*	1.2±0.63^{ab}	0	2	9/10	9/10	90
	CMA0509*	1.3±0.95^{ab}	0	3	9/10	10/10	100
	F1CM0809*	1.0±0.00^{ab}	1	1	10/10	9/10	90
C	102943*	1.3±0.67^{ab}	1	3	10/10	10/10	100
	102947*	0.8±0.42^{ab}	0	1	8/10	9/10	90
	115757*	1.1±0.74^{ab}	0	3	9/10	9/10	90

[A] Clinical score in the same column with different superscript was significantly different ($P < 0.05$).

***** The isolates were used for 16S rRNA sequencing

3. Disk diffusion test

Twenty-two antimicrobial drugs were used for antimicrobial susceptibility test to 18 field isolates of *Av. paragallinarum* by disk diffusion test. All isolates were susceptible to amoxicillin-clavulanic acid and lincospectin, whereas more than 90% were susceptible to furazolidone and tylosin. The other, more than 70% of the isolates were susceptible to amoxicillin, cefotaxime, ceftiofur, fosfomycin, gentamicin, norfloxacin and spectinomycin. However, there was a high prevalence of resistance to erythromycin and streptomycin. All isolates were resistant to cloxacillin, lincomycin and neomycin (Table 4-3).

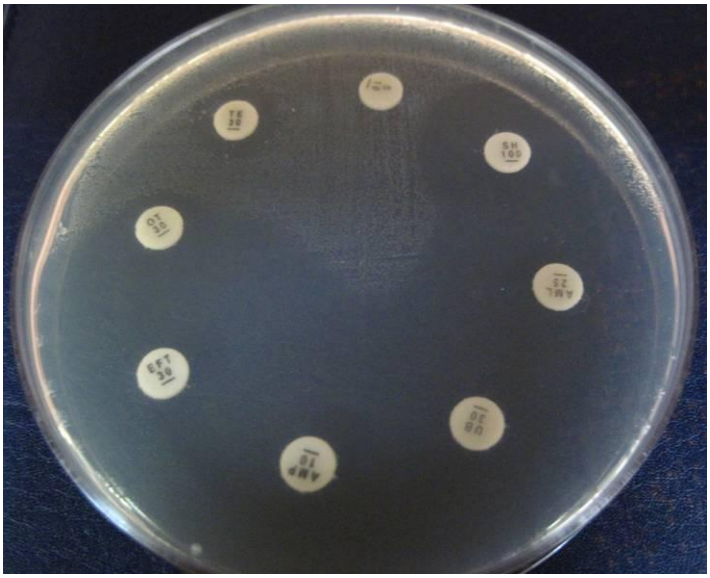

Figure 4-15 The disk diffusion test was performed on TM/SN agar. The inhibition zones were measured with a venier caliper and were recorded in millimeters.

Table 4-3 Disk diffusion test of the antimicrobials against 18 field isolates of *Av. paragallinarum* from Thailand.

Antibiotic (µg)	No of isolates classified as			% Sensitive
	Sensitive	Intermediate	Resistant	
Amoxycillin (25)	13	0	5	72
Amoxycillin-Clavulanic Acid (20+10)	18	0	0	100
Ampicillin (10)	11	1	6	61
Cefotaxime (30)	14	0	4	78
Ceftiofur (30)	13	0	5	72
Ciprofloxacin (5)	12	2	4	67
Cloxacillin (5)	0	0	18	0
Doxycyclin (30)	7	4	7	39
Enrofloxacin (5)	11	2	5	61
Erythromycin (15)	1	3	14	6
Fosfomycin (50)	14	0	4	78
Furazolidone (15)	17	0	1	94
Gentamicin (10)	15	2	1	83
Lincomycin (2)	0	0	18	0
Lincospectin (10)	18	0	0	100
Nalidixic acid (30)	7	1	10	39

Neomycin (30)	0	0	18	0
Norfloxacin (10)	13	1	4	72
Oxytetracyclin (30)	7	1	10	39
Penicillin (10)	8	5	5	44
Sulfamethoxazole-trimethroprim (23.75+1.25)	6	0	12	33
Spectinomycin (100)	16	0	2	89
Streptomycin (10)	1	1	16	6
Tylosin (150)	17	1	0	94

Part 2: The study of capsule, lipopolysaccharide and outer membrane protein of *Av. paragallinarum*

1. The sample extraction

Capsule extraction

All of three field isolates were found capsule by using India ink stain and observed the clear zone around bacteria cells. The capsule was extracted by NaCl extraction and % yield of extraction were approximately 11–14% of whole bacteria. The appearance of capsule extracts was gel-like pellet and was dissolved in 0.85% NaCl solution before use.

Lipopolysaccharide extraction

The LPS was extracted using LPS extraction kit from iNtRON Biotechnology Inc. (Kyungki-Do, Korea) and % yield of extraction were approximately 11 – 17% from whole bacteria. The appearance of LPS extracts was gel-like pellet and could be completely dissolved in Tris-HCl buffer (pH 8.0) with boiling.

Outer membrane protein extraction

Percent yield of OMP extraction were approximately 21 - 25 % from whole bacteria. The appearance of OMP extracts was solid pellet and was colloidal suspension when resuspended in 10mM HEPES.

Table 4-4 % yield of capsule, LPS and OMP extraction of *Av. paragallinarum*.

Extraction	% yield (approximately)
Capsule	
112179	12.45
CMA0509	13.64
115757	11.22
Lipopolysaccharide	
112179	11.37
CMA0509	17.88
115757	13.21
Outer membrane protein	
112179	21.20
CMA0509	25.42
115757	22.80

2. Characterization

Hemagglutination test

The capsule, OMP and LPS extracts of 3 fields *Av. paragallinarum* isolates were performed hemagglutination test using GA-fixed chicken erythrocytes. The result was found that OMP extracts of all 3 field isolates were positive with hemagglutination test (Figure 4-16), whereas capsule and LPS extracts were negative.

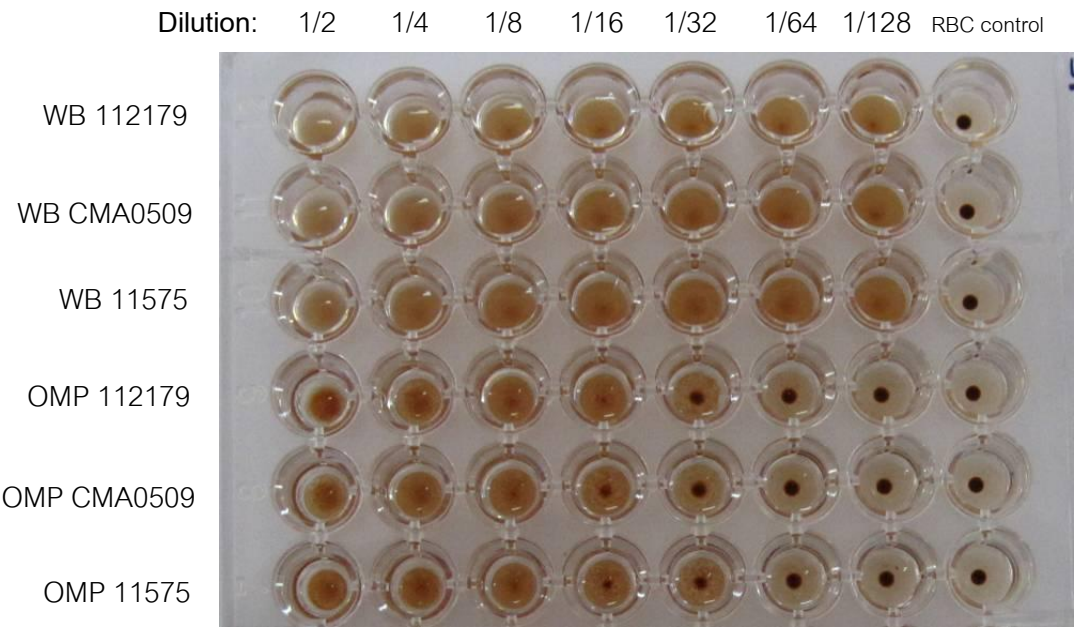

Figure 4-16 The HA test was performed and hemagglutination of RBCs were observed. The upper 3 rows were whole bacteria and lower 3 rows were OMP extracts.

3. Virulence study

***In ovo* study**

The mortality of chicken embryos by capsule, LPS or OMP extracts was performed by inoculating in 11 days old chicken embryonated eggs via yolk sac route. Most chicken embryos that were inoculated with all 3 field isolates of capsule and OMP extracts in any dilution (10 mg/ml to 10 µg/ml) were not died except OMP that extracted from 112179 at the concentration of 1 mg and 100 µg but only one egg of both dilution (data not shown), while LPS extracts of all 3 isolates could cause embryos death within 48 hr after inoculation. The highest concentration of LPS that cause the embryo death was 10 mg/ml while the lowest concentration was 10 µg/ml. Some dead embryos have been found hemorrhage on the body surface (Figure 4-17) but not severe as whole

bacteria of *Av. paragallinarum* inoculation. The 50% chicken embryo lethal dose (ELD_{50}) was shown in Table 4-5.

Table 4-5 The 50% chicken embryo lethal dose (ELD_{50}) of chicken embryos by capsule, LPS or OMP extracts from *Avibacterium paragallinarum* at various concentrations.

Isolates	50% egg lethal dose (ELD_{50})*		
	capsule	LPS	OMP
112179	> 1000 µg	316 µg	> 1000 µg
CMA0509	> 1000 µg	229 µg	> 1000 µg
115757	> 1000 µg	204 µg	> 1000 µg

* Concentration of extract substance that inoculate via yolk sac per egg.

Figure 4-17 The LPS extracts were inoculated in 11 days old embryonated eggs, the hemorrhage on body surface of some dead embryos was found.

In vitro study

The Vero and MDCK cell lines (Figure 4-18) were used for observed morphological changes and destruction of cell lines that affected from capsule, LPS and OMP extracts. The result found that all sample extracts at of all 3 isolates in any concentration did not cause morphological changed and destruction of both cell lines when 96 hr incubation ends. In addition, there were still not found difference with negative control when the incubation time was extended (7 days).

To clarify the result from above, the whole bacteria of all 3 isolates were performed invasion assay test by using both Vero and MDCK cell lines. The protocol was described as above in the cell culture invasion assay part. The results were the same as using chicken fibroblast cells that all 3 isolates could not invaded Vero cell line and MDCK cell line after end of incubation time (3 and 6 hr).

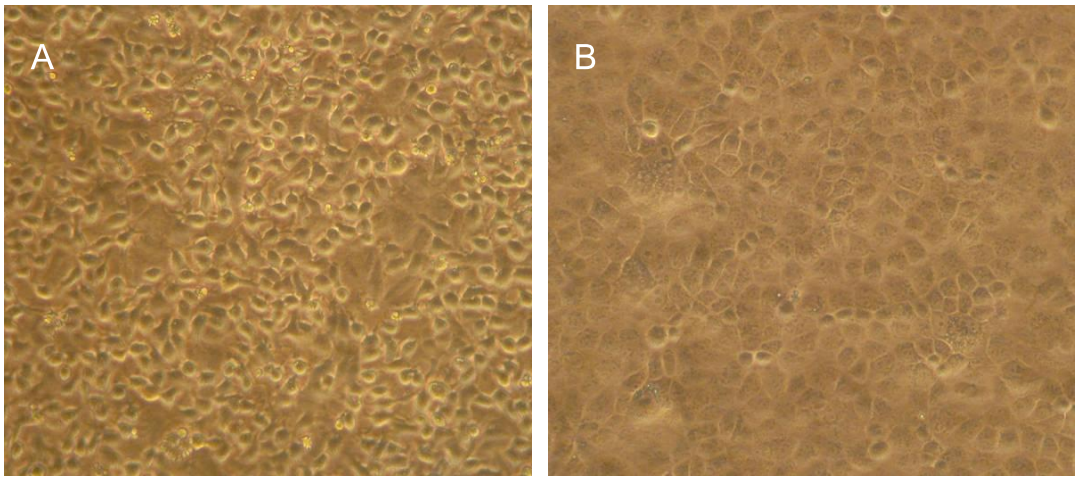

Figure 4-18 The typical elongated shape of fibroblast-like cells morphology of vero cell line (**A**). And the normal polygonal shape of MDCK cell line (**B**).

CHAPTER V

DISCUSSIONS AND CONCLUSIONS

In Thailand, the previous reports of infectious coryza outbreaks due to serovars A, B and C have been reported (Neramitmansuk and Neramitmansuk, 1985; Chukiatsiri and Chansiripornchai, 2007; Chukiatsiri et al., 2009; Chukiatsiri et al., 2010). All *Av. paragallinarum* isolates of Thailand in this study are NAD-dependent as same as in many countries except South Africa and Mexico that can be found NAD-independent strains (Mouahid et al., 1992; Garcia et al., 2004). In the current study, most of the isolates were serovar A (10 isolates) with the remaining isolates being serovar B (5) or C (3). From this study, the serovar of Thailand isolates are the same as the reports from Mexico, Ecuador, Argentina and China that mainly found serovar A (Chen et al., 1993; Terzolo et al., 1993; Sandoval et al., 1994; Soriano et al., 2001; Cabrera et al., 2010). Whereas Indonesia and Brazil have been found serovar A and C (Blackall et al., 1994; Poernomo et al., 2000). This continued presence of the three Page serovars in Thailand confirms the need for the use of vaccines that contain all three serovars unless there is very specific information available about the challenge situation on particular farms. As many of the isolates in the current study came from vaccinated flocks, it would appear that even vaccinated birds can continue to be colonized by *Av. paragallinarum.*

Protein profile of 18 field isolates and reference strains *Av. paragallinarum* by using SDS-PAGE showed that all of them in three serovars had similar profile and could not use as a tool for serovar identification. The result was similar as previous study by Blackall and Yamamoto (1989) but in contrast to studied of Neumann and Hinz (1977) that reported strains-specific profiles. The differences of media, protein preparation techniques and electrophoretic conditions may cause the differing results (Blackall and Yamamoto, 1989).

The 16S rRNA nucleotide sequence of 10 *Av. paragallinarum* isolated in Thailand was clustered into two distinct groups. Group 1 contained all 3 serovars (A, B and C) that all 3 isolates of serovar C were clustered in this group and group 2 clusterd with both serovar A. From this study, this technique could not be used as a tool for *Av. paragallinarum* serotyping. Due to, the 16S rRNA was a conserved region of the

bacteria. However, the 16S rRNA nucleotide sequence could be used to identify the species of genus Avibacterium. As our study, the non *Av. paragallinarum* isolates which showed satellitic growth on blood agar and cross streak with *S. hyicus* were sequenced and blasted. Three of them were *Av. volantium* and another two were *Av. avium*. This result was agreed to the previous study (Blackall et al., 2005; Christensen et al., 2009) which used 16S rRNA nucleotide sequence to identify *Av. paragallinarum* from other *Avibacterium* spp. but could not use as serotyping tool because they could not found the relationship of each serovar within same group. However, the 16S rRNA sequencing is a beneficial tool for identified the unknown species of any organism.

According to our study, all isolates of *Av. paragallinarum* could not invade primary cells of chicken fibroblasts even with a prolonged incubation time (24 hr). However the previous study from Ueda et al. (1982) that used CEF to study the adhesion of *Av. paragallinarum* under the scanning electron microscope. The virulence strains could adhere chicken fibroblast by attach to the plasma membrane of CEF. They were enclosed in membrane-limited vesicles and appeared morphologically intact. In fact, there are no reports about invasion of *Av. paragallinarum* to CEF. As chicken respiratory epithelial cells are the targeted cells of the natural disease, such respiratory cell types are more relevant for adherence and invasion studies. However, chicken respiratory epithelial cell lines or primary cell cultures are not available at this time.

Some isolates could survive in the chicken macrophages for up to 5 hr with one isolate able to survive for 13 hr (Table 4-1). As the incubation time increased, there was a clear reduction in the numbers of surviving bacteria. These results were similar to those reported by Kramer et al. (2003) who found an initial uptake of *S*. Enteritidis by macrophages which was then followed by a clearing of *S*. Enteritidis immediately after incubation. While only one isolate of *Av. paragallinarum* could be recovered at 13 hr incubation, *Salmonella* are still found in the macrophage cell lines 24 to 48 hr after phagocytosis (Chadfield et al., 2003; Kramer et al., 2003; Chadfield, 2004). It would appear that *Av. paragallinarum* isolates lack the ability or the capacity to survive within these macrophages. Important virulence factors known to be active in other pathogens that protect against phagocytosis (Quinn et al., 2002a; Wilson et al., 2002) would appear to be missing in *Av. paragallinarum*. Other bacteria such as *S.* Typhimurium can survive inside macrophages by developing defense mechanism against the reactive forms of

oxygen, host defensins or low pH (Rathman et al., 1996). The fact that infectious coryza is essentially a mucosal surface infection suggests that intra-cellular survival is not a key prerequisite for *Av. paragallinarum.* As there is one report that isolates of *Av. paragallinarum* have been recovered from normally sterile sites such as liver, spleen and hock joints (Sandoval et al., 1994), there is clearly the potential, in some isolates, to survive normal host defense mechanisms. Further studies, including examination of isolates known to have been recovered from internal tissues, are required.

Another factor of bacteria that related to the macrophage phagocytosis was capsule. Although capsule of the bacteria can protect the bacteria from phagocytosis but the previous study found both encapsulated highly virulent and less virulent strains of *Pasteurella multocida* could be uptake and were killed by macrophages. However, the virulent strains of *P. multocida* were more resistant to phagocytosis and phagocytic killing by chicken macrophages when compared with the less virulent strains (Poermadjaja and Frost, 2000). From our study, all field isolates could found capsule but the results of survival inside macrophage were different, may be it due to the different among capsule composition or other factors that should be further study.

The results from nitric oxide production from HD 11 cell line which were incubated with various isolates of field *Av. paragallinarum* were revealed. The isolates with higher intracellular survival also gave a higher level of NO production. Due to the nitric oxide are act as microbicidal substance that produce from macrophage among phagocytosis process. It may be the isolates that were killed rapidly by macrophages, would produce less amount of nitric oxide than the isolates that longer survive in macrophage. In addition, the level of nitric oxide from 3 hr incubation of some lived *Av. paragallinarum* isolates in this study was higher than from 72 hr incubation of formalin killed *Enterobacter cloacae*, *Klebsiella pneumonia*, *Staphylococcus chromogenes*, *Enterococcus gallinarium*, *Bacillus megaterium* that approximately 40.6 -47.9 μM (Crippen et al., 2003).

The clinical signs seen in the experimentally infected chickens were similar to the common clinical signs of infectious coryza seen in field outbreaks. No mortalities occurred in this study and % infection of all isolates was over 80%. From the average clinical sign score, isolates 102984 and 112179 which belonged to serovar B and A, respectively had significantly higher clinical sign score ($P < 0.05$) than isolate CMU1009

which belonged to serovar A. Therefore, there was no correlation between serovar and severity of the disease found in this study. Soriano et al. (2004b) examined the virulence of Kume serotyping scheme, reference strains and showed that all nine reference strains could cause clinical signs in challenged chickens and that serovar C-1 showed the highest virulence while serovar C-4 showed the lowest virulence. In contrary to Bragg (2002a), South African field isolates of serovar C-3 showed more virulent than A-1, B-1 and C-2 isolates. In the current study, some Thailand field isolates of serovar A were more virulent than serovar C isolates while others were not. While further studies are needed, it seems to have no consistent association between serovar and virulence.

The antibiotics commonly used in Thailand for treatment of infectious coryza are erythromycin, tetracycline derivatives and sulfonamides. Enrofloxacin had been used until 2008 at which time the Thai Food and Drug Administration prohibited the use of this antibiotic in food animals. Our results (>90% resistance, Table 2) suggest that the use of erythromycin to treat infectious coryza in Thailand is not advisable. In addition, the other antibiotics which are commonly used to treat coryza in Thai poultry (doxycycline, oxytetracycline and sulfamethoxazole-trimethroprim) are likely to fail as resistance was present in >60% of isolates. The selection of appropriate antimicrobial drugs, supported by sensitivity testing of isolates, will help poultry veterinarians to prescribe the most efficacious antibiotics. Furthermore, accurate doses, duration of treatment and methods of administration are also important for a successful antimicrobial treatment program (Chansiripornchai, 2009).

Our findings of high levels of resistance to neomycin, streptomycin and erythromycin (>75%) match those reported in other countries (Fernández et al., 2000a; Poernomo et al., 2000; Hsu et al., 2007a). The increasing level of antibiotic resistance against *Av. paragallinarum* within a short time period observed in Taiwan has been suggested to be due to the presence of a multi-drug resistance plasmid (Hsu et al., 2007a). Further study looking at the role of multi-drug resistance plasmids in Thai isolates of *Av. paragallinarum* is warranted.

From capsule, OMP and LPS extract study, the HA activity could be found only in outer membrane extraction for all 3 isolates. OMP extractions of this *Av. paragallinarum* contain hemagglutinin while capsule and LPS extractions cannot found HA activity. Chicken embryonated egg has been used to observe the effect of *Av.*

paragallinarum secreted proteins. The mortality of chicken embryos was observed when secreted proteins were inoculated via yolk sac (Pérez-Márquez et al., 2008). In some bacterial species, chicken embryonated egg has also been used for lethal effect study of endotoxin or LPS and a 50% chicken embryo lethal dose (ELD_{50}) were calculated. From this study LPS concentration of 204-316 µg of all 3 isolates could cause the mortality in 50% egg lethal dose (ELD_{50}). Whereas, the outer membrane protein and capsule extracts could not induce mortality of chicken embryos in most isolates and concentration. The effect of LPS extract to mortality of chicken embryos may be due to endotoxin effect of LPS (Quinn et al., 2002a; Wilson et al., 2002). The low potency of LPS of *Av. paragallinarum* in this study was seen as a quite high ELD_{50} when compare with other bacterial species. Previous study on the effect of LPS or endotoxin to chicken embryos, a 50% chicken embryo lethal dose (ELD_{50}) was difference in different bacterial species for example, *Capnocytophaga sputigena* (15.6 µg via intravenous (IV) route) (Stevens et al., 1980), *Escherichia coli* 0127-B8 (0.66 µg via chorioallantoic membrane (CAM) route), *Shigella paradysenteriae* (4.2 µg, CAM), *Serratia marcescens* (100 µg, CAM), *E. coli* (62 µg, CAM)(Smith and Thomas, 1956), *E.coli* 0111 (0.45 µg, IV) (De Azavedo et al., 1985), *Serratia marcescens* (0.008 µg, IV), *Vibrio cholera* (0.005- 0.14 µg, IV) (Finkelstein, 1964). From the previous study, different bacterial species, LPS extraction procedures, route of inoculation or age of chicken embryos were also effect to the mortality of chicken embryos (Smith and Thomas, 1956; Stevens et al., 1980). The action of endotoxin is damage to the blood vessel. Smith and Thomas (1956) was studied the lethal effect of Meningococcal endotoxin by inoculated endotoxin via CAM route. The chicken embryos were found congestion of vessels and numerous perivascular hemorrhages in almost embryos. Even the highest concentration of OMP extract from this study, no dead chicken embryos had been found. It may cause by the toxic substance in this extraction may not be enough to cause chicken embryo death. The extraction procedure may influence to the chemical composition and biological activity of the extracts. While the effect from capsule extract also could not cause the embryo death, it may be the important functions of capsule are about adhesion to host cell and protect the bacteria from phagocytosis (Quinn et al., 2002a; Wilson et al., 2002). The capsule does not play an important role as toxic substance to host cell.

All isolates of LPS, OMP and capsule extract could not cause any change in Vero and MDCK cell lines even the infected time was extended. The effect of *Av. paragallinarum* to these cell lines was clarified by incubating live bacteria with Vero or MDCK cell lines and total plate count was done at the end of incubation. Even Vero and MDCK cell lines has been used for invasion study in some bacterial such as *Leptospira interrogans*, *Salmonella* spp., *Shigella* spp., *Escherichia coli* (Finlay and Falkow, 1988; Barrow and Lovell, 1989; Leung and Finlay, 1991; Merien et al., 1997) but *Av. paragallinarum* could not invaded into Vero and MDCK cells. From this result, it may conclude that *Av. paragallinarum* is extracellular bacteria. However, chicken respiratory epithelial cells are probably more relevant for *Av. paragallinarum* studies.

Overall of this study, all 3 serovars of *Av. paragallinarum* could be found in Thailand and all of them can cause the clinical signs in experimental chickens. Then trivalent vaccine maybe needs to use for prevention infectious coryza disease in Thailand. From drug sensitivity test, the resistance too many kinds of antibiotics have found. The laboratory testing should be concerned for the treatment of coryza outbreaks. The virulence of field isolates, there is no consistent association between serovar and virulence. In addition, there is no correlation between clinical sign scores and survival in macrophage of each serovar. The study of capsule, OMP and LPS extraction at concentration 1000 µg/egg via yolk sac route, LPS of virulent isolates *Av. paragallinarum* can cause chicken embryo death whilst OMP and capsule cannot. About cell culture usage, chicken fibroblast, Vero cell line and MDCK cell line are not appropriate to use for invasion assay of *Av. paragallinarum*. Finally, the SDS-PAGE and 16S rRNS cannot use as serotyping tool for *Av. paragallinarum* but 16S rRNA can use for unknown bacterial species identification.

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Appendices

Appendix A

Media

Blood agar

INGREDIENTS	500 ml	1000 ml
Blood agar base (OXOID®, England)	40 g	80 g
Distilled water add to	500 ml	1000 ml
Sheep blood	25 ml	50 ml

Dissolve blood agar base in distilled water and boil until fully dissolve. Then sterilize by autoclaving at 121 C for 15 minutes and when the media temperature is decrease to 45-50 C, mix sheep blood and pour the media into sterile petri dishes.

CMI broth (chicken meat infusion broth)

(1) Ingredients for CMI broth.

INGREDIENTS	500 ml	1000 ml
Chicken meat infusion[A]	450 ml	900
Vitamin assay casamino acid (difco,USA)	2.5 g	5 g
Bacto-soytone (difco,USA)	2.5 g	5 g
NaCl	2.5 g	5 g

(2) Supplements for CMI broth

INGREDIENTS	500 ml	1000 ml
Chicken serum[B]	25 ml	50 ml
Fresh yeast extract[C]	25 ml	50 ml
1% β-NAD[D]	5 ml	10 ml

Dissolve vitamin assay casamino acid, Bacto-soytone and NaCl in chicken meat infusion and adjust pH to 7.0 with 10% NaOH. Then sterilize by autoclave at 121 C, 15

minutes and when the media temperature is decrease to 45 C, add chicken serum, fresh yeast extract solution and β-NAD. The CMI broth should be keep at 4 C until use.

[A] chicken meat infusion

Use one kilogram of chicken breast muscle per 2 liter of distilled water. Blend chicken meats with blender, add distilled water and incubate at 4 C overnight. Boil the mixture on medium fire approximately 45 min and filter through Whatman® filter paper no.1 (2 µm) for 2 times and follow by filter through 0.45 µm nitrocellulose membrane filter with sterile technique. The chicken meat infusion should be keep at -20 C until use.

[B] Chicken serum

Sterilize chicken serum by filter through 0.45 µm nitrocellulose membrane filter, inactivate by boil in water bath at 56 C for 30 min and keep at -20 C until use.

[C] Fresh yeast extract

Blend five hundreds milligram of fresh yeast for bread baking using blender and add 1.5 liter of distilled water. Boil the mixture in water bath at 100 C for 30 min, cooling down and then centrifuge at 5,000 x g. Filter the supernatant through Whatman® filter paper no.1 (2 µm) and follow by filter through 0.45 µm nitrocellulose membrane filter with sterile technique. The yeast extract solution should be keep at -20 C until use.

[D] β-NAD

β-NAD is prepared to 1% by diluting 100 mg with 10 ml of distilled water and sterile by filter through 0.45 µm nitrocellulose membrane filter. The β-NAD solution should be keep at -20 C until use.

GC agar base

INGREDIENTS	500 ml
GC agar base (OXOID®, England)	18 g
Soluble haemoglobin (OXOID®, England)	5 g
Vitox (OXOID®, England)	1 vial
Distilled water	

1. Suspend 18g of GC Agar Base in 240ml of distilled water and bring gently boiling to dissolve the agar. Sterilize by autoclaving at 121 C for 15 minutes.

2. Prepare a 2% solution of Soluble Haemoglobin Powder (LP0053) by adding 250ml of warm distilled water to 5g of haemoglobin powder. Continually stir the solution during the addition of water. Sterilise by autoclaving at 121 C for 15 minutes.

3. Dissolve the contents of one vial of Vitox (SR0090) as directed on the vial label.

4. Aseptically add the Vitox solution to 240ml of sterile GC Agar Base cooled to 50 C.

5. Aseptically add the 250ml of sterile haemoglobin solution, cooled to 50 C to the GC Agar Base-Vitox-Antibiotic Supplement solution. Mix gently to avoid trapping air bubbles in the agar and pour into sterile petri dishes.

TM/SN

(1) Ingredients for TM/SN agar.

INGREDIENTS	500 ml	1000 ml
Biosate Peptone (BBL, USA)	5 g	10 g
NaCl	5 g	10 g
Starch (Merck, Germany)	0.5 g	1 g
Glucose (Merck, Germany)	0.25 g	0.5 g
Yeast extract (Merck, Germany)	0.25 g	0.5 g
Bacteriological Agar No. 1	7.5 g	15 g
Distilled Water	500 ml	1000 ml

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

2. Add the required volume of water and suspend well.

3. Adjust the pH to 7.5 using NaOH solution.

4. Dispense into 500 ml bottles and sterilize by autoclaving at 121 C for 15 minutes.

5. Remove agar from autoclave and place in 50 C waterbath to cool for at least 30 minutes.

(2) Supplements for TM/SN agar.

SUPPLEMENTS	500 ml	100 ml
1% NADH (Sigma, USA)	1.25 ml	0.25 ml
0.05% Thiamine HCl[A]	5 ml	1 ml
Heat Inactivated Chicken Serum (56^0C/30 minutes)	5 ml	1 ml
O-A Complex[B]	25 ml	5 ml

6. In a biohazard cabinet, aseptically combine the supplements for the required volume of agar as in Table 2, in a sterile container.

7. Remove molten agar from 50°C waterbath and within the biohazard cabinet add supplements, mix and pour the plates.

8. Allow plates to dry in the cabinet for 30 minutes and store in sealed plastic bags at 4°C.

9. Incubate one plate at 37°C for 48 hours for a sterility check.

10. Discard unused plates after 2 weeks.

[A]0.05% Thiamine solution

Accurately weigh 0.05 g of thiamine (also called Aneurine Hydrochloride) into a 200 ml beaker and add 100 ml of RO water and dissolve thiamine. In the biohazard cabinet sterilize the solution using a 0.22 µm filter and a 50 ml syringe. Store at 4^0C.

*The solution always dispense aseptically in the biohazard cabinet.

[B]Oleic acid – Albumin (O-A) complex

Make a sodium oleate solution by combining 0.3 ml of oleic acid (any general purpose grade reagent quality will do) with 25 ml of 0.05N NaOH. Make an albumin solution by dissolving 23.75 g of bovine albumin fraction V (Merck, Germany) in 475 ml of normal saline (add gradually and avoid heating). To prepare O-A complex add 25 ml of sodium oleate solution to albumin solution. Adjust pH to 6.8. Filter (depth filtration best) and dispense aseptically in 100 ml amounts. Incubate in 37^0C water bath overnight and incubate in 56^0C water bath for 30 minutes. Store at 4^0C.

*Dispense aseptically in biohazard cabinet as required.

TMB

(1) Ingredients for TMB.

INGREDIENTS	100 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 ingredients from table 1.
2. Add the volume of water required and mix.
3. Adjust the pH to 7.5 using NaOH solution.
4. Dispense into 500 ml bottles and autoclave.
5. Remove the broth from autoclave and cool for at least 30 minutes.

(2) Supplements for TM/SN agar

SUPPLEMENTS	100 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 (56^0C/30 minutes)	1 ml	5 ml
O-A Complex[B]	5 ml	25 ml

[A & B] as previously described at TM/SN preparation.

6. In a biohazard cabinet, aseptically combine the supplements for the required volume of broth as in Table 2, in a sterile container. Add combined supplements to broth and mix.

7. Dispense to volumes required.

8. Incubate 3 mls of broth at 37°C for 48 hours for a sterility check.

9. Store unsupplemented broth at 4°C.

10. Discard unused plates after 2 weeks.

Mist. desiccans

A. horse serum	300	ml
(Sterile by filter through 0.45 µm nitrocellulose membrane filter)		
B. glucose	30	g
Soy peptone	1.3	g
Distilled water add to	100	ml

Sterilization by autoclave at 121 C for 15 minutes.

Before use, A and B are mixed together at ratio of 30: 10.

For Hemagglutination test

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% Volume 100 ml

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 3,000 x g for 7 min, 4 C.

3. Removed 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 cold 4% glutaraldehyde in PBS at 1:1 final ratio.

5. Stirrer the mixture overnight at 4 C on magnetic stirrer.

6. Then centrifuge at 3,000 x g for 10 min and wash with PBS 3 times.

7. Dilute the glutaraldehyde fixed erythrocytes with PBS to 10% final concentration and add thiomerosal to the final concentration of 0.01%.

8. Store at 4 C until use.

PBS-BSA (Phosphate Buffered Saline-Bovine Serum Albumin) Volume 1 liter

Na_2HPO_4-$2H_2O$	0.45	g
NaH_2PO_4-$12H_2O$	2.53	g
NaCl	8	g
Bovine serum albumin	1	g
NaN_3	4	g
Distilled water add to	1,000	ml

Dissolve all chemical substances in distilled water, adjust pH to 6.9-7.1 and filter through 0.45 μm nitrocellulose membrane filter. Keep at 2-5 C and should be use within one month.

For SDS Page

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

Tris (1.5M)	18.171	g
2 Na EDTA (0.008M)	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 (0.008M)	0.296	g

Dissolve in 70 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 them all and adjust to 100 ml with distilled water.

[A]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 (freshly prepared) Volume 100 ml

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

Other

PBS (Phosphate Buffered Saline) pH 7.2 Volume 1 liter

NaCl	8	g
KCl	0.2	g
Na_2HPO_4	1.44	g
KH_2PO_4	0.25	g
Distilled water add to	1,000	ml

Sterilize by autoclaving at 121° C for 15 minutes.

Appendix B

Table 5-1 Properties of *Avibacterium paragallinarum* isolates in Thailand.

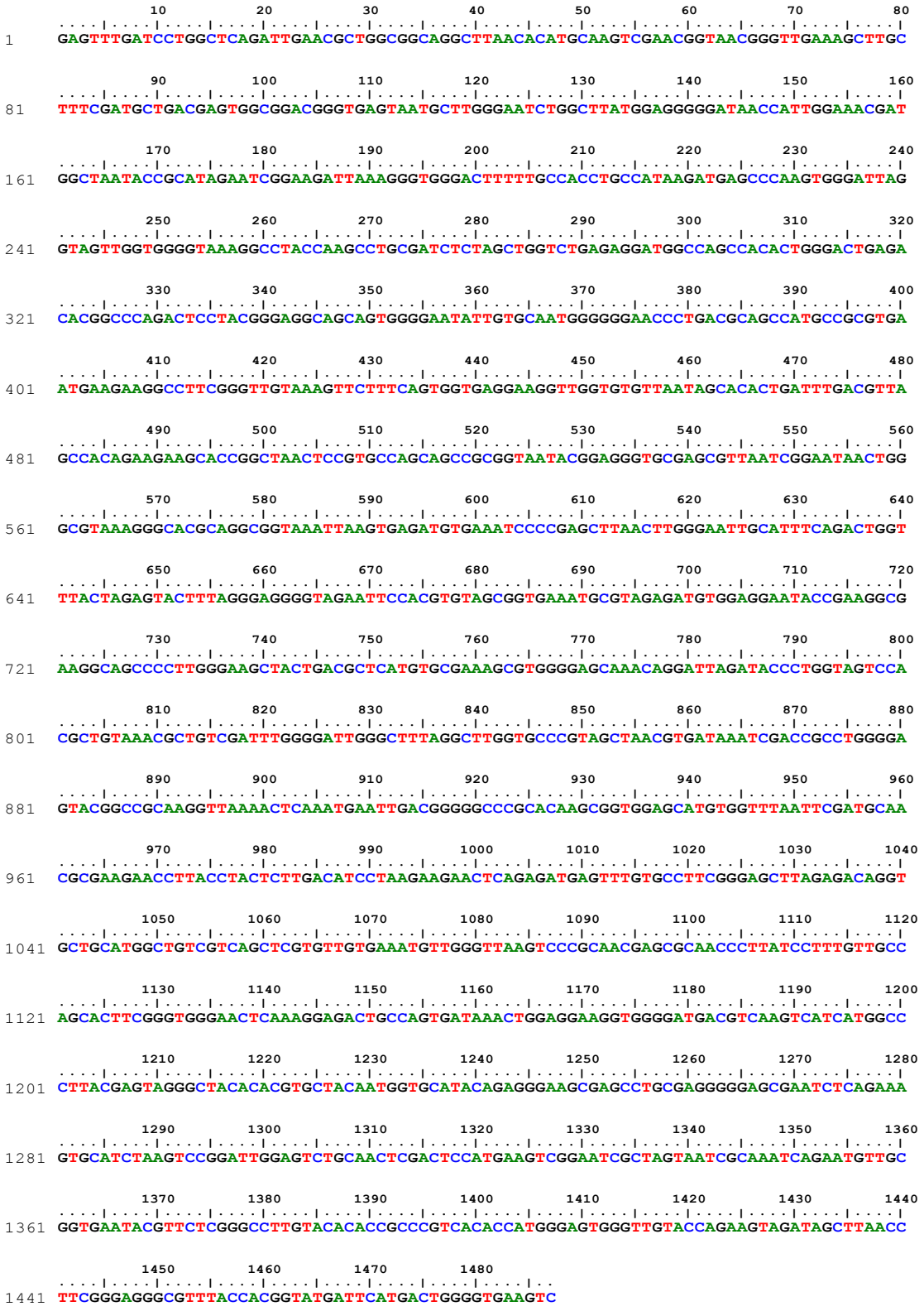
Strain	Source	Type	Date	PCR	Satellite growth	Identified
B1E1	NIAH*	Layer	2526	+	+	Apg serovar A-2
423	NIAH*	-**	~2530-35	+	+	Apg serovar A-2
111492	NIAH*	Breeder	2550	+	+	Apg serovar A
102947	NIAH*	-**	2550	+	+	Apg serovar C-2
1687	NIAH*	-**	2539	+	+	Apg serovar B
746	NIAH*	-**	~2530-35	+	+	Apg serovar A
102984	NIAH*	Breeder	2551	+	+	Apg serovar B
112179	NIAH*	-**	2549	+	+	Apg serovar A
102090	NIAH*	Native	2550	+	+	Apg serovar A
98	NIAH*	Breeder	2527	+	+	Apg serovar A
115757	NIAH*	Layer	2551	+	+	Apg serovar C
102943	NIAH*	-**	2550	+	+	Apg serovar C
211108	Chonburi	Layer	11/21/2551	+	+	Apg serovar B
IR1	Chaiyaphum	Layer	2/26/2549	+	+	Apg serovar A-2
CMA0509	chiangmai	Layer	16/5/2552	+	+	Apg serovar B
F1CM0709	chiangmai	Layer	15/7/2552	+	+	Apg serovar B
CHS0809	Chachoengsao	Layer	15/7/2552	+	+	Apg serovar A
CMU1009	chiangmai	Layer	30/9/2552	+	+	Apg serovar A

*NIAH (National Institute of animal health)

**No records

16S rRNA sequencing of 11 *Av. paragallinarum* isolates.

1) IC_IR1



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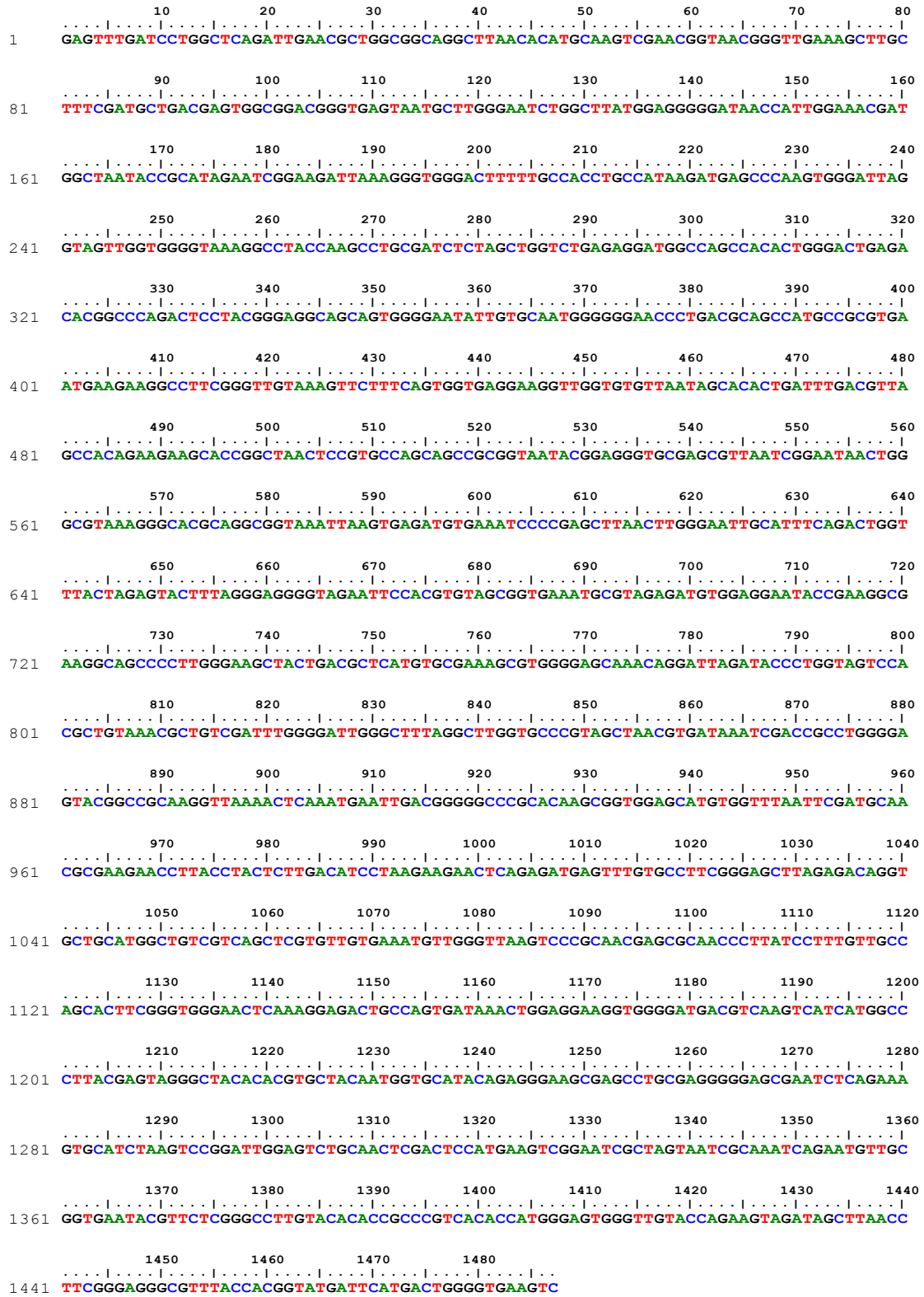
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3) IC_102947


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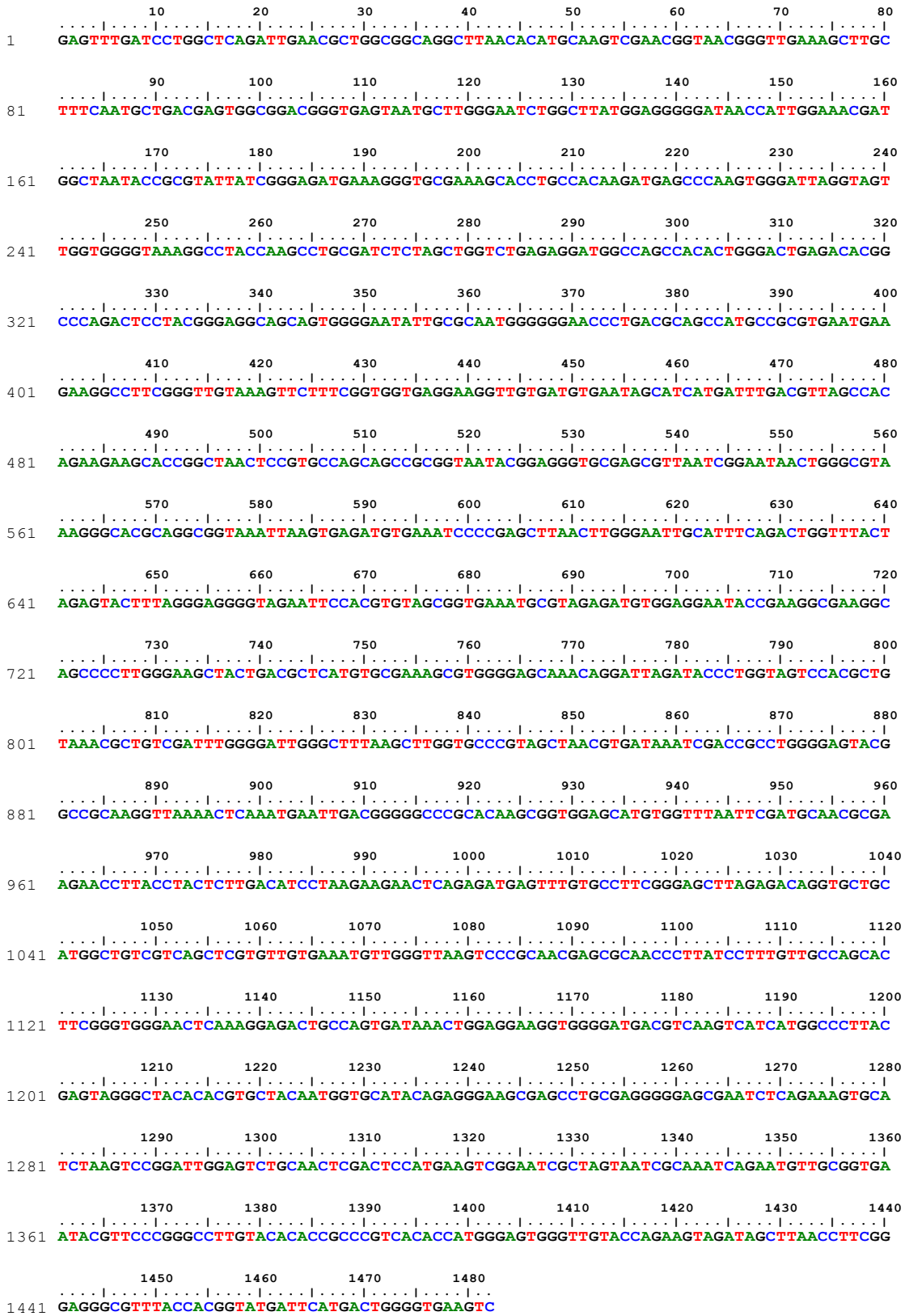
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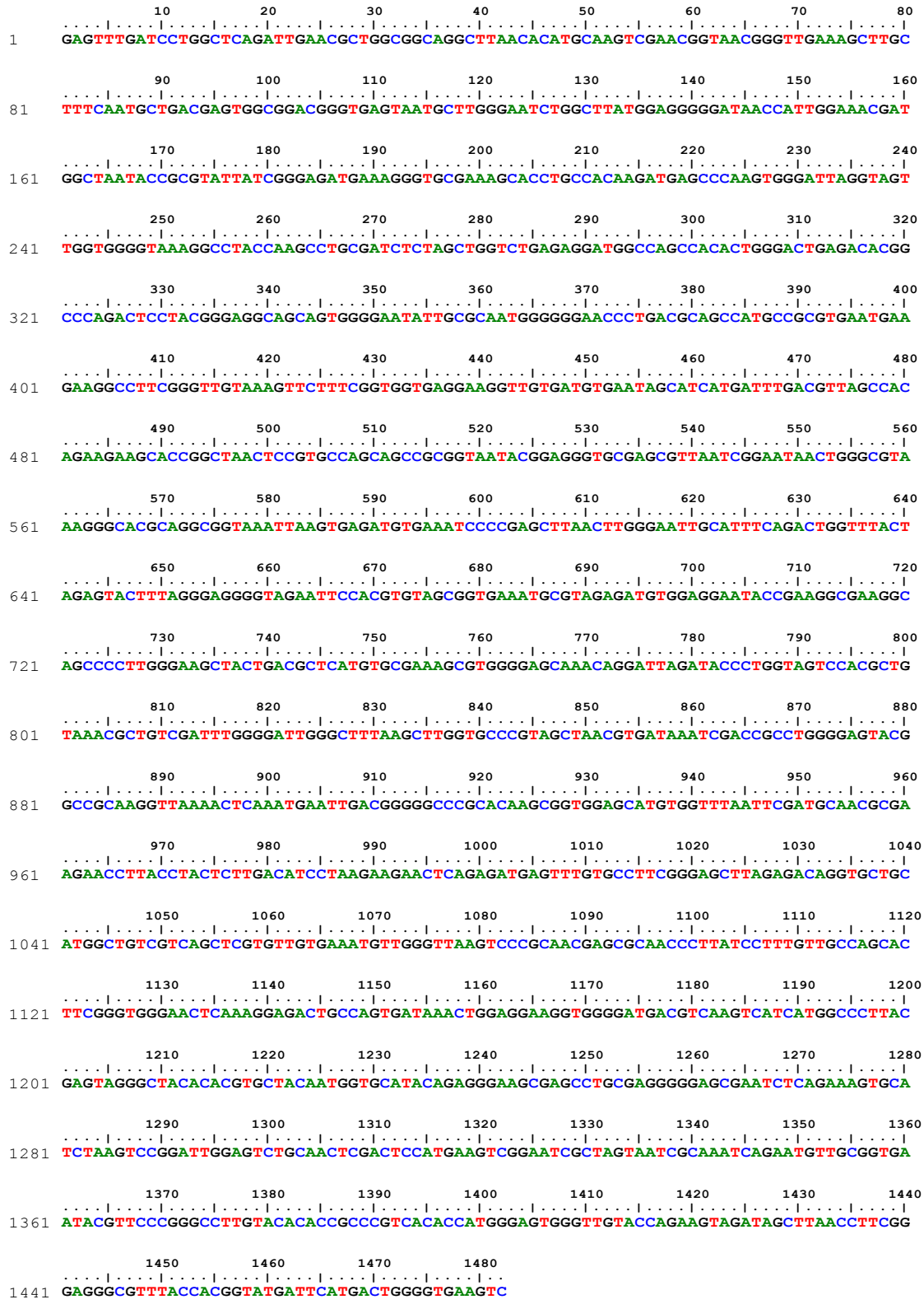
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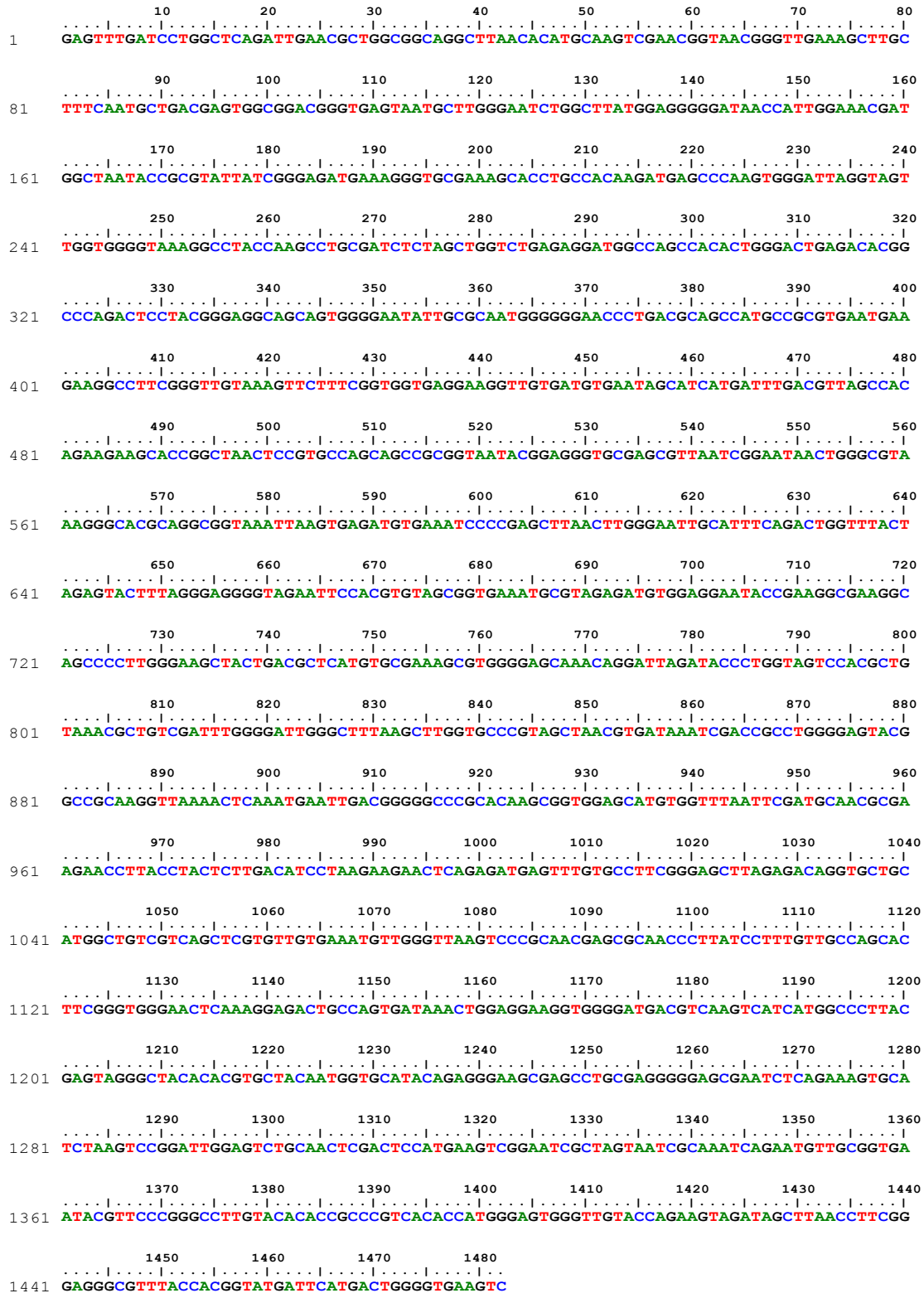
6) IC_102090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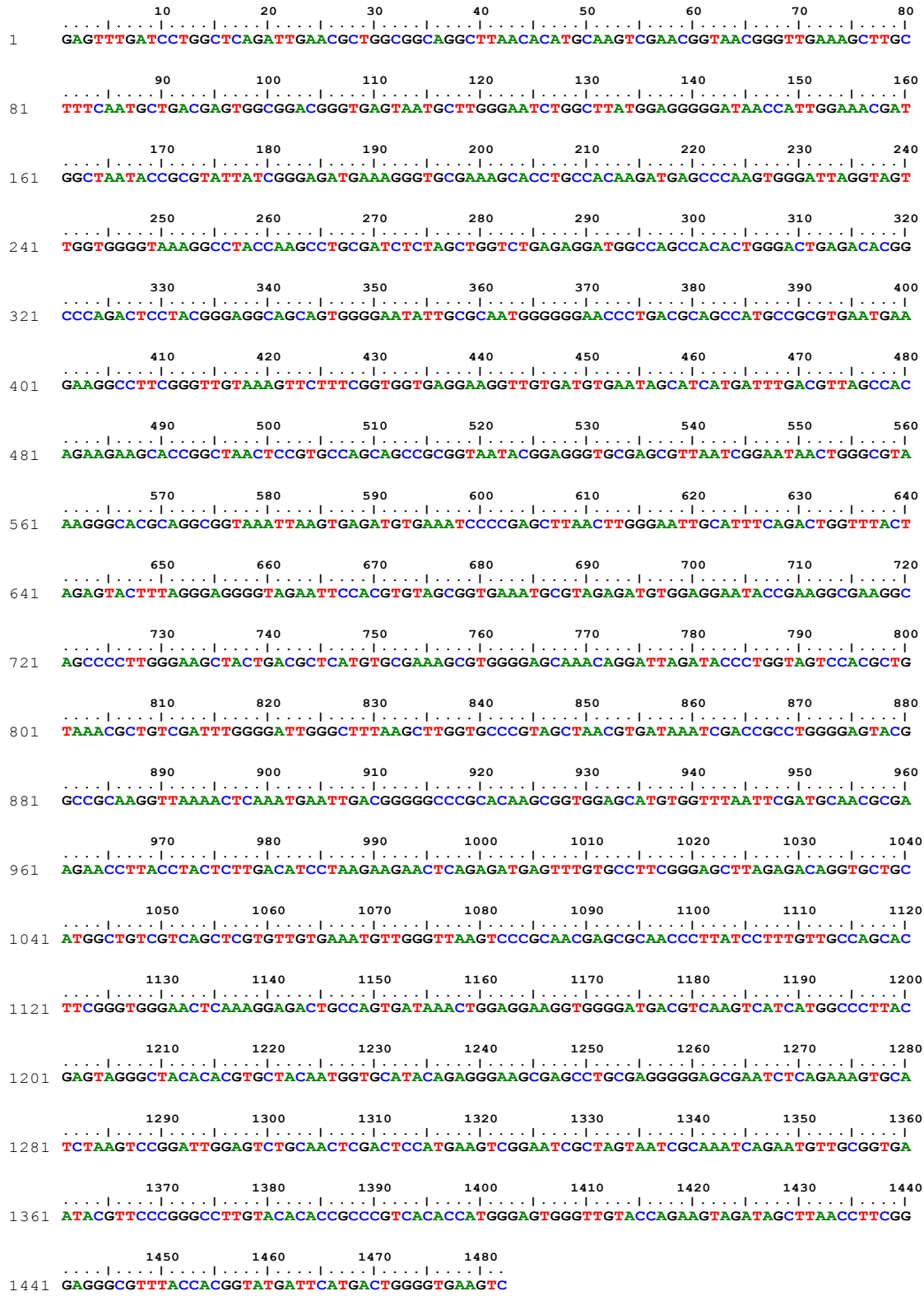
7) IC_115757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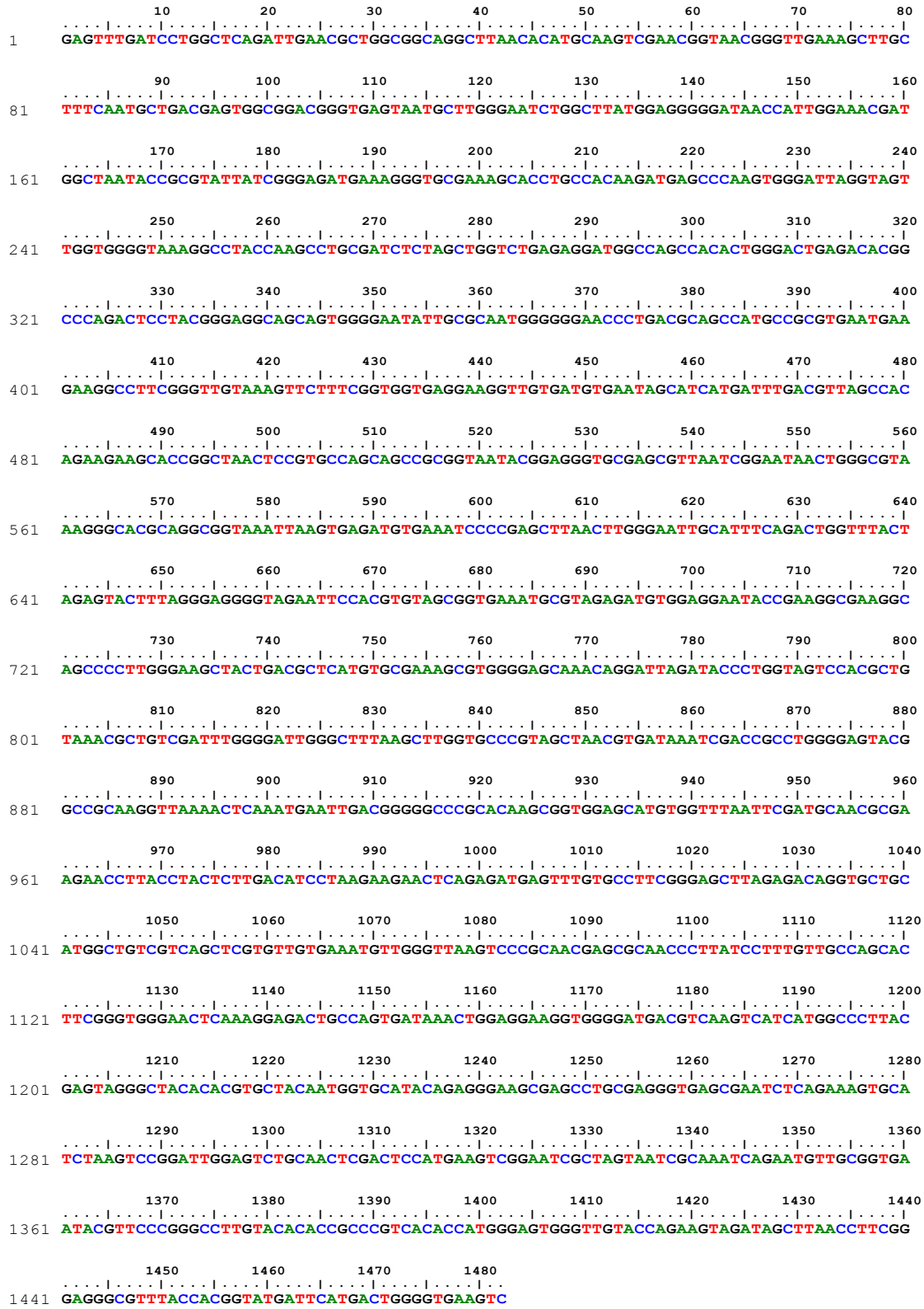
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1281 TCTAAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCAAATCAGAATGTTGCGGTGA
1370 1380 1390 1400 1410 1420 1430 1440
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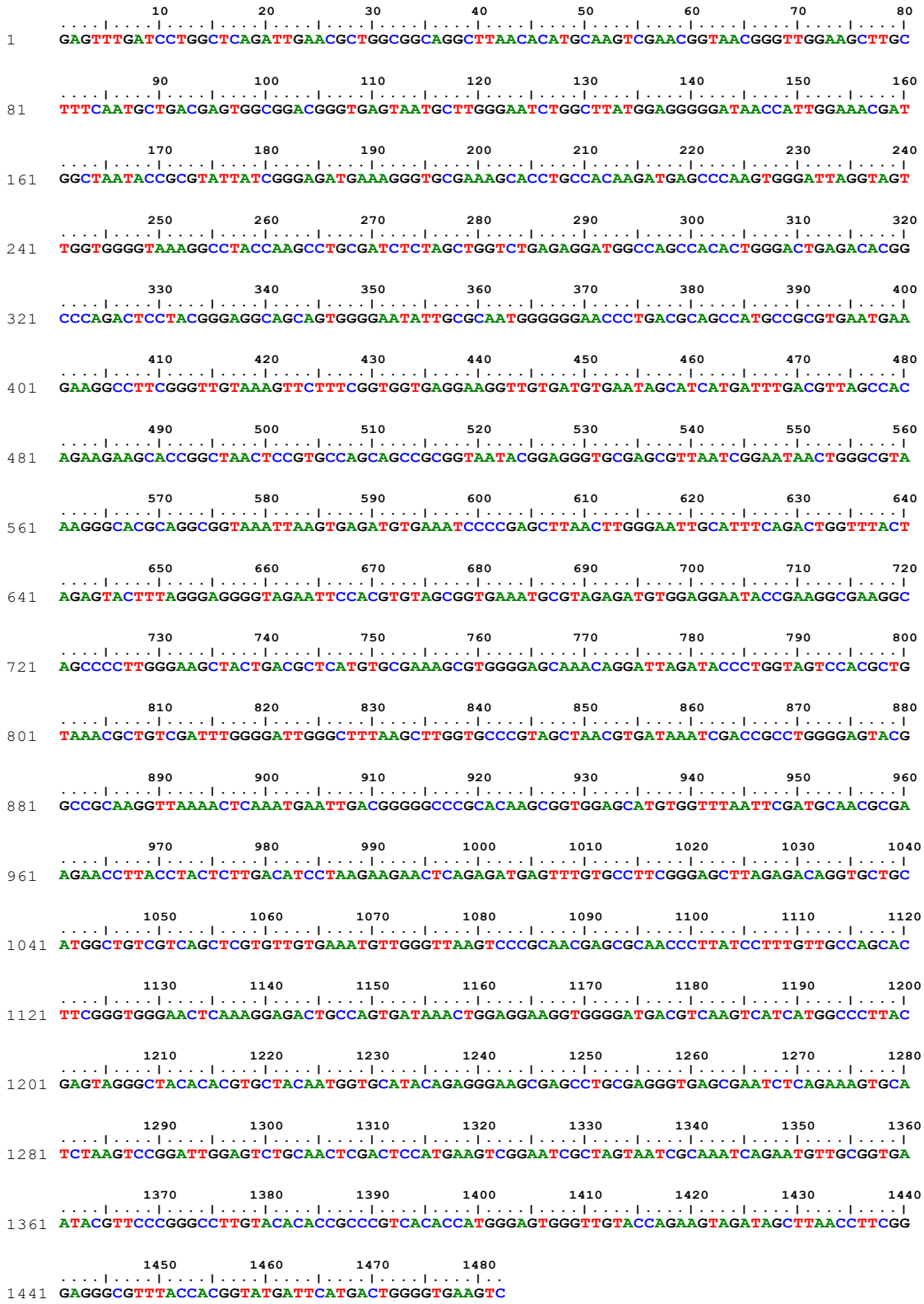
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481 AGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATAACTGGGCGTA
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801 TAAACGCTGTCGATTTGGGGATTGGGCTTTAAGCTTGGTGCCCGTAGCTAACGTGATAAATCGACCGCCTGGGGAGTACG
881 GCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGA
961 AGAACCTTACCTACTCTTGACATCCTAAGAAGAACTCAGAGATGAGTTTGTGCCTTCGGGAGCTTAGAGACAGGTGCTGC
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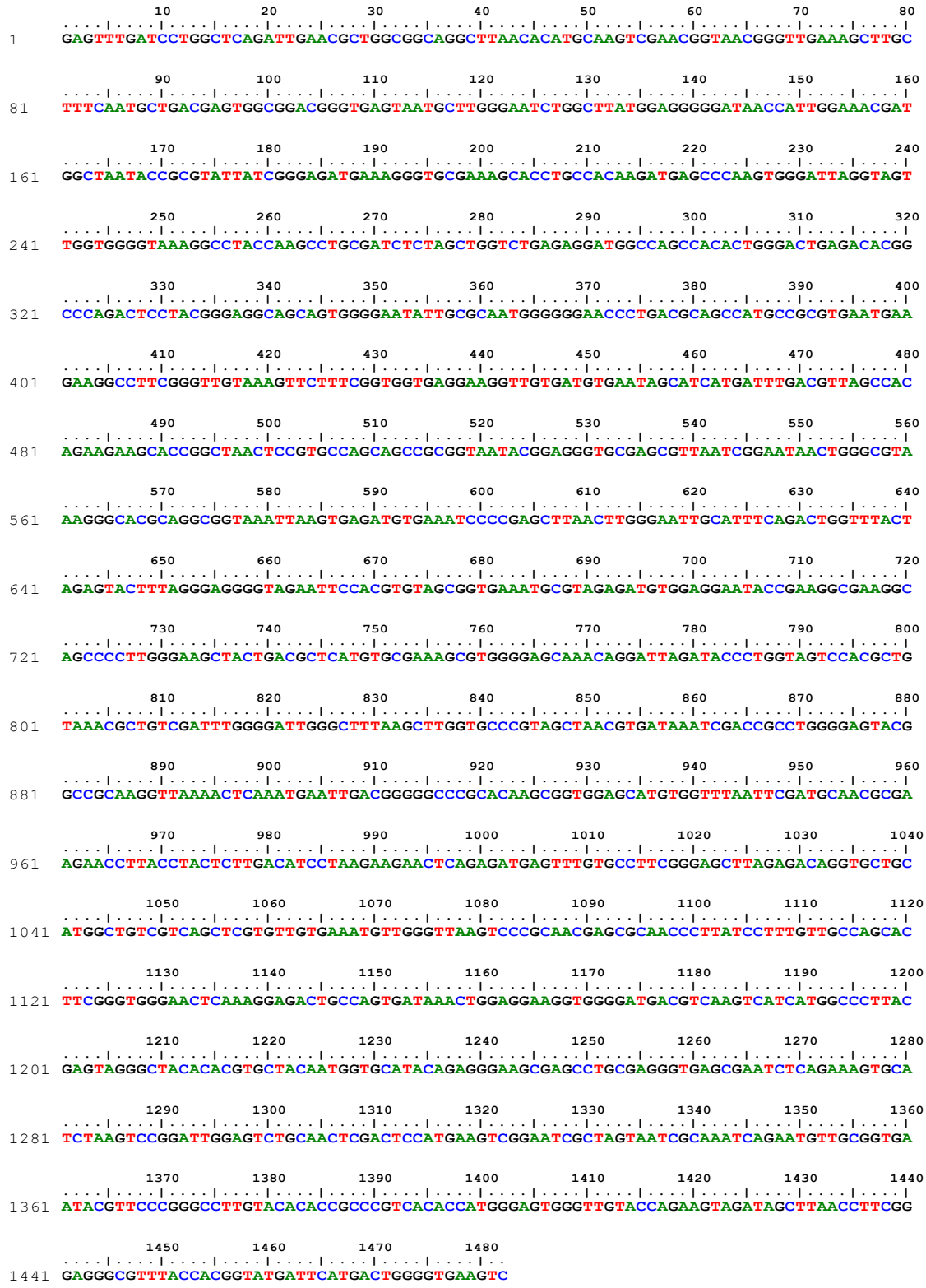

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